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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.

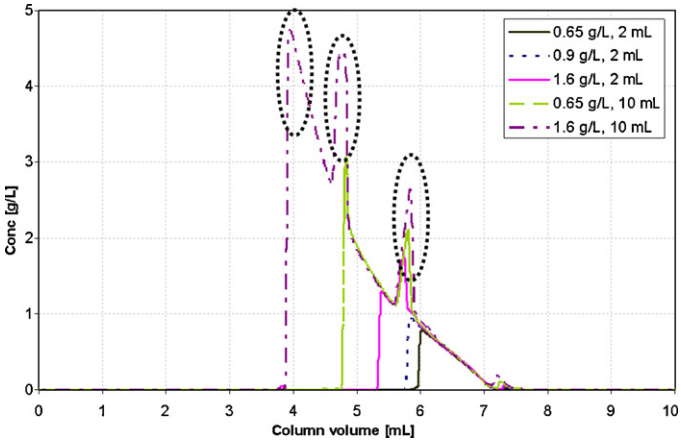

Fig. 1. Elution profiles with increasing mass injections of pure Calcitonin. Splits in the peak start appearing at high solute overloading. The concentrations (g/L) and injection volumes (mL) corresponding to each of the peaks are shown in the legend. Three distinct peaks of pure Calcitonin, caused by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

Table 3
Solvent gradient methods using solvent compositions shown in Table 1.

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
Y	100	0
Y + 0.01	100	0
Y + 20.0	40	60
Y + 20.01	10	90
Y + 40.0	10	90

Y = additional loading period (4, 7, 10, 13, 16 and 20 min).

the column entrance even if the loading modifier concentration is continued indefinite even after all the peptides are loaded. In the rest of this report, such practice of continuing with the loading modifier concentration, even after the completion of the sample feeding, will be referred to as "additional loading period" to differentiate it from the time period of actually loading the sample mixture. The time comprising the sample feeding and the additional loading period will be referred to as the "net loading" time. It can be expected from this discussion that even if the net loading time is varied, it should result into the same elution profiles as long as the same mass of the peptide is injected. But under the current conditions, there were significant (but repeatable) variations in the profiles caused by differences in net loading time. The results of five successive experiments are presented here to clarify the point. All the experiments were carried out using the same solvent gradient method listed in Table 3, but the only difference between these five experiments was their *net* loading times. Although in all the experiments, the same feed concentration and the same feeding time was used, the loading modifier concentration (101 g/L ACN or ~13%, v/v) was continued for additional (different) time lengths. The resultant profiles from these experiments (Fig. 2) show marked differences in their shapes. It can be also observed that (a) the variations in peak deformation, based on the net loading time, are not random rather follow a particular pattern, (b) the longer the additional loading time is, the smaller the size of the middle peak (among the three distinct peaks of Calcitonin) becomes, till it completely disappears, and (c) once the middle peak disappears the profile remains almost unaltered even with longer additional loading, which can be verified by comparing the peaks corresponding to 16 and 20 min of additional loading period, respectively.

Both the above observations can be explained based on (a) the equilibrium between the TFA and the di-hydrogen phosphate anions in forming ion-pair with the basic groups of Calcitonin molecule, and (b) the stage-like operation in a chromatographic column. Calcitonin, which is available as ion-paired with TFA from the synthesis step is gradually transformed into phosphated Calcitonin in the course of interaction with the mobile phase, used in the experiments described above. Now, as the transformation of all the Calcitonin molecules is not instantaneous (and not complete) because of the presence of several equilibrium stage operations in chromatographic columns, the stages of transformation gave rise to all the effects observed in this study.

For a broader explanation let us theorize that the strongly adsorbed Calcitonin molecules act like dynamic ion-exchangers to the TFA and the di-hydrogen phosphate anions, as schematically illustrated in Fig. 3. This illustration, particularly the possible orientation of the Calcitonin molecule (Fig. 3), can be supported by the facts that (a) Calcitonin is cylindrical in shape, and (b) following the Kyte–Doolittle method [27] of determining average hydrophobicity of a peptide molecule, four out of the five amine groups attached to Calcitonin is likely to be exposed to the mobile phase. [Several softwares are available in the Internet to determine the hydropathy plot of peptides and proteins using the Kyte–Doolittle method.

