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Mixed Mode Chromatographic Stationary Phases in Pharmaceutical Peptide Analysis

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***Abstract**: The mixtures of pharmaceutical peptides generally include peptide fragments and similar peptides. Reversed-phase HPLC (RP) is the most frequently utilized method for pharmaceutical peptide analysis. These stationary phases could not sometimes separate complicated peptide mixtures. Free silanols can additionally lead to trailing peaks that reduce peak resolution. These challenges leas to explore the advantages of mixed-mode stationary phase in peptide analysis. Particulate or monolithic mixed-mode stationary phases contains polar and hydrophobic groups. Polar groups are inserted into the alkyl chains "embedded" or used mask the residual silanols "Polar end-capping". This review aimed to discuss the role of particulate and monolithic mixed-mode stationary phases in the analysis of pharmaceutical peptides. Detailed description of these phases were presented. Examples of peptide separation using these phases were shown.*

***Keywords:** Peptides, Analysis, Quality, Chromatographic Mixed-Mode.*

Abbreviations:
HPLC: High-Performance Liquid Chromatography
SEC: Size Exclusion Chromatography
CZE: Capillary Zone Electrophoresis
SAX: RP/Strong Anion Exchange Phases
ECC: Capillary Electrochromatography
EOF: Electroosmotic Flow
SCX: RP/Strong Cation Exchange
CMC: Critical Micelle Concentration
PEDAS: Pentaerythritol Diacrylate Monostearate
SEMA: 2-Sulfoethyl Methacrylate

I. INTRODUCTION

Analyzing pharmaceutical peptides is crucial to ensure their quality and biological effects [1]. The mixtures of pharmaceutical peptides generally include peptide fragments and similar peptides [2]. Multiple techniques can be employed to separate such intricate mixtures, including high-performance liquid chromatography (HPLC) [3], size exclusion chromatography (SEC) [4], capillary zone electrophoresis (CZE) [5], and capillary isoelectric focusing (cIEF). HPLC is often preferred for pharmaceutical peptide evaluation [6]. Among its techniques, reversed-phase HPLC (RP) using C18 stationary phases is the most frequently utilized for peptide analysis [7]. These stationary phases sometimes fail to separate complicated peptide mixtures, including highly hydrophobic and hydrophilic peptides and peptides with similar structures (related peptides) [8]. Additionally, remaining silanols can form electrostatic bonds with peptides, resulting in trailing peaks that degrade peak resolution [9]. These challenges necessitate exploring alternative column types with different selectivity than RP columns [10]. The use of mixed stationary phases: Polar groups are used to mask the residual silanols "Polar end-capping" or polar group insertion into the alkyl chains were also invented to obtain new RP [11]. The role of the mixed chromatographic stationary phases in analyzing peptide mixtures will be detailed in this review.

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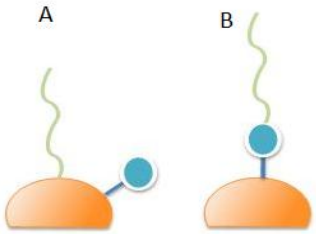
II. DESCRIPTION OF MIXED STATIONARY PHASES

Monolithic or particulate mixed phases contain two domains: polar groups that can be neutral, ionizable, positively or/and negatively charged, and hydrophobic groups that can be aliphatic or aromatic (C4, C8, C12, C18, phenyl) [12]. Depending on the nature of the polar groups present in the mixed stationary phases, they can be classified into: a) mixed stationary phases with non-ionizable neutral polar groups: these phases contain neutral polar groups (amide, hydroxyl, carbamate) that offer hydrophilic interactions (dipole-dipole, hydrogen bonds) with the analytes. These mixed phases can be called RP/HILIC [13]. (b) mixed stationary phases with negatively charged polar groups. These polar groups may have permanent negative charges (sulfonate) (mixed RP/strong cation exchange phases, RP/SCX) or dependent on the pH of the mobile phase (carboxylate) (mixed RP/weak cation exchange phases, RP/WCX) [14]. (c) mixed stationary phases with positively charged polar groups: these polar groups may have permanent positive charges (quaternary ammonium) (mixed RP/strong anion exchange phases, RP/SAX) or dependent on the pH of the mobile phase (amine) (mixed RP/weak anion exchange phases, RP/WAX). (d) mixed stationary phases with both positive and negative charges: these mixed phases are of the RP/zwitterionic type [15].



