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Review

Advances in Therapeutic Peptides Separation and Purification

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Abstract: Peptides are gaining prominence in various fields, including the pharmaceutical industry. To meet regulatory requirements, they must achieve a certain purity threshold to ensure safe administration. Numerous purification technologies have been employed to purify peptides, aiming to reduce cost and time while being sustainable and efficient. These include chromatography, magnetic nanoparticles, isoelectric focusing, and membrane filtration. The physicochemical properties of peptides are the main driving element behind these technologies. While chromatographic separation remains the gold standard for peptide separation and purification, with various models to predict the elution behaviors of peptides, other technologies have demonstrated their capability to meet the performance of established chromatographic methodologies, with better productivity and reduced cost. This opens the door for further investigational studies to assess these outcomes and potentially introduce new techniques for peptide purification. In this review, we examine these technologies in terms of their efficiency and their ability to meet sustainability requirements, concluding with remarks and an outlook on future advancements.

Keywords: peptides; proteins; separation; purification; magnetic nanoparticles; chromatography; membrane filtration; sub/supercritical fluid chromatography; isoelectric focusing; sustainability

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1. Introduction

Peptides are one of the key medications in the pharmaceutical market. Their selectivity, safety profile, and the ability to tackle unmet medical needs have led to them being embraced by various fields, including immunology [1], drug discovery [2,3], and materials [4,5], among others [6]. A total of 31 peptides were approved between 2019 to 2023 by the United States Food and Drug Administration (FDA) [7]. This has been the major driving force for shifting attention towards the downstream processes [8].

Peptides can be obtained from different sources, including (i) natural and (ii) chemical synthesis and (iii) the peptide libraries screening approach [9], where phage display is considered an effective tool for this purpose [10]. The harmonization between disciplines such as medicinal chemistry, biology, and pharmaceutics has allowed for diversifying peptide families, with a plethora of applications [11]. Three main technologies are considered for synthesizing peptides: (i) classical solution peptide synthesis (CSPS), (ii) solid-phase peptide synthesis (SPPS), and (iii) liquid-phase peptide synthesis (LPPS) [12]. No matter through which route peptides are obtained, it is inevitable to have impurities in the crude peptide, and it is thus deemed necessary to carry out a purification endeavor to meet a purity criterion for safe administration. Impurities can be classified into two categories: (i) product-related impurities, which have chemical similarity to the target product, and (ii) process-related impurities, which arise from the production method used, such as salts, DNA fragments, or cell debris, among others [13,14]. There have been continuous calls for increased understanding of the process and products to ensure the defined quality

attributes are met [15]. For example, quality by design (QbD) discipline was introduced in 2004 in order to achieve a desired state for pharmaceutical manufacturing [15]. Doubtlessly, advancements in synthetic methodologies are considered constantly; however, purification is always needed, albeit to different extents [16].

The main challenge of peptide purification lies in the structural homology between the main peptide and its impurities. For instance, impurities often share the same amino acid constituents but may have fewer amino acids (deletion sequences), epimers (due to racemization), β-peptides resulting from Asp and Ser isomerization, or modified peptides resulting from the alkylation of Met, Trp, and Tyr or the oxidation of Met and Cys [17]. Hence, several research efforts have focused on understanding the properties of peptides, including their hydrophobic character, charge, sequence composition, and other factors, to better comprehend the elution patterns of these molecules in reversed-phase liquid chromatography (RPLC) [18–20] and in hydrophilic interaction liquid chromatography (HILIC) [21–23]. Petersson and colleagues conducted a captivating study to create a column characterization database [24]. This study assessed several of those phenomena, which are herein discussed later [24].

Separation and purification are interconnected processes in analytical chemistry. The fundamental distinction between them is that separation involves transforming a mixture of substances into two or more distinct product mixtures, while purification focuses on removing impurities from an analyte sample. Table 1 summarizes the separation and purification technologies discussed in this review along with their main applications according to the authors' perspective.

Table 1. Separation and purification technologies and their applications.

#	Technology	Applications
1	RPLC	Separation, identification, and purification
2	HILIC	Separation, identification, and purification
3	MMC	Separation, identification, and purification
4	2D	Separation, identification, and purification
5	SFC	Separation, identification, and purification
6	MNPs	Separation and purification
7	IEF	Separation and purification
8	Membrane filtration	Purification

RPLC, reversed-phase liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; MMC, mixed-mode chromatography; 2D, two-dimensional methods; SFC, sub/supercritical fluid chromatography; MNPs, magnetic nanoparticles; IEF, isoelectric focusing.

In this review, we discuss the variety of the available separation and purification techniques for peptides in terms of their efficiency, sustainability, and future perspective by the authors.

