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Overview of the Role of Chromatographic Modes in Pharmaceutical Peptide Analysis

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***Abstract**: Peptides represent promising candidates for medical treatments. They can be designed for high specificity, which minimizes the adverse effects. Ensuring their purity, identity, and quality is crucial to their therapeutic efficacity. Peptides exhibit different physical and chemical properties (such as charge, pI, hydrophobic nature, size, etc.). These characteristics complicate the separation of a mixture of peptides. Various analytical techniques were used for peptide purification, peptide mapping, or peptide identification. HPLC continues to be the preferred method for analyzing peptides. Different HPLC modes were applied to separate peptides from their impurities and related substances. Among these modes, ion exchange, reversed-phase, normal-phase, and HILIC were performed to achieve peptide separation. This review will discuss chromatographic techniques and their role in peptide analysis, including analyzing peptide mixtures, creating peptide mapping, or isolating the peptide of interest from associated compounds.*

***Keywords:** Peptides, Analysis, Quality, Chromatographic Modes.*

I. INTRODUCTION

Peptides have varied physicochemical characteristics (charge, pI, hydrophobicity, size, etc.) [1]. This diversity of characteristics makes separating a complex mixture of peptides (peptide fragments, related peptides, etc.) difficult [2]. Indeed, several methods can be used to separate this complex mixture: High-performance liquid chromatography (HPLC) [3], Size exclusion chromatography (SEC) [4], Capillary zone electrophoresis (CZE) [5], and capillary isoelectric focusing (cIEF) [6].

HPLC remains the method of choice for peptide analysis. Reversed-phase HPLC (RP) with octadecyl stationary phases (C18) is the most widely used mode for peptide analysis [7]. It offers good selectivity and high efficiency [8]. Unfortunately, sometimes these phases do not allow complete separation of complex mixtures of peptides, containing both very hydrophobic and very hydrophilic peptides and peptides with a similar structure (related peptides) [9]. In addition, residual silanols, by establishing electrostatic bonds with peptides, lead to the formation of trailing peaks, which alters the resolution between peaks [10]. The poor resolution will cause problems in determining the purity of a therapeutic peptide for example [11].

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Electrostatic interactions distort the peaks, which can lead to the co-elution of certain impurities with the peptide of interest [12]. These limitations require the search for other types of columns to present an alternative selectivity to RP columns [13]. These columns are based on different interaction mechanisms: ion exchange, hydrophilic interactions, or even the coupling of several interaction mechanisms [14].

In this review, we will present the different chromatographic methods and their interest in the analysis of peptides, whether for the study of peptide mixtures, the establishment of peptide mappings, or the separation of the peptide of interest from its related substances.

II. ION EXCHANGE CHROMATOGRAPHY

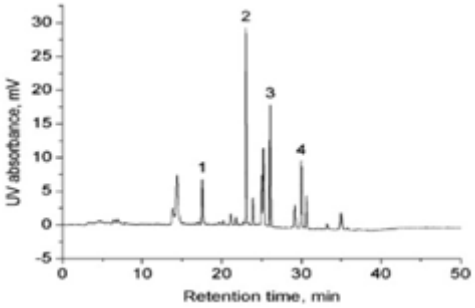
Ion exchange chromatography is a liquid phase chromatography where the stationary phase is constituted either by an insoluble solid (resins, silica, or glass beads) on which ionizable or ionized functional groups are grafted or by monoliths obtained using monomers carrying ionizable or ionized functional groups [15]. The separation of compounds is based on an ion exchange process [16]. Theoretically, the separation phenomenon based on this exchange property can only be applied to compounds with ionizable or ionized groups, it is therefore applicable for the separation of peptides or proteins for purification, peptide mapping, or quality control [17].

There are two types of ion exchange phases. Cation exchange phases (CEX) will be chosen to analyze cationic species [18]. This stationary phase contains sites (negatively charged) capable of exchanging cations [19]. These cation exchange sites can be strong, as the case for phases grafted with sulfonate anions, or weak, as the case for phases grafted with carboxyl groups [20]. The second type is called the anion exchange phase (AEX) [21]. Anionic column will be chosen for the analysis of anions [22]. The anion exchange sites (positively charged) can be strong, as the case for phases grafted with quaternary ammonium groups, or weak, as the case for phases grafted with tertiary amine groups [23].

