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Review

Static vs. Dynamic Electrostatic Repulsion Reversed Phase Liquid Chromatography: Solutions for Pharmaceutical and Biopharmaceutical Basic Compounds

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Abstract: Many efforts have been made to separate basic compounds, which are challenging to resolve in reversed phase liquid chromatography. In this process, they are strongly retained and the peak shape undergoes significant distortion. The principal origin of this has been identified with the non-negligible interaction with residual deprotonated silanols. Consequently, all solutions that efficiently shield silanols are being sought. This review is an upgrade on the use of the electrostatic repulsion reversed phase (ERRP) approach: retention of bases, in protonated form, can be achieved by modulating the charge repulsion caused by the presence of positive charges in the chromatographic system. This study successfully (i) introduced fixed positive charges in the structure of stationary phases, (ii) used cationic and hydrophobic additives in the mobile phase, and (iii) used the ERRP-like approach employed at the preparative level for peptide purification.

Keywords: basic compounds; static ERRP; dynamic ERRP; hydrophobic cation additive

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1. Introduction

An efficient resolution of basic compounds by reversed phase HPLC (RP-HPLC) often requires fine tuning of unconventional mobile phases. In fact, their difficult separation is mainly due to the interaction of basic nitrogen with the residual, non-negligible acid silanols found on the stationary phase. Indeed, considering this, at most operating pHs, basic sites are protonated while silanols are in anionic form, and the strong resulting electrostatic interaction increases retention times of analyte and produces a peak shape distortion, including tailing, fronting, and splitting. In addition, taking into account that a large number of compounds of pharmaceutical interest are bases, and that biopharmaceutical and biological samples (e.g., peptides) also have multibasic sites, the HPLC analysis of basic samples has received particular attention [1–3]. Reversed phase HPLC remains the first choice of work, offering a set of possible solutions. Some of these were proposed by Snyder in 1988, and they are still valid: (i) a careful selection of the stationary phase with a reduced number of acidic sites; (ii) working at low pH of the mobile phase to suppress the anionic form of silanols and (iii) addition of amines (such as triethylamine) in the mobile phase to shield silanols [4].

Many hundreds of RP packed columns are commercially available, and suppliers recommend those dedicated to the analysis of basic molecules. In fact, to be competitive, vendors provide column performance tests, often including additional elution profiles of strong basic probes [5,6].

Regardless of the anchoring chemistry (often not stated in detail) to prepare silica-based stationary phases for RP-HPLC, it should be noted that about 50% of original silanols present on the silica surface remain unreacted after derivatization with organo-silane. Surface coverage of a typical C18 ligand span between 4 and 4.5 micromoles/m^2, while the

surface concentration of silanols on bare silica is about 8.0 micromoles/m^2 [7]. Moreover, the additional "end-capping" procedure, with smaller silylating agents, fails to inactivate the residual silanols [8]. In the last 20 years, with the diffusion of ultra-high-pressure liquid chromatography (UHPLC), a lot of attention has been paid to innovative silica bulks with a reduced number of silanols. High-purity silica and hybrid silica particles were routinely used to develop stationary phases. The reduced amount of metals on the surface and the organic moieties in the siliceous skeleton enhance the peak performance, reducing the unwanted secondary selector/selectand interactions and increasing mechanical resistance. Hybrid silica, in particular, causes a reduction of nearly one-third of the number of silanol sites [9–13]. Unfortunately, in resolving bases, the use of recently developed silica bulks alone does not circumvent the problem. Therefore, working at a low pH (usually pH < 3.5) in the mobile phase represents one of two suitable solutions that allow the use of the standard RP commercially available columns. In these elution conditions, silanols are uncharged and basic solutes protonated. Sample peaks are sharp and their retention times are shortened. Alternately, the addition of amines to the eluent, preferably tertiary ones, can efficiently shield residual silanols, achieving similar effects. In this case, amines are positively charged at acidic pH and can interact electrostatically with the surface anionic silanols, while the hydrophobic moiety of the molecule can associate with the alkyl fragment of the stationary phase. Despite the two approaches having limitations (in some cases very low retention and consequent loss of selectivity are achieved; additives and acid mobile phases are not perfectly well-matched with MS detection), they have gained renewed interest due to the mixed-mode electrostatic repulsive interaction combined with the hydrophobic one. The two approaches, which have, to date, only focused on the suppression of silanols to enhance hydrophobic interaction and peak shape, were recently revised to investigate the effect of electrostatic repulsion. The modulation of the amount of fixed (herein called static) or adsorbed (herein called dynamic) positive charges on the stationary phase can efficiently contribute to retention of bases. Looking to the literature, a similar interaction has been provided in electrostatic repulsion hydrophilic interaction chromatography, named ERLIC. This elution mode involves a combination of the ion-exchange stationary phase with the same charge of analytes and the high organic solvent concentration as classical hydrophilic interaction liquid chromatography (HILIC) conditions [14,15]. In reversed phase liquid chromatography, the expression "electrostatic repulsion reversed phase" (ERRP) was introduced by Gritti in 2013 by studying the absorption behaviors of ionizable compounds in the presence of static positive charges on the C18 stationary phase [16]. This review aims to describe the recent results obtained with ERRP from two different points of view: the "static-ERRP", when a fixed positive charge is part of stationary phase, and oppositely, the "dynamic-ERRP", when the reversed phase is accomplished with the assistance of hydrophobic and UV-transparent cationic additives in the eluent. As the final part, the static ERRP-like preparative approach was also presented due to its importance in the purification of peptides.