2. Reversed-Phase Liquid Chromatography (RPLC)

High-performance liquid chromatography (HPLC) is the gold standard for separating and purifying peptide molecules [16,25]. RPLC is the most widely used approach for separating and purifying peptides. However, due to the complexity in their structure, other paradigms are also considered. For instance, ion exchange chromatography (IEX) is an important separation technique for charged peptides, providing better resolution and overall separation efficiency. IEX operates by differentiating peptides based on their charges [26]. In this method, the stationary phase is functionalized with anionic groups that can bind and purify positively charged peptides and vice versa [26].

Lin and co-workers concluded that adding 0.1% m-nitrobenzyl alcohol to the mobile phase as a supercharging agent enhances the sensitivity and charge state of the sialylated glycopeptides and disulfide bridge in disulfide-containing peptides. This improvement was observed in the investigated acidic and high-molecular-weight glycopeptides [27] and equine Interleukin-5 (eIL5) model protein [28], resulting in a better resolution. It is

worth mentioning that the supercharging agent also improved the MS1 signal intensity of disulfide bridge peptides and *C*-terminal peptides with His-Tag. These findings are important because many peptide therapeutics feature single or multiple disulfide bridges in their structures [6].

Interestingly, Ma et al. developed three silica stationary phases modified with peptides containing Phe and Pro [29]. They examined these phases using 11 racemic compounds under normal-phase HPLC mode [29]. The study successfully separated the enantiomers of furbiprofen and naproxen. The enhanced enantioselectivity was influenced by both the length of the peptide chiral selector and the configuration of the chiral amino acids within the peptide [29].

Petersson and co-workers published a series of intriguing papers that investigated 43 RPLC columns to establish a column characterization database to help identify backup columns for existing methods and highlight complementary stationary-phase combinations with significant selectivity differences for method development [30,31]. The same group then described strategies for assessing the peak purity of therapeutic peptides, where they focused on the selection of columns and mobile phases [32]. After their developed protocol for column characterization, they established a column characterization database. The study investigated various phenomena, including the impact of oxidation, alterations in negative charge, steric interactions like racemization and amino acid sequence changes, aromatic and phenolic interactions, and variations in positive charge [24]. Moreover, they investigated 51 mobile phases to maximize the chromatographic selectivity of peptide separations using RPLC [33]. Their work also included a rapid RP (UHPLC) method development screening strategy for the purity determination of peptide-based pharmaceuticals [34]. In their various series, they utilized up to 29 peptides such as angiotensin I, insulin, melittin, ubiquitin, and bradykinin, among others.

Lenčo et al. thoroughly presented a fascinating tutorial publication on peptide separation using RPLC. The tutorial includes the fundamentals of chromatography, gradient separation of peptides, and factors influencing the overall separation quality [35].

3. Hydrophilic Interaction Liquid Chromatography (HILIC)

A significant drawback of RPLC is its inadequate retention of polar species. The term hydrophilic interaction liquid chromatography (HILIC) was coined by Dr. Alpert in 1990 [36]. HILIC uses a polar stationary phase such as silica and a high percentage of organic solvents containing a mobile phase to boost the retention of polar compounds [36]. HILIC serves as a suitable alternative, offering sufficient retention for polar peptides and potentially improving peptide identification compared to RPLC.

The mobile-phase conditions, including pH, salt concentration, and organic solvent composition, can significantly affect selectivity and peak shape [37]. To address this, Alpert carried out a study to investigate the influence of different salts on the retention behavior of peptides utilizing two sequences: WWGSGPSGSGGDGGGK and WWGSGPSGSGG(pSer)GGGK [38]. The study demonstrated that well-hydrated counterions facilitate the partitioning of charged solutes into the immobilized aqueous layer. Conversely, poorly hydrated counterions inhibit this partitioning. For neutral solutes, the impact was more modest, with retention times either remaining unchanged or slightly increasing as the concentration of any salt increased [38].

HILIC is employed not only in analytical applications but also in pretreatment steps, such as solid-phase extraction, followed by RPLC separations. Ikegami explored all of these intriguing applications along with the limitations [39]. An interesting review for the advancement in the HILIC field was also carried out by Dr. Alpert [40].

4. Mixed-Mode Chromatography (MMC)

Multimodal or mixed-mode chromatography (MMC) relies on media supports functionalized with ligands that enable multiple interaction modes, including ion exchange, hydroxyapatite, affinity, size exclusion, and hydrophobic interactions. Thus, multiple

retention mechanisms operate within a single chromatographic system [41]. MMC utilizes RPLC and IEX to work coherently, in which the peptide has multiple interaction spots with the stationary phase, allowing for better separation output [41]. MMC has been demonstrated to perform better than the previous individual paradigms on their own [42]. MMC aids the detection of trace amounts of the analyte and separates polar species that cannot be separated otherwise with the standard RPLC [41,42]. MMC has four categories depending on the chemistry design as well as bimodal media [41]: RP/anion exchange (AEX), RP/cation exchange (CEX), HILIC/AEX, and HILIC/CEX bimodal phases, as well as RP/AEX/CEX and HILIC/AEX/CEX trimodal materials [41]. Washburn and colleagues identified 1484 proteins and 5540 peptides from yeast using RP/CEX [43].