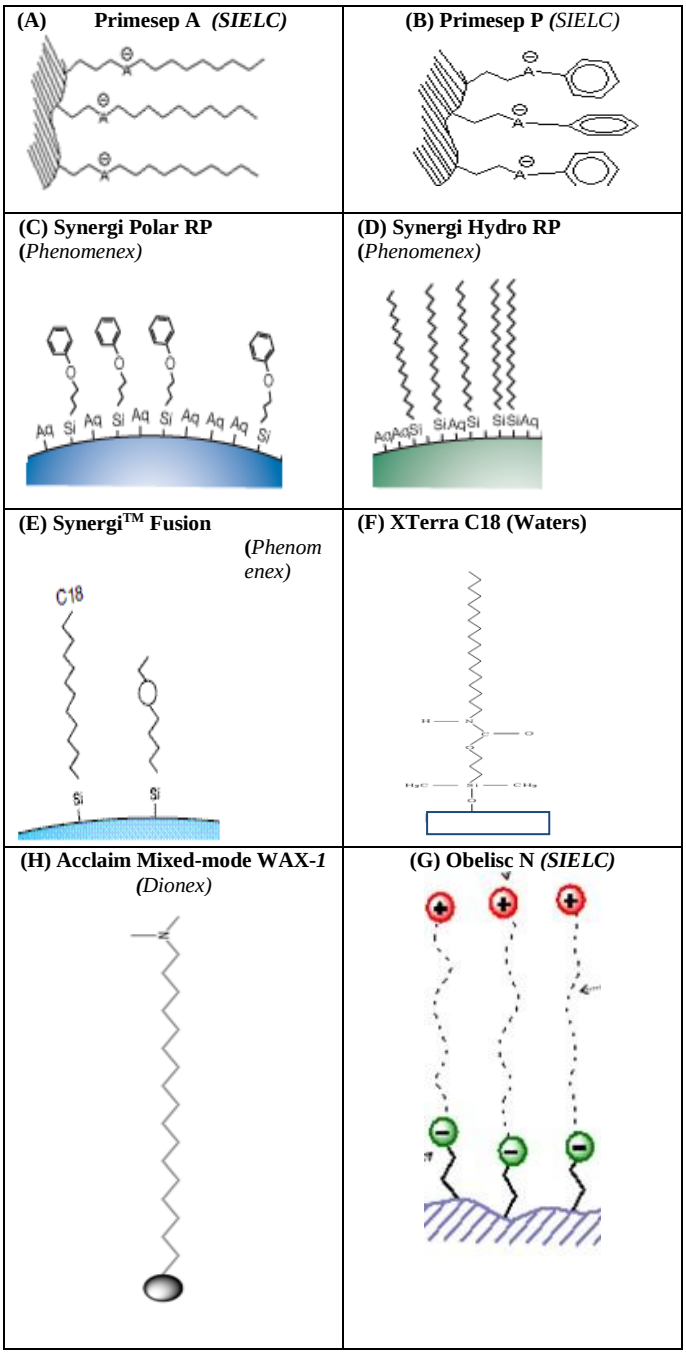
[Fig. 9: Schematic Representation of the Two Types of Mixed Stationary Phases. (A) with a Polar "End-Capping". (B) with a Polar Group Inserted into the Hydrophobic Graft ("Embedded") [15]]

A. Particulate Mixed Stationary Phases

Particulate mixed stationary phases are traditionally divided into two categories with different chromatographic characteristics in HPLC [16].

Phases where polar and apolar groups are grafted side by side on the stationary phase support. They generally have a polar end-capping (Figure 1A), on the "residual" silanols of the silica.