2. Static ERRP-HPLC

The designing of alternative stationary phases to resolve basic compounds is an ambitious goal. Being the most intuitive and immediate solution, mixed-mode ion-exchange/reversed phase stationary phases (weak anion exchange/strong anion exchange, WAX/SAX, as well as weak cation exchange/strong cation exchange, WCX/SCX, and RP) were optimized to be used in these fields, exploiting the interaction due to the opposite charges if cation exchange supports were employed or the shielding of silanols using anionic exchange supports. For example, D.V. McCalley and coworkers reported the use of mixed-mode RP/embedded ion-exchange stationary phases. In this work, the elution of a set of bases (e.g., codeine, amphetamine and benzylamine) on different mixed-mode/RP stationary phases has been investigated: Primesep phases containing the strong carboxylate functionality (totally ionized over the normal pH range of RP working conditions) and a Hypersil Duet made of a mixture of 50% discrete C18 silica particles and 50% discrete

sulfonic acid cation-exchange functionalized particles [17]. Different amounts of acetonitrile/phosphate buffer and different pHs were evaluated choosing low pH values (pH < 3) as optimal to obtain favorable retention. Premisep phases give good peak shapes due to the shielding of silanols by embedded ionic groups. Moreover, an additional and significant advantage of these phases lies in the increase in loadability for basic compounds. This behavior is amenable in the purification step of basic compounds.

The concept of electrostatic repulsion was applied with Waters Company's introduction of a new family of charged surface hybrid (CSH) mixed-mode phases for UHPLC applications. They possess a permanent positive surface charge together with the hydrophobic portion, such as octadecyl or phenyl moiety, and were specifically designed for elution of basic compounds [18,19]. The manufacturer declares that a controlled number of basic groups have been tethered on surfaces and, at pHs lower than 3, these groups are quantitatively protonated. The surface of the adsorbent is positively charged, as are the basic compounds that should be eluted for electrostatic repulsion in combination with the adsorption mechanism due to the hydrophobic fragment. Gritti et al. studied the resulting mixed-mode interaction behaviors of these stationary phases in terms of adsorption isotherms [20,21]. The authors found that the overall binding constants (electrostatic and chemical contributions) on each adsorption site decreased with increasing surface concentration of the fixed charges [22]. However, as the pH increases and the surface charge density decreases, electrostatic repulsion decreases, and the cationic sample regains access to the surface of CSH increasing retention. They observed that at a pH larger than 4.0, the electrostatic repulsion is no longer effective, and the severe tailing of peak shapes happens.

