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DOCTORAL COURSE IN CHEMICAL SCIENCES
CYCLE XXXIII

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From batch to continuous chromatography for the purification of therapeutic peptides, with particular emphasis on Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)

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List of papers

This thesis is based on the following papers. Reprints are appended at the end of the thesis.

I **Modeling the nonlinear behavior of bioactive peptide in reversed-phase gradient elution chromatography**, C. De Luca, S. Felletti, M. Macis, W. Cabri, G. Lievore, T. Chenet, L. Pasti, M. Morbidelli, A. Cavazzini, M. Catani, A. Ricci, *J. Chromatogr. A*, 1616, **2020**.

II **Determination of the thermodynamic behavior of a therapeutic peptide in overloading conditions in gradient elution chromatography**, C. De Luca, S. Felletti, G. Lievore, A. Buratti, T. Chenet, L. Pasti, M. Morbidelli, A. Cavazzini, M. Catani, M. Macis, A. Ricci, W. Cabri, *J. Chromatogr. Sep. Tech*, 11, **2020**, 1-4.

III **From batch to continuous chromatographic purification of a therapeutic peptide through multicolumn countercurrent solvent gradient purification**, C. De Luca, S. Felletti, G. Lievore, A. Buratti, S. Vogg, M. Morbidelli, A. Cavazzini, M. Catani, M Macis, A. Ricci, W. Cabri, *J. Chromatogr. A*, 1625, **2020**.

IV **Modern trends in downstream processing of biotherapeutics through continuous chromatography: the potential of Multicolumn Countercurrent Solvent Gradient Purification**, C. De Luca, S. Felletti, G. Lievore, T. Chenet, M. Morbidelli, M. Sponchioni, A. Cavazzini, M. Catani, *Trends Analyt. Chem.*, 132, **2020**.

V **Oligonucleotides: Current trends and innovative applications in the synthesis, characterization and purification**, M. Catani, C. De Luca, J. Medeiros Garcia Alcântara, N. Manfredini, D. Perrone, E. Marchesi, R. Weldon, T. Müller-Späth, A. Cavazzini, M. Morbidelli, M. Sponchioni, *J. Chromatogr. A*, 1454, **2016**, 86-92.

VI **Boosting the purification process of biopharmaceuticals by means of continuous chromatography**, C. De Luca, S. Felletti, G. Lievore, A. Buratti, M. Sponchioni, A. Cavazzini, M. Catani, M. Macis, A. Ricci, W. Cabri, *LCGC*, 6, **2020**, 30-34.

VII **Process Intensification for the Purification of Peptidomimetics: the case of Icatibant through Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)**, C. De Luca, S. Felletti, D. Bozza, G. Lievore, M. Morbidelli, A. Cavazzini, M. Catani, W. Cabri, M. Macis, A. Ricci, *in preparation*.

Papers not included in this thesis

The way to Ultrafast, High-Throughput Enantioseparations of Bioactive Compounds in Liquid and Supercritical Fluid Chromatography, O. H. Ismail, S. Felletti, C. De Luca, L. Pasti, N. Marchetti, V. Costa, F. Gasparrini, A. Cavazzini and M. Catani, *Molecules*, 23, **2018**, 2709-2720.

Recent Achievements and Future Challenges in Supercritical Fluid Chromatography for the Enantioselective Separation of Chiral Pharmaceuticals, S. Felletti, O. H. Ismail, C. De Luca, V. Costa, F. Gasparrini, L. Pasti, N. Marchetti, A. Cavazzini, M. Catani, *Chromatographia*, 82, **2019**, 65-75.

On the effect of chiral selector loading and mobile phase composition on adsorption properties of latest generation fully- and superficially-porous Whelk-o1 particles for high-efficient ultrafast enantioseparations, S. Felletti, C. De Luca, O. H. Ismail, L.Pasti, V. Costa, F. Gasparrini, A. Cavazzini, M. Catani, *J. Chromatogr. A*, 1579, **2018**, 41-48.

New frontiers and cutting edge applications in ultra high performance liquid chromatography through latest generation superficially porous particles with particular emphasis to the field of chiral separations, M. Catani, S. Felletti, O. H. Ismail, F. Gasparrini, L. Pasti, N. Marchetti, C. De Luca, V. Costa, A. Cavazzini, *Anal. Bioanal. Chemistry*, 410, **2018**, 2457-2465.

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Investigation of mass transfer properties and kinetic performance of high-efficiency columns packed with C18 sub-2μm fully and superficially porous, S. Felletti, C. De Luca, G. Lievore, L. Pasti, T. Chenet, G. Mazzoccanti, F. Gasparrini, A. Cavazzini, M. Catani, *J. Sep. Sci.*, 43, **2020**, 1737-1745.

Shedding light on mechanisms leading to convex-upward van Deemter curve on a cellulose tris(4-chloro-3-methylphenylcarbamate)-based chiral stationary phase, S. Felletti, C. De Luca, G. Lievore, T. Chenet, B. Chankvetadze, T. Farkas, A. Cavazzini, M. Catani, *J. Chromatogr. A*, 43, **2020**, 1737-1745.

Contents

1 Introduction

Production of biopharmaceuticals

Protein-based drugs have become an important class of therapeutics starting from the Eighties. The term "biopharmaceutical" was first used in that period to describe a class of therapeutic proteins produced by modern biotechnological techniques, especially via genetic engineering. Biopharmaceutical drugs structurally mimic compounds found within the body and are produced using biotechnologies. Recent advances in genomics, proteomics, recombinant expression technologies and peptide synthesis have led to an increased development of protein and peptide therapeutics [1–4].

By 2002, around 120 biopharmaceutical products had gained marketing approval in the USA and Europe, leading to a global pharmaceutical market of $15 billion [1]. In 2014, it was estimated that biopharmaceuticals generate global revenues of $163 billion, making up about 20 percent of the pharma market [5]. This value keeps into account hormones, blood factors, vaccines, monoclonal Antibodies, oligonucleotides, etc. Due to the chemical similarity between proteins and peptides, which are both constituted of amino acids chains, often peptides are classified into the category of biopharmaceuticals, though they are chemically synthesized.

Bioactive proteins and peptides are investigated as novel therapeutic compounds with several biological properties for potential medical applications. Development of protein- and peptide-based therapeutic products for medical use is growing continuously as they are receiving an increasing rate of approval by the United States Food and Drugs Administration [6]. Also, in the last months, the interest towards the potential of biopharmaceuticals has turned on, because many of the therapeutics under testing for the treatment of COVID-19 symptoms are based on biopharmaceuticals, such as monoclonal antibodies and oligonucleotides [7–9].

Biopharmaceutical drugs such as antibodies, peptides and recombinant proteins have

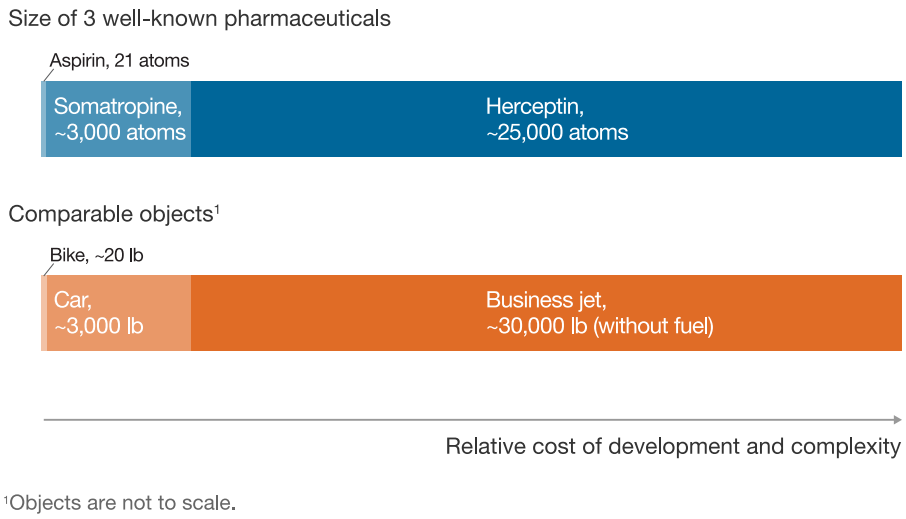

Figure 1.1: Comparison between sizes of aspirin, a traditional "small molecules" employed as pharmaceutical, and of two examples of biomolecules with very different molecular weight (somatropine and herceptine). Reproduced with permission from [5].

