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Role of HILIC Stationary Phases in Pharmaceutical Peptide Analysis

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***Abstract**: Hydrophilic interaction chromatography (HILIC) is characterized by a polar stationary phase and an organic (apolar) mobile phase containing a small proportion of water. The HILIC retention mechanism is based on solute partitioning between the mobile phase (rich in organic solvents) and the aqueous phase layer. This separation method had already been used in 1975 for the analysis of oligosaccharides. The term HILIC was proposed by Alpert in 1990 during a study on the separation of amino acids and peptides. Stationary phases specifically developed for HILIC approaches can be particulate (pure silica or polar groups grafted onto silica-based or polymeric supports) or monolithic. This chromatographic mode has proven useful, particularly for hydrophilic peptides exhibiting low retention on RP columns. This review aimed to present the role of HILIC particulate and monolithic phases in the analysis of pharmaceutical peptides. Detailed descriptions of different HILIC phases were presented. Examples of peptide separation by HILIC mode were shown.*

Keywords: Peptides, Analysis, Quality, Chromatographic HILIC Mode.

Abbreviations:
HILIC: Hydrophilic Interaction Chromatography
SEM: Scanning Electron Microscopy
EDMA: Ethylene Di Meth Acrylate
ACTH: Adrenocorticotropic Hormone

I. INTRODUCTION

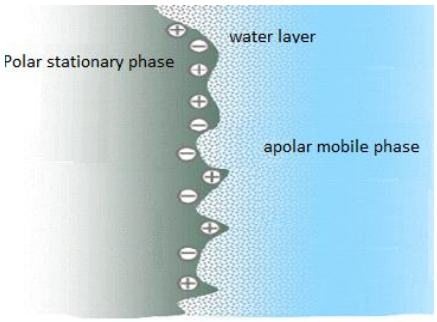
Hydrophilic interaction chromatography (HILIC) was first described by Alpert in 1990 [1]. This chromatographic mode is characterized by a polar stationary phase and an organic (apolar) mobile phase containing a small proportion of water [2]. In HILIC mode, the mobile phase mixture has a much greater eluting force than the mobile phases traditionally used in the normal-phase [3]. It generally consists of an aqueous phase mixed with acetonitrile or methanol [4].

The organic part of the mobile phase (usually acetonitrile) behaves as a weak solvent, while the aqueous phase is considered a strong solvent [5]. Regarding stationary phases, a wide range of materials available can be used for HILIC separations on the market [6]. This chromatographic separation method can be likened to "normal aqueous phase chromatography" without the drawbacks associated with using water-immiscible solvents [7]. Since compounds are Eluted in order of increasing hydrophilicity, polar compounds are retained more than apolar compounds [8]. The stationary phase is apolar, and elution is performed in order of increasing hydrophobicity in RP-HPLC [9].

Hydrophilic interaction chromatography can separate highly hydrophilic compounds [10]. These compounds exhibit low retention in reversed-phase liquid chromatography, and consequently, low resolution [11]. Therefore, hydrophilic interaction stationary phases, by allowing the retention of hydrophilic compounds, are of great interest [12]. This separation method had, in fact, already been used in 1975 for the analysis of oligosaccharides [13]. The term HILIC was proposed by Alpert in 1990 during a study on the separation of amino acids and peptides. Early applications included the analysis of amino acids and peptides [14] and carbohydrates [15]. Their use was later extended to other low molecular weight polar molecules such as cytosine, uracil, and adenosine [16], drugs such as vancomycin and cephalosporins [17], bacterial toxins [18and other compounds in the food industry [19].

II. HILIC RETENTION MECHANISMS

The distinction between normal-phase liquid chromatography and hydrophilic interaction chromatography has long been discussed [20]. However, it is currently accepted that "normal-phase chromatography" relies primarily on an adsorption phenomenon [21]. HILIC chromatography is based primarily on solute partitioning between the mobile phase (rich in organic solvents) and the aqueous phase layer adsorbed on the surface of the polar stationary phase, as illustrated in Figure 1.



[Fig.1: Schematic Representation of HILIC Retention Mechanism]

The hypothesis of a partition between the aqueous phase, partially immobilized at the stationary phase, and the mobile phase was proposed by Alpert in 1990.

This hypothesis was based on the work of Orth and Engelhardt in 1982 [22]. They studied the variation of the retention factor of oligosaccharides on phases of aminated silica, diaminated ethylene, and triaminated ethylene [23].