The most commonly used phases in ion exchange chromatography are porous particles based on silica, polystyrene/divinylbenzene, or polysaccharides on which grafted charged functional groups. Studies conducted by Mant and Hodges in 1985 [24] showed that highly cation exchange columns (Synchropak S300) can separate complex mixtures of basic peptides consisting of 10 peptides, with a net charge (+2 to +10 at pH 3 and pH 6) and sizes ranging from 9 to 36 amino acids. Other peptide separations based on the ion exchange mechanism have been successfully applied to basic peptides from the tryptic digestion of casein using an S-HyperD column, containing sulfonate groups [25].

In the European Pharmacopoeia, ion exchange chromatography was used for the research and determination of the content of the related substances of aprotinin and salmon calcitonin [Monograph: Aprotinin, 01/2009: 0580, Monograph: Salmon calcitonin, 01/2008: 0471]. In addition, a strong anion exchange column (Pharmacia Mono Q HR 5/5) was used.

Monolithic columns have been gaining popularity in recent years. Firstly, these phases were developed in micro or nano HPLC to avoid the step of filling the capillary with stationary phase particles and especially to circumvent the difficulties related to frits. Organic ion-exchange monolithic stationary phases represent a significant type of monolith [26]. In the analysis of peptides or proteins, several studies have demonstrated the interest brought by these stationary phases in capillary HPLC [27]. These studies have mostly been carried out on monoliths presenting sulfonate groups as strong cation exchange sites. Gu et al. developed a strong cation exchange monolith by photopolymerizing 2-acrylamido-2-methylpropanesulfonic acid (AMPS) and polyethylene glycol diacrylate PEGDA monomers [12]. This phase showed a high resolving capacity to separate peptide fragments produced after tryptic digestion of β-casein. In addition, this monolith separated four synthetic peptides from their degradation products. Figure 1 shows the separation of four peptides from their degradation products. Another strong cation exchange monolithic stationary phase prepared by photopolymerization was developed based on sulfopropyl methyl acrylate and ethylene glycol diacrylate (SPMA-co-EDMA) monomers by Chen *et al.* [13]. This column allowed the separation of complex mixtures of synthetic and natural peptides by capillary HPLC.



[Fig. 1: Analysis of a Mixture of Four Synthetic Peptides, by Capillary Liquid Chromatography [Gu 2006]. Column: A Monolith of AMPS and Polyethylene Glycol Diacrylate (PEGDA), 16,5 cm ×75 um. Buffer A: NaH_2PO_4 5 mM (pH 2,7) and Buffer B: Buffer A + NaCl 0,5 M, the two Buffers Contain 40% (v/v) ACN. Gradient: 0-2 min: 1% B, linear gradient AB (5% B/min), 1-100% B; 10 min and then 100% B: 40 min. Flow rate: 100 nL/min. Detection: 214 nm. Peptides: [1] Ac-Gly-Gly-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-Lys-amide, [2]Ac-Lys-Tyr-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-Lys-amide, [3] Ac-Gly-Gly-Ala-Leu-Lys-Ala-Leu-Lys-Gly-Leu-Lys-amide, [4] Ac-Lys-Tyr-Ala-Leu-Lys-Ala-Leu-Lys-Gly-Leu-Lys-amide]

Other monolithic ion exchange columns have been prepared by post-synthesis modification. This is the case, for example, of the photografting of AMPS monomers onto monoliths based on polyethylene glycol methacrylate and glycidyl methacrylate monomers [15]. This photografting has made it possible to obtain strong cation exchange columns (sulfonate groups of AMPS) allowing the separation of peptide fragments resulting from the tryptic digestion of cytochrome c. Recently, Chen *et al.* synthesized cation exchange monoliths based on monomers of 2-hydroxyethyl methacrylate phosphoric acid (PAHEMA) and bis [2-(methacryloyloxy) ethyl] phosphate (BMEP), polyethylene glycol diacrylate (PEGDA), polyethylene glycol acrylate (PEGA) [14]. The cation exchange sites in these monolithic phases contain phosphate groups instead of sulfonate groups. This phase separated a mixture of synthetic and natural peptides (Val-Tyr-Val, Gly-Tyr, methionine enkephalin, leucine enkephalin, angiotensin I). Ion exchange stationary phases allow the separation of peptides or proteins according to their charges, which gives them a selectivity complementary to reversed phases (RP). This is why they have been used in two-dimensional or multidimensional chromatographic systems for an optimal separation of macromolecules such as proteins [28].