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high specificity and potency compared to small molecules. These features arise from their macromolecular composition, which provides the structural complexity that is often required for specificity. It must be highlighted that targeting a specific site of action often requires the aid of a delivery vehicle, the choice of which depends on the specific properties of the protein or peptide to be delivered, as well as the unique properties of the tissue being targeted.

Compared with traditional chemical pharmaceuticals, proteins are considerably larger molecular entities with inherent physiochemical complexities, from their primary amino acid sequences through higher-order secondary and tertiary structures and, in some cases, quaternary elements such as subunit associations [10–12]. To get the idea, aspirin, a small molecule, is made of 21 atoms, whereas a biopharmaceutical species can contain from 2000 to 25000 atoms (see Fig.1.1).

This structural complexity means that biopharmaceutical drugs are large and susceptible to degradation, which makes it challenging also to formulate and deliver them [13, 14]. Moreover, industrially it is impossible to obtain them as a pure compound and therefore their mixtures are usually quite complex from the chemical point of view. To ensure product safety and efficacy, every kind of drug must meet defined quality characteristics, imposed by regulatory agencies, such as Food and Drug Administration (FDA). Among these characteristics, a high degree of purity is fundamental for compounds with pharmaceutical scope. There are no general purity re-

quirements for biopharmaceuticals; anyway, even if the correlation between protein structure and function is under investigation, the impact of various impurities on the biological and pharmaceutical activity of a drug remains uncertain and can only be assessed through experimental measurements [2, 15].

The process of manufacturing a biopharmaceutical product entails two fundamental steps, called upstream and downstream processing respectively. *Upstream processing* includes the steps associated with the production of the biomolecules. Some of them are produced from mammalian or microbial cells (e.g. recombinant *E. coli* or yeast cultures), plant cell cultures and moss plants in bioreactors, while small peptides are usually produced via Solid Phase Peptid Synthesis. On the other side, *downstream processing* includes the chemical and physical separations necessary for the isolation and purification of the product itself from the complex culture or synthesis mixture [16,17].

Preparative liquid chromatography in batch conditions (operating with a single column), usually, is the technique of choice for the purification of therapeutic peptides and other biopharmaceuticals [18]. To isolate big amount of compound, large volumes of crude mixture are injected, which clearly contain many different species, considering the target product and its product- and process-related impurities. Thus, in this context, chromatography is operated in overloading (nonlinear) conditions, and this implies on the one hand that the peak resolution is limited, and on the other hand that the retention of the analytes depends on the concentration of every other species in solution, as it will be explained in the next chapters. Moreover, usually the separation is conducted under gradient conditions, because the retention of biomolecules is strictly related to the composition of the mobile phase (percentage of organic modifier, salt concentration, etc.) [19–25].

Knowing the thermodynamic behavior of a biomolecule in particular chromatographic conditions can help developing an optimized batch method for its purification in preparative chromatography, allowing to save time and chemicals (feed, solvents, etc.). Therefore, the first part of this thesis is related to the study of the adsorption mechanism of a peptide in reversed-phase gradient elution liquid chromatography, in overloading conditions.

In biopharmaceutical manufacturing, process development accounts for around 30% of costs, upstream processing for around 20%, but the highest outlay is attributed to downstream processing [26], which is responsible for around 50% of the total costs. The demand for new and effective biotherapeutic production methods has resulted in new technologies associated with both the production and the purification of protein-

based drugs. The latest innovations in the upstream of biopharmaceuticals, however, have not been equaled by corresponding improvements in the downstream process, which still represents the bottleneck in the whole production of biotherapeutics [27]. Therefore, currently there is a considerable pressure to reduce the costs of downstream processing, and also to improve the current production ways by introducing new concepts such as continuous manufacturing [28, 29]. Indeed the possibility of operating each one of the production steps in the continuous mode has been considered and investigated [30, 31].
Particularly, the second part of this thesis focuses on a purification process which works continuously and follows the principle of countercurrent chromatography. This technique is called Multicolumn Countercurrent Solvent Gradient Purification and, in this work, it has been applied for the purification of two real industrial peptide mixtures, which will be illustrated in the next chapters.

1.1 Modeling the chromatographic adsorption behavior of biomolecules in preparative conditions

As already said, liquid chromatography (LC) is one of the most used techniques industrially employed for the purification of biomolecules. Particularly, preparative conditions are used with the scope to purify and collect for future uses the target product. Differently than chromatography in analytical (*linear*) conditions, in preparative chromatography the analytes do not elute as gaussian-shaped, symmetrical peaks. On the contrary, their shape is asymmetrical and fronting or tailing phenomena can occur and become more evident when the sample amount injected increases. This happens because in *nonlinear* conditions the retention of a compound is concentration-dependent and this behavior is related to the shape of the adsorption isotherm of the compound of interest. In linear chromatography, the concentration of the analyte adsorbed on the stationary phase is directly proportional to its concentration in the mobile phase, therefore the isotherm is also linear. Moreover, in diluted conditions, the retention time does not change with the concentration of the compound. In overloading conditions, the concentration of the analyte adsorbed depends on the concentration of every species in solution. The analyte isotherm is not linear anymore and its peak shows a different shape depending on the isotherm model followed, which in turn depends on the chromatographic system. Therefore, in preparative chromatography, different species contained in the sample compete for the adsorption and influence the adsorption of each other. Industrially, working conditions for their purification are decided

on a trial-and-error basis, and this causes possible waste of time and of compound. Modeling the thermodynamic behavior involved in the adsorption of the target peptide is the basis to obtain information useful to optimize large-scale purification processes, such as the maximum loading. These data could be exploited in pharmaceutical industry, where the single column chromatographic methods are being substituted by continuous or semi-continuous processes to overcome the drawback of the purity-yield trade-off, which will be discussed in the following [27,32–37].

1.2 The downstream processing of biopharmaceuticals

During the last years, manufacturing of biopharmaceuticals has been intensively improved. As far as the upstream step of the biomolecules manufacturing is concerned, recombinant technology has been introduced recently, especially to obtain monoclonal antibodies, hormones and blood factors. With this respect perfusion bioreactors working in continuous are becoming more and more common and they are going to replace the traditional batch methods. But this is not the only upstream method feasible to obtain biomolecules.

Peptides are a class of biomolecules that can have a biological activity into the human body, such as they can act as neurotransmitters, hormones and regulators. Their bioactivity is mainly determined by their amino acid sequence. Also, they have high specificity for their target tissues and do not accumulate in the human organism [38]. For what concerns the production of peptides, they are usually chemically synthesized through Solid Phase Synthesis, a technique developed by Merrifield [39]. It consists of a series of step of coupling-wash-deprotection-wash. Basically, the first step is to bond the first amino acid to an insoluble resin; then one amino acid at a time is added and it bonds to the free extremity of the growing chain. Therefore, the chain lengthens from the C- to the N-terminal residue by means of a series of coupling reactions. The advantage of using this technique is the possibility to recover the product by filtration at the end of the synthesis; anyway, it is not possible to perform intermediate purifications. Nowadays, solid phase methodologies are often carried out in automated synthesizers.

As said, the downstream steps tend to dominate overall economics of the whole manufacturing of biotherapeutics, in terms of costs and also time. The industrial mixtures of peptides synthesized with the approach of Solid Phase Synthesis, for instance, can contain dozens of impurities chemically very similar to the target product. The number of impurities, in addition, increases with the complexity and size of the peptide

synthesized, because secondary reactions can occur at the level of the upstream step. The most common by-side products are species where one or more amino acids have reacted in the wrong position, or species containing chiral centers.
The purification methods chosen must distinguish between molecules which have similar chemical behavior, size, hydrophobicity or charge. Usually, a single method for the separation of the target compound from impurities is not sufficient to fulfill the purity requirements imposed [40,41]; thus, more purification steps orthogonal to each other are applied in the downstream processing. Preparative liquid chromatography is the most versatile technique suitable for this scope, generally conducted in batch conditions, meaning with the use of a single column.