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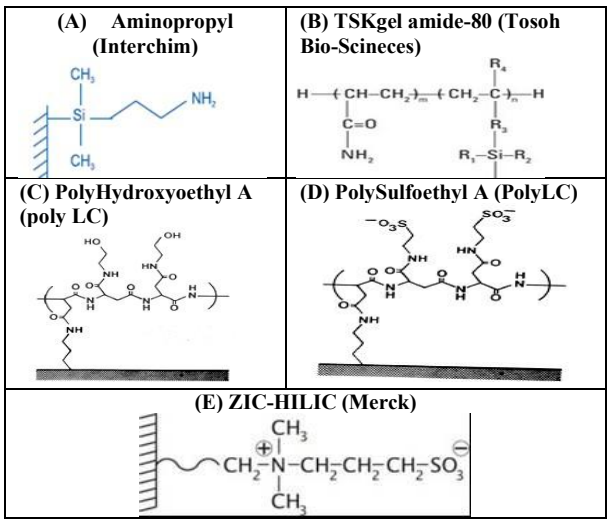
They thus showed that the retention of compounds decreased when the difference in polarity between these two phases was reduced (stationary phase/mobile phase), favouring a partitioning phenomenon [24]. Other work carried out subsequently focused on the study of the effect of temperature on retention (evaluated according to the Van't Hoff equation) [25]. This work measured the retention of model compounds, such as aspirin, salicylic acid, and cytosine, as a function of temperature (20 to 70°C) on four different types of columns (unmodified silica, zwitterionic, amide, and amine) [26]. The measured enthalpy was negative, indicating an exothermic process during the solute transfer from the mobile phase to the stationary phase [27]. A weak specific interaction between solute and functional groups of the stationary phase is remarked in agreement with the hypothesis of a partition mechanism rather than an adsorption on the stationary phase [28]. Regarding the enthalpy value of aspirin obtained on the HILIC column with amino groups, the enthalpy value was positive. The authors concluded that the retention was related to partition and ion exchange [29]. Other authors suggest a retention mechanism combining a partitioning phenomenon with hydrogen bonds [30] and electrostatic interactions between the analytes and the stationary phase [31]. It appears, based on all the work carried out in HILIC mode, 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 [32].

III. CLASSIFICATION OF HILIC STATIONARY PHASES

Stationary phases specifically developed for HILIC can be particulate (pure silica or polar groups grafted onto silica-based or polymeric supports) or monolithic. A wide variety of functional groups exists. Figure 18 shows examples of the chemical structure of HILIC stationary phases [33]. Each type of HILIC phase interacts with analytes through different mechanisms (hydrogen bonds and/or electrostatic interactions). The different implicated mechanisms lead to different selectivity toward similar compounds. The polarity and ionisation of the analyte and the stationary phase must be considered to understand and optimise retention/separation in HILIC mode.



[Fig.2: Examples of HILIC Stationary Phases]

A. Pure Silica

These are particulate stationary phases based on unmodified silica, the purity of which depends on the silica preparation method. Due to the ionisation of silanols, this silica phase is likely to establish hydrogen bonds with analytes and/or to present cation exchange mechanisms at high pH. It is particularly suitable for the analysis of small polar molecules. This type of column, made of ungrafted silica, has often been preferentially chosen for studies requiring coupling with mass spectrometry [34]. HILIC-MS/MS coupling has been used for the analysis of drug substances such as atenolol [33] and acyclovir [34]. However, this type of phase can lead to a high retention of compounds with a basic character. Irreversible adsorption of analytes on silica has sometimes been observed [35]. However, recent work by McCalley has shown that it is possible to analyze basic compounds (nortriptyline, diphenhydramine, benzylamine, and procainamide) with good efficiency on this type of phase (Halo HILIC) using a mobile phase consisting of a mixture of a high percentage of ACN and an aqueous solution of ammonium formate at basic pH [36].

B. Silica-Modified with Diol-Cyano-Amine Groups

Silica-modified phases with cyano groups do not form hydrogen bonds with analytes and therefore offer low retention for polar compounds compared to other HILIC stationary phases. Furthermore, they are not very stable in the presence of intermediate-polarity solvents. This type of stationary phase is therefore rarely used in HILIC mode. Studies have shown that peptides are poorly retained in this type of stationary phase [37].