A new MMC approach called electrostatic repulsion RPLC (ER-RPLC) was developed by Gritti and Guiochon [44]. ERRP was developed to resolve basic compounds, many of which are produced by the pharmaceutical and biopharmaceutical industries. In ER-RPLC, the retention of bases in their protonated form can be achieved by modulating the charge repulsion caused by the presence of fixed (static) or adsorbed (dynamic) positive charges within the chromatographic system [45]. In static ER-RPLC, a fixed amount of positive charge is chemically anchored to the stationary phase. In dynamic ER-RPLC, cationic additives are added to the mobile phase. ER-RPLC circumvents problems associated with complex surface structure of the stationary phase, including the silanol problem, which is exacerbated at pH > 3.5 [46,47]. Both static and dynamic ER-RPLC play a crucial role in resolving basic compounds, offering enhanced performance compared to conventional RPLC [45].

Gritti and Guiochon tested static ER-RPLC using two peptides, bradykinin and β-lipotropin, along with six other proteins [44]. They utilized BEH-C_{18} column with low, medium, and high surface-charge densities [44]. As a result of the electrostatic repulsion between the charged analytes and the surface charge, the retention times decreased with increasing surface-charge density [44]. They also observed poor retention at low analyte concentrations due to repulsion from the stationary phase, while higher concentrations resulted in better retention [44]. This approach resulted in a reduction in the peak tailing of protonated bases under acidic pH conditions and shorter analysis times [44].

Mazzoccanti and colleagues utilized dynamic ER-RPLC and achieved unprecedented separation of a basic glucagon peptide (pI 7.5–8.5) [48] from its impurities, including its [D-His]^1-GLUC epimeric impurity and other degradation impurities, in a single process [49]. The same task was not achievable with the conventional AEX strategy [49].

According to Mazzoccanti et al., dynamic ER-RPLC demonstrated superior performance not only in resolving elusive impurities but also in producing symmetrical peaks and enhancing the longevity of the chromatographic column [50]. The epimeric [L-Arg]1-Icatibant impurity of Icatibant was efficiently resolved using dynamic ER-RPLC, which outperformed both static ER-RPLC and ion-pair RP (IP-RP) chromatography [50].

It is worth highlighting that a similar approach was adopted in HILIC, called electrostatic repulsion HILIC (ER-HILIC), for the isocratic separation of phosphopeptides [51] and applications in the proteomic field [52].

Doping RPLC (DRPLC) is an advanced mode of MMC, but it utilizes both RP and IEX ligands in doping quantities. In this setup, the IEX ligands are responsible for electrostatic interactions, while the RP ligands facilitate hydrophobic interactions [53]. DRPLC offers the ability to precisely choose and change the amount of each component. Unlike traditional MMC, DRPLC uses a smaller amount of the IEX ligand compared to the hydrophobic ligand. As a result, the interaction is not equally distributed along the surface (Figure 1).

Interestingly, DRPLC exhibits both attractive and repulsive interactions between the analyte and the ligand, whereas MMC considers only attractive interactions. The overall separation mechanism in DRPLC depends on the concentration of both the RP and IEX ligands [53]. The performance of this stationary phase was exemplified by purifying various peptides with diverse molecular weights (1.2 to 3.4 KDa), isoelectric points (pIs) (4.9 to 11.5), and amino acid compositions. Adjusting the amount of IEX ligands enhanced the

separation of these industrially relevant peptides, including goserelin and insulin. The attractive–repulsive interaction mode proved to be superior to the attractive–attractive interaction mode.

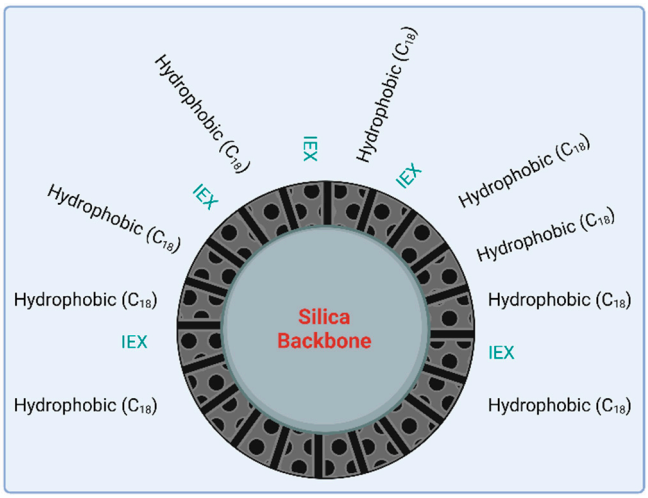

Figure 1. Schematic representation of DRPLC. C_{18} is used as an example.