1.2.1 Different kinds of impurities

The impurities contaminating the feed can be gathered in two groups.
The first kind of impurities is often referred to as *process-related impurities*, which are species not related to the target product from a chemical point of view, e.g. nucleic acids, host cell, components of the cell culture media, salts, etc. These species derive from the upstream process and can be removed quite easily by means of affinity chromatography, in a bind-and-elute mode [42]. For the purification of monoclonal Antibodies (mAb), for instance, the main ligand used as stationary phase is ProteinA, able to bind specifically but reversibly the mAb [43].
Let's consider Figure 1.2. In affinity chromatography, the feed is loaded into the column in big amount, until the saturation of the resin and the breakthrough of the product. The product binds specifically to the stationary phase, whereas all the other species are washed away by the mobile phase. Then, the column is washed and, successively, elution conditions are changed in order to recover the product and to clean the column. During this phase, what is important is to recover the maximum amount of the target protein from the biological feed injected, whereas it is not necessary to satisfy strict purity requirements. At the same time, it is advisable to obtain the pre-purified product with a high concentration, in order to reduce the volume to be treated in the following steps. This first step is called *Capture* and it is applied to mixtures of biomolecules, especially proteins, obtained starting from cells cultures; it is not performed for peptides synthesized through Solid Phase Synthesis. It is to be noted that stopping the loading when the breakthrough begins avoids the product loss but also limits the utilization of the resin and the productivity. This is the main disadvantage of performing the capture step in batch conditions.

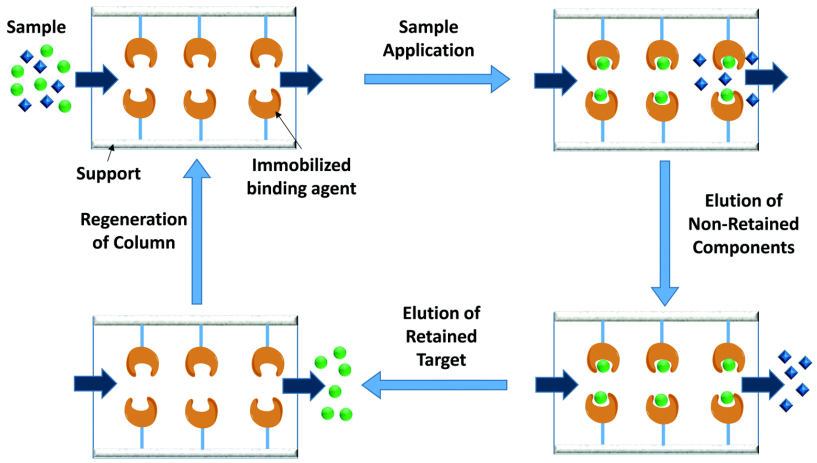

Figure 1.2: Example of a batch process exploiting the principles of affinity chromatography. On the stationary phase, a binding agent (such as ProteinA) is immobilized (top left). The feed is loaded into the column; only the target product (such as an antibody) can interact with the binding agent, whereas all the impurities are not retained and elute (on the right). Then the mobile phase composition changes, in order to release the target and to regenerate the column (bottom left).

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The second group of impurities gathers the so-called *product-related impurities*, which are removed with a number of *Polishing* steps. Examples of product-related impurities are fragments, aggregates and variants of the protein or peptide of interest, which means species that vary only slightly in their primary sequence as compared to the target [44]. The latter are usually the most difficult species to remove because of their high similarity with the target. In this case, impurities and product contain similar functional groups [2, 43], thus affinity chromatography is not useful at this level; reversed-phase, ion-exchange, size exclusion or hydrophobic interactions chromatography are typically employed because they exploit differences in charge, size or hydrophobicity of the analytes to separate [33, 45]. The similarity of the target product and the impurities leads to a situation where the compound of interest elutes halfway between two other groups of impurities: weakly (early eluting) and strongly (later eluting) adsorbing impurities. Moreover, in preparative conditions an overload of the feed occurs, and this contributes to worsen the overlap between the main peak and the adjacent impurities peaks [46]. Lower loadings, on the one hand, improve the separation but, on the other hand, cause a decrease in productivity, which can be detrimental for companies from the economical point of view [47]. The slope of the gradient has also an

impact on the separation: a steep gradient risks to cause the co-elution of the product with other impurities. On the other hand, a shallow gradient might improve the separation, but the productivity would be negatively affected by the increase of the time.

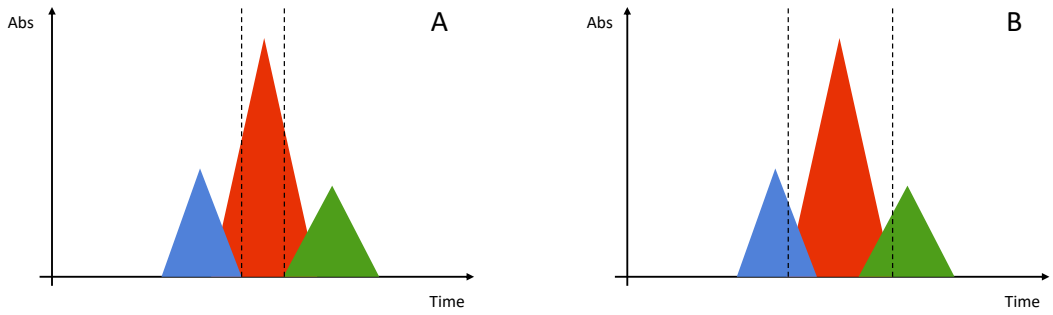

Figure 1.3: Purity-yield trade-off in a preparative batch chromatogram. The first group of species eluting are the weakly adsorbing impurities (red peak), then the target, which is intermediate (blue peak) and last the strongly adsorbed impurities (green peak). The product peak is not completely resolved, therefore one can choose to collect either a narrow window (case A), containing the product with high purity but scarce recovery, or a broad collection window, with high recovery but limited purity (case B).

Because of this not negligible overlap, one must choose whether it is preferable to collect a small amount of very pure product (narrow collection window) or a large amount of product with low purity (broad collection window). In the first case, high purity comes at expenses of the recovery, whereas in the second case the purity must be sacrificed to obtain a high recovery. This dualistic behavior is the so-called *"purity-yield trade-off"* and corresponds to the main drawback intrinsic to batch chromatography (see Fig.1.3) [32, 48].
For particularly valuable and expensive compounds, which is most often the case of biopharmaceuticals, the overlapping parts of the chromatogram cannot be simply discarded and wasted, but they must be recycled to recover the product contained in them. In batch chromatography, the recycling is performed manually by the operator, with risks of errors and waste of time. This means that the fractions containing impure product are collected, pooled and reinjected during a successive run, together with some fresh feed.

1.2.2 The advantages of continuous chromatography

The limitations encountered during both the capture and the polishing steps can be addressed by means of continuous chromatographic techniques [47, 49]. The reason is that they are based on the principles of countercurrent chromatography, where it is simulated that the stationary phase moves in the opposite direction with respect to the mobile phase.

The concept behind countercurrent chromatography is to exploit the different affinity of two compounds for the stationary phase. Figure 1.4 shows a simple scheme representing what happens to a binary system in countercurrent chromatography [31]. A slow turtle and a fast rabbit represent two different analytes, moving to the right in the direction of the arrow (mobile phase). At some point they come into contact with a conveyor belt (stationary phase) which moves in the opposite direction. The animals have different velocities with respect to the conveyor belt. The slow turtle cannot overcome the velocity of the conveyor belt, thus it is transported to the left. The turtle moves together with the conveyor belt; it represents a strongly adsorbed compound, which moves slowly into the column because of its great affinity to the stationary phase. On the contrary the fast rabbit can continue running to the right, in the same direction as the mobile phase; it represents a compound only weakly adsorbed into the column. Therefore, at the end of the process, the animals have been separated at the two sides of the conveyor belt, whose movement simulates the opposite movement of the stationary phase with respect to the mobile phase. In principle, a complete separation of the two solutes can be achieved.