In the silica-modified phases with Diol groups, the diol groups are bonded to the silica surface by either a short alkyl (propyl) or a longer chain. The 2,3-dihydroxypropyl phase is a stationary phase where the diol groups are bonded to the surface of the support by a short alkyl chain [38]. Its structure provides high polarity and a good capacity to form hydrogen bonds with the compounds to be analyzed. Undecyl-1,2-diol stationary phases are long alkyl chains with diol groups at their ends.

This last example of a HILIC-diol stationary phase can be used as either a reversed-phase stationary phase or a HILIC phase, depending on the composition of the mobile phase [38]. These stationary phases were among the first standard grafted silica phases developed. The aim was to limit the adsorption phenomena sometimes observed on silica phases [39]. These stationary phases are rarely used. However, a recent publication shows that this type of stationary phase has proven helpful for the separation of a mixture of purine and pyrimidine bases using a mobile phase consisting of a water/hexane/isopropanol mixture, thanks to the combination of partitioning and adsorption phenomena [40].

In the silica-modified phases with amine groups, the amine groups are linked to the silica surface by a short alkyl chain (Figure 2A). These stationary phases are mainly composed of amino-propyl groups linked to the silica. Depending on the pH of the mobile phase, they allow the analyzed compounds to be retained by hydrogen bonds and by an anion exchange

mechanism. Acidic compounds are therefore more retained, unlike what is observed with a pure silica phase. They can be used for studies in normal-phase chromatography and HILIC mode. They are used for the separation of carbohydrates or peptides [41]. Although these stationary phases have proven helpful for separating complex mixtures, they have many disadvantages. Indeed, the reactivity of the amine group can lead to the irreversible adsorption of compounds containing a carboxyl group, forming a Schiff base with them, which modifies the functionality of the stationary phase. In addition, in an aqueous medium, this phase is not very stable [42].

C. Silica-Modified with Amide Groups

The amide groups, linked to the silica surface by a short alkyl chain, do not possess basic properties. Thus, compound retention will be relatively independent of the pH of the mobile phase compared to other HILIC phases containing ionisable amine groups. Because the amide group is relatively unreactive, these stationary phases exhibit good chemical stability. TSK gel amide 80 (Figure 2B) has been widely used for the separation of sugars and peptides [43].

D. Silica-Modified with Poly (Succinimide) Groups

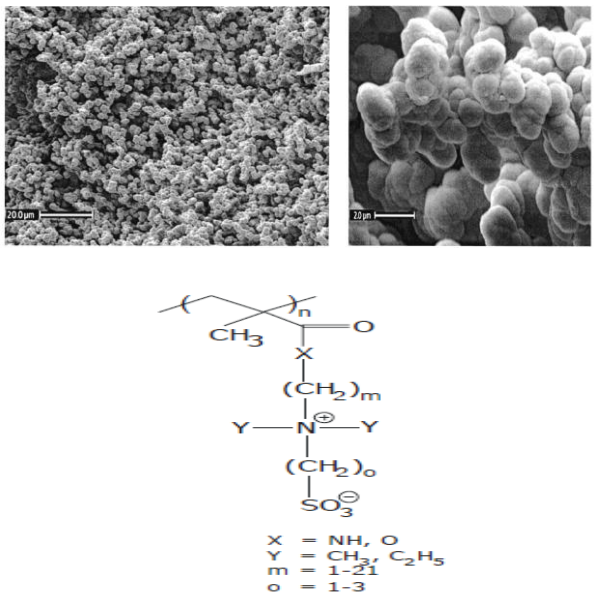
A covalent reaction between poly succinimide obtains these stationary phases and silica functionalized with an aminopropyl group. The reactions can lead to three types of stationary phases, namely poly(2-sulfoethyl) aspartimide, poly(2-hydroxyethyl) aspartimide, and poly(aspartic acid). The poly(2-sulfoethyl) aspartimide phase (PolySulfoethyl A, PolyLC, Columbia, MD, USA) is an amide-type stationary phase with sulfonate groups, which allows the combination of HILIC and cation exchange (Figure 2D). This phase has been successfully applied to the separation of complex mixtures of peptides [44]. However, it has been observed that this column is not very stable over time (1 month) [45]. The poly-aspartic acid phase (Poly CAT A, Poly LC, Columbia, MD, USA) is an amide-type stationary phase with carboxyl groups from the side chains of aspartic acid. This phase is also mixed (two retention mechanisms: HILIC and cation exchange).