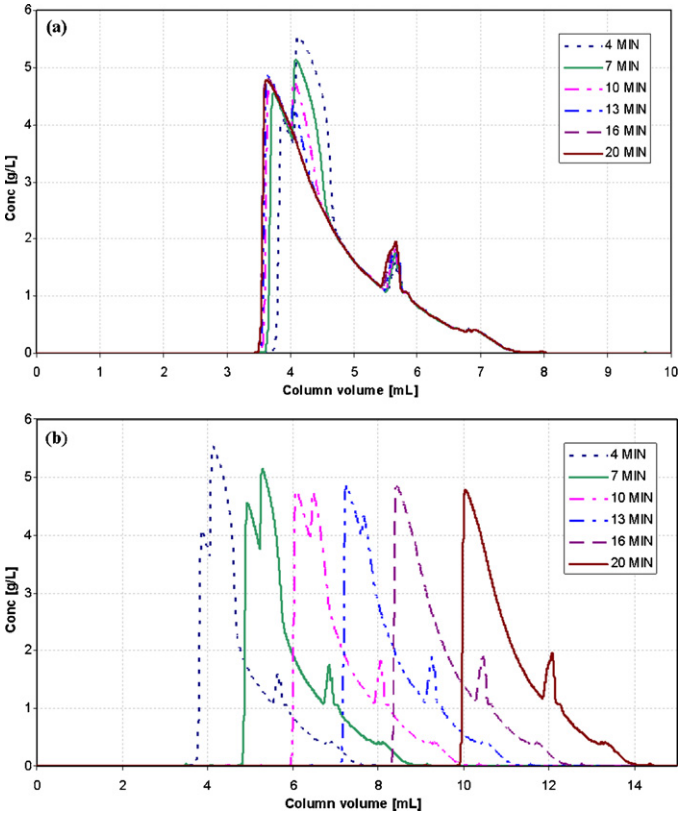

Fig. 2. Profiles with different additional loading times, having sample loading time of 3 min at 1 mL/min and 5.5 g/L sample concentration. Profiles of (a) were plotted starting from the beginning of the gradient elution, whereas those of (b) were plotted from the beginning of the additional loading time. It can be noted that the profiles with additional loading times of 16 and 20 min respectively are the same indicating an absence of any effect beyond 16 min.

For Calcitonin, such a plot (Fig. 4) was developed using the software available online at fasta.bioch.virginia.edu/fasta/grease.htm.] It can be noted (Fig. 4) that Calcitonin is overall hydrophobic till the 12th amino acid and during the adsorption process it is quite likely that this part of the molecule will be attached to the stationary phase. Now if this information is transported to the actual Calcitonin molecule (Fig. 5), it can be noted that while adsorbed, most of the amine groups of Calcitonin are likely to stay exposed to the mobile phase. This particular orientation of Cal-

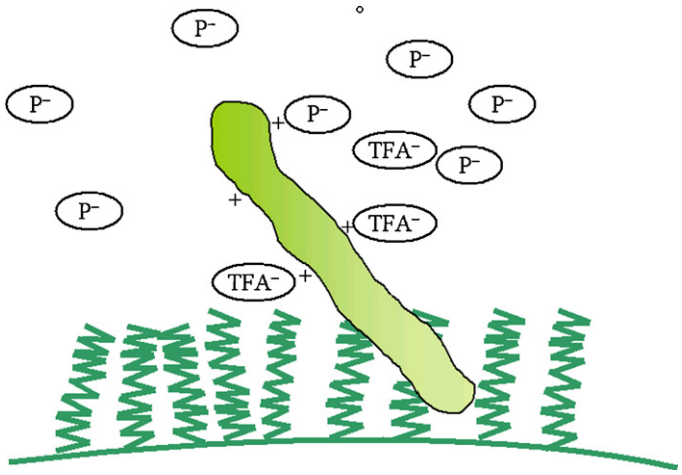

Fig. 3. A plausible schematic orientation of Calcitonin molecule, working as ion-exchange ligand for the tri-fluoroacetate (TFA^-) and the di-hydrogen phosphate (P^-) anions.

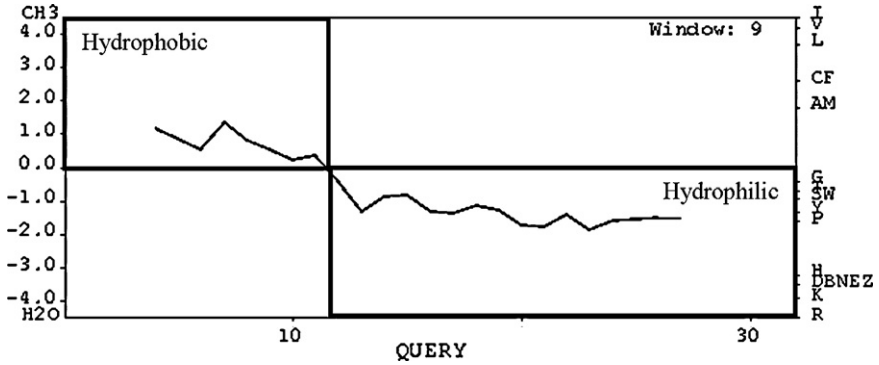

Fig. 4. Hydropathy plot of Calcitonin measured with the Kyte–Doolittle method, which shows that for Calcitonin there are two distinct zones of hydrophobicity.

citonin molecule (Fig. 3) can strongly facilitate the possibility of Calcitonin acting as ion-exchange ligands for the TFA and hydrogen phosphate anions. The overall phenomenon which is most likely happening during the loading period can be described as follows. Calcitonin molecules when loaded on to the column are mostly associated with TFA anions. At the loading modifier concentration the Calcitonin molecules form a zone of ion-exchange ligands at the entrance of the column. With the continuation of the loading modifier concentration, the TFA anions attached to the Calcitonin molecules start eluting from that zone, facing competition from their di-hydrogen phosphate counterparts. Once the TFA anions are outside the Calcitonin zone they are quickly eluted from the column as they do not adsorb on reversed-phase material. Even during the elution period this process is continued. This physical behaviour is similar to the observation in ion-pair reversed-phase chromatography [3]. In ion-pair chromatography (IPC), special chemicals called ion-interaction reagents (IIR) are used. These reagents basically have a strong hydrophobic group attached to an ionisable functional group. In reversed-phase systems these reagents form ion-pair complex with other counterions and can greatly modulate their retention behaviour. Two different interactions are mainly theorized to explain the activities of IIR [3,28–31] within the framework of stoichiometric models, which suggests reaction schemes between the IIR and the ionic analytes, leading to formation of chemical complexes. Now these complexes can form either within the mobile phase, leading to neutralization of the IIR molecule and subsequent adsorption, or, the IIR first getting adsorbed on the reversed-phase and acting as dynamic ion-exchangers with respect to the solute molecules. Most of the recent reports suggest that a combination of both the mechanism is possible depending upon the experimental conditions. In the present study the role played by Calcitonin as an IIR with multiple ionisable sites can be easily comprehended. The ion-interaction mechanism follows the model of dynamic ion-exchanger during the loading period, whereas both the mechanisms are possible during the elution period.