III. REVERSED-PHASE POLARITY CHROMATOGRAPHY

Reversed-phase liquid chromatography (RP-HPLC) is the most widely used separation method [29]. Briefly, reversed-phase chromatography consists of a polar mobile phase, usually water or buffer to which an organic solvent such as methanol, acetonitrile, or tetrahydrofuran is added, and an apolar stationary phase, for example, an alkyl chain of variable length, grafted to a pure or hybrid silica support [30]. Polar compounds have a greater affinity for the mobile phase and are eluted quickly [31]. Conversely, less polar compounds have a greater affinity for the stationary phase and are eluted later [32].

Stationary phases with reversed-phase polarity are often based on silica on which hydrophobic groups (for example C8, C12, or C18 alkyl chains) are grafted to silanol functions on the surface of the support [33].

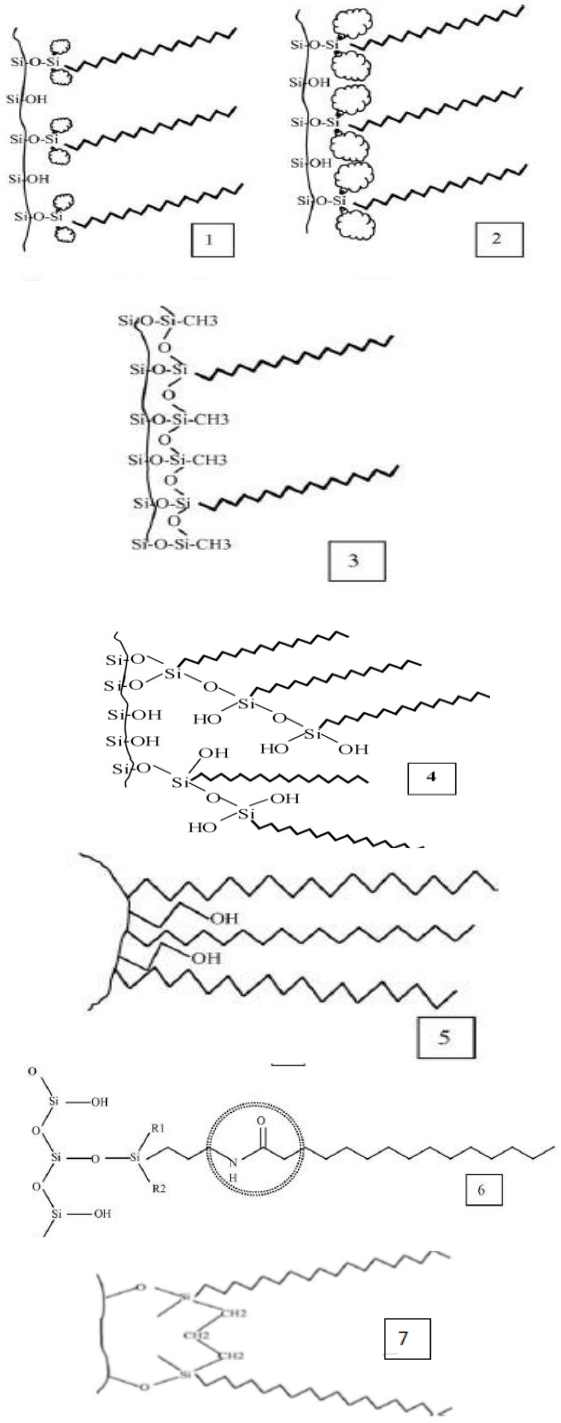
Their performance depends on many parameters. Two main properties are important because they determine the choice of columns [34]. Among them, the hydrophobicity and the residual silanols (SiOH) activity are determinants [35].