E. Cyclodextrin-Modified Silica

These cyclodextrin-based stationary phases possess chiral properties and are therefore used in HILIC mode to separate chiral compounds. These are cyclic stationary phases consisting of glucosidic units (five or more) (e.g., α-D glucopyranoside) [46]. These stationary phases have been applied to the separation of oligosaccharides [47], coupled with mass spectrometry detection [48].

F. Zwitterionic Stationary Phases (Sulfoalkylbetaine)

These are characterized by the presence of positively and negatively charged groups. A short alkyl chain separates these two groups. Compared to stationary phases dedicated to ion exchange chromatography, the electrostatic interactions between these groups and oppositely charged analytes are weaker. These HILIC stationary phases allow several mixed retention mechanisms to be combined: HILIC, anion, and/or cation exchange [49]. These Sulfoalkylbetaine (ZIC-HILIC) phases contain positively charged groups close to the silica surface (quaternary ammonium) and negatively charged groups on the outside (sulfonates). These phases were developed by Irgum et al. [50] and then by the company SeQuant [51] (ZIC®-HILIC, Umeå, Sweden) were first prepared by chemical modification of the silica surface (Figure 2E). Other supports have been proposed for carrying out analyses at higher pH, such as polymers, based on particles obtained from 2-hydroxyethyl methacrylate (HEMA) monomers [52]. Thus, SeQuant markets a zwitterionic polymer column (ZIC®-pHILIC) whose support is composed of porous polymer grains. This type of HILIC column is currently one of the most used. It has been used for the analysis of various compounds such as aminoglycosides, morphine, and its derivatives (reviewed in Ikegami 2008). This support has also proven very useful for metabolome studies [53]. In addition, these columns have the advantage of being relatively insensitive to changes in the pH of the mobile phase [54]. Sulfoalkylbetaine-type zwitterionic monoliths have also been developed. For example, the monolith (SPE-co-EDMA) results from the copolymerization of N, N-dimethyl-N-methacryloxyethyl-N-(3-sulfopropyl) ammonium betaine (SPE) and ethylene dimethacrylate (EDMA) monomers [55]. Figure 3 shows the structure and image of a monolithic column (SPE-co-EDMA) [56], taken by scanning electron microscopy (SEM) in which the mesopores and macropores of the monolith are visible.



[Fig.3: SEM (Scanning Electron Microscopy) Photography of a Zwitterionic Monolith Prepared by Copolymerization of SPE and EDMA Monomers]

IV. PEPTIDE ANALYSIS IN HILIC MODE

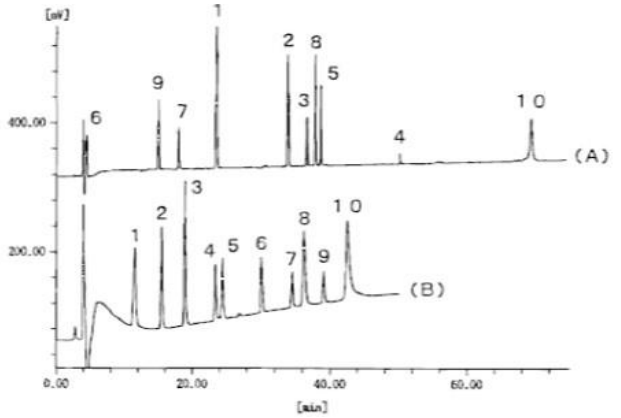
This chromatographic mode has proven useful, particularly for hydrophilic peptides exhibiting low retention on RP columns. Table 1 summarizes examples of using different types of HILIC phases described in the literature for separating peptides, proteins, and amino acids.