Based on this theory, the observed elution behaviours discussed above can be explained in the following way:

(a) Peak splitting is basically caused by ion-pairing of Calcitonin molecules with multiple counterions, i.e. the TFA and the hydrogen phosphate anions. As TFA is more hydrophobic than di-hydrogen phosphate [4,14,21], it can be expected that the higher the association of Calcitonin with TFA, the higher its retention will be. Additionally, the saturation capacities of the TFA-ed Calcitonin and the phosphated Calcitonin are markedly different, which can lead to forming peaks with different shapes (Figs. 1 and 2). Now, at low solute injections the peak splits may generally not be visible as the elution volume of the di-hydrogen phosphate concentration is high enough to transform most of the TFA ion-pair to mostly phosphated ion-pair in the elution profile. Here the elution volume of di-hydrogen phosphate indicates the net volume of the solvent containing di-hydrogen phosphate anions used for eluting the Calcitonin molecules completely. At higher solute concentrations, however, the di-hydrogen phosphate concentration and its elution volume are not sufficient to replace all the TFA-ed ion-pairs. This resulted into separate peaks of mostly TFA-ed Calcitonin and mostly phosphated Calcitonin and appeared as peak splits (Figs. 1 and 2). It may be mentioned here that the small peaks at the extreme end of the profiles presented in Figs. 1 and 2 were created by the presence of impurities and will not be discussed further in the report.

(b) The gradual deformation of the elution profiles, more specifically the gradual disappearance of the middle peak, when

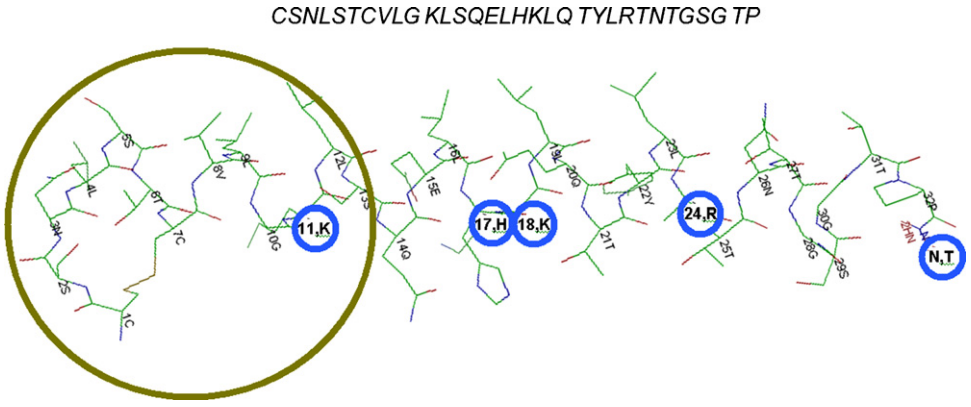

Fig. 5. Molecular structure of Calcitonin showing the hydrophobic zone with the large open circle. The smaller open circles show the presence of the amine groups in the molecule. The amino acid sequence of Calcitonin is shown at the top.

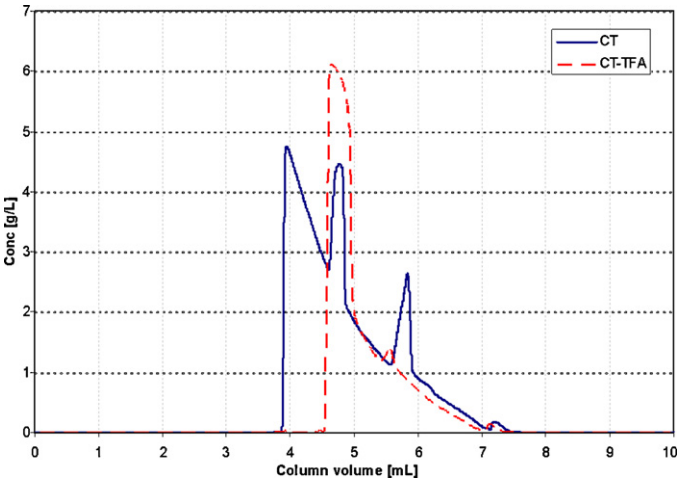

Fig. 6. Elution profile of the sample (CT–TFA), where additional TFA was added to Calcitonin, shows marked difference from the profile of an earlier experiment with CT sample (Fig. 1) without additional TFA. The consolidation of the CT–TFA profile around the middle peak indicates the influence of TFA in forming the same.

increasing the additional loading time of the feed (Fig. 2) can also be easily explained. Although additional continuation of loading mobile phase does not alter the adsorbed positions of the Calcitonin molecules at the column entrance, the TFA ion-pairs are continually replaced by di-hydrogen phosphate ion-pairs during this operation. The middle peak, which is most likely formed by Calcitonin molecules ion-paired with TFA, gradually disappears due to this gradual transformation to phosphated molecules.