The importance of the activity of the residual silanols depends on the number of accessible silanols and their relative acidic character (i.e. their ionization depends on the pH of the mobile phase) which relies on the method of obtaining and the purity of the silica of the support [36]. It is also important to consider the involved type of silanols: isolated, geminal, or/and vicinal silanols because this also influences their “acidic character” [37]. The presence of accessible silanols on the surface of the grafted silica will promote not only the retention of polar compounds, through hydrogen bonds, but also that of compounds possessing basic nitrogen, by establishing electrostatic bonds [38]. These parasitic hydrophilic interactions often lead to peak tailing and poor resolution, especially for strong basic molecules. To overcome this problem, new stationary phases have been developed [39].

Figure 2 shows examples of the chemical structure of different reversed-phase polarity stationary phases

with alkyl grafts.

These new phases can be obtained by high-density grafting of the silica, to reduce as much as possible the number of accessible residual silanols [40]. In this high-density grafting method, the alkyl chains are very close preventing access to residual silanols (Figure 2-1).



[Fig. 2: Schematic Representation of Stationary Phases consisting of Alkyl-grafted Silica (1) Monofunctional Phases, (2) Monofunctional Phases with Steric Protection, (3) Horizontal Polymerization, (4) Vertical Polymerization, (5) Hydrophilic End-capping, (6) Insertion of a Polar Amide Group in the alkyl chain, Near the Grafting Site ("Embedded" Phase), and (7) Bidentate Phase]

Another method to obtain these new reversed phases was to add bulky grafts (such as tert-butyl groups) near the grafting site, resulting in the masking of the silanols by steric hindrance. This grafting is carried by a monochlorosilane [41] (Figure 2-2).

"Polymeric" grafted silicas (using a di- or trichlorosilane) were also used to obtain new RP. There are two types of polymeric phases: phases exhibiting horizontal polymerization (Figure 2-3) and phases exhibiting vertical polymerization (Figure 2-4).

Masking or "end-capping" of the "residual" silanol groups by short alkyl chains such as trimethylsilyl was also applied to obtain new RP [42]. The use of mixed stationary phases: polar groups are used to mask the residual silanols "polar end-capping" (figure 2-5) or inserted into the alkyl chains (figure 2-6) were also invented to obtain new RP.

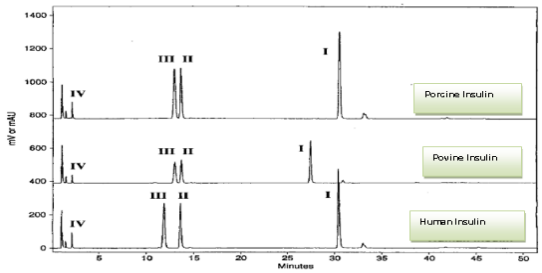
Another type of stationary phase is represented by the grafting of the alkyl chain using bidentate groups "bidentate grafts" (figure 2-7). This concept was developed by Kirkland *et al.* [43]. The experiments carried out showed that the analyses were reproducible, the column obtained is efficient and has great stability at extreme pH (less than 2 or greater than 7).

In addition to particulate stationary phases, silica-based monolithic phases are also available for reversed-phase separations [44]. They are often prepared by the sol-gel process and then chemically functionalized by protocols equivalent to those already described for silica-based particulate phases [45]. Indeed, publications on reversed-phase silica-based monoliths mostly describe phases chemically modified by octadecyl dimethyl-N, and N-diethylaminosilane (ODS-DEA) [46]. Silica-based monolithic phases can also be treated after their functionalization to mask residual silanols by "end-capping" [47]. Reversed-phase stationary phases can also be completely organic. In this case, they are made of polymers based on polystyrene, polymethylacrylate, or polyacrylamide [48]. The advantage of these phases compared to monolithic stationary phases based on silica is the possibility of working at basic pH.