Table-I: Examples of Different Types of HILIC Phases for Separating Peptides, Proteins, and Amino Acids

	Column	Supplier	Peptide
Silica	Atlantis HILIC Silica	Waters (Milford, MA, USA)	Analysis of therapeutic peptides using an online two-dimensional chromatographic approach: Neurotensin A, Neuromedin B, Neurokinin A, adrenocorticotropic hormone (ACTH) fragment 1–10, Ranakinin, Dynorphin A, Galanin, vasoactive intestinal peptide, parathyroid hormone PTHrP (1–34), PTHrP (1–36), ACTH hormone Identification of hydrophilic peptides with angiotensin-converting enzyme (ACE) inhibition activities in milk hydrolysate using an offline two-dimensional approach
	Hypersil Silica	Agilent Palo Alto, CA, USA	Analysis of glutathione and its oxidized form. High-temperature separation of amino acids, peptides, and their Amadori, Gly, Di-Gly, Tri-Gly, Glucose Gly, Glucose Di Gly, Glucose-tri Gy compounds
	Zorbax RX Silica		
Sulfo Alkyl Betaine	ZIC-HILIC	SeQuant (Southborough, MA, USA)	Separation of peptides resulting from tryptic digestion of human serum albumin (HSA) Analysis of model peptides, GPFPIIV, LVTDLTK, ADLAK, FQpSEEQQQTEDELQDK, LKECCDKPLLEK, proteins, and peptides from the cell nuclear lysate Separation of peptides resulting from tryptic digestion of alpha-1-glycoprotein (AGP) and IgG Purification of bacteriocins obtained from E. faecium
Amide	TSKgel Amide-80	Tosohaa, PA, USA	Isolation and detection of peptides resulting from tryptic digestion of Hela cells (phosphorylated peptides) Separation of model peptides or peptides resulting from tryptic digestion of Cytochrome C and β-Lactoglobulin
Poly (2-Sulfo Ethyl ...	Poly-Sulfoethyl A	PolyLC (Columbia, MD, USA)	Separation of peptide mixtures differing in hydrophobicity and net charge
			Separation and characterization of peptides with helical conformations differing by amino acid substitutions with more hydrophobic or hydrophilic amino acids.
			Purification of synthetic peptides with α-helix conformations from their cognate impurities exhibiting Ser side-chain acetylation

A. Peptide HILIC Retention Mechanism

HILIC liquid chromatography, based on a mobile phase partially composed of an aqueous phase, is a promising alternative to reversed-phase chromatography for analysing polar peptides. It offers the advantage of using a polar mobile phase, whose composition is similar to that used in reversed-phase, and a polar stationary phase, whose composition is often identical to that used in normal-phase columns. Comparing these two chromatographic modes, one can expect to observe inversions in the elution order for peptides. The initial work carried out by Alpert in 1990 showed that the elution order of peptides was reversed compared to reversed-phase chromatography [55]. However, a comparative study of the separation of a complex mixture of peptides in the reversed phase (Figure 4A) (TSKgel ODS-80Ts (C18)), and in the HILIC phase (Figure 4B) (TSKgel amide-80 (carbamate), the structure is shown in Figure 2B), was conducted later [56].

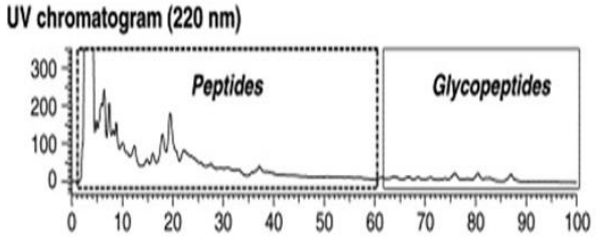


[Fig.4: Chromatograms of a Mixture of Peptides on (A) Column: TSK gel ODS-80Ts. Mobile Phases: A = Water + 0.1% TFA: B = ACN + 0.1% TFA. Linear Gradient of ACN from 5 to 55% (0.6% ACN/min) and (B) Column: TSK gel Amide-80. Mobile Phases: A = Water + 0.1% TFA: B = ACN + 0.1% TFA. Linear Gradient of Water from 3 to 45% (0.6% water/min). Peptides: 1, FY; 2, Fggf; 3, Fleei; 4, Dymgwmdp-Nh2; 5, Nftyggf; 6, Agsq; 7, Waggda sge; 8, Ygg fm tsqksqtplvt; 9, Astttnyt; 10, Vl sege wqlv lhvwak vead vaghgq dilirl fksh petlekf dr fkhlk teaem]