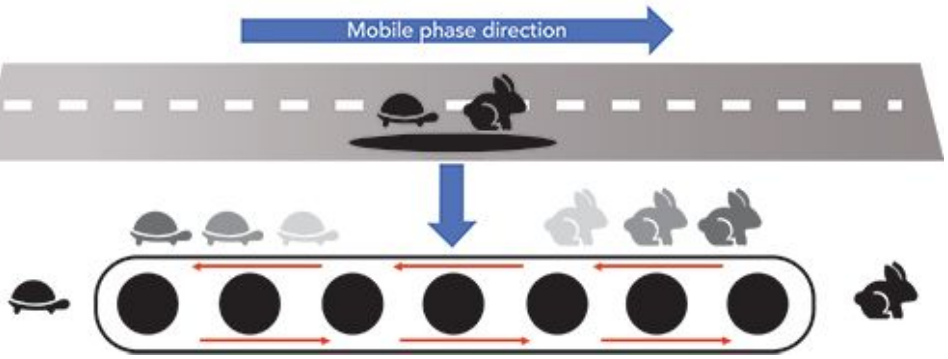

Figure 1.4: Schematic representation of the countercurrent chromatography. The turtle (strongly adsorbed compound) has a higher affinity towards the stationary phase than the rabbit (weakly adsobed compound), therefore apparently it moves slowlier. Their different velocity causes their separation at the edges of the conveyor belt. Modified with permission from [31]

Figure 1.5: Comparative scheme between True Moving Bed (on the left) and Simulated Moving Bed (on the right). In SMB, the countercurrent movement of the stationary phase is simulated through the switching of the valves, which connect the columns of the multicolumn system. Modified with permission from [2].

The theory of countercurrent chromatography can find different applications. For example, in the process called *True Moving Bed* (TMB) the solid phase moves phisically in the opposite direction than the mobile phase [50]. This technique is usually unpractical because of the technical difficulty to move the the solid phase without generating multiple flow paths and particle attrition [2]. Apart in the case of TMB (True Moving Bed), which anyway is hardly ever employed, countercurrent chromatography is always realized by employing multicolumn systems. In this cases, instead of moving physically the chromatographic medium, the outlet and inlets of the columns are moved with respect to the chromatographic medium [51–53]. This means that the movement of the injection and collection points, opposite to the movement of the eluent, allows simulating the countercurrent movement of the stationary phase. Clearly, the injection and collection points cannot be moved continuously along the chromatographic column. Therefore, several columns are used in series and the positions of the inlet and outlet streams are moved intermittently between the columns at regular time intervals. Basically this is accomplished through some switching valves, which change the connection between the columns and thus regulate the path accessible to the mobile phase and, as a consequence, to the analytes. This leads to great advantages in terms of recovery and productivity of the process [31, 47, 54, 55].

Different techniques are based on this mechanism of separation. One of the most well-known is *Simulated Moving Bed* (SMB), a general scheme of which is reported in

Fig.1.5. This technique, anyway, can only be applied for the separation of two species (binary separations) having different affinity towards the stationary phase. For example, since the '90s, SMB has been intensively applied for the separation of enantiomeric mixtures, where only one of the components interacts with the stationary phase [24]. Moreover, it can be used only for purification in isocratic conditions [56–59]. For further information about countercurrent chromatography and SMB the reader is referred to the literature [52, 60–62].
Continuous chromatographic processes find application both in the capture and in the polishing steps of biopharmaceuticals manufacturing.

Continuous processes for the Capture step

When the capture step is executed on a single column, for example for the purification of mAbs (monoclonal antobodies) with proteinA stationary phase, the feed is loaded in big amount to saturate the column [63]. Thus the product is accumulated on the stationary phase until the beginning of its breakthrough. However, if the mass transfer resistance is elevated, which is often the case of large biomolecules, the breakthrough curve broadens. Therefore, to prevent product loss, the column cannot be loaded until its dynamic binding capacity has been reached (see Fig.1.6, on top). On the other hand, interrupting the loading well before reaching the maximum saturation capacity leads to scarce resin utilization, with consequent decrease in productivity and increase in solvent consumption [47].
The solution to this issue in batch is a two columns countercurrent process called Capture-SMB [65]. Let's consider the bottom part of Fig.1.6. Two affinity columns are connected in series during the loading step, so that the first one can be saturated to a higher degree than in batch, beyond its dynamic breakthrough capacity. Successively, the first column is washed with a buffer, so that the portion of product that is not retained from the first column is not wasted but flows into the second column and adsorbs there [42, 64]. The columns are successively disconnected: the first is eluted to recover the product purified and equilibrated, the second keeps being loaded until it reaches 1% breakthrough. In the third step, the second and first columns are interconnected and loaded in series analogously to the first step. At this point, it is the second column which is being loaded. In the fourth step, the second column is washed, eluted, cleaned, and reequilibrated while loading continues on the first column. This step represents the end of one cycle.
This process allows to maximize the resin utilization and improves the productivity.

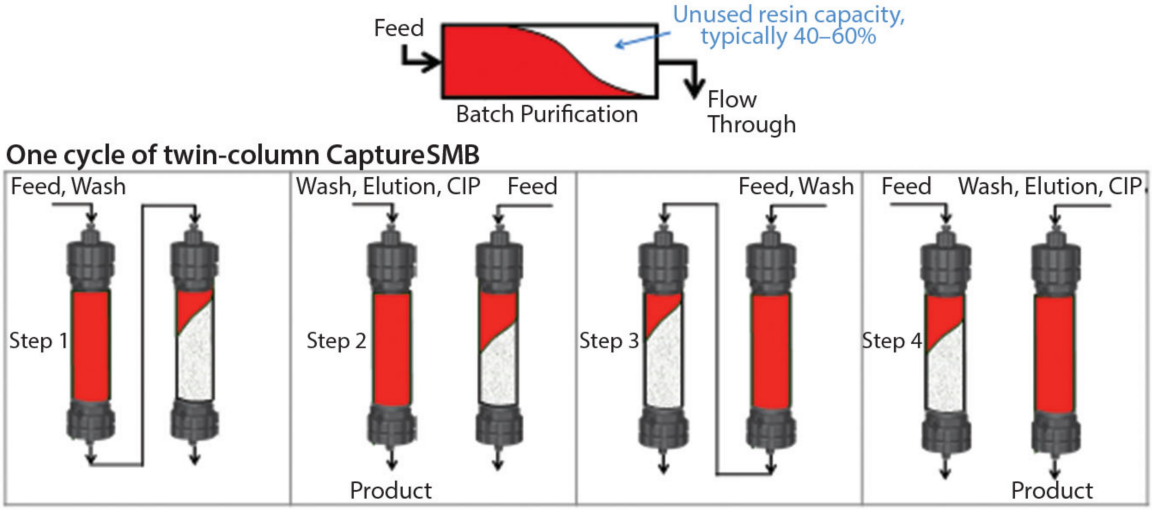

Figure 1.6: Scheme of the batch capture process (on top) and of one cycle of twin-column Capture-SMB (bottom). CIP means Cleaning-In-Place. Reproduced from [64].

In the case of Capture-SMB, the internal recycling occurs during the step of the loading [47, 49].
It must be highlighted that in Capture-SMB it is not possible to work in gradient conditions and only isocratic conditions can be applied [66]. Moreover, only binary mixtures can be purified with this technique, since the stationary phase only distinguishes between species which exhibit an affinity for the ligands and species that don't.

Continuous processes for the Polishing steps

Differently from capture step, the scope of polishing steps is to obtain the product with a high purity, by removing all the product-related impurities, produced during the upstream part of the process. As already stated above, the similarity between the product and these impurities leads to the impossibility of using affinity chromatography for this kind of purification. Moreover, their chemical similarity does not allow a good resolution of the peaks in overloading conditions, causing the so-called "purity-yield trade-off". The overlapping regions in batch chromatography are manually recycled. On the contrary, continuous (or semicontinuous) chromatography allows the *internal* recycling of the portions of the chromatogram where product and impurities coelute, and this step is performed automatically by the instrument.
Nowadays, the most suitable semicontinuous technique for the polishing steps in biopharmaceutical industry is the twin-column Multicolumn Countercurrent Solvent

Gradient Purification (MCSGP), because it can be applied to ternary separations, where the product elutes as intermediate between two other groups of impurities, and, what's more, it works also for separations requiring an organic modifier gradient, which is the case of large biomolecules [20–22,24,26,42,60,67]. Contrary to the capture process, where the recycling takes place during the loading, in MCSGP it occurs during the elution.