An interesting paper published in 2012 [23] compares the elution of a small family of compounds of pharmaceutical interest on UHPLC hybrid bridged ethyl hybrid (BEH) stationary phases (Acquity CSH C18, Acquity CSH Phenyl, BEH C18, BEH C8, BEH Phenyl and BEH Shield RP18). Compounds are bases, acids and neutral and working conditions were optimized at pH = 3 and pH = 10 by gradient elution. On CSH phases, as expected, the bases weakly retained a low pH, while the acids showed the opposite trend. In addition, these stationary phases showed a good chemical stability after repeated switching of pHs (chromatographic traces B and C in Figure 1). The authors, moreover, demonstrate that the CSH phases outperform the others when formic acid and low concentration of buffer (ammonium formate) at pH 3 were employed in the resolution of the target mixture. These elution conditions agreed with the LC–MS applications [23]. Therefore, from a practical point of view, the success of a hybrid CSH stationary phase lies in the elution of weak bases at a pH smaller than 3 and in the potential MS compatibility.

Moving to the other stationary phases bearing positive charge fragments in their structures, embedded quaternary ammonium groups confined to the surface proximity [24–26] and ionic liquid-modified silica were developed by resolving peptides and nucleotides, which are also subject to the effect of residual silanols [27–29]. It is interesting to note that the potential of these supports on elution of bases was evaluated in terms of both electrostatic anionic attraction (for acid compounds) and hydrophobic interactions (mainly for neutral and uncharged bases) in the elution condition where the basic portions of compounds are uncharged. Few exceptions of studies concerning repulsion mechanisms are present, as reported by Abbood et al. They developed a quaternary ammonium-embedded C23 stationary phase that improved the separation of a mixture of peptides positively charged at pH 7.5. By varying pH and ionic strength, the authors clearly demonstrated the involvement of both electrostatic repulsion and attraction with the stationary phase [30]. A structurally comparable stationary phase has been presented by Ihara et al. [31].

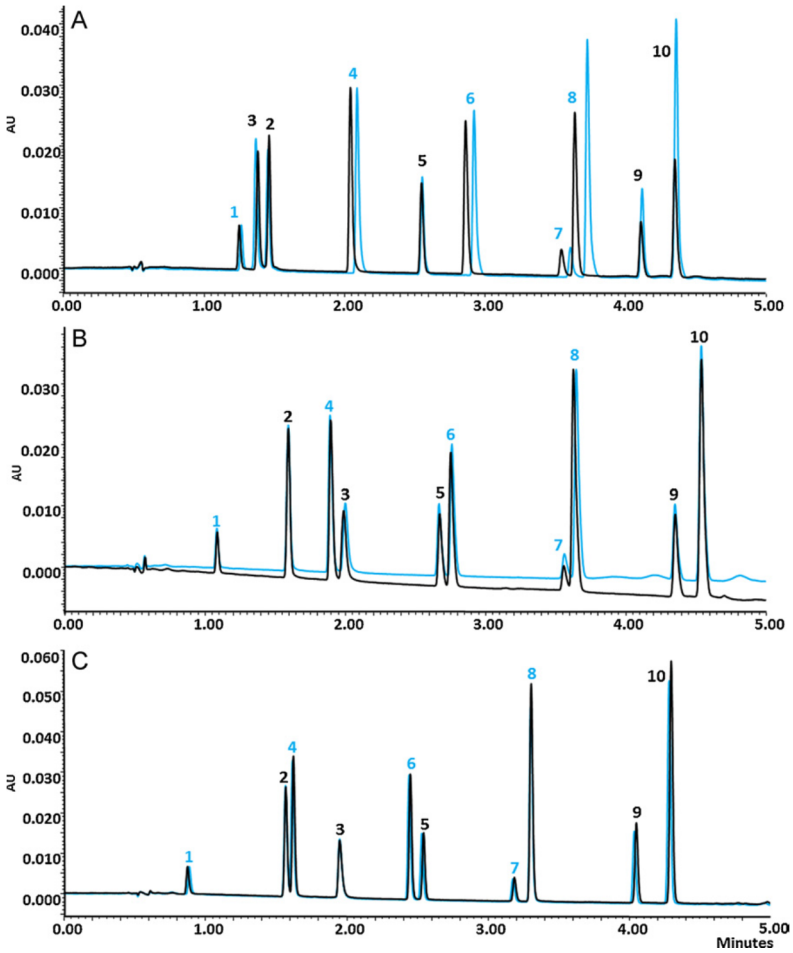

Figure 1. Chemical stability after repeated switches of pHs. Overlaid chromatograms obtained during experiments at pH 3.0 recorded before (black line) and after (blue line) those at pH 10.0. CSH stationary phases (Acquity CSH C18, trace **B** and Acquity CSH Phenyl, trace **C**) compared with Acquity BEH C18 (trace **A**). Reproduced with permission from [23].