The results obtained show that, as expected, the elution order of the peptides is modified between the two columns due to the differences in interaction (solutes-stationary phase) between the two columns (polar interactions with the HILIC column and hydrophobic interactions with the C18 phase). Hydrophobic peptides such as FLEEI (peptide 3) and DYMGWMDP-(NH2) (peptide 4) containing hydrophobic residues (Val, Leu, Ile, Trp, and Phe) are more retained in the reversed-phase than in the amide phase. On the other hand, hydrophilic peptides such as AGSQ (peptide 6) are eluted very early on the ODS column, while they are well retained on the amide column. This work, showing the separation of peptides according to their hydrophilicity, supports the existence of a hydrophilic interaction phenomenon. However, the elution order of the peptides in this study is not completely reversed between the two types of stationary phases. Different selectivity, concerning the mixture of peptides analyzed, was observed. Additional studies carried out by the same team on the same stationary phase suggest that the amino acid composition and the secondary structure of the peptides also have a determining influence on the retention of peptides in HILIC mode [56]. This is in agreement with the work of Mant carried out in 1998 [55Using large amphipathic peptides (50 amino acids), presenting an α-helix structure on a polysulfoalkyl stationary phase (HILIC / cation exchange). Substituting an amino acid on the hydrophilic side modified the retention in HILIC mode. Substituting an amino acid on the hydrophobic side modified the retention in reversed-phase chromatography. Thus, for large peptides, peptide retention is based on the regions of contact with the stationary phase rather than on their overall hydrophilicity or hydrophobicity, which would explain why the elution order between these two chromatographic modes, is not completely reversed when analyzing mixtures of peptides of different sizes.

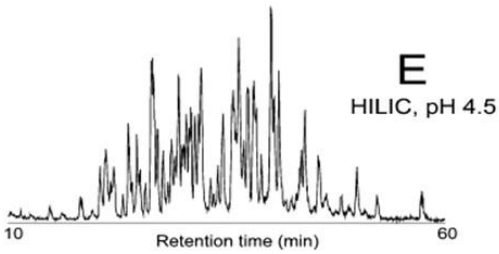
B. Applications: Peptide Mapping

Hydrophilic interaction chromatography, using a mobile phase composed of water or volatile buffers (acetate or formate), makes this separation method compatible with mass spectrometry. This chromatographic method is therefore helpful for analysing complex mixtures of peptides resulting from the enzymatic digestion of proteins [56], particularly for establishing peptide maps of hydrophilic proteins. A peptide map of immunoglobulin G (IgG), a glycoprotein, was thus established using a ZIC-HILIC stationary phase [57] (Figure 5).



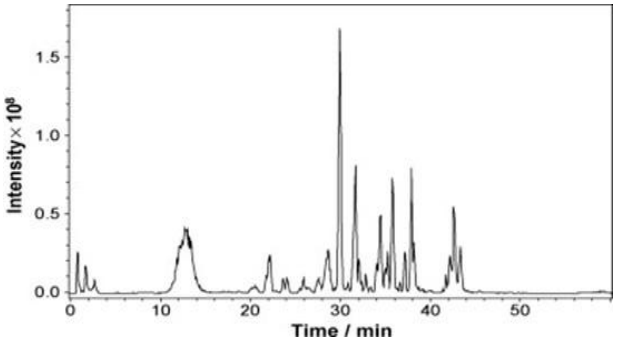
[Fig.5: Separation of Peptides Resulting from Tryptic Digestion of IgG in HILIC Mode. λ : 220 nm. Column: ZIC-HILIC (150 mm × 2.1 mm, dp 3.5 µm). Flow Rate: 200 µL/min. Mobile Phase: Gradient (A/B/C = 36/59/5 (0 min) → 64/31/5 (120 min)). Solvent A: 50% ACN, Solvent B: ACN, Solvent C: 100 mM Ammonium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

The peptide fragments resulting from the tryptic digestion of IgG contain both peptides and glycopeptides. When analyzing peptides resulting from the tryptic digestion of this glycoprotein, glycopeptides are eluted after non-glycosylated peptides, because glycosylated peptides, being more hydrophilic, are retained by hydrophilic interactions on the ZIC-HILIC phase more than non-glycosylated peptides, allowing for the separation of the two groups of peptides. Other authors have demonstrated the value of HILIC stationary phases for the separation of a complex mixture of peptides resulting from the enzymatic digestion of proteins [58]. For example, Gilar et al. analyzed peptide fragments resulting from the digestion of a mixture of proteins (enolase, phosphorylase b [59], hemoglobin, BSA, antidiuretic hormone) and six peptides (Rasg-1, angiotensin 1-7, bradykinin, angiotensin 2, angiotensin 1, renin substrate, melittin) using a pure silica-based stationary phase (Figure 6).