Reversed-phase HPLC is a method that allows reproducible, selective, and sensitive analyses to be obtained. Therefore, reversed-phase HPLC is the most widely used method for the separation of complex mixtures of peptides, whatever the objective: purification, isolation, purity determination, and peptide mapping [49]. Reversed-phase HPLC allows the separation of peptides or proteins with small modifications in their chemical structure [50]. Therefore, Reversed-phases are often used for the purification of peptides or proteins or the control of their purity [51].

For example, goserelin is a synthetic, anticancer hormone, the analogue of the gonadotropin-releasing hormone GnRH. It is synthesized by solid-phase chemical synthesis. This method produces a complex mixture containing goserelin, its impurities, and related peptides. The separation and characterization of these peptides were carried out in RP-HPLC, with a C8 column, under experimental conditions compatible with mass spectrometry (mobile phase: ACN/water + 0.1% TFA, 25/75, v/v) (Figure 5) [27].

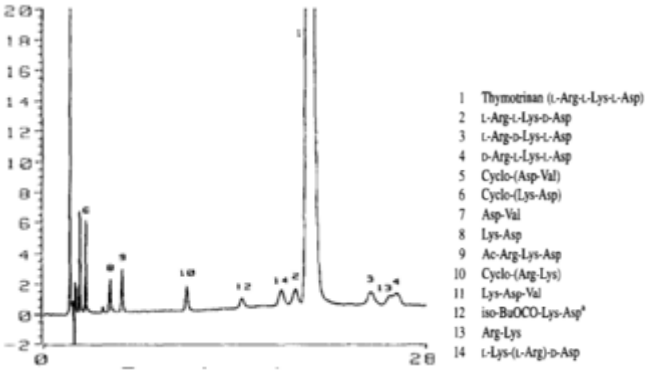
In the European Pharmacopoeia, the purity control of most therapeutic peptides with a monograph is carried out by reversed-phase HPLC. This is the case for protirelin, buserelin, felypressin, goserelin, glucagon, leuprorelin, and desmopressin. Similarly, the establishment of a peptide mapping of a protein, to carry out its quality control, is often carried out by reversed-phase HPLC [1]. The same is true for other therapeutic peptides or proteins such as insulin, interferon-α 2, salmon calcitonin, human glucagon, and somatropin. For example, reversed-phase HPLC is recommended for monitoring insulin. Insulin is a hormone consisting of 2 polypeptide chains, an A chain of 21 amino acids and a B chain of 30 amino acids, linked together by two disulfide bridges and an intrachain disulfide bridge in the A chain. It is used in the treatment of diabetes. It can be of human origin (human insulin) or animal origin (porcine or bovine insulin). Bovine insulin differs from human insulin by 3 amino acids, while porcine insulin differs from human insulin by one amino acid. In the European Pharmacopoeia, RP-HPLC (Spherisorb ODS) is used to establish the peptide mapping of insulin (Figure 3). It is noted that the retention time of fragment I is identical for porcine insulin and human insulin, those of fragments II and IV are identical for all insulins, and that of fragment III is identical for bovine and porcine insulins.



[Fig. 3: Peptide Mapping Chromatograms of three Types of Insulin: Human, Porcine, and Bovine [Ph. Eur. 6th edition]. Mobile phase A: a Mixture of 100 mL ACN, 200 mL Sulfate Buffer Solution pH 2.0, and 700mL water. Mobile phase B: a mixture of 200 mL sulfate buffer solution pH 2.0, 400 mL ACN, and 400 mL water. Gradient: 0 to 60 min; 90 to 30% phase A, 60 to 65 min: 30 to 0% phase A, 65 to 70 min: 0% A. Temperature: 40 °C. λ: 214 nm. Injection Volume: 50 µL. Column: Spherisorb ODS (end-capped), 100 x4.6 mm, dp: 3 µm [Monograph, European Pharmacopoeia, 01/2008: 0838)]

Reversed-phase HPLC does not always allow the separation of peptides with similar structures. Several strategies can be used to improve the separation, such as the addition of ion-pairing reagents to the mobile phase or the use of multidimensional systems [52].