4. Experimental verification of ion-pairing effect

The objective of this section is to experimentally demonstrate that the observations related to peak deformations, described above, and the supporting explanation of these observations based on the ion-pair effect at overloaded conditions, are valid. In this direction two different sets of experiments are presented which demonstrate the presence and the role of TFA ion-paired with Calcitonin molecules as the prime factor.

4.1. Experiment 4a

A pure Calcitonin sample was prepared having Calcitonin concentration of 1.6 g/L (0.463 mmol/L) and TFA concentration of 0.15 g/L (1.3 mmol/L). This sample is basically the same one used for generating the profile with the highest peptide loading in Fig. 1, except the addition of TFA in the sample mixture. The experiment was carried out using the same solvent gradient method described in Table 2, following a sample injection of 10 mL. The resultant profile is shown in Fig. 6 along with the corresponding profile from Fig. 1. It can be clearly noted that in the new profile the addition of TFA has resulted into a mass accumulation around the middle peak at the expense of the two other peaks. The result certainly indicates the influence of TFA in forming the middle peak, and also a stronger retention of Calcitonin due to the increased ion-pairing with TFA anions, which imparts increased hydrophobicity compared to phosphate anions as mentioned earlier.

4.2. Experiments 4b

This experiment actually comprises three separate experiments following the method described in Table 2. A purified Calcitonin sample of 1.3 g/L with an injection volume of 3 mL was used in all

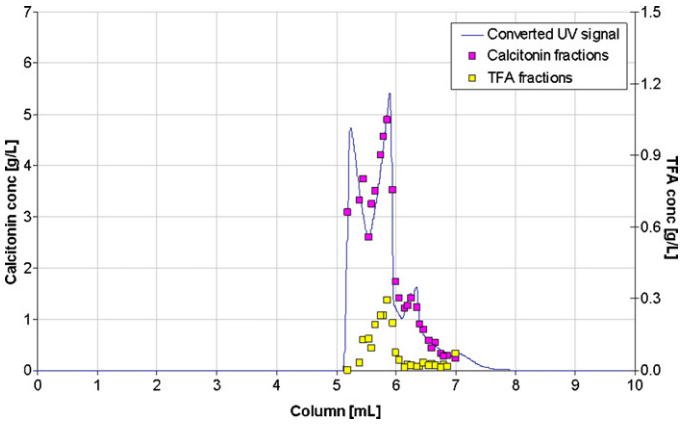

Fig. 7. The chromatogram and fraction analysis results shown for an injection without any additional loading period. The higher concentration of TFA along the middle peak clearly indicates the association of this compound with the Calcitonin eluting at that time.

the experiments. The first experiment was carried out without any additional loading period. For the next two experiments, on the other hand, additional loading periods were 4 and 14 min respectively. Fractions were collected for each experiment at an interval of 0.25 min using an automatic fraction collector (GILSON FC203B). Each of the fractions were analysed using a method described in Appendix A, leading to the concentration values of Calcitonin and TFA. Results of these experiments are shown in Figs. 7–9 respectively. It can be noted that in all the experiments significantly high presence of TFA was detected in the fractions collected along the middle peaks. It can be also noted that with increasing additional loading time, which resulted into the decrement of the size of the middle peak, the net presence of TFA also decreased. It can be inferred from these experiments too that the middle peak is formed by Calcitonin molecules ion-paired with the TFA anions, and, with increasing elution volume of the di-hydrogen phosphate buffer, TFA association with Calcitonin is gradually replaced with the former.

From the main inferences of these two experiments that (a) some Calcitonin molecules have stronger ion-pair association with TFA than the others (forming the middle peak), and (b) with increasing net loading time (or in general, with increasing net elution volume of the Calcitonin molecules) these TFA ion-pairing is gradually reduced, the role of ion-paring in causing the two observations described before can be clearly apprehended.

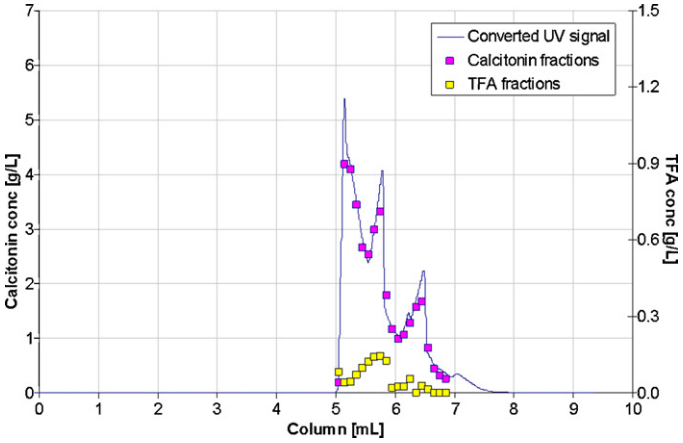

Fig. 8. The chromatogram and fraction analysis results shown for an injection with additional loading time of 4 min. It can be noted that the size of the middle peak as well as the TFA concentration has reduced in this process.