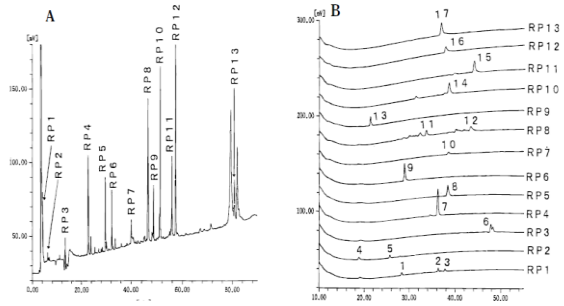
[Fig.6: Chromatogram of Peptide Fragments from Tryptic Digestion of a Mixture of Proteins and Peptides using LC-MS]

Other authors have also separated peptide fragments from the tryptic digestion of ß-lactoglobulin [57]. Using an amide-type stationary phase (TSKgel Amide-80) and using gradient elution, the mobile phase consisting of a mixture of ammonium formate and ACN (Figure 7).



[Fig.7: HILIC Separation of Peptide Fragments From β-lactoglobulin Digestion. Column: TSKgel Amide-80 (50×2.0 mm, dp: 3 µm). Sample Concentration: 0.5 mg/mL, Injection Volume: 2 µL. Mobile Phase: Solvent A: ACN, Solvent B: 20 mM HCOONH4. Linear Gradient: 10–50% B in 40 min, 50–90% B: in 15 min. Flow Rate: 100 µL/min]

Sometimes, it is not always possible to obtain a satisfactory separation for all peptide fragments resulting from the digestion of a protein using a single separation mode. Therefore, it is sometimes necessary to resort to a two-dimensional separation. Most two-dimensional separations are based on the RP/SCX coupling [54]. However, HILIC mode chromatography, which has a selectivity orthogonal to reversed-phase chromatography, can also be used in multidimensional systems, either online or offline, to separate all peptide fragments. A first example of two-dimensional separation, combining the RP and HILIC modes, is presented below (Figure 8).



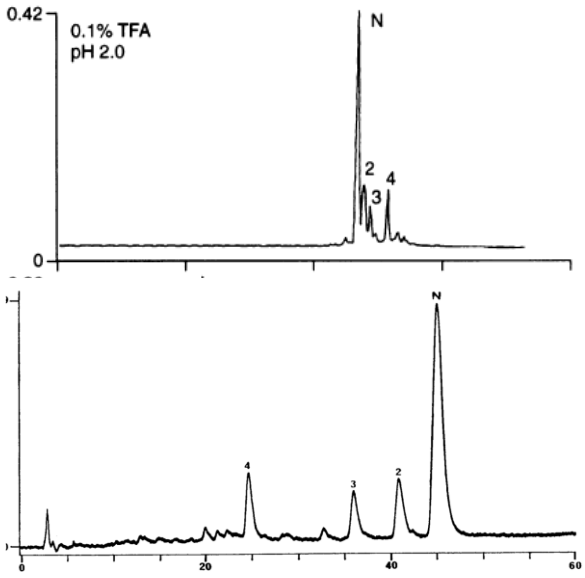
[Fig.8: (A) Chromatograms of Tryptic Digestion of Concanavalin A on TSKgel ODS-80Ts. Mobile Phase: Linear Gradient of ACN from 5 to 55% in 0.1% TFA (0.56% ACN/min). 13 Fractions are Obtained. (B) Chromatograms of the 13 Obtained Fractions (RP1 to RP13) on TSKgel Amide-80. Mobile Phase: Linear Gradient of Water from 3 to 45% in 0.1% TFA (0.6% water/min)]

In this experiment, a two-dimensional separation of a mixture of peptides resulting from the tryptic digestion of concanavalin A was performed [55]. Reverse-phase HPLC first separated the mixture of peptides resulting from the digestion. The 13 fractions obtained at the column outlet were collected (chromatogram 24A). Each collected fraction was then concentrated and analyzed in HILIC mode using an amide stationary phase. HILIC exhibits a selectivity complementary to RP-HPLC, making it possible to separate the peptides in the same fraction by HILIC. Thus, Three peptides (TAK, DQK, and SK) from fraction 1 (RP1) were

separated. Fraction 2 (a single peak RP2) presented a mixture of two peptides (LK and SVR). Other authors have shown that another type of HILIC stationary phase, based on unmodified silica, due to its orthogonal selectivity with RP, allowed a two-dimensional separation of a complex mixture of peptides resulting from the enzymatic digestion of a mixture of proteins [56].