They prepared a long-chain octadecyl-modified silica where the cationic moiety located close to the silica network is represented by the ionic liquid (IL) imidazolium. Additionally, in this paper, the direct comparison with the standard C18 stationary phase showed that this support could interact with solutes according to a mixed mechanism with ion–dipole and electrostatic interactions. As an example, separation of bases and nucleosides was achieved by using pure water as the mobile phase (Figure 2). In the last 10 years, other ILs have been immobilized on monoliths or silica particles to be used for HPLC applications. A review of 2015 summarizes the stationary phases developed with different ILs that are able to resolve mixtures of samples due to mixed-mode interactions [32]. However, most applications concern the elution in the reversed phase/anion exchange mode; in addition to the hydrophobic interaction, the electrostatic attraction between the positively charged IL and negatively charged analyte (mainly acids) was explored. Even the most recent works are along the same line [33–36] and the use of these phases in electrostatic repulsion modality to improve the elution of basic compounds remains unexplored.

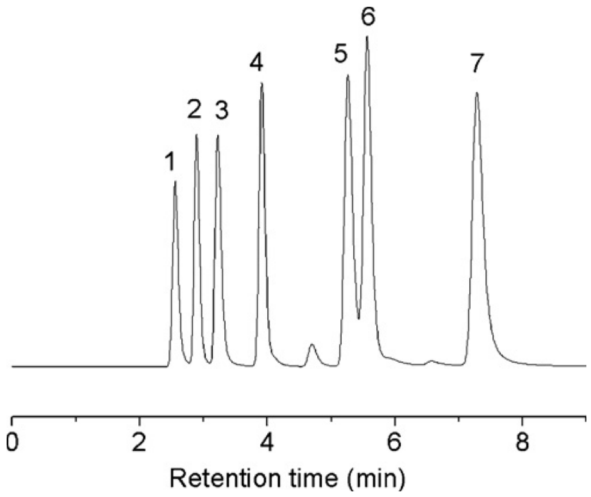

Figure 2. Separation of bases and nucleosides on imidazolinium-embedded C23 stationary phase. Sample mixture: cytidine (1), uracil (2), uridine (3), thymine (4), guanosine (5), xanthosine (6), and adenosine (7). Reproduced with permission from [31].

3. Dynamic ERRP-HPLC

Charged amphiphilic or liophilic additives in the mobile phase have been successfully used to enhance the retention of basic compounds. When these additives are anionic, the modulation of retention could be affected by a combination of mechanisms: ion-pairing or ion-exchange chromatography. In the first case, protonated bases and anionic additives (inorganic as well as organic) provide the formation of neutral adducts and can be resolved by a reversed phase mechanism. In the second case, the anionic additives, especially those bearing a hydrophobic portion, can be adsorbed onto the stationary phase, promoting an electrostatic interaction similarly to a strong or weak anion exchange stationary phase [37–41]. In these elution conditions, the anionic silanols are not shielded by counterions; however, their effect on the peak distortion can be negligible. As an alternate approach, the addition of cationic and hydrophobic additives at low pHs of mobile phase can also help the resolution of bases. Now, the effect of these positively charged additives will provide an efficient suppression of silanols and, at the same time, the retention of protonated bases will be obtained by electrostatic repulsion on the surface of the positively charged stationary phase. The additive is adsorbed on the surface, mimicking, in a dynamic way, a fixed charged stationary phase, as seen in Figure 3. Actually, two classes of additives were employed: hydrophobic ammonium salts and ionic liquids [42–47]. It should be noted that to realize the better electrostatic repulsion effect, the counterion of the additive should not interact with the stationary phase nor with the analyte. For this purpose, highly hydrated anions (such as chloride, bromide and hydroxy anions) were used, having low affinities for the alkyl-chain stationary phase [48].