Phases having a polar group inserted into a hydrophobic graft, most often an alkyl chain (Figure 9B). When the particulate phases have this type of grafting, they are called "embedded". These phases are called PEG Polar Embedded Group). Figure 2 and Table 1 show examples of commercially available "embedded" and "end-capped" mixed stationary phases.



[Fig.2: Schematic Representation of Mixed RP-type Stationary Phases]

Table-I: Some Examples of Mixed Stationary Phases Available on the Market

Stationary Phases	Company	Hydrophobic groups	Polar groups
Prism RPN	ThermoScientific	C12	Polar
Xterra C18 RP	Waters	C18	Carbamate
Symmetry Shield C18	Waters	C18	Carbamate
Zorbax Bonus RP	Agilent	C14	Amide
HyPurity Advance	Thermo separation	C8	Amide
COSMOSIL 5-C18-PAQ	Nacalai tesque	C18	Polar End-capping
ProntoSIL 120-5-C8 ace-EPS	Bischoff	C8	Polar
ProntoSIL 200-5-C18 ace-EPS	Bischoff	C18	Polar
ProntoSIL 120-5-C18 Aqplus	Bischoff	C18	Polar end-capping
Aqua C18	Phenomenex	C18	Polar end-capping
YMC ODS-Aq	YMC	C18	Polar end-capping
Synergi Fusion RP	Phenomenex	C18	Polar "embedded"
Synergi Polar-RP	Phenomenex	Phenyl	Ether between silica and phenyl with Polar end-capping
Inertsil ODS-Ep	GL Science	C18	Hydroxyl
Discovery Amide C16	Supelco	C18	Amide
Polaris Amide C18	Varian	C18	Amide
Supelco ABZ+	Supelco		Amide
Obelisc-R	SIELC	-	Positively and negatively charged groups
Obelisc-N		-	Positively and negatively charged groups
Primesep A		-	# negatively charged groups "embedded"
Primesep B		-	Positively charged groups "embedded"
Primesep P		phenyl	Positively charged groups "embedded"

Compared to reversed-phase stationary phases, the position of polar groups relative to apolar groups leads to different chromatographic characteristics. Several studies have compared the characteristics of mixed phases with polar end-capping or an embedded polar group to conventional reversed-phase stationary phases [17].

Polar end-capping stationary phases are the closest to reversed-phase columns in terms of their retention mechanisms [18].

This suggests that polar groups have less effect on column selectivity when grafted directly onto

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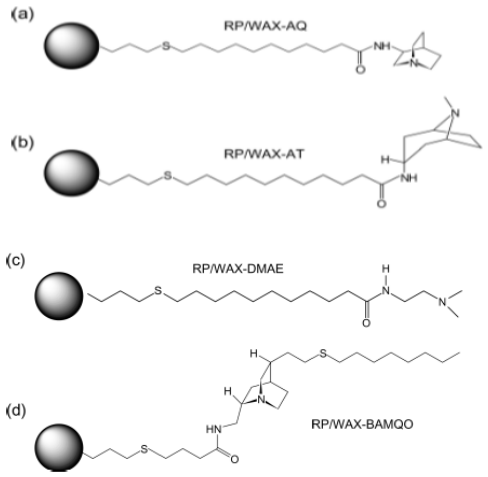
silanols ("polar end-capping") than when inserted into the hydrophobic domain [19]. From various studies, it can be concluded that the presence of embedded polar groups gives the stationary phase several properties compared to a conventional stationary phase: improved wettability, masking of residual silanols, lower hydrophobicity, and different selectivity [20].

If the mixture to be separated contains hydrophilic compounds, a mobile phase with a low organic modifier content in the mobile phase must be used [21]. The presence of the polar group in the stationary phase then makes it possible to work with high percentages of aqueous phase in the mobile phase, while maintaining good solvation of the alkyl chains of the stationary phase [22].