Kadlecová and co-workers demonstrated the benefits of MMC columns for the separation of peptides [54]. They evaluated three MMC columns with RP/anion-exchange mechanisms: two RP octadecyl columns and one column with MMC RP/anion-exchange characteristics only within a defined pH range [54]. They selected a set of peptides varying in polarity, length, amino acid sequence, and charge state, which included dipeptides, *N*-blocked dipeptides, and oligopeptides [54]. They demonstrated the potential of MMC columns for analyzing differently charged peptides in a single run [54].

A novel MMC, HILIC/CEX chromatography, was developed to enhance the separation and analysis of peptides and proteins. This innovative method leverages the complementary strengths of hydrophilic interaction and CEX mechanisms [55]. Interestingly, in the characteristic conditions of HILIC/CEX, characterized by high concentrations of acetonitrile in the mobile phase, certain "self-assembly" peptides are anticipated to adopt secondary α-helical structures. This facilitates interaction of their charged, hydrophilic face with the CEX matrix, as observed in various studies utilizing increased acetonitrile levels [55,56]. Hence, HILIC is widely regarded as a versatile and preferred technique for separating a significant class of peptides known as self-assembly peptide sequences.

5. Two-Dimensional Separation Methods

Peptide species that coelute make it difficult to detect one of the species; therefore, a separation technique that acts as a first dimension complementing the second dimension, which is RPLC, is required to facilitate detection. The first dimension includes either an "in-line" technique such as MMC methods or an "off-line" technique such as isoelectric focusing [57] or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [58]. In 2D chromatography, the injected sample is separated by passing through two different separation stages, achieved by injecting the eluent from the first column onto a second column [59].

Aebischer et al. developed theoretical models to predict the effectiveness of dilution and determine the minimum dilution value required, if applicable [60]. The theoretical approach was experimentally validated on a variety of representative small molecules, including coumaric acid, atropine, bumetanide, amitriptyline, diphenhydramine, and peptides YNSFDEWKCTFSW and KEHWDMWSHL [60]. This approach enabled determination of dilution conditions where peak distortion or broadening occurs, allowing avoidance of such conditions. The theoretical predictions closely matched experimental results for small molecules, while acceptable differences were observed for peptides [60].

Guillarme et al. also investigated the influence of dilution factor on maximizing peak capacity within a specified analysis time. They compared one-dimensional RPLC with two-dimensional RPLC × RPLC approaches [61]. The study provided the optimal column specifications and dilution factors for RPLC × RPLC to achieve maximum performance in terms of peak capacity and sensitivity [61].

Petersson et al. published a study focused on defining the second-dimension chromatographic gradient conditions and demonstrating the 2D-LC-MS methods developed using this approach [62].

6. Sub/Supercritical Fluid Chromatography (SFC)

Conditions of sub- and supercritical fluid refer to fluid behavior in liquid- or gas-like states, respectively [63]. A significant advantage of SFC is that it allows for a reduction in the amount of organic solvent used during purification processes. This is possible because SFC utilizes CO_2 as a major mobile-phase component [64]. Schiavone et al. successfully conducted preparative-scale SFC purification of bovine insulin and bradykinin using a mobile phase composed of CO_2 along with a mixture of methanol and acetonitrile, 0.2% trifluoroacetic acid (TFA), 5% water, and a 2-picolylamine column [65].

Tognatelli et al. successfully conducted an analytical-scale SFC purification of a mixture of peptides, including V8376, G3502, Leu-enkephalin acetate, Met-enkephalin acetate, and angiotensin II acetate. They employed a mobile phase composed of methanol and CO_2 supplemented with 0.1% TFA using a 2-ethyl pyridine column [66]. Using a Luna HILIC stationary phase in conjunction with methanol supplemented with TFA or ammonia, Ventura reported several advantageous applications of SFC for the separation of peptide libraries and crude therapeutic peptides, including Leu-enkephalin acetate, Met-enkephalin acetate, angiotensin II acetate, neutral, acidic, basic, and macrocyclic peptides, with peptides up to 40 mer long [67]. Makarov and Regalado recently achieved successful separation of cyclic therapeutic peptides, namely linaclotide and gramicidin S., at both analytical and preparative scales by employing a chaotropic effect mechanism in SFC. They used ammonium hydroxide in water-rich modifiers with a poly(4-vinylpyridine)-based stationary phase (Dcpak SFC-B, P4VP) [68].

Results from various studies have demonstrated that the addition of small amounts of water to the CO_2/methanol mobile phase enhances SFC chromatography by improving the solubility of polar analytes [65,68–71]. Along these lines, Govender et al. studied the effect of combining methanol with water, TFA, and CO_2 on the mobile-phase ability to purify crude peptides using SFC. Their study focused on peptides associated with cardiovascular diseases and diabetes, such as angiotensin II, insulin β chain (15–18), and (15–23) peptides [72].