The MCSGP process will be discussed in detail in Chapter 3.

2 Adsorption equilibria in liquid chromatography

2.1 Model-based optimization of a chromatographic process

Reversed-phase (RP) chromatography represents one of the most important techniques for analysis and purification of proteins and peptides [68–70]. Purification processes at industrial level are developed under strong time limitation and with small amount of compound available. Those constraints often obstruct the use of detailed process modeling. Usually, the optimization of chromatographic processes is done empirically, on a trial-and-error basis. This empirical approach generally leads to suboptimal process performance. However, lately strong efforts have been made to incorporate process modeling in the optimization of chromatographic processes [71–74]. The results of investigation of thermodynamic equilibria influencing the separation in overloading condition, for instance, could be used to optimize the transfer of a batch method to the MCSGP technology, since it has been demonstrated that the oucome of a twin-column MCSGP method is strictly related to the outcome of the batch process [66].
Proteins and peptides bear chemical groups with physical and chemical properties significantly different (acidic or basic groups, hydrophobic or hydrophilic groups etc.). The mobile phase composition has a great influence on the conformation assumed by these macromolecules and, thus, on their retention. As a consequence, even by changing the content of organic modifier in the mobile phase by few percentage points, the retention factor can be greatly increased or decreased [19, 21, 75–78].
In this thesis, thermodynamic data, namely the adsorption isotherms *in isocratic conditions*, were collected through the so-called Inverse Method (IM), a numerical approach which is particularly appealing when the compound to purify is available in very low amount or extremely expensive. In these cases, IM results more suitable than tradi-

tional techniques, such as Frontal Analysis. Nevertheless, as already said, the purification of biomolecules (in the case of this study, peptides) is carried out *in gradient conditions*, meaning that the mobile phase composition changes during the elution. Thus, the modeling of the chromatographic behavior during gradient elution must keep into account the correlation of the retention with the variation of the mobile phase composition. Basically, it must be considered that the isotherm parameters are not constant along the gradient.
In the next sections, an overview of some thermodynamic aspects of adsorption in linear and nonlinear chromatography will be provided.

2.2 Definitions

First of all, the distinction between linear and nonlinear chromatography must be made. In linear chromatography, the concentrations of a species in the mobile and stationary phase are proportional at equilibrium. This means that the adsorption isotherm (the equation correlating the concentration of a component in the stationary phase with its concentration in the mobile phase) is a linear curve. The peaks are gaussian, symmetrical, and their retention times do not depend on the composition of the sample injected. Linear chromatography generally can explain phenomena observed in analytical applications.
On the other side, nonlinear chromatography keeps into account phenomena contributing to the asimmetry of the peaks, which most often occurs in overloading and preparative applications. In this case, the adsorption isotherm of a component is not linear, and this means that the concentration of a compound in the stationary phase is not proportional to its concentration in the mobile phase, at equilibrium. Also, the equilibrium isotherm of a species is dependent on the concentration of all the other species in solution. Therefore, the band profile and retention time of a component depends on the composition and amount of the sample injected. Preparative applications are part of the nonlinear chromatography. The issues related to the nonlinearity of the isotherm are clearly complex because of the interdependence of the concentrations of all the components in the stationary and mobile phases.
Considering the mass transfer kinetics, another classification can be made. If the column efficiency and the rate of mass transfer kinetics can be considered infinite, meaning that the axial dispersion is negligible, this is the case of ideal chromatography. It means that the stationary phase is always at equilibrium with the mobile phase, and the only factor affecting the peak shape is the thermodynamics of adsorption. Other-

wise, in nonideal chromatography the column efficiency is limited. Different assumptions can be made regarding the reasons behind band broadening phenomena; every assumption corresponds to a different chromatographic model.

2.3 Chromatographic models

2.3.1 Mass balance equation

In every chromatographic separation, there is one parameter which is conserved: the mass of every component injected. This axiom is resumed in the Mass Balance Equation (MBE).

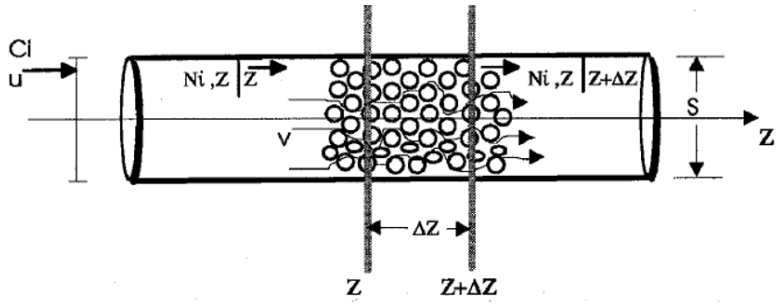

Figure 2.1: The mass of a component is conserved within a column slice. Reproduced from [19].

Let us consider Fig.2.1. The column is assumed to be radially homogeneous; the concentrations of the components do not change along the diameter. Therefore, the only independent variables are the time t and the length of the column z. C_i represents the concentration of the compound i in the mobile phase, whereas q_i represents the concentration of the compound i in the stationary phase. Also, it is assumed that different species do not react with each other during the chromatographic run.
The MBE adfirms that the difference between the mass of compound i which enters a portion of column of thickness Δz during the time Δt and the quantity of i leaving the slice during the same time is equal to the amount of i accumulated in the slice. The differential form of MBE for a component, obtained for Δz and Δt which tend toward 0, is expressed as:

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_{L,i}\frac{\partial^2 C}{\partial z^2} \tag{2.1}$$

where C and q are the concentrations of the analyte in the mobile and stationary phases respectively. $F = (1 - \epsilon_t)/\epsilon_t$ is the phase ratio and $\epsilon_t = V_0/V_{col}$ is the total porosity of

the column (with V_0 and V_{col} the thermodynamic void volume and the column voume, respectively); u is the local average mobile phase velocity and $D_{L,i}$ is the axial dispersion coefficient of the compound i in the mobile phase. The first two terms on the left correspond to the accumulation terms in the mobile and stationary phase, the third term is the convective term and on the right there is the diffusion term.
One MBE must be written for each component of the system, including the mobile phase. However, knowing the MBE of a component is not sufficient to predict its band profile shape. First, a set of initial boundary conditions is required. Another information necessary to solve the MBE is the correlation between the local concentrations of a compound in both phases.
To simplify the problem, some assumptions can be made, which correspond to different chromatographic models. The chromatographic behavior of a species can be modeled through equilibrium or kinetic theories. When mass transfer and adsorption processes are considerably fast, their contribution to band broadening are negligible and istantaneous equilibrium between the mobile and stationary phases is reached. If it can be assumed that the two phases are always at equilibrium, the relationship between the stationary phase concentration of a compound and its concentration in the mobile phase is given by the equilibrium isotherm. On the contrary, if non-equilibrium effects take place because of slow mass transfer or adsorption processes, it is better to use kinetic theories to describe the chromatographic process.
In the following, the ideal model and the Equilibrium-Dispersive model will be illustrated. During the years, other more complex models, such as lumped kinetic model and general rate model, have been developed but will not be further considered in this thesis.

2.3.2 The ideal model

The simplest model of chromatography is the ideal model. As said in Sect.2.2, the ideality lies in assuming the column efficiency infinite. Axial dispersion is nil and the stationary and mobile phases are always in equilibrium. Therefore, the diffusion term in Eq.2.1 is zero:

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = 0 \tag{2.2}$$

The ideal model considers as negligible the mass transfer kinetics and axial dispersion. The band broadening is due only to the non-linearity of the adsorption isotherm.