On silica-based supports, the presence of residual silanols leads to electrostatic interactions with basic compounds, which results in peak broadening phenomena [23]. Masking of residual silanols can occur either by electrostatic repulsion of analytes with polar groups inserted in the mixed phases or through interactions between the polar group of the stationary phase and the residual silanols, the silanols of the stationary phase then preferentially forming hydrogen bonds with the polar group incorporated within the chain rather than with the analytes [24]. This improves the efficiency of the peaks of basic compounds [25].

Incorporating the polar groups in the alkyl chain reduces the hydrophobic character of the stationary phase [26]. On the other hand, polar end-capping does not influence the hydrophobic character of the stationary phase [27]. Comparison of the hydrophobic characteristics of three groups of stationary phases, mixed columns with polar end-capping, mixed columns with polar groups embedded within the alkyl chains, and conventional columns were performed [28]. Hydrophobicity was estimated by calculating the butylbenzene retention factor (kBB), using an ACN/20 mM KH_2PO_4- K_2HPO_4 mobile phase, pH 7, (65/35, v/v). No significant difference was found in hydrophobicity between conventional stationary phases (k_{BB} =7.87 ± 2.37) and those with polar end-capping (k_{BB} =7.43 ± 1.80). In contrast, stationary phases with polar groups embedded within the alkyl chains showed lower hydrophobicity (k_{BB} = 4.42 ± 1.57).

Conventional stationary phases with reversed phase polarity mainly exhibit hydrophobic interactions between the analyte and the stationary phase [29]. Mixed phases can exhibit in addition to hydrophilic interactions, hydrogen bonds, dipole-dipole interactions, and/or electrostatic interactions []. This influences separations and improves the retention and selectivity of polar compounds [30]. The number and diversity of commercial stationary phases containing polar groups inserted into an alkyl chain have sharply increased in recent years [31]. In most of these phases, the polar groups are linked to the support surface by a short alkyl chain (often propyl) [32]. These polar groups can be of very varied nature [33]. They can be neutral (uncharged) such as urea [Prism C18 RP], carbamate [Symmetry Shield C8], amide [HyPURITY Advance], a sulfonamide, an alkyl ether (Polaris C18 Ether). Other mixed phases have groups that are permanently positively or negatively charged (quaternary ammonium, sulfonate) or whose charge state depends on the pH (amine, carboxylate) [34]. The position of the polar groups on the alkyl chain can influence the relative importance of the different types of interactions observed with mixed phases [35]. Some mixed particulate stationary phases have polar groups at the end of the alkyl chain. For example, the Acclaim Mixed-mode WAX-1 column [Dionex] has a polar group (tertiary amine) at the end of the C18 alkyl chains (Figure 3H). Other particulate stationary phases have polar groups incorporated into the alkyl chains (close to the silica surface) and polar groups at the end of these chains. This is particularly the case for a series of mixed particulate stationary phases, developed. These phases were obtained by chemical modification of the silica surface (Kromasil) in two steps: activation of the silica surface by grafting thiol groups, then chemical reaction with one of the following reagents: (a) N-undecanoyl-3-aminoquinuclidine, (b) N-(10-undecanoyl)-3-amino tropane, (c) N, N-dimethyl-N9-10-undecanoyl-1,2-ethanediamine, (d) N-butanoyl-(2S,4S,5R)-2-aminomethyl-5-[(2-octylthio)ethyl]-quinuclidine (BAMQO (Figure 3). These chemical modifications of the silica surface made it possible to obtain a set of stationary phases (Figures 3a to 3c) composed of a hydrophobic domain (alkyl chain), embedded polar groups (thioether and amide), and a weakly anion-exchange group (WAX) located at the end of the hydrophobic domain (amine groups). These RP/WAX stationary phases (Figures 11a to 11c) contain terminal tertiary amines. In the RP/WAX phase (d), the anion-exchange site is inserted inside the hydrophobic part, with added additional thioether group (Figure 3).