Indeed, computational tools play a crucial role in the separation of peptides, offering savings in time, effort, and cost. Neumann and colleagues utilized the DryLab 2000 software (Molnar-Institute) to predict the retention times (RTs) of peptides such as bacitracin (Bac), colistin, tyrothricin (Tyro), and insulin analogues [73]. Despite the demonstrated applicability of currently available modelling software for predicting high-modifier SFC separations, there are limitations in predicting peak widths and slight drifts in RTs. However, accurate predictions were successfully validated through verification experiments [73]. The authors subsequently applied their developed model and method development approach using design of experiments (DoE) principles to human serum albumin (HSA) and six analogues with molecular weights up to 6 kDa [74]. Their results from SFC provided sufficient resolution, suggesting it could serve as a potential alternative or complementary method for determining impurities in this mass range [74].

7. Magnetic Nanoparticles (MNPs)

Nanostructures like magnetic nanoparticles (MNPs) hold promise for the purification and separation of biomolecules such as proteins and peptides. This is attributed to their manufacturability, biocompatibility, and the ability to manipulate them using an external

magnetic field [75–81]. Unlike well-established chromatographic methods, MNPs are still in the early stages of development despite their use in the separation of various biomolecules [82,83]. Fe_3O_4 nanoparticles are the most commonly used MNPs, capable of binding to proteins through non-covalent interactions, covalent bonds, physical absorption, or bioconjugation [84–87]. Other types of MNPs, including hematite (α-Fe_2O_3), maghemite (γ-Fe_3O_4), $NiFe_2O_4$, $CoFe_2O_4$, and silica-coated SiO_2, have been employed in various studies [88–90].

Charges of proteins at physiological pH vary depending on their isoelectric points (pI); therefore, positively or negatively charged nanoparticles will selectively interact with proteins [91]. Nanoparticle size plays a significant role in the adsorption of proteins by magnetic nanocomposites. Generally, the larger the MNPs, the larger the adsorption of protein [92].

Figure 2 depicts some metal-functionalized MNPs used for selectively binding proteins. MNPs can be functionalized with biomolecules and/or synthetic polymers to facilitate target purification [75].

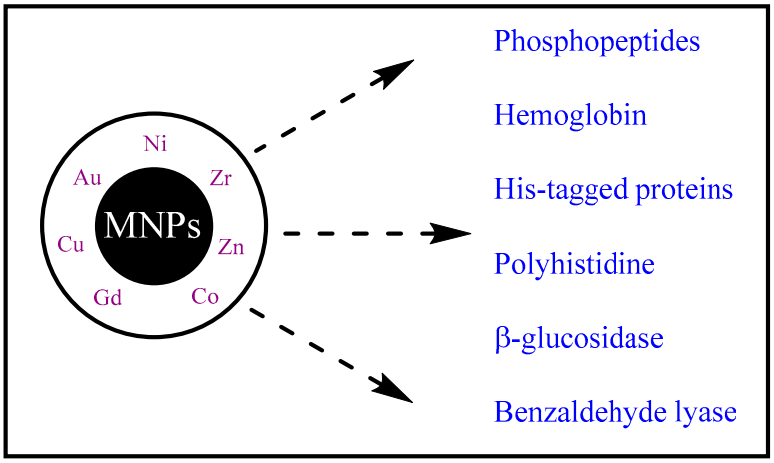

Figure 2. Metal-functionalized magnetic nanoparticles used for selective binding of proteins.

Liu and colleagues developed multifunctional magnetic mesoporous core/shell heteronanostructures designed to address several limitations of conventional magnetic nanoparticles (MNPs). These innovations aim to overcome issues such as low magnetic response, complex fabrication processes, and poor recyclability [93]. They were successful in efficiently separating and purifying His-tagged cyan fluorescence protein (His-tagged CFP) from a cell lysate of *E. coli*. Additionally, they demonstrated the selective enrichment of low-molecular-weight biomolecules from tryptic protein digest solutions and complex biosamples such as human serum [93].

Wan and colleagues developed an integrated protocol combining nanoparticle protein coronas with liquid chromatography–tandem mass spectrometry (LC/MS/MS) to analyze small open-reading frame-encoded peptides (SEPs) in human serum [94]. Typically, the detection of SEPs using mass spectrometry (MS)-based proteomic assays is hindered by the wide dynamic range of serum/plasma protein abundance. By employing three types of nanoparticles—TiO_2, Fe_3O_4@SiO_2, and Fe_3O_4@SiO_2@TiO_2—they successfully identified 164 new SEPs in human serum samples [94]. Their outcomes reaffirm that MNPs can be utilized for high-throughput parallel protein separation before LC/MS. This technique is fast, efficient, reproducible, and simple to operate with 96-well plates and centrifuge tubes [94].