2.3.3 The Equilibrium-Dispersive model

The Equilibrium-Dispersive model (ED) describes processes where the mass transfer kinetics is fast but not infinite. Therefore, the MBE (Eq.2.1) can be modified in

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_a\frac{\partial^2 C}{\partial z^2} \tag{2.3}$$

where $D_{a,i}$ is an apparent dispersion coefficient that lumps together all the contributions to band broadening of the component i, such as diffusion phenomena and finite rate of mass transfer kinetics (adsorption-desorption). Also, it is assumed that D_a is independent on the concentrations of the components in the sample. The apparent dispersion coefficient can be expressed as

$$D_a = \frac{uL}{2N} \tag{2.4}$$

where u is the mobile phase linear velocity, L the length of the column and N the number of theoretical plates.

2.4 Thermodynamic aspects of adsorption

For the resolution of the mass balance equation of a component, whichever its mathematical form is, it is necessary to correlate its concentration in the mobile phase with its concentration in the stationary phase. Moreover, different species can compete for the adsorption, and this factor must be kept into account. Therefore, in liquid chromatography, the problem of determining the stationary phase concentration of each component is extraordinarily complex, because the concentration of every component in solid phase must be related to the concentration of all the other species in the mobile phase.

This correlation, at constant temperature and pressure, is given by an adsorption isotherm. Several adsorption models have been developed to describe different possible situations, depending on the interactions established between the molecules of adsorbate and depending on the homogeneity of the adsorbent surface. The isotherm model regulating the adsorption of the components can be revealed by the peak profile shape (gaussian or asymmetric, with fronting or tailing phenomena).

In the following, the most used isotherm models will be illustrated.

2.4.1 Linear isotherm model

The simplest isother model for a single component is the linear isotherm. The concentration of the analyte in the stationary phase is proportional to its concentration in the

mobile phase. The slope of this curve, a, is the Henry's constant of adsorption, which in turn is related to the retention factor of the component and to the phase ratio F:

$$q = aC = \frac{k'}{F}C \tag{2.5}$$

The linear isotherm model applies for low concentrations injected, therefore it is usually encountered in analytical applications. Since the mass loaded is low, the molecules of analyte do not compete for the adsorption on the stationary phase.
In this case, peaks are symmetrical, gaussian, and the retention time does not change with the concentration of the species.

2.4.2 Langmuir isotherm model

In this single-component model, the solid surface is assumed to be energetically homogeneous, meaning that all the adsorption sites are equivalent. The molecules of analytes can adsorb only in a monolayer configuration, they cannot interact with each other. The Langmuir isotherm equation is:

$$q = \frac{aC}{1 + bC} = \frac{q_s bC}{1 + bC} \tag{2.6}$$

where b and a ($= q_s b$) are the equilibrium and Henry constants of adsorption, respectively. q_s represents the saturation capacity, which is the maximum concentration of analyte that can adsorb on the stationary phase. These values are dependent on the temperature and on the content of organic modifier in RP-LC.
It can be demonstrated that if C tends to 0, the Langmuir isotherm becomes a linear curve, as in the Eq.2.5, where $a = q_s b$.
The Langmuir isotherm is a curve convex upward; this model leads to peaks that show a shock in the front and a diffuse boundary in the tail. On the other side, isotherms convex downward are defined "anti-langmuirian", and their peaks show a diffuse boundary in the front and a shock in the rear.
Even if this model is quite simple, it has been demonstrated that it is a good approximation for different kind of single-component chromatographic processes in nonlinear conditions. In the next chapters of the thesis, the attention will be focused on this particular adsorption isotherm rather than on other simpler or more complex ones.

2.4.3 Bilangmuir isotherm model

This model assumes the the existence of two different adsorption sites on the stationary phase. The component can interact differently with the two sites; its concentration

on the stationary phase depends on the adsorption of the compound on both the sites:

$$q = \frac{q_{s,1} b_1 C}{1 + b_1 C} + \frac{q_{s,2} b_2 C}{1 + b_2 C} \tag{2.7}$$

$q_{s,1}$ and $q_{s,2}$represent the saturation capacities of the two sites; b_1 and b_2 are the adsorption constants of the sites. This model is often employed to fit enantiomers separations on chiral stationary phases, where it is assumed that one site has enantioselective features while the other is non-selective.

2.4.4 Tóth isotherm

The Tóth isotherm accounts for the heterogeneity of the solid surface through the parameter t and it assumes no adsorbate-adsorbate interactions. The adsorption sites show a unimodal adsorption energy distribution. The equation is

$$\theta = \frac{q}{q_s} = \frac{C}{(b + C^t)^{\frac{1}{t}}} \tag{2.8}$$

The Tóth isotherm becomes the Langmuir isotherm for $t = 1$.

2.4.5 Bi-Moreau isotherm

Sometimes, it can happen that the peptide peak changes shape with increasing the loading. For example at relatively low loading the peak has a shock in the front and a dispersed rear, similarly to what happens if the isotherm is Langmuirian. But when the column is further overloaded, the peak profile changes shape, showing a shock in the rear and a dispersed front. This behavior at higher concentrations indicates that adsorbate-adsorbate interactions establish, as it happens for the Moreau isotherm. Therefore, in order to describe the peptide adsorption, it must be considered that there are two different adsorption sites on the surface: the first one is Langmuirian and dominates at low peptide concentrations, whereas the second site is a Moreau type and is predominant at higher loading. The overall adsorption isotherm becomes

$$q = \frac{q_{s,1} b_1 C}{1 + b_1 C} + \frac{q_{s,2} b_2 C (1 + I b_2 C)}{1 + 2 b_2 C + I b_2^2 C^2} \tag{2.9}$$

where I is a parameter which represents the adsorbate-adsorbate interaction; b_1 and b_2 are the adsorption constants for the two sites and $q_{s,1}$ and $q_{s,2}$ are the saturation capacities for the two sites. Clearly, this model is very complex because it requires the knowledge of 5 parameters for every species involved and therefore more simplex isotherms can often be employed to simplify the calculus.

2.5 Adsorption isotherm determination: the Inverse Method

The Inverse Method (IM) is a numerical method which allows to determine the isotherm parameters from experimental profiles in overloading conditions. In this thesis, IM has been used to calculate the isotherm parameters in isocratic conditions, when the content of organic modifier in the mobile phase does not change during the run. Only small quantities of sample are necessary to perform this study [79–81].
Firstly, the detector must be calibrated. To do this, the column is replaced with a zero-dead-volume connector and 500 μL of every standard of peptide, with a different concentration each, are injected. The plateau absorbance found at the wavelength of interest is reported in a graphic in function of C (concentration of the component in the mobile phase). *Experimental* profiles at different concentrations in isocratic conditions are recorded; their profiles (Abs vs. time) are converted in C vs. time, using the slope of the calibration curve. Then, on the basis of the peaks shape, an isotherm model and a set of initial parameters are chosen. To obtain a *calculated* profile, a system of equations (including the mass balance equation and the isotherm model chosen) needs to be solved. The calculated and the experimental profiles are then compared and, if the agreement is not satisfactory, the set of parameters is iteratively changed until the match between the profiles is good. The numerical optimization was made using the Simplex method, minimizing the sum of the squares of the differences between the simulated and experimental profiles [19, 82–84]. In the end, a different set of isotherm parameters is found for every fraction of organic modifier in the mobile phase. A correlation between the parameters and the experimental conditions used is therefore necessary to model the adsorption behavior in gradient elution.