[Fig.3: Mixed RP/WAX Phases Grafted with: (a) N-undecanoyl-3-amino quinuclidine, (b) N-(10-undecanoyl)-3-amino tropane, (c) N, N-dimethyl-N9-10-undecanoyl-1,2-ethanediamine, (d) N-butanoyl-(2S,4S,5R)-2-aminomethyl-5-[(2-octylthio)ethyl]-quinuclidine (BAMQO)]

Mixed particulate zwitterionic stationary phases have also been developed. They have charged groups near the silica surface and charged groups, of opposite charge, outside the hydrophobic domain. Originally, they were obtained by adsorption of hydrophobic zwitterionic/amphoteric surfactants on a hydrophobic support. The most commonly used surfactants are of the sulfobetaine type [36]. Carboxybetaine surfactants

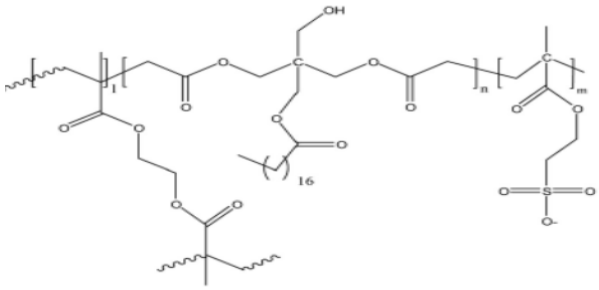
have also been used [37]. Recently, these phases have been obtained by chemical modification of the silica surface [38]. This is the case of the Obelisc N column [SIELC] which has negative charges inside the alkyl chain and positive charges outside the chain (Figure 3G).

B. Monolithic Mixed Stationary Phases

Monolithic mixed stationary phases RP-type mixed phases in the form of monoliths have mainly been developed for capillary electrochromatography (ECC) [39]. In ECC, interest has focused on these phases carrying an ionizable or ionized group because they ensure a fairly intense electroosmotic flow (EOF) and limit the influence of pH on the EOF. These charged groups have also been used as sites of electrostatic interactions with analytes (repulsion, ion exchange). In addition, the apolar groups (classically linear alkyl chains) ensure retention according to a reversed-phase polarity partition mechanism [40]. Most studies of these types of monoliths describe RP/strong cation exchange (SCX) monoliths with permanently negatively charged groups such as sulfonate [41]. Other monoliths have been studied, including those with the following interactions: RP/weak anion exchange (RP/WAX) [42], RP/strong anion exchange (RP/SAX) [43], RP/zwitterion [44], and RP/HILIC [45]. Their performances in micro/nano chromatography are very important. However, they are little used in liquid chromatography [46].

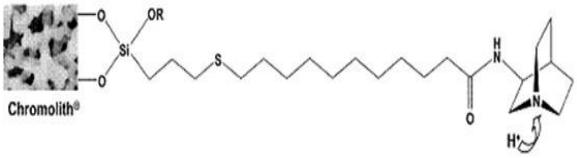
Several methods have been developed to introduce a charged site into the monolith skeleton [47]: copolymerization or post-synthesis modification (grafting, coating). Copolymerization with monomers carrying ionizable or ionized groups is the most direct and easiest method [48]. The monomers providing charges to the monoliths can be the following: positively charged monomers: 2-(acryloyloxy) ethyl trimethylammonium chloride, diethyl aminoethyl (DEAE), 2-(acryloyloxy) ethyl trimethylammonium methyl sulfate (AETA), 3-(acryl amido propyl) trimethylammonium chloride (MAETA), and negatively charged monomers: 2-acryl amido propane sulfonic acid (AMPS) (most commonly used monomer), vinyl sulfonic acid (VSA), sulfoethyl methyl acrylate (SEMA), sulfopropyl methyl acrylate (SPMA), methacrylic acid. The hydrophobicity of the monoliths obtained using this method is mainly provided by monomers with short alkyl chains (hexyl, butyl, propyl) or by crosslinking agents. Indeed, a compromise between the solubility of hydrophobic monomers with long chains and that of relatively polar monomers with charged groups is difficult to find. Monoliths synthesized by copolymerization have long alkyl chains (C12, C17). Mixed monoliths with long alkyl chains in an aqueous medium in a 75 µm internal diameter capillary for micro HPLC and ECC were successfully synthesized [49]. They used a water-soluble detergent monomer: an alkyl ammonium salt (3-allylamino-2-hydroxypropyl dodecyl dimethyl ammonium chloride) where the alkyl groups provide the hydrophobic domain of the phase and the ionic groups provide the electrostatic interaction sites [50]. However, the phase obtained after polymerization of this monomer showed low hydrophobicity. Hydrophobic monomers (e.g., hexylacrylate) were added to the polymerization mixture To increase its hydrophobicity. The alkyl ammonium salt concentration increased to a concentration higher than the critical micelle concentration (CMC). This concentration allowed solubilization of the additional hydrophobic monomers.