C. Separation and Identification of Impurities in Synthetic Peptides

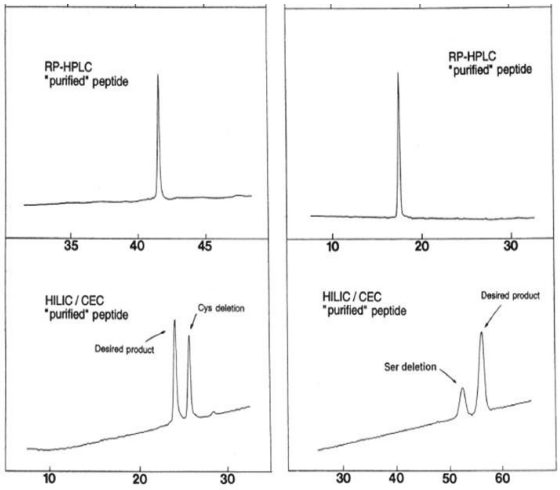
HILIC chromatography is also frequently used to purify or separate synthetic peptides to ensure their purity and quality for therapeutic use. Indeed, HILIC experimental conditions are compatible with mass spectrometry detection, which can allow the separation of the peptide from its impurities and their identification. Thus, a method for purifying synthetic peptides using a poly(2-sulfoethyl) aspartimide stationary phase has been proposed [58]. In this study, the authors demonstrated that it was possible to separate a synthetic peptide containing 21 amino acids from its synthetic impurities. The secondary structure of this peptide corresponds to a α-helix with a hydrophobic and a hydrophilic portion. Its analysis by LC/MS demonstrated that impurities 2 and 3 correspond to an acetylation of the Ser residues of the peptide, while impurity four results from the deletion of a Lys residue.



[Fig.9: Separation of a Peptide Labeled N (Ac-KIS-ALKEKIS-ALKEKIS-ALKE-NH2) from its Synthetic Impurities. A)- Column: Zorbax Eclipse XDB C8. Conditions: Linear-Gradient (1% ACN/min). Flow Rate: 1 mL/min. Eluent A: Aqueous Phase + 0.1% TFA and Eluent B: ACN + 0.1% TFA, pH 2.0]

The authors found that separating the peptide of interest (peptide N) from its synthetic impurities was not complete using RP-HPLC (Figure 9A). However, using a PolySulfoethyl A column, a stationary phase combining HILIC/cation exchange, allowed for satisfactory separation of the peptides from the mixture (Figure 9B). The peptides were eluted in order of increasing hydrophilicity. Peptide 4, the least hydrophilic, was eluted first (deletion of a Lys removes a positive charge, thus decreasing the peptide's hydrophilicity). Peptides 3 and 2 were eluted next. They are more hydrophobic than the peptide of interest due to acetylation at the hydrophilic part of the peptide, which also decreases their hydrophilicity.

Another example presented by Mant et al. demonstrates the usefulness of the HILIC mode for the analysis of synthetic peptides. Indeed, they analyzed two peptides, one of which consists of 35 residues with a Cys in position 2, and the other is composed of 17 residues with a disulfide bridge between two Cys residues [50]. Analysis of these peptides in reverse-phase chromatography shows a single peak. On the other hand, analysis in HILIC mode using a PolySulfoethyl A phase (HILIC/cation exchange) makes it possible to demonstrate the presence of synthetic impurities (Figure 10).



[Fig.10: Analysis of Two Synthetic Peptides: A: 35-Residue Peptide with a Cys in Position 2, B: 17-Residue Peptide with a Disulfide Bridge Between Two Cys residues. (RP-HPLC): Linear Gradient of Two Solvents A and B (1% B/min, Equivalent to 1% ACN/min). Eluent A: Water + 0.05% TFA, Eluent B: ACN + 0.05% TFA. Flow rate: 1 ml/min]

V. CONCLUSION

In conclusion, it appears that HILIC mode can be used for many applications, such as protein quality control or the purification of synthetic peptides. Detailed descriptions of different HILIC phases were presented. Examples of peptide separation by HILIC mode were shown.

DECLARATION STATEMENT

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.
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