2.6 Modeling of overloaded profiles in gradient conditions

As said in Sect.2.4.2, the isotherm parameters are strongly related to the experimental conditions chosen, such as the content of organic modifier in the mobile phase. In gradient elution chromatography, in reversed-phase conditions, the fraction of organic modifier (ϕ) is increased constantly with time. If the range of ϕ in which the compound of interest elutes is quite narrow, it is possible to consider that the isotherm type is not affected by changes in the mobile phase composition, but its parameters change with ϕ [21,79,85]. The linear solvent strength model correlates the variation of the retention

factor with the content of ϕ [86–88]:

$$lnk(\phi) = lnk_0 - S\phi \tag{2.10}$$

k_0 represents the retention factor extrapolated in absence of organic modifier in the mobile phase ($\phi = 0$). S is a coefficient characteristic of the system solute-solvent.
By considering the Langmuir equation (Eq.2.6) and remembering that $a = \frac{k'}{F}$, from the Eq.2.10 it can be derived that a has an exponential dependence from ϕ:

$$a(\phi) = a_0 e^{-S\phi} \tag{2.11}$$

The same relation connects b and ϕ if the range of ϕ is narrow enough, because q_s can be considered constant [19,89,90]:

$$b(\phi) = b_0\, e^{(-S\phi)} \tag{2.12}$$

If Eq.2.12 is combined with the Langmuir isotherm (Eq.2.6), a new Langmuir isotherm is obtained, which keeps into account the variation of the parameters with ϕ during elution:

$$q(\phi) = \frac{q_s\, b_0\, e^{(-S\phi)} C}{1 + b_0\, e^{(-S\phi)} C} \tag{2.13}$$

The mass balance equation of the Equilibrium-Dispersive model (Eq.2.3) can be numerically solved by applying a finite difference method based on the so-called backward-backward scheme [19,21].
Lastly, boundary and initial conditions need to be defined in order to solve the mass balance equation. The Danckwerts-type boundary conditions have been applied [19, 91,92] while the gradient in the inlet feed has been simulated as follows:

$$\phi(t,0) = \begin{cases} \phi_0 & 0 \le t \le t_{inj} \\ \phi_0 + \frac{\Delta\phi}{t_g}(t - t_{inj}) & t_{inj} \le t \le t_{inj} + t_g \\ \phi_0 + \Delta\phi & t \ge t_{inj} + t_g \end{cases} \tag{2.14}$$

where t_{inj} is the length of the rectangular injection profile, ϕ_0 is the initial fraction of organic modifier and t_g is the duration of the gradient.
The equations just described have been used to model the chromatographic behavior in overloading conditions of an octapeptide, during a gradient elution.

3 Fundamentals of MCSGP

As mentioned before, it frequently happens that batch purifications, especially when many product-related impurities are present, are affected by a yield-purity trade-off. This trade-off states that a high purity comes at the price of a high recovery and vice versa. Clearly, the resolution of the main peak is the main factor affecting the performance of the whole process. In order to modulate the peaks separation, it is advisable to work in gradient conditions, since the retention of biomolecules is hugely dependent on the composition of the mobile phase, on the salt concentration or on the percentage of organic modifier [24, 25]. The slope of the gradient has also an impact on the separation: a steep gradient risks to cause the co-elution of the product with other impurities. On the other hand, a shallow gradient, as well as a lower loading, might improve the separation, but the productivity would be negatively affected by the increase of the time. Alternatively, stationary phase with smaller particles could be employed, but this would cause higher backpressures. Therefore, these options are quite impractical [66, 93].

To overcome the limits intrinsic of batch chromatography for polishing applications, an appealing possibility is to use the Multicolumn Countercurrent Solvent Gradient Purification process, a semicontinuous technique. To realize it, the instrument must be equipped with two or more identical column which are connected or disconnected through a system of valves [43]. In its very first arrangement, MCSGP was realized with six columns [25, 48, 94]. Later on, its setup has been more and more simplified until arriving at the version with three [95, 96] or even two columns [97, 98]. The fewer the columns, the simpler the instrument is, in terms of tubings and valves. Nowadays, the MCSGP process can be performed on a unit called ContinChrom CUBE combined (YMC ChromaCon AG, Zürich, Switzerland).

3.1 The *Design Batch Chromatogram*

The first operation to develop an MCSGP process is to define a batch method where at least a portion of the chromatogram shows a sufficient purity. This is the so-called *Design Batch Chromatogram*.

Let's consider Fig.3.1, which represents a chromatogram obtained in batch conditions. In batch, the loading of the feed happens in a single step (step 1). During the elution, 5 different windows can be recognized. First, weakly adsorbing impurities (W) elute; the target product is not present in this window (step 2). In the region W/P (step 3), while W are still eluting, also the product starts eluting. In this portion of the chromatogram, the purity is rather scarce, but the amount of peptide is not negligible and therefore it cannot be wasted. During the step 4, the product P elutes as a pure or almost pure window. Successively, another overlapping region is present, where P elutes together with strongly adsorbing impurities (S), during the step 5. In the end, the column is stripped with a high percentage of organic solvent to remove S and finally equilibrated (step 6). The unresolved side portions of the peak, corresponding to step 3 and 5, need to be recycled to recover the product contained in them. When

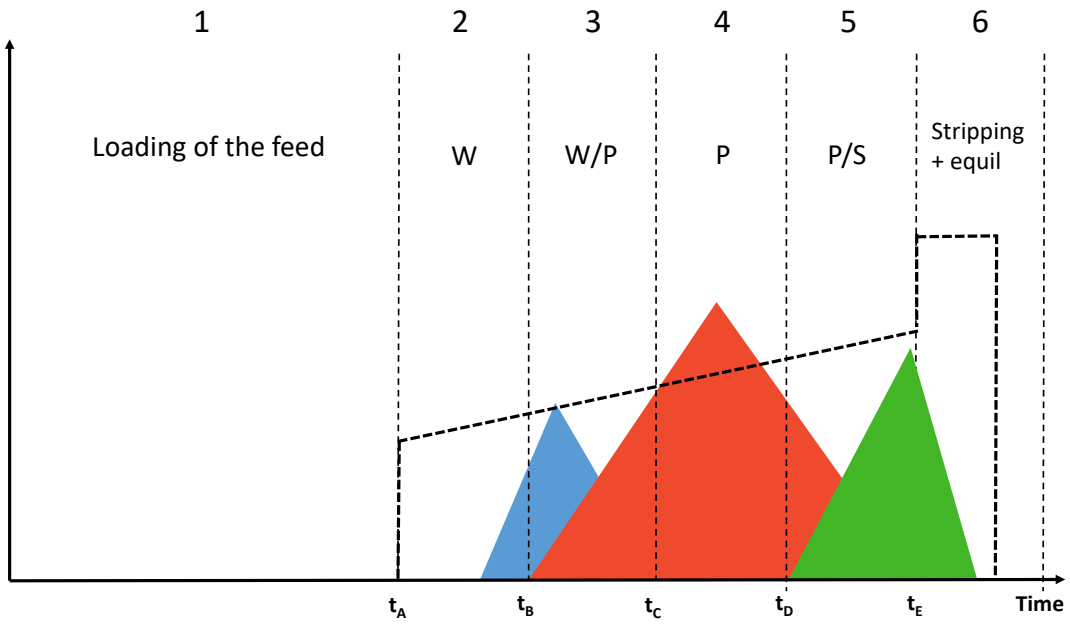

Figure 3.1: Scheme of the regions in which a batch chromatogram can be divided. Reproduced from [99].

working in batch, the recycling is performed manually. The experimental conditions employed in the design batch run will be maintained in the MCSGP process, such as loading, gradient slope and the stripping and equilibration protocol.

3.2 Principles of MCSGP

As said above, MCSGP is a technique which was developed to perform the automatic recycling of the overlapping regions, improving the productivity at the same time. Therefore, the two columns must be working connected or disconnected alternatively. When a batch method is transferred to MCSGP, the columns accomplish the same tasks of the batch, but one column is shifted of half a cycle with respect to the other. In Fig.3.2, a scheme is reported, which shows how the connection between the columns changes step by step. Column-1 is in the upstream position while column-2 is in the downstream position. This means that the overlapping regions elute from column-1 and are recycled in column-2 during the interconnected steps.