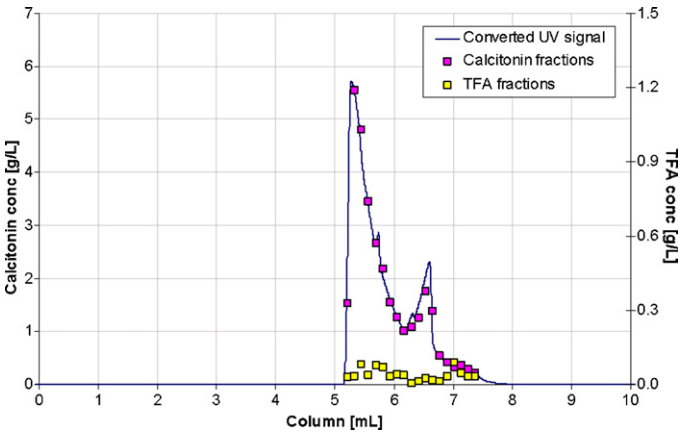

Fig. 9. The chromatogram and fraction analysis results shown for an injection with additional loading time of 14 min. The middle peak has almost disappeared in this case, along with the presence of TFA.

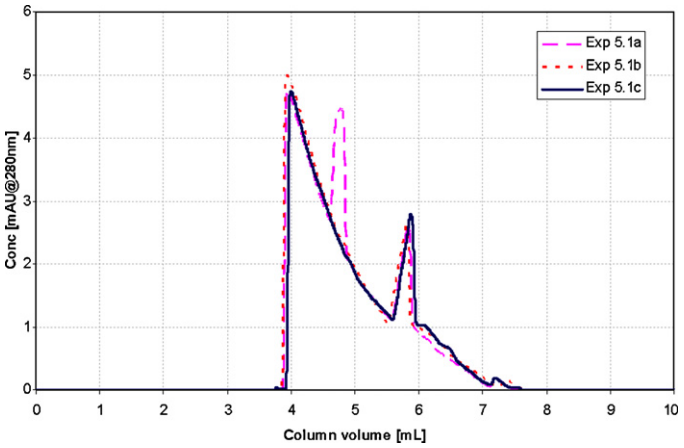

Fig. 10. Experimental results to show that additional loading period brings a permanent change to the Calcitonin molecules. The detailed experimental conditions corresponding to the profiles are available at the experimental sections shown in the legend.

5. Investigating other plausible causes

Although, the above experiments clearly show the role played by ion-pairing with multiple anions as the cause of the unusual peak profiles, at highly overloaded peptide elution, other plausible effects under the current experimental conditions should be investigated.

Broadly speaking, the elution behaviours described before, especially the effect of additional loading time on the peak deformations, are truly intriguing. This is because the behaviour of solute molecules in reversed phase, as function of modifier concentrations, is sufficiently well understood otherwise. Although the debate over the nature of the retention mechanism (adsorption or partitioning) could not be closed decisively, there is no doubt that at sufficiently low (initial) modifier concentrations, lipophilic molecules get strongly attached to the solid phase at the entrance of the column and there should not be any change in the elution profile even if the operation (with the low modifier concentration) is continued indefinitely. This view is supported by the basic thermodynamic understanding in a chromatographic column. Several additional phenomena other than ion-pairing, however, may also occur during the experimental procedure and influence to generate the discussed elution behaviours. There are possibilities of movement or reorientation of the peptide molecules, through solid diffusion or other phenomena, even at highly retained condition [32]. There could be myriads of other factors, like viscous fingering, column degeneration, other kinetic effects [11], etc., leading to peak splits. It is also possible that several of these factors are acting together to result into such situations. To develop a clear understanding of the plausible roles of these factors in the current situation, if any, the following sets of experiments were carried out.

5.1. Effect of column properties

This set of experiments was carried out in order to detect if the peak deformations are influenced by any artefact of the column, e.g. some defect or damage in the stationary phase or some special property of the reversed-phase ligands, etc. All the experiments were carried out from a single solution of 1.6 g/L Calcitonin.

5.1.1. Experiment 5.1a

This experiment was carried out using the solvent gradient method described in Table 2, with a sample injection of 10 mL. The resulting profile, shown as the dashed line in Fig. 10, can be observed to have splits at multiple points.

5.1.2. Experiment 5.1b

This experiment follows the same method and injection volume as in experiment 5.1a, except that there was an additional loading period of 20 min. The profile obtained from this experiment, shown by the dotted lines in Fig. 10 is almost identical to the previous profile except the middle peak, which had expectedly disappeared.

5.1.3. Experiment 5.1c

To conduct this experiment, the entire eluted mass of Calcitonin, from experiment 5.1b, was collected in a flask. The modifier concentration associated with this collection was reduced down to the loading modifier concentration level in a vacuum rotary evaporator (Buchi Rotavapor R-200) using a temperature bath controlled at 25 °C and pressure at 100 mbar, controlled by Buchi vacuum controller V-800. This solution was then loaded onto the column as feed, following the same procedure as in experiment 5.1a, i.e. *without* any additional loading period. The elution profile of this experiment is presented as the solid line in Fig. 10. It can be observed that although this experiment used the method of experiment 5.1a, i.e. without any additional loading time, its profile looks like that of experiment 5.1b, which used additional loading time.

The result of experiment 5.1c clearly shows that the peak deformations are not resulting from any column artefact. Had it been the case, the peak shapes could be reproduced by following the same experimental conditions. But that did not happen. Experiment 5.1c could not reproduce the profile of experiment 5.1a, although the experimental conditions were the same. The similarity in profiles, from experiments 5.1c and 5.1b, rather shows a change in the Calcitonin sample itself, occurred during experiment 5.1b.

5.2. Effect of adsorption kinetics

The main objective behind these experiments was to verify if the peak deformations are also controlled by any time dependent phenomenon, occurring during the varying additional loading periods. All these experiments were carried out from a single solution of 3.47 g/L Calcitonin, having a loading volume of 3 mL.