Ding and Yan et al. successfully developed hydrophilic poly(N, N-methylenebisacrylamide/1,2-epoxy-5-hexene)-coated magnetic nanospheres functionalized with 2-aminopurine (Fe_3O_4@poly(MBA/EH)@2AP) for the enrichment of glycopeptides and glycosylated exosomes using HILIC [95]. This innovative approach enabled the analysis of 290 glycosylated peptides and 184 glycosylation sites, corresponding to 185 glycoproteins in the serum of

uremic patients. Additionally, 42 glycopeptides were enriched from the saliva of healthy individuals, demonstrating promising efficiency in terms of sensitivity (0.01 fmol/μL), loading capacity (125 μg/mg), high selectivity (BSA = 1000:1), and repeatability (over 10 times) [95].

Reusability poses a significant challenge for MNPs, with limited available information on this aspect. Apart from reusability and the challenges associated with large-scale optimization, the recovery of MNPs is crucial. Efforts have been invested in addressing this issue. For instance, Powell and colleagues optimized a magnetic nanoparticle recovery device (MagNERD) designed to separate, capture, and reuse superparamagnetic Fe_3O_4 from treated water under continuous-flow conditions [96]. For further details about MNPs, readers are encouraged to refer to other reviews [75,97,98].

8. Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) is an efficient method developed for the electrophoretic analysis of proteins [99]. Since the net charge of proteins depends on pH, in this method, electrophoresis is achieved by pH changes [99]. In IEF, a sample containing a mixture of peptides or proteins is injected into a chamber where a pH gradient is established. An electric field is applied, causing basic species to migrate towards the cathode and acidic species towards the anode. Each peptide or protein halts migration when it reaches a zone in the gradient where the pH matches its pI. Subsequently, the peptide or protein is moved to a detection window where it can be identified [100]. IEF has been developed in several modes, including analytical and preparative scales, in either a cellulose-based separation medium [101] or solution [102].

Shen and colleagues developed conditions for high-efficiency capillary isoelectric focusing (CIEF) of peptides. They demonstrated these methods by separating a complex yeast cytosol tryptic digest employing conventional UV detection [103].

Pirmoradian and colleagues introduced a novel online multijunction capillary isoelectric focusing fractionator (OMJ-CIEF) for separating proteins and peptides in solution [104]. They subsequently optimized the OMJ-CIEF as a micropreparative device for fractionating more than 10 μg of complex peptide mixtures based on their pI [105].

Truman and colleagues reported enhanced yields for separating elastin-like proteins using pI-based phase separation (pI-BPS) [106]. This method leverages the proteins' pI, exposing them to conditions where their net charge is zero. This induces aggregation or phase separation of the proteins from the solution, facilitating their purification [106].

pIChemiSt is a free tool for calculating pIs of modified peptides. The authors examined 29 modified peptides and 119,093 natural peptides, achieving an improvement in R^2 values from 0.74 to 0.95 and 0.96 compared to conventional sequence-based approaches for the two studied pKa prediction tools, ACDlabs and pKaMatcher, respectively [107].

9. Membrane Filtration

A membrane serves as a barrier with selective permeability for certain species based on their charge, size, shape, or other characteristics. This selective permeability prevents the passage of some ionic and molecular components through the membrane (Figure 3) [108].

Membrane filtration is an efficient process for the purification of peptides, offering a level of purity comparable to that achieved through chromatography. Membranes are more productive and cost-effective than chromatographic methods. Enzymatic membrane reactors enable simultaneous production and separation of peptides, while electrodialysis–ultrafiltration provides ultra-selective peptide separation. Membrane filtration based on size selectivity can be grouped as nanofiltration (NF), ultrafiltration (UF), microfiltration (MF), or reverse osmosis (RO). NF and UF are the technologies of choice for the purification of bioactive peptides with a molecular-weight cut-off (MWCO) of 200–8000 Da and 1000–300,000 Da, respectively [109,110]. Various polymers are used as membrane material, including (1) polyethersulfone (PES) polymer, which is hydrophilic and has a higher negative surface charge [111,112]; (2) polyacrylonitrile [113]; (3) regenerated cellulose [114]; cellulose acetate [115]; and polyamides [116] for the isolation of proteins and peptides.

Membrane filtration can be successfully applied in a continuous mode and integrated into other separation methods [110].