In a paper published last year, our research group investigated the role of tetrabutylammonium hydroxide and tetrabutylammonium hydrogensulfate in the elution of 11 basic pharmaceutical compounds [42]. Different RP columns (Ascentis Express C18 2.0 µm, Acquity BEH C18 1.7 µm, Titan C18 1.9 µm) for ultra-high-performance chromatography (UHPLC) were evaluated together with the CSH column as reference of the static ERRP approach. All tested UHPLC columns, based on either pure silica or hybrid organic silica, benefited from the use of a cationic additive. Figure 4 shows the elution profiles of some compounds in the individually optimized RP and dynamic ERRP conditions. In the presence of TBA, peaks are very symmetric like to a perfect Gaussian even with even more retained compounds. In addition, high values of efficiency were maintained.

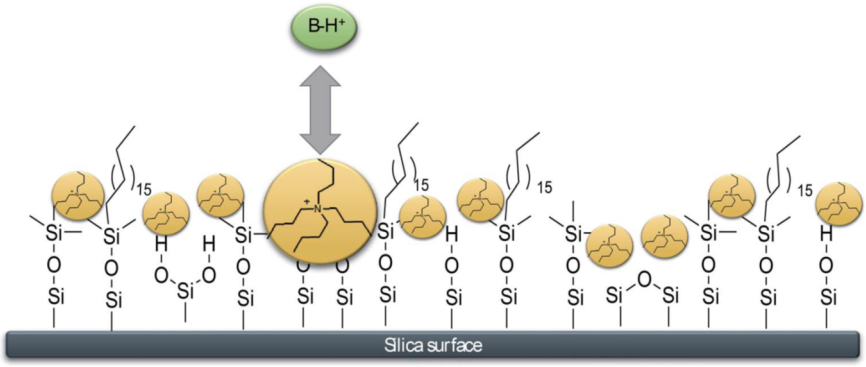

Figure 3. Schematic representation of dynamic ERRP conditions. The hydrophobic positively charged additive (here tetra-butyl ammonium ion, TBA) is adsorbed into the nonpolar stationary phase due to its lipophilic alkyl chains. Reproduced with permission [46].

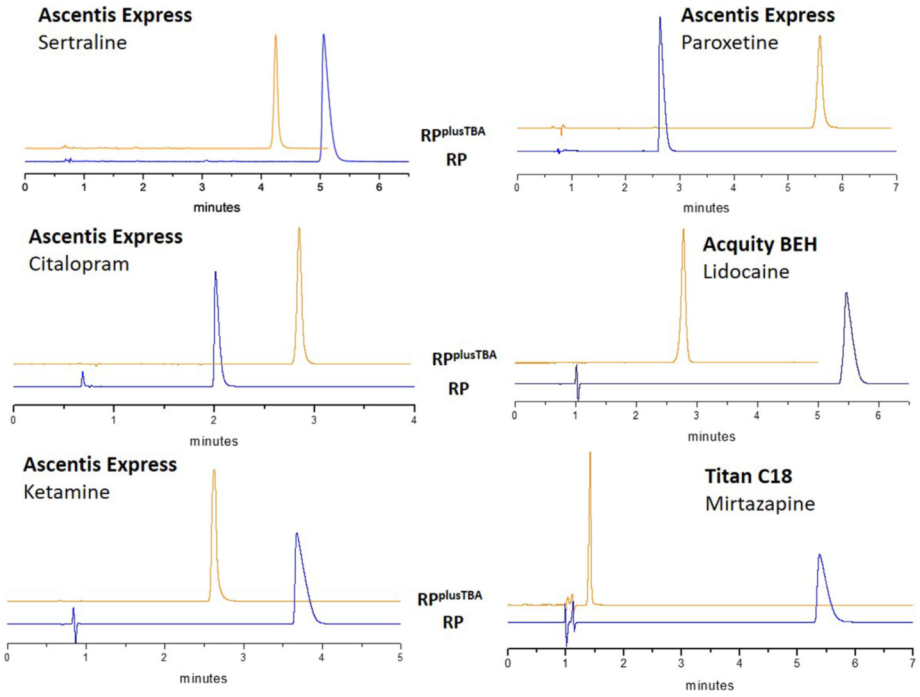

Figure 4. Dynamic ERRP in different commercially available UHPLC columns. Reproduced with permission from [42].