5.2.1. Experiments 5.2a

The first set of experiments was carried out following the solvent gradients method listed in Table 4. It can be noted that the main difference between these methods is their flow rates, which are 1, 0.75, 0.5 and 0.25 mL/min respectively. The time ranges of the gradients (Table 4) used in these experiments were adjusted to

Table 4
Solvent gradient methods for different flow rates, using solvent compositions shown in Table 1.

Time (min)				Solvent A (%)	Solvent B (%)
1 mL/min	0.75 mL/min	0.5 mL/min	0.25 mL/min		
0.0	0.0	0.0	0.0	100	0
20.0	26.67	40.0	80.0	40	60
20.01	26.68	40.01	80.01	10	90
40.0	53.34	80.0	160.0	10	90

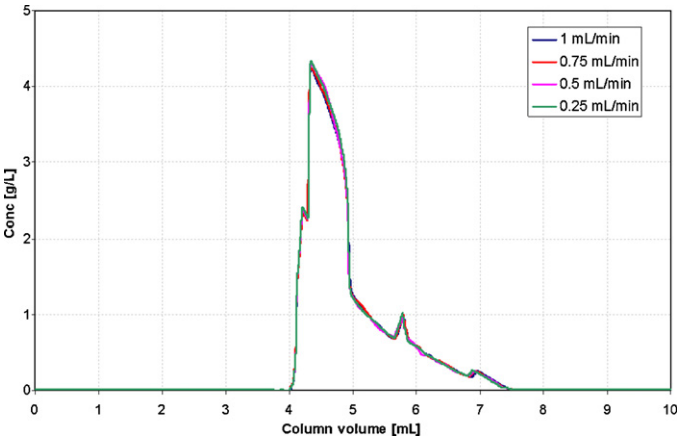

Fig. 11. The experiments, carried out with flow rates 1.0, 0.75, 0.5 and 0.25 mL/min respectively, used the same solvent gradient w.r.t. the elution volumes. Injected sample concentration was 3.47 g/L and injection volume 3 mL. The profiles show that their shapes did not change, irrespective of the solute residence time in the column.

maintain the same gradient according to their elution volumes. All these experiments were carried out *without* any additional loading period. It can be observed (Fig. 11) that irrespective of the column residence time, which varied by multiples, the profiles remained absolutely the same.

5.2.2. Experiments 5.2b

Another set of experiments was carried out to understand the kinetic effect exclusively during the additional loading period. This was done by changing the solvent flow rates during the additional loading period only, while maintaining the same experimental conditions during the gradient operation. Fig. 12 shows the resultant profiles where all of them used the gradient method shown in Table 2 in the elution phase. For the profiles shown with the dotted line and the dashed line the additional loading time was 10 min, whereas for the profile shown with the solid line, it was 7 min. For the dashed profile, the first 5 min of additional loading time was carried out with a flow rate of 0.4 mL/min, whereas for the next 5 min the flow was 1 mL/min. It may be observed (Fig. 12) that the profiles having the same additional elution volume ($1 \times 7 = 0.4 \times 5 + 1 \times 5 = 7$ mL) during the additional loading period look the same rather than the profiles with the same additional loading times. From the above results it can be concluded that the peak deformations are not affected by any time-dependant phenomenon; rather solely depends on the volume of the mobile phase coming in contact with the solute, indicating chemical equilibrium, more specifically the secondary equilibrium (ion-pair effect in this case), as the controlling factor.

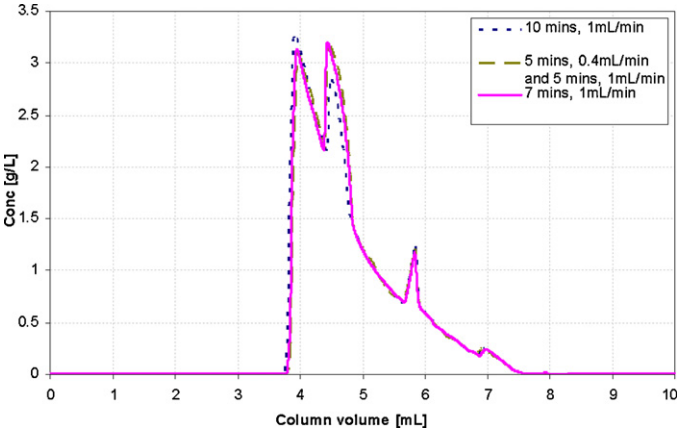

Fig. 12. Experiments showing the dependence of the shapes of the profile on the elution volume of the additional loading period. Both the profiles with the dotted and the dashed line had 10 min additional loading time, but with different flow rates as indicated in the legend. The profile with solid line had 7 min additional loading time at 1 mL/min. Injected sample concentration was 3.47 g/L and injection volume 3 mL for all the cases.