The first approach consists of adding reagents to the mobile phase to increase the retention of peptides. Ion-pairing reagents can be anionic or cationic. Anionic reagents are the most commonly used in peptide analysis. Indeed, trifluoroacetic acid (TFA) is the most commonly used ion-pairing reagent for peptide and protein analysis [52]. TFA homologs have been used for the optimization of peptide separation such as pentafluoro propionic acid (PFPA) and heptafluorobutyric acid (HFBA) [53]. Other anionic ion-pairing reagents can be used in peptide analysis: alkyl sulfonates (hexane, heptane sulfonate). All the ion-pairing reagents used allow, on the one hand, the suppression of interactions of basic peptides with residual silanols and on the other hand the increase in the retention of hydrophilic peptides through the formation of ion pairs [54], which allows the improvement of the selectivity and therefore the optimization of the separation of a complex mixture of peptides in reversed-phase HPLC. Shibue *et al.* studied the effects of the nature and concentration of anionic ion-pairing reagents (TFA, PFPA, and HFBA) on the retention and separation of synthetic peptides in reversed-phase HPLC [30]. They observed a difference in peptide selectivity depending on the nature and concentration of the ion-pairing reagent. Indeed, the separation of a mixture of 12 synthetic peptides is improved by increasing the concentration and degree of hydrophobicity of these reagents (TFA < TFPA < HFBA). In addition, by increasing the concentration of these reagents, the width of the peaks decreased, which improved the resolution between the peptides [55]. This effect is marked when the peptide has a significant net positive charge. Thus, the addition of ion-pairing reagents improves the separation of a peptide of interest from its related substances. Another example concerns the separation of thymotrinan from its related peptides which was carried out by ion-pairing chromatography. Thymotrinane (L-Arg-L-Lys-L-Asp) is a peptide fragment of thymopoietin (49 amino acids). It can stimulate the immune system. The separation of thymotrinan from its related peptides was carried out on a C18 column by adding hexane-sulfonate to the mobile phase (Figure 4) [29].



[Fig. 4: Separation of Thymotrinan from its related Peptides (each at 1%) using Hexane-Sulfonate as an Ion-Pairing Agent. Column: Ultrasphere C18 IP, C-18, 5 µm, 250 x 4.6 mm (Beckman), Mobile Phase: 30 mM Sodium Hexane-Sulfonate and 20 mM sodium phosphate buffer in water/MeOH, 70:30 v/v, pH 3.0. Temperature: 40 °C [29]]

However, reversed-phase HPLC does not always allow a complete separation of a complex mixture of peptides, even when using ion-pairing reagents. Therefore, interest has turned to other strategies: multidimensional systems and mixed stationary phases.

Multidimensional systems are based on combining two or more retention properties of chromatographic columns to obtain an optimal resolution, compared to the one-dimensional system [56]. In these systems, stationary phases with reversed-phase polarity often represent the second dimension, thanks to

their resolving capacity. The most widely used two-dimensional approach for peptide analysis consists of using strong cation exchange stationary phases (separation is based on the difference in peptide charge and size) in the first dimension and particulate reversed-phases (separation is based on the difference in peptide hydrophobicity) in the second dimension [57]. This approach is widely used in proteomics [58]. Since monolithic stationary phases provide efficient and rapid separations, studies have evaluated the contribution of these stationary phases in the analysis of a complex mixture of peptides in multidimensional systems. Kimura et al. used a cation exchange column (in the first dimension) and a monolithic C18 capillary column based on silica (in the second dimension) for the separation of a mixture of peptides resulting from the tryptic digestion of bovine serum albumin [18]. They found that the peak capacity was, under these conditions, 700 in 40 min, thanks to the use of the monolithic capillary columns in the second dimension. Although two-dimensional systems based on different types of stationary phases offer orthogonal selectivity, this strategy can lead to disadvantages: a small peak capacity [3], the incompatibility of the mobile phases either between the two stationary phases [4], or mass spectrometry, as a detection system [1]. To address these drawbacks, studies have been carried out on two-dimensional approaches using reversed-phase stationary phases in both dimensions (RP/RP) to improve peak capacity [1] or by coupling hydrophilic interaction phases with RP phases exhibiting complementary selectivity and excellent compatibility with the mobile phases used when coupling with mass spectrometry.