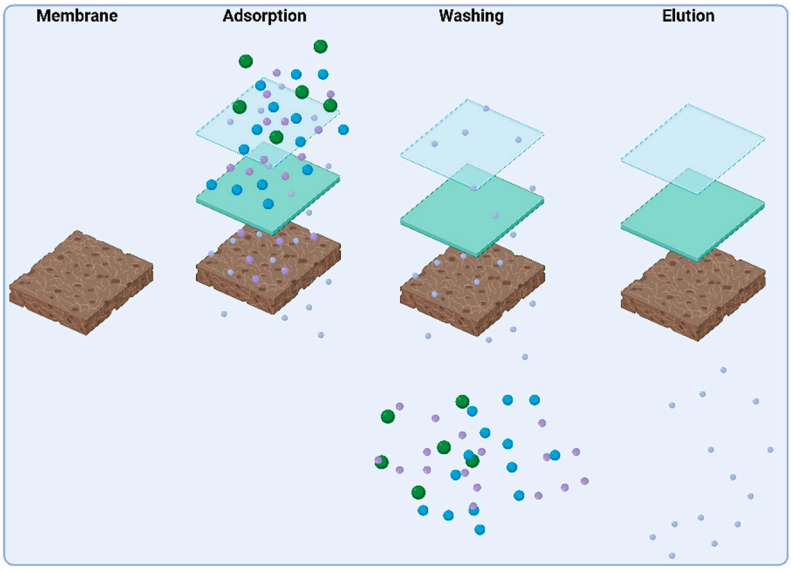

Figure 3. Schematic representation of the membrane filtration mechanism. Green/blue, impurities; purple, analyte.

Several bioactive peptides have been purified using membrane filtration. Beaubier et al. reported a successful purification of an antimicrobial peptide called neokyotorphin (NKT) using a regenerated cellulose UF membrane with 1 and 3 kDa MWCO [114]. Electrodialysis with ultrafiltration membranes (EDUF) has shown promise as an ultra-selective process for separating bioactive peptides. EDUF combines the charge selectivity of electrodialysis (ED) with the size-based exclusion capabilities of UF. On the other hand, enzymatic membrane reactors (EMRs) are recognized as effective technologies for online peptide purification and enzyme recycling. These reactors integrate enzymatic reactions with membrane filtration, allowing continuous purification of peptides while enabling efficient enzyme reuse [110].

Tight NF membranes with MWCO ranging from 200 to 1000 Da are frequently used for concentrating or desalting peptide solutions following UF separation. This process enables the production of highly stable, desalinated bioactive peptides suitable for various industrial applications. The peptides were obtained by performing peptic and tryptic hydrolysis on the primary proteins present in MPC, as simulated through in silico proteolysis. The sequences include β-casein (P02666) and αs2-casein (P02663), among others [117,118].

Roblet and co-workers reported successful peptide separation using ED with a filtration membrane (EDFM) [119]. They managed to isolate peptides rich in Glu, Lys, Arg, and His, achieving a 40% enhancement in glucose uptake for the final feed fraction at 1 ng/mL, synergistically with insulin [119]. They also assessed the impact of pH on the unfractionated salmon protein hydrolysate (SPH) to evaluate EDFM separations and to concentrate insulin-modulating peptides from a complex mixture [119]. The data indicated that pH had no significant effect on conductivity or peptide concentration in either the anionic (APC) or cationic peptide compartments (CPC) during the EDFM process of a salmon frame protein hydrolysate [119]. However, it was observed that the final feed compartment (FFC) and CPC after EDFM treatment at pH 6 significantly enhanced glucose uptake in L6 muscle cells grown in tissue culture [119].

Dibdiakova and co-workers studied different filtration processes for concentrating dipeptidyl peptidase IV (DPP-IV)-inhibiting bioactive peptides from chicken byproduct protein hydrolysates, including MF, UF, NF, and RO. Among the four membranes studied, the UF membrane exhibited the best separation properties for maximizing the yield and concentration of bioactive peptides. Overall, UF was demonstrated to be an effective

technology for removing undesired high-molecular-weight substances and concentrating small-molecular-weight bioactive peptides from chicken byproduct hydrolysate [120].

10. Sustainable Peptide Separation and Purification

There is a continuous emphasis on the need for innovative solutions, especially in synthetic methodologies, to produce peptides of the highest possible purity. This reduces the burden on necessary purification efforts and establishes a sustainable model for peptide synthesis and purification [121].

Acetonitrile is the primary solvent used in RPLC for peptide separation and purification, but it presents significant issues due to its cost and toxicity. However, greener alternatives such as isopropanol and ethanol have been proven effective in ion-pair-based chromatography as well [122]. We are also witnessing new advancements in this field, with Cabri et al. introducing dimethyl carbonate as a green alternative [123,124] to acetonitrile for purifying therapeutic peptides [125]. Promising data in terms of purity and recovery were obtained, along with a reduced analysis duration [125].