6. The last peak

The reason behind the formation of the last peak, among the three distinct peaks of Calcitonin (Figs. 1, 7–9) has to be separately discussed. From the above discussion it can be clearly inferred that the occurrence of this peak is also controlled by the ion-pair effect. However, it can be observed (Figs. 7–9) that unlike the middle peak, this peak is not associated with TFA anions. This follows also from the observation (Figs. 7–9) that with increasing additional loading times, when the middle peak gradually disappears, the last peak actually grows in size. This in fact indicates the possibility that the last peak is resulting from phosphate ion-pairing only. But the same reasoning is also applicable for the first peak. So what can then lead to the formation of two different peaks out of the same ion-pairing? A possible answer may again lie in the ionogenic nature of the Calcitonin molecules [22]. Carr and co-researchers [21,22] have conclusively reported that even under very high counterion concentrations, not all the basic sites of the ionogenic solutes are ion-paired. This actually gives rise to the possibility of having an additional form of the basic group, i.e. no ion-pairing, along with TFA and phosphate ion-pairing. Now, Calcitonin has multiple charged sites and each of these sites can occur in one of these three states discussed above. So, in a standard situation a Calcitonin molecule may actually exist in any form out of a very high number of possible combinations, leading to multiple retention characteristics. This also indicates that even if all the TFA Calcitonin ion-pairs are replaced with phosphate Calcitonin ion-pairs, we still may have Calcitonin molecules with varying degree of ion-pairing and hence varying retention characteristics. To verify this possibility a set of three experiments were carried out, using the same method (shown in Table 2) but with different concentrations of the phosphate buffer in the solvents. The acetonitrile and water composition in the solvents were kept the same as listed in Table 1, but the phosphate compositions for the three experiments were taken as 2, 6 and 12 g of H_3PO_4/kg of solvent, respectively. In each of the experiments, the results of which are shown in Fig. 13, an additional loading time of 20 min was employed as previously it was found that beyond 16 min of additional loading virtually no change of profile occurs, indicating a dispelling of the undesirable ion-pair effect by that time.

Table 5
Solvent compositions used for generating modifier gradient.

Experiment	Solvents	Acetonitrile (g)	Water (g)	H_3PO_4 (g/kg)	TFA (g/kg)
1	A	100.5	868.1	2	–
2	A1	100.5	868.1	–	0.15
3	A2	100.5	868.1	–	0.3
All	B	455.6	414.5	2	–

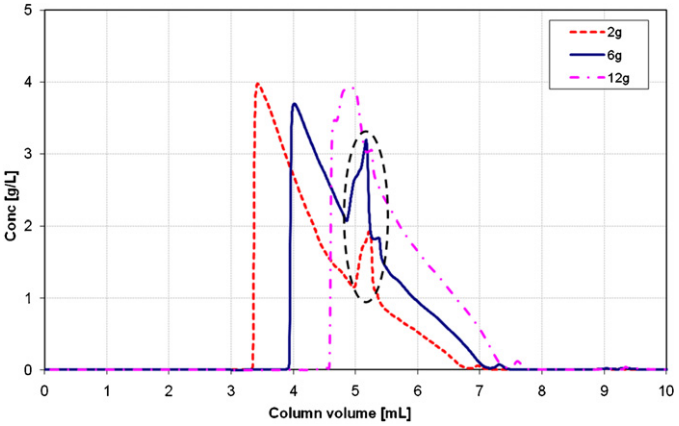

Fig. 13. The profiles were created with increasing concentration of phosphoric acid in the solvents (2, 6, and 12 g H_3PO_4/kg of solvent), whose ACN and water compositions are described in Table 1. Each of these experiments had an additional loading time of 20 min, which was sufficient to ensure the absence of any peak deformations due to the presence of TFA (Ref. Fig. 2).

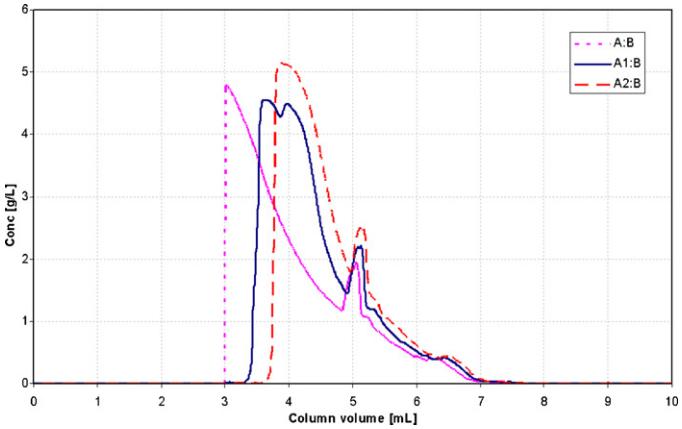

Fig. 14. The profiles were created using the solvents A, A1, A2 and B, with different combinations (A:B, A1:B and A2:B). Full details regarding the composition of these solvents are presented in Table 5.

It can be observed from Fig. 13 that the profile with 2 g H_3PO_4/kg of solvent looks the same as in the earlier experiments, i.e. complete disappearance of the middle peak, leaving behind two prominent peaks. The profile with 6 g H_3PO_4/kg of solvent, on the other hand, shows a significant increase of the last peak and a reduction in the size of the first peak. Finally, with a phosphate concentration of 12 g H_3PO_4/kg of solvent, the last peak further grows in size and the sharp divide between the first and the last peak disappears, making it almost look like a single peak. The inference which can be drawn from this set of experiments is that the last peak is constituted by the Calcitonin molecules ion-paired with the phosphate anions, but with much higher degrees of ion-pairing, compared to the ion-pairing of the molecules constituting the first as well as the middle peak. However, this should be regarded as a qualitative observation as the Calcitonin molecules may still occur in various degrees of ion-pairing even within these two divides.