IV. NORMAL-PHASE CHROMATOGRAPHY

Normal-phase chromatography was the most widely used separation method before the development of reversed-phase chromatography. This separation method is based on ta polar stationary phase (made of silica or alumina) and a non-polar mobile phase made of organic solvents. With these phases, polar compounds exhibit strong interactions with the stationary phase which can lead, under certain conditions, to very significant retentions. To reduce the retention of these compounds, stationary phases containing polar groups such as amine, diol, or nitrile groups were then developed. This technique is based on the interaction of compounds with polar functional groups present on the surface of the stationary phase leading to their retention. Compounds of interest are eluted according to their polarity: nonpolar molecules are eluted more quickly while polar molecules are retained more. Normal-phase chromatography has the advantage of allowing a large number of solvents to be used to obtain ideal selectivity during a separation [1]. Studies have shown that separating a group of membrane peptides (4 to 50 amino acids) was possible using a stationary phase of silica grafted with aminopropyl groups. The mobile phase consisted of a mixture of methanol/chloroform/isopropylamine [3]. However, normal-phase chromatography remains little used in the analysis of peptides due to the poor solubility of polar compounds in organic solvents [4]. In addition, it has the disadvantage of being not very robust when using traditional silica phases (the percentage of water on the surface of the stationary phase varies from day to day depending on climatic conditions, significantly influencing retention), which has posed problems in terms of reproducibility. Furthermore, many solvents potentially usable in the normal-phase absorb UV below 250 nm, making peptide detection difficult.

V. HYDROPHILIC INTERACTION CHROMATOGRAPHY (HILIC)

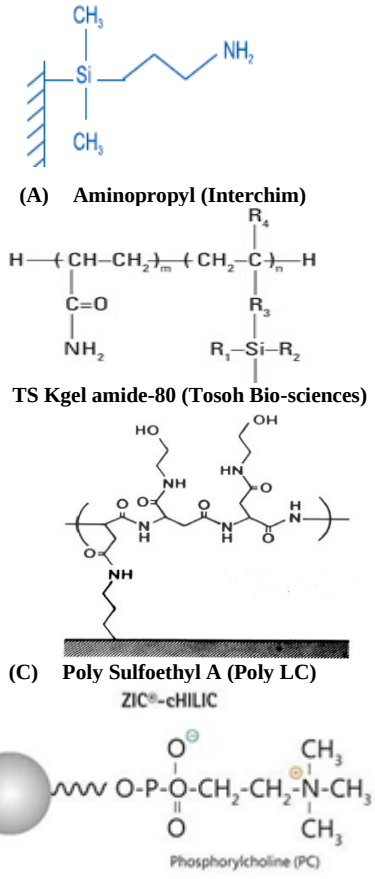
Hydrophilic interaction chromatography (HILIC) was first described by Alpert in 1990 [57]. This chromatographic mode uses a polar stationary phase and an organic (apolar) mobile phase containing a small proportion of water. In HILIC mode, the mixture used to prepare the mobile phase has a much greater eluting force than the mobile phases traditionally used in the normal phase. It generally consists of an aqueous phase mixed with acetonitrile or methanol. The organic part of the mobile phase (usually acetonitrile) behaves as a weak solvent, with the aqueous phase being considered the strong solvent. As for stationary phases, a wide range of materials available on the market can be used for HILIC separations. This chromatographic separation method can be compared to "aqueous normal-phase chromatography" without the drawbacks associated with solvents that are immiscible with water. Since the compounds are eluted in increasing order of hydrophilicity, polar compounds are retained more than apolar compounds. This is the opposite phenomenon to that of RP-HPLC where the stationary phase is apolar and elution is carried out in increasing order of hydrophobicity [59].

Hydrophilic interaction chromatography allows, in particular, the separation of highly hydrophilic compounds. These compounds have low retention and consequently low resolution in reversed-phase liquid chromatography. This is why hydrophilic interaction stationary phases, by allowing the retention of hydrophilic compounds, are of great interest. This separation method had, in fact, already been used in 1975 for the analysis of oligosaccharides. The term HILIC was proposed by Alpert in 1990 during a study of the separation of amino acids and peptides. The first applications were in the analysis of amino acids and peptides and carbohydrates. Their use was then extended to other low molecular weight polar molecules.