Intriguingly, Peyrin and Lipka demonstrated that preparative-scale SFC can be considered a green method, as it fulfills the six principles of green analytical chemistry: (i) Prevent waste through reduced solvent consumption: SFC uses CO_2 as a major solvent, reducing the overall solvent consumption compared to traditional liquid chromatography methods. (ii) Use safer solvents and processes: CO_2 is non-toxic, non-flammable, and readily available. It is considered safer compared to many organic solvents used in liquid chromatography. (iii) Increased energy: The use of CO_2 as a main solvent in SFC requires lower energy inputs compared to traditional liquid chromatography methods, where heating and cooling cycles are often necessary for solvent evaporation and separation processes. This efficiency contributes to reduced energy consumption and operating costs in laboratory and industrial settings. (iv) Avoid chemical derivatives: SFC typically uses minimal additives or derivatization steps, avoiding the use of unnecessary chemicals. (v) Analyze sample in real time to prevent pollution: SFC allows for real-time analysis, minimizing the need for extensive sample preparation steps that could lead to pollution. (vi) Minimize the potential for accidents: CO2 used in SFC is non-flammable, reducing the risk of accidents associated with flammable solvents. Preparative-scale SFC aligns well with these principles, making it a sustainable and environmentally friendly technique for chromatographic separations [126].

Ding and Yan considered an oxygen–amino ring-opening reaction to incorporate 2-aminopurine onto the surface of their developed nanomaterial [95]. This reaction is fast, mild, and catalyst-free, aligning with the principles of green chemistry [127].

11. Challenges

Various production routes were considered to isolate bioactive peptides, including fermentation of proteins by proteolytic microbes, proteolysis by enzymes from plants or microorganisms, and proteolysis by gastrointestinal enzymes [128]. Depending on the source of peptides, isolating them could sometimes lead to rendering them inactive. From a separation point of view, this will further complicate the process, increasing the cost. For example, peptides could develop a preferred domain of binding with the non-polar stationary phase [129], and denaturation could take place as well [130], resulting in an expected elution behavior [19]. The aforementioned obstacles could also be exacerbated during the successive purification steps. Therefore, the process must be validated in advance and with the aid of computational tools to explore the extent of these processes on the properties of the peptides being investigated [128].

Sustainability remains a paramount concern, with adherence to legislation adding significant burdens to processes like purification. While these innovations have hugely positive benefits for the health of humankind, it remains incumbent upon the pharmaceutical industry to ensure that their products are delivered in a sustainable manner.

Indeed, we have seen growing interest in this aspect when introducing new separation and purification techniques.

12. Conclusions and Future Prospect

With the noticeable increased demand of peptides, there is a clear need from the purification discipline to improve the purity standard of peptides, making them fit for purpose. Significant efforts have been invested to deliver pure peptides with sufficient yield that minimizes the need for carrying out additional syntheses. Most importantly, in some cases where peptides are originated from natural sources, it might not be easy to obtain additional amounts, as they are scarce, and this necessitates efficient purification techniques to obtain most of the isolated quantities and not to lose them. Therefore, advancement in the purification field has never stopped. Various properties of these valuable molecules have been considered to efficiently separate and purify them. Hydrophobicity is an important physicochemical property of peptides, and it has been exploited for their purification; a lot of computational algorithms have been developed with the aim of enhancing the overall purification task both at separation and purification scales.

It is evident that the exploitation of various techniques such as MMC and OMJ-CIEF has proven efficient in achieving synergistic effects for better purification. Moreover, DRPLC is an appealing technique, as it considers both the attractive and repulsive interaction forces between the analyte and the stationary phase. This dual-interaction approach provides flexibility in separating peptides with varying charges within a single setting. Second-dimensional separation techniques are not only effective in chromatography but also in other methods, such as combining electrophoresis with IEF, leveraging the advantages of both mechanisms [131]. These advanced methods enhance the separation and isolation of target peptides, improving the overall efficiency and effectiveness of the purification process.

A multistage UF process can be employed to obtain bioactive peptides with high purity and activity. EDFM is a promising technology due to its excellent flexibility and selectivity, showing potential for large-scale separation of BPs in the future.

Preparative chromatography is the large-scale liquid chromatography that aims at purifying larger quantities of the crude peptide. Multiple successive steps are generally considered to purify the crude sample, where the pure compound is collected, and the waste is discarded. Scaling up the analytical scale all the way to semi-preparatory and full-preparatory scales requires careful design to achieve the intended purpose with an optimum efficiency [8].

Sustainable purification, like other sustainable approaches, is crucial for protecting both the environment and human health [132]. These methods are gaining more traction, as they can be adopted under various circumstances and align with the regulations imposed by regulatory agencies. By prioritizing sustainability, the pharmaceutical industry can ensure compliance with legislation while minimizing environmental impact [124].

Progress in peptide separation and purification techniques is evident across various methods and not limited to HPLC alone. Continuous optimization and the adoption of second- and even third-dimensional techniques promise to enhance overall process throughput in terms of efficiency and time. Hence, it is crucial to not underestimate or overly favor any single technique at the expense of others.

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