Ruiz-Angel and coauthors have investigated the performance of amines and imidazolinium-based ILs as additives in RP conditions. They demonstrated that silanols were blocked and the ability to shield is related to the size of the cation and its adsorption capacity. In particular, in the elution of the beta-blocker family by using ILs, the authors evaluated the effects of different cations at different concentrations and with counterions of different natures on retention. The retention upon addition of the ILs with the same counterion was compared and a larger effect was observed for the imidazolium cations with a longer side chain (1-hexyl-3-methylimidazolium). From an electrostatic repulsion point of view, the concentration of ILs is responsible for the thickness of the surface charge film—higher density surface charge achieves shorter retention times [43,44].

Many scientists believe that the main disadvantage of using cationic additives (either amine-based or ILs) lies in the reproducibility of elution conditions after long use. The nonreproducibility is due to the strong adsorption of the cations on the stationary phase, which prevents its regeneration. Although in some cases a long re-equilibration time may be necessary, especially in gradient elution, the dynamic ERRP approach can be used in

routine methods such as, for example, quality control of pharmaceutical samples. A recent paper reports the ICH validation of an analytical method to determine the contents of both terbutaline and salbutamol in tablets [45]. 1-Ethyl-3-methylimidazolium bromide ([EMIM][Br]) was selected as an additive whilst using an aqueous mobile phase free of organic cosolvent and a C8 stationary phase. The gain in quality of separation (peak shape and retention time) is clearly shown in Figure 5: B chromatographic trace refers to elution at pH = 3 and in absence of the IL additive, while C trace was recorded using the EMIM additive. Finally, the chromatographic method was compared to the official methods described in the USP 39 and BP 2013 monographs, which were more selective.

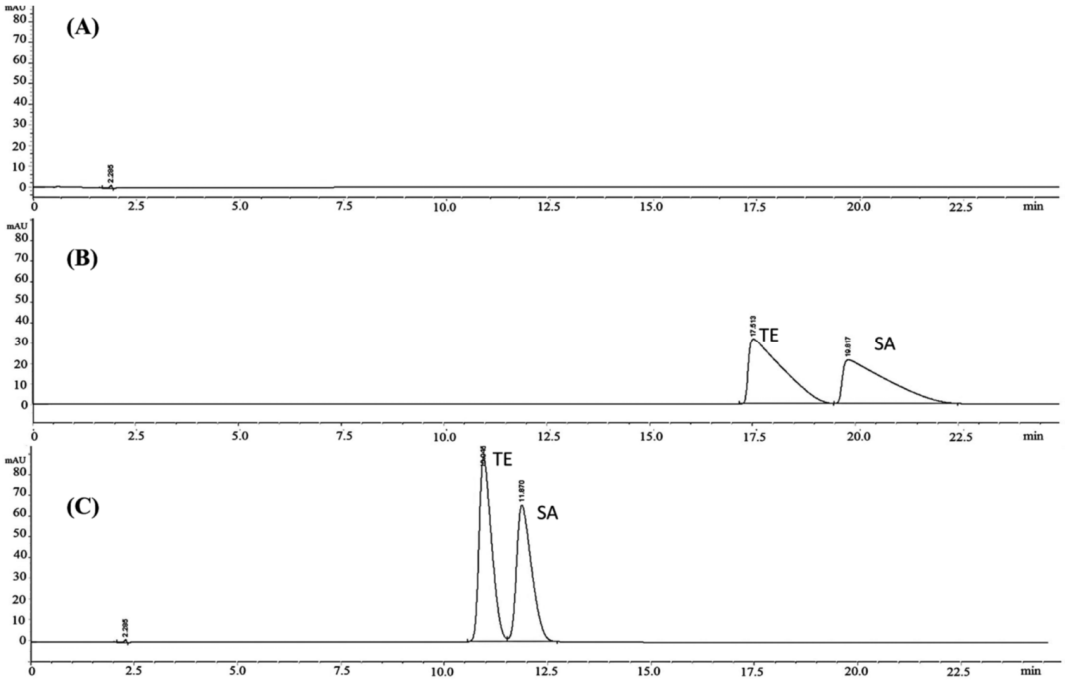

Figure 5. Typical chromatograms of blank (**A**); standard mixtures eluted with mobile phase without the addition of RTIL (**B**) and with [EMIM][Br] (**C**). Reproduced from [45].