Recently, a series of monolithic stationary phases by copolymerization of pentaerythritol diacrylate monostearate (PEDAS), 2-sulfoethyl methacrylate (SEMA), in the presence or absence of ethylene glycol di methacrylate (EDMA) were developed (Figure 4) [51]. These monoliths contain a C17 alkyl chain and a cation exchange site (sulfonate).



[Fig.4: Schematic Representation of a Negatively Charged Monolithic Stationary Phase: poly (PEDAS-co-SEMA-co-EDMA), Pentaerythritol Di acrylate Monostearate (PEDAS), 2-Sulfonyl Methacrylate (SEMA), Ethylene Glycol di Methacrylate (EDMA)]

The surface modification of monoliths can be achieved using the "post-synthesis" method. For example, a new mixed monolithic column of the RP/WAX type for HPLC prepared by chemical modification of the surface of a silica-based monolith was described (Chromolith®). The phase was prepared in two steps [52]. The first step consists of activation of the surface of the silica monolith by reagents containing thiol groups, and then an in situ mobilization of the N-(10-undecenoyl)-3-amino quinuclidine selectors by radical reactions (Figure 5). The chromatographic characteristics of this mixed monolithic phase were compared with a monolithic-C18 column, using acidic and neutral organic compounds, dipeptides, and tripeptides [53]. The results showed that the mixed monolithic phase is more polar than the C18 phase. Moreover, it exhibited complementary selectivity to C18 monolithic columns due to the combination of two mechanisms (reverse phase polarity partitioning and electrostatic interactions) in retention [54].



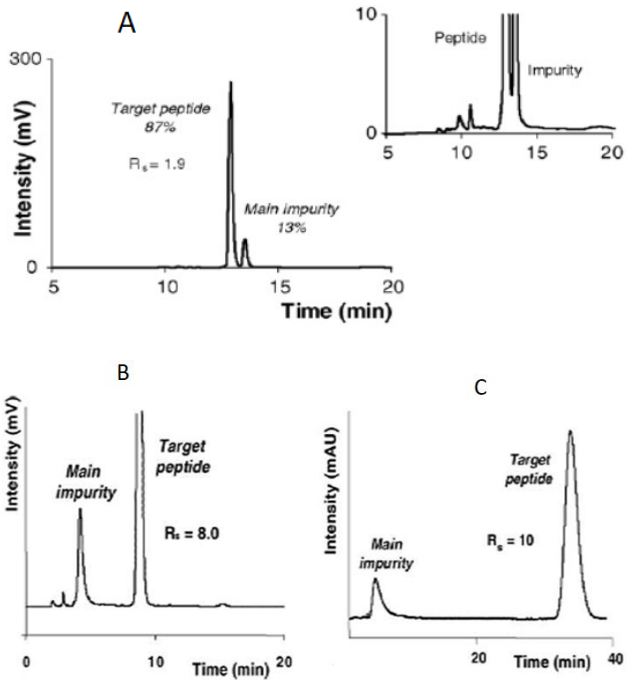
[Fig.5: Schematic Representation of a Monolithic Silica-Based Stationary Phase of the RP/WAX Type]

III. ANALYSIS OF PHARMACEUTICAL PEPTIDES BY MIXED-PHASE CHROMATOGRAPHY

The different properties of mixed stationary phases (masking residual silanols, different interaction mechanisms, improvement of wettability, different selectivity) can be useful in the peptides'