7. Using secondary equilibria for better separation—a possibility

Another way of looking at the competing ion-pairing effect between the counterions of two different buffer species is to employ them in creating another dimension of separation possibility. In the current study, the unwanted and uncontrolled presence of a counterion (TFA), in addition to the standard buffer of the solvent (di-hydrogen phosphate), had resulted into undesirable consequences. In a more desirable or controlled condition, more than one counterion species can be employed in the solvent to deliberately deform the peaks. We have recently shown [26] how peak deformation, or more specifically peak splitting, in overloaded solvent gradient chromatography can be used advantageously for better separation. In preparative condition a split in the peak of the target component can lead to generating better separation by creating an extended zone of purified component. In a similar way, a selection of multiple buffers can be used as another dimension of separation, along with the variation of modifier concentration. To demonstrate this possibility a set of three experiments was carried out, using the same method (shown in Table 2) but with different solvent compositions, shown in Table 5. In all the experiments an additional loading time of 20 min was employed to avoid any undesirable effect from unaccounted ion-pairing. The resultant profiles are shown in Fig. 14. The profile with solvent combination A:B is the standard one, which was used in all the previous experiments, and mostly has phosphate ion-paired with Calcitonin. The profile with the combination A1:B has TFA in Solvent A in place of the phosphate buffer. The right combination of TFA and phosphate anions (from Solvent B) in the mobile phase, in this case, could create a split in the peak which can be noted from Fig. 14. A higher amount of TFA in the solvent as in the combination A2:B, however, resulted into a stronger TFA ion-pairing effect which resulted again into a single peak. These results are presented just to demonstrate a possibility of creating controlled splits in the peaks, which may be used for better separation in certain cases. A detailed study towards understanding the full potential of using multiple buffers is not within the scope of this paper.

8. Conclusion

The work presented in this paper demonstrates the effect of ion-pairing at high column overloading during the elution of an industrial peptide, Calcitonin, in a reversed-phase system. At high column loading the elution peaks of Calcitonin can get splitted, and more interestingly, the elution profiles can get gradually deformed as a result of increasing column loading times. It was proved, backed by a series of experiments, that competing ion-pairing effect between Calcitonin and the TFA and di-hydrogen phosphate counterions was the reason behind such behaviour.

The insights, developed through this study, into the role of multiple counterions in creating unexpected elution behaviour of peptides (or more generally of the ionogenic molecules), are relevant to other similar situations. For example, in industrial separation of peptides, several successive chromatographic steps are

generally employed, using different buffers at each step. As in most of the operational pH ranges the peptides occur in zwitterionic form, at each step of separation they mostly transform to the salts of the respective buffer counterions. Now this process of transformation, to the salts of the new buffer counterion, depends on the relative equilibrium of the ion-pair associations between the new buffer and the previous one. If the new buffer is weaker than the previous one, e.g. phosphate and TFA or acetate and phosphate, etc., the process of transformation is gradual, depending upon the elution volume of the mobile phase and the concentration of the buffer. So, depending upon the relative equilibrium of ion-pairing, several of these buffer counterions can be carried over to the successive steps, resulting into competing ion-pairing effect. This ultimately may lead to unexpected peak profiles especially at the region of overloading where industry works. One of the main contributions of this work is to draw attention to such phenomena and develop an experimental protocol to systematically understand the true nature behind such effects.

Another important contribution of this work is to highlight the role played by secondary equilibria, e.g. ion-pair effect, in preparative chromatography. The primary separating tool in preparative and industrial chromatography has always been the equilibrium distribution of the solute molecules between the mobile and the stationary phase. The working domain of secondary equilibria has been confined to designing better resolutions in analytical separation. This paper first detects the role played by secondary equilibria in deforming the peak profiles in highly overloaded conditions and then indicates the possibility to utilize this phenomenon in developing another dimension in designing processes for better separation in chromatographic systems.

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Appendix A. Experimental procedure for detecting TFA in eluted peak fractions

Several methods have been discussed in the literature for detecting TFA in collected fractions. The suggested methods range from using techniques involving ion-exchange chromatography [33], head-space gas chromatography [34], electrophoresis [35], and also reversed-phase chromatography [36]. In the current context, the most convenient method found was the method suggested in Ref. [36]. A short description of the experimental setup and the method is provided below.

A.1. Apparatus

Chromatographic experiments were performed using the same Agilent HPLC modular setup HP1100, described before in the main text. The column used was: 150 mm × 4.6 mm I.D. SB300 from Agilent. The mobile phase was formed by mixing solvent A and solvent B, following the gradient method described below. The composition of solvent A was 4.5 mmol/L tetrabutylammonium-hydrogen-sulfate (TBAHS), as the ion-interaction reagent (IIR), dissolved in water–methanol mixture (60:40, v/v), and solvent B was pure methanol of HPLC grade. The spectra of TFA have been obtained on line, using a multiple wavelength UV detector, at the wavelength of 220 nm. The TFA response was calibrated taking the average response of experiments carried out with three different TFA solutions, with known concentrations, using three different injection volumes. All the reagents used were of analytical-reagent grade and were used without further purification. Water was purified using a Millipore water purification system, Methanol was obtained from Fischer Scientific and extra-pure TBAHS was obtained from Acros Organics. All the experiments were performed at flow rate 1 mL/min. The column was stabilized by passing through it a mobile phase for 30 min prior to the main chromatographic run.

Solvent compositions used for the analytical procedure of detecting TFA in elution profile fractions:

0–8 min	100% A + 0% B
8–10 min	From 100% to 10% A (0–90% B)
10–50 min	10% A + 90% B
50–51 min	from 10% to 100% A (90–0% B)
51–60 min	100% A + 0% B

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