As a corollary, we noted that the dynamic ERRP approach begins to be efficiently applied even in the analytical control of peptides and related impurities as an alternative to the ERLIC [46,49]. However, the interest in this field mainly concerns purification, as reported in the following section.

4. Applying Static ERRP Approach at the Preparative Scale for Peptide Separation

The chromatographic strategies described up to now have been mainly applied to the separation of small basic charged molecules. However, peptides and proteins, given their structures (namely, molecules of various lengths made up of charged units based on isoelectric point, pI), are the ideal candidates to be analyzed in ERRP, both in static and dynamic versions. Actually, from an analytical point of view, there are very few studies in the literature compared to the ERRP analysis of small basic molecules. For this reason, in this review, we introduce the more representative data concerning the ERRP approach for peptide separation at the preparative level. Indeed, since the discovery of insulin [50], new production methods for peptide-based drugs have been a great deal of interest. The chemical synthesis of peptides is undoubtedly noteworthy; thanks to this technique, it is possible to build peptides with unnatural amino acids and pseudo-peptide bonds with unexplored therapeutic potential [51,52]. However, this involves many impurities, strictly related to the peptide of interest, that must be eliminated before the drug enters the market. Biopharmaceutical companies spend much time and economic resources on downstream

processes (i.e., product purification and isolation) [53] and, nowadays, the choice technique for purification is preparative RP—LC. It has proven to be an effective and reliable, albeit expensive, technique. However, it may not be enough in the purification of peptides, which, given their complexity and the presence of different charged groups in their backbone, are notoriously difficult to purify.

Several strategies have been developed to enhance the purification of peptides [54], many of which involve the synthesis of stationary phases characterized by bimodal chemical surfaces [55–66]. In fact, by exploiting the concept of orthogonality, namely, using two different separation methods, it is possible to increase the separation power of a chromatographic system, as is evident in multidimensional chromatography [67].

From the perspective of this review, an interesting development is undoubtedly the one made by Morbidelli and coworkers in 2015 [68]. A reversed phase and ion-exchange (IEX) doped mixed-mode stationary phase has been described in this article. Although mixed-mode stationary phases that carry both types of interactions (hydrophobic and electrostatic ones) on a single ligand represent a preferred solution at the analytical level [24–26,30,31], the peculiarity of the one described by Morbidelli and coworkers lies in mixing two separate ligands, each exhibiting an interaction. Indeed, the reversed phase ligands (i.e., C18 chain), which display hydrophobic interactions, and ion-exchange ligands (such as quaternary amines or sulfonate groups), which display electrostatic interactions, are both separately present on the silica surface. The latter consists of the coexistence of the hydrophobic group (van der Walls interactions are always considered attractive) and the IEX group that can be used either as attractive ligands (ligand and analyte have opposite charges) or as repulsive ligands (ligand and analyte have the same charge) [68]. These mixed-mode chromatographic media, also called stochastic mixed mode [69,70], show two main advantages. Since there are two distinct ligands, the former advantage regards the possibility of choosing the ion-exchange ligand density, modulating the intensity of the electrostatic interactions.