From all the work carried out in HILIC mode, it appears that the relative importance of the different mechanisms involved in the retention of compounds depends on the type of stationary phase used and the mobile phase employed, in particular the nature and percentage of the organic solvent, the pH, the concentration and the nature of salts in the buffer.

Stationary phases particularly developed for HILIC approaches can be particulate (pure silica or polar groups grafted onto silica-based or polymeric supports) or monolithic. There is a wide variety of functional groups. Figure 5 shows examples of chemical structures of HILIC stationary phases. Each type of HILIC phase interacts with analytes by different mechanisms (hydrogen bonds and/or electrostatic interactions) and exhibits different selectivity towards similar compounds. The polarity and ionization state of the analyte and the

stationary phase must be taken into account to understand and optimize retention/separation in HILIC mode.



(A) Aminopropyl (Interchim)

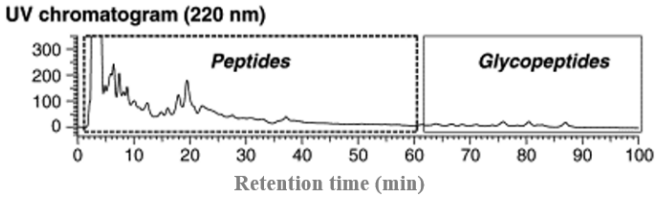
(B) TS Kgel amide-80 (Tosoh Bio-sciences)

(C) Poly Sulfoethyl A (Poly LC)

(D) ZIC-HILIC (Merck)

[Fig.5: Schematic Representation of HILIC-type Stationary Phases]

This chromatographic mode has proven to be very useful, particularly for hydrophilic peptides exhibiting low retention on RP-type columns. Hydrophilic interaction chromatography based on a mobile phase composed of water or volatile buffers (acetate or formate) makes this separation method compatible with mass spectrometry. This is why this chromatographic mode is interesting for analyzing complex mixtures of peptides resulting from the enzymatic digestion of proteins [Jandera 2008, Mant and Hodges 2008a], particularly for the establishment of peptide mappings of hydrophilic proteins. A peptide mapping of immunoglobulin G (IgG), which is a glycoprotein, was thus established using a stationary phase of the ZIC-HILIC type (Figure 6).



[Fig. 6: Separation of Peptides Resulting from Tryptic Digestion of IgG in HILIC mode. λ : 220 nm. Column: ZIC-HILIC (150 mm × 2.1 mm, dp 3.5 µm). Flow rate: 200 µL/min. Mobile Phase: Gradient (A/B/C = 36/59/5 (0 min) → 64/31/5 (120 min)). Solvent A: 50% ACN, Solvent B: ACN, Solvent C: 100 mM Ammonium Acetate Buffer (Solvent C)]

VI. CONCLUSION

Peptides are a promising therapeutic class in the treatment of different diseases. Ensuring their quality is crucial to obtaining therapeutic efficacity. Different techniques were used to assess the peptide quality, such as CZE, HPLC, SEC. etc. HPLC is the most widely used method for separating peptides. The normal-phase method has limitations in the analysis of peptides. To overcome these limitations, hydrophilic interaction chromatography was developed. Reversed-phase HPLC is the most used method in the separation of peptides. In this review, the principle of different modes of HPLC were detailed including normal-phase, reversed-phase, ion-exchange and HILIC mode. The contribution of the different modes of HPLC in the analysis of peptides were discussed.

DECLARATION STATEMENT

After aggregating input from all authors, I must verify the accuracy of the following information as the article's author.

- **Conflicts of Interest/ Competing Interests:** Based on my understanding, this article has no conflicts of interest.
- **Funding Support:** This article has not been sponsored or funded by any organization or agency. The independence of this research is a crucial factor in affirming its impartiality, as it has been conducted without any external sway.
- **Ethical Approval and Consent to Participate**: The data provided in this article is exempt from the requirement for ethical approval or participant consent.
- **Data Access Statement and Material Availability**: The adequate resources of this article are publicly accessible.
- **Authors Contributions**: The authorship of this article is contributed only to the author.

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