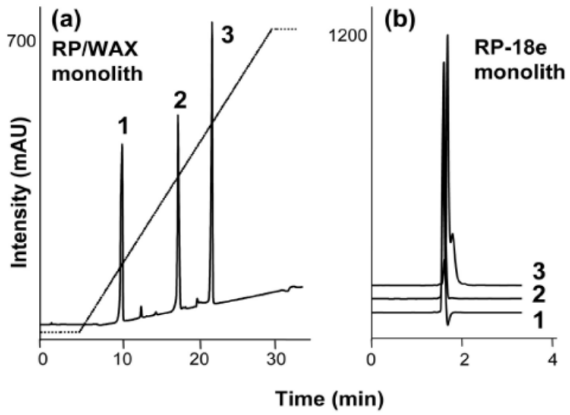
analysis. However, they are still little used in the analysis of complex mixtures of peptides, particularly those resulting from the enzymatic digestion of proteins [55]. For the analysis of complex mixtures of peptides, reversed-phase HPLC remains the most frequently used methodology, although it is difficult to separate all the peptides present under these conditions [56]. As we have previously reported, peptides have varied physicochemical properties; in particular, in the case of basic peptides, interactions can be established with residual silanols, which can cause trailing peaks and can lead to insufficient resolution [57]. This is why the use of certain mixed stationary phases, on the one hand by masking residual silanols, on the other hand by the different interaction mechanisms that they can present, can be an interesting alternative to reversed-phase HPLC. Currently, most of the work on peptide analyses using this type of mixed stationary phases has been carried out in ECC [58]. This work, for the most part, shows that it is possible to separate mixtures composed of about ten peptides by ECC. Recent work reports the interest of these mixed stationary phases in the analysis of peptides by HPLC. Indeed, studies carried out showed the interest of using a new mixed column of the RP/WAX type for the separation of a target peptide (N-acetyl-Ile-Glu-Gly-Arg-p-nitroanilide) from its major impurity [59]. This column, described in Figure 11a, comprises a hydrophobic domain (alkyl chain), embedded polar groups (thioether and amide), as well as a weak anion exchange group (WAX) at the end of the hydrophobic domain (amine) [60]. The presence of these two domains (hydrophilic/hydrophobic) makes it possible to combine electrostatic interactions and reversed-phase mechanisms.

Figure 6A shows the separation of a peptide of interest from its major impurity on a conventional C18 column. We note a low resolution between these two compounds (R_S = 1.9). On an RP/WAX column (Figure 6B), the combination of different mechanisms, such as reverse phase and anion exchange, improves the separation of the peptide of interest from its impurity with a notable increase in resolution (RS = 8) and also with a change in selectivity. The presence of polar groups in the mixed phase also made it possible to carry out the analyses, on this mixed stationary phase, with a purely aqueous mobile phase (100% aqueous phase containing 1% acetic acid) (Figure 6C), which makes it possible to obtain an improvement in the resolution between the peptide of interest and its impurity (RS = 10). The same team also studied the contribution of a new mixed monolithic stationary phase prepared by in situ fixation of the N-(10-undecenoyl)-3-aminoquinuclidine selectors on the surface of a silica monolith (Figure 5). As previously explained, this phase showed that it presented a combination of different interaction mechanisms that could explain the retention of the studied compounds (a reversed-phase polarity partition and electrostatic interactions). This stationary phase was used for the analysis of peptides. The results showed that it is particularly effective for separating hydrophilic and acidic peptides.