Why does this chromatographic medium find its main application in the purification of peptides? It is essential to underline that the peptides produced through chemical synthesis can be characterized by impurities that differ for single amino acids. Moreover, peptides containing a more significant number of charged amino acids are less hydrophobic. On the other hand, more hydrophobic peptides are less charged. This allows both the attractive–attractive and attractive–repulsive approaches to be described. In Figure 6, a peptide pool is shown: peptide P is the peptide of interest, peptide W is more charged (less retained in RP), and peptide S is more hydrophobic (more retained in RP). Working in attractive-attractive mode, ligand and analyte have opposite charges; thus, W has the most significant increase in retention, the retention of P increases slightly, and that of S is unaffected. In contrast, in attractive-repulsive mode, ligand and analyte have the same charge, the retention of W decreases considerably, P has a marginal decrease in retention, and retention of S is unaltered. The co-occurrence of hydrophobic and repulsive interactions is the focus of this review, and we can go so far as to include this kind of stationary phase used in attractive-repulsive mode in the static ERRP category.

Nevertheless, what are the differences and similarities between these stationary phases and those used in the reversed phase? As regards the retention factor, it can be controlled in these chromatographic media through three strategies:

(i) Basing them on stationary phases RP, thus modifying the concentration of organic modifier;
(ii) Working on the concentration of buffer used in the mobile phase, increasing (or decreasing) the concentration of counterions that shield the IEX groups makes it possible to modulate the retention of the charged analytes;
(iii) Modulating the concentration of the IEX group on the surface of the silica particle during the synthesis of the stationary phase. The more groups present, the more the charged molecule is repulsed in attractive-repulsive mode.

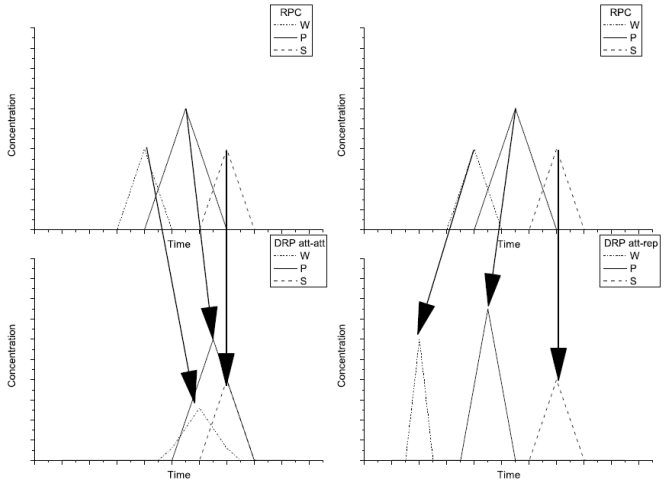

Figure 6. RP phase vs. doped RP phases, as explained by Morbidelli et al. Reproduced with permission from [68]; published by Elsevier, 2015.

Selectivity, defined as the ratio of the retention factors of two analytes, was studied using insulin and desamido-insulin as probes in isocratic elution. The attractive-repulsive mode provided higher selectivity values than those of the attractive-attractive and reversed phase. It is noteworthy that the highest values were obtained by working with a buffer whose pH was lower than the pI of insulin and using a stationary phase whose groups were positively charged at the same pH, which is, in practice, a mode of work that can be compared to that of the static-ERRP described above.

However, the ultimate purpose of these chromatographic media is to be used on a preparative and industrial scale. Therefore, in over-loading working conditions. The attractive-repulsive working model was compared using the more common RP stationary phases used in preparative chromatography (e.g., RP C4 and RP C8). In this case, the stationary phases proposed showed decidedly better values in terms of yield and productivity. Finally, fixed charges on the stationary phase represent an advantage in preparative chromatography over dynamic ERRP. In this way, the mobile phase additive will not present and it must not be eliminated.

5. Conclusions

This review focuses on electrostatic repulsion reversed phase chromatography in the elution of basic compounds. Chromatographic separation of bases requires substantial experience in analytical method development to obtain well-resolved and symmetric peaks. The use of chromatographic systems where the hydrophobic interactions are combined with the electrostatic repulsions stands out as a valid solution. In fact, at acidic pH of the mobile phase, positively charged and hydrophobic molecules (bonded or adsorbed on stationary phase) allow the retention of ionized basic compounds and, at the same time, they shield residual silanols, improving chromatographic performances. Static ERRP is preferred when MS detection is needed or for large-scale purifications. Dynamic ERRP is useful in the optimization of analytical methods that require a selection of additives and concentrations. Despite the fact that the two approaches appear to be niche topics in the literature, we believe that they will play important roles in the future.

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