[Fig.6: Separation of N-acetyl-Ile-Glu-Gly-Arg-p-nitroanilide from its Major Impurity. (A) C18 Column, Beckman Ultrasphere (ODS) (5 µm, 150 mm x 4. 6 mm). Mobile Phase: A Water + 0.1% TFA/ (B) ACN + 0.1% TFA, Gradient: 5 to 60% B for 20 min, (B) RP/WAX Stationary Phase (5 µm, 250 mm x 4 mm), Mobile Phase; ACN/Water/1 M Formic Acid, pH 4.5 (20/70/10) (pH Adjustment with Ammonia). (C) RP/WAX Stationary Phase (5 µm, 150 mm x 4 mm), Mobile Phase; Aqueous Phase Containing 1% (v/v) Acetic Acid at pH 4.5, (pH Adjustment with Ammonia)]

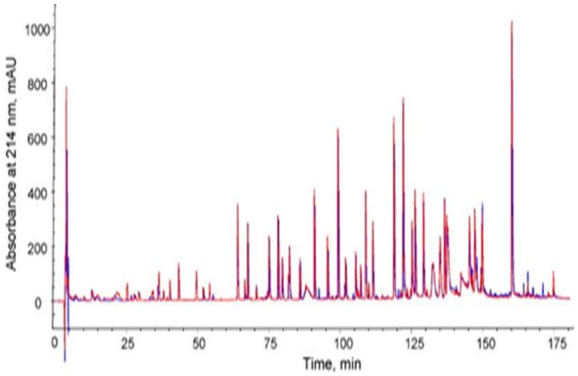
Figure 7 shows the separation of 3 acidic peptides: Gly-Asp (1), Asp-Asp (2), Asp-Asp-Asp (3), obtained on the mixed monolithic column and on a C18 column. These peptides were not separated on the C18 column, however on the mixed monolithic column, these peptides were well separated.



[Fig.7: Separation of Acidic Peptides on Two Monolithic Columns: RP-C18 and Mixed RP/WAX Type. The Mixture Contains 0.3 mg/mL of Gly-Asp (1), Asp-Asp (2), Asp-Asp-Asp (3). Injection Volume: 60 µL. Stationary Phase: RP-C18 Phase (100 * 4.6 mm) and Mixed RP/WAX Type (100 * 4.6

mm). (a) Mobile Phases: (A) water, (B) ACN, (C) 200 mM Phosphoric Acid pH 3.0 Adjusted by ATddition of triethylamine. Gradient: 0-5 min: 10% B v/v, 5-30 min: 0 to 60% C (0-120 mM), Flow Rate: 1mL/min, (b) Mobile Phases: (A) water + 0.1% TFA, (B) ACN + 0.1% TFA. Gradient: 0-5 min: 3% B v/v, 5-35 min: 3 to 90% B, flow rate: 1mL/min. Temperature: 25°C. Detection: 214nm]

Mixed stationary phases have also been used for peptide mapping, for example, for peptide mapping of the monoclonal antibody (immunoglobulin IgG). This (Figure 8) was carried out under experimental conditions compatible with mass spectrometry on a mixed Polaris C18 Ether column (Varian, Torrance, CA) containing ether groups placed between a C18 alkyl chain and the silica surface.



[Fig.8: Peptide Mapping of IgG after Tryptic Digestion. Column: Polaris C18 Ether (250 mm×2.0 mm, Varian, Torrance, CA). Gradient: 0% to 65% B in 195 min. Solvent A: water + 0.1% TFA, Solvent B: 10% water, 90% Acetonitrile + 0.085% TFA. T = 50 °C]

IV. CONCLUSION

Analyzing pharmaceutical peptides is crucial to ensure their quality and biological effects. HPLC is the most widely used method for analyzing peptides. Reversed-phase is the most used stationary phase in the separation of peptides. Mixed-mode stationary phases present an alternative of revered-phases for analyzing peptides. Mixed-mode stationary phases contain polar and apolar groups. Thes phases could be particulate and monolithic. In this review, description of particulate and monolithic mixed-mode stationary phases were detailed. Examples of the contribution of these phases in peptide analysis were given.

DECLARATION STATEMENT

I must verify the accuracy of the following information as the article's author.

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- **Ethical Approval and Consent to Participate:** The content of this article does not necessitate ethical approval or consent to participate with supporting documentation.
- **Data Access Statement and Material Availability:** The adequate resources of this article are publicly accessible.
- **Authors Contributions:** The authorship of this article is contributed solely.

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