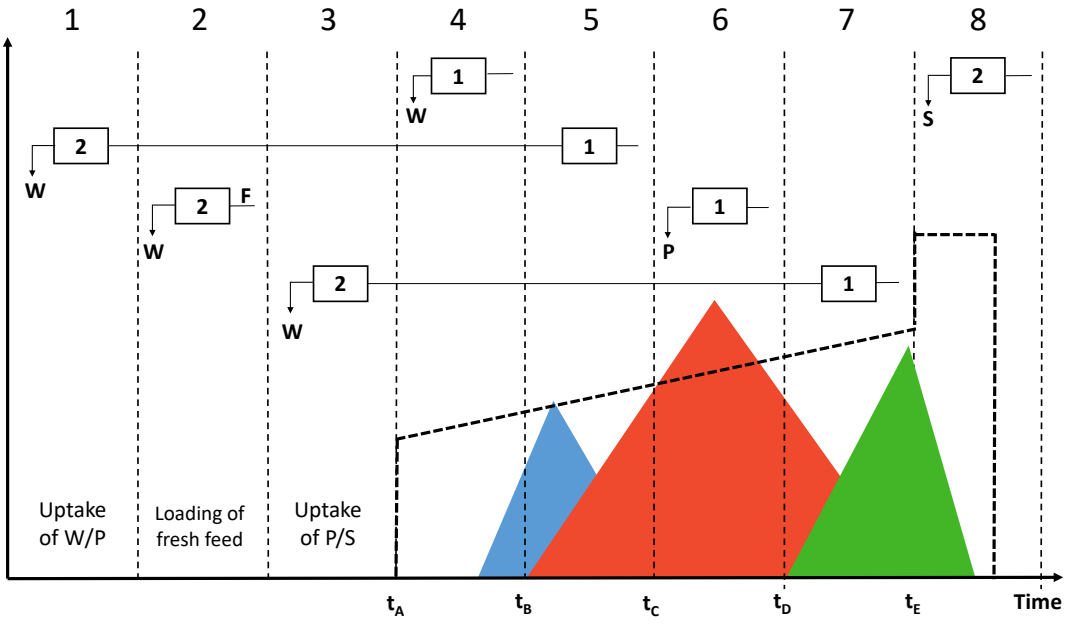

Figure 3.2: Schematic representation of the tasks accomplished by the two columns when the batch method is transferred to MCSGP. Reproduced from [54].

At the beginning of the process, column-1 is loaded with fresh feed as in the batch process. Then a cycle starts:

- When the gradient starts in column-1 (zone 4), weak impurities W start eluting and are sent to the waste outlet; this fraction is discarded since it does not contain the product P. Contemporarily, column-2 is being stripped and washed (zone 8) Therefore, the columns are working in parallel and are disconnected.

- When the first overlapping region (W/P) elutes from column-1 (zone 5), it is directly loaded into column-2 (zone 1), after the valves have switched position. Now the columns are connected.

- In zone 6, a fraction with product purity satisfying the requirements imposed is recovered from column-1. At the same time, some fresh feed is loaded in column-2. The columns are working again in batch mode.

- After that, the switching valves connect again the columns, and the overlapping region P/S leaves column-1 (zone 7) to be loaded into column-2 (zone 3).

- At this point, column-1 must be stripped to remove all the strong impurities and re-equilibrated to the percentage of organic modifier that is necessary at the beginning of the gradient (zone 8). Column-2, on the other side, has been fully loaded and can undergo the solvent gradient (zone 4).

Fig.3.3 can help understanding how the connection between the columns changes during a switch.

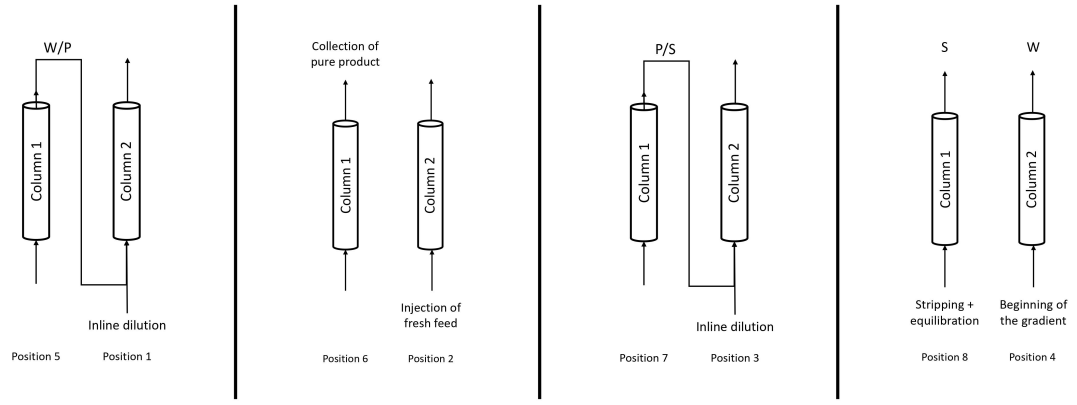

Figure 3.3: Schematic representation of the connection between the columns during a switch. Column-1 is in the upstream position. Reproduced from [99].

The procedure just described represents a switch of the MCSGP process, meaning that the columns have exchanged position (now column-1 is in the downstream position). When they exchange position again and return to the initial configuration a cycle is completed. Thus, one cycle consists of two switches; one pool of pure target product is collected for each switch, during zone 6 of Fig.3.2. Usually, after a couple of switches, a steady-state is reached; in other words, cycle after cycle, the UV profiles do not change and therefore result superimposable. Also, at steady-state conditions, purity and recovery of the pools collected switch after switch are almost identical. This means that the number of cycles to carry out depends uniquely on the amount of fresh feed that is to be purified, since the performance are identical for every cycle which is at steady-state.

It can be noted that the windows which have been previously discussed are delimited by five characteristic times (from t_A to t_E), which correspond to the switch of the inlet and outlet valves that regulate the path accessible to the mobile phase. Particularly, t_A indicates the beginning of the gradient; from t_B to t_C the first overlapping region elutes; t_D defines the end of the collection pool and t_E the end of the overlapping window P/S. If all the other operating conditions are kept identical as those of the batch, this set of switching times is the only degree of freedom of the MCSGP process. The path followed by the eluent stream during a switch is shown in Fig.3.3

An important aspect to consider is that the overlapping regions contain a percentage of organic modifier higher than the percentage present in the mobile phase at the beginning of the gradient. Thus, when they are recycled, they need to be diluted with an inline dilution stream, so that the product can adsorb on the stationary phase of the downstream column. As a rule of thumb, the fraction containing W/P is diluted to reach the organic modifier concentration that can be found at t_B, so that the product adsorbs on the stationary phase while the weak impurities start moving along the column. On the other side, the window containing P/S is diluted to reach the percentage of organic modifier at the beginning of the gradient (in correspondence with t_A), because both the product and the strong impurities must be retained in the downstream column.

Also, it must be specified that the amount of fresh feed injected switch after switch during the disconnected zone 2 in Fig.3.2 is calculated based on the difference between the quantity of product loaded in the batch run and the amount of product recycled within the overlapping regions, which does not leave the system. By doing so, cycle after cycle the same mass of target product is loaded into each column.

3.3 Development of an MCSGP process

During the gradient of the design batch run, fractions are collected periodically. They are then analyzed offline by means of a suitable HPLC method, to evaluate their purity and recovery. After the HPLC analysis, it is possible to build a graphic called *Pareto curve*, which reports how purity varies as a function of the recovery along the main peak. This purity profile can be employed to decide which portion of the peak fulfills the purity requirements. This part of the chromatogram will represent the product elution window. Starting from the purest fraction, which also shows a limited recovery, one imagines to pool together one after the other all the neighboring fractions, in order of decreasing purity. By doing so the recovery increases, because the hypothetical pooling window is being broadened, while the purity decreases. This trend is described by the Pareto curve.

To have a fair comparison between the performance of the batch and the MCSGP, it is necessary that the total column volume of the two processes is comparable. Therefore, the batch method must be run also on a column approximately long twice as much as the column used in MCSGP. The batch run on the long column is then used as a reference to evaluate the outcome of the MCSGP process. The batch method must be run also on one of the columns used in MCSGP to set the switching times.

The switching times are the only variables of the process if all the other experimental conditions are kept constant. Therefore, the results obtained in MCSGP strongly depend on how they are chosen. The first trial is usually done by choosing the values of t_C and t_D corresponding to a certain hypothetical pool in which purity fulfills the requirements and recovery is sufficiently high. t_B and t_E must be set in order to minimize the amount of product eluting in the waste windows (meaning the zones where W and S elute). Fig.3.2 represents the ideal case where the waste zones (zone 4 and zone 8 respectively) do not contain at all the target product, but in real cases, this hardly ever happens. In more realistic situations, it is preferred to waste a small amount of very impure product in W and S streams rather than risking to accumulate impurities into the system during the recycling steps.

For all the cycles that are at steady-state, MCSGP performance translate into a unique pair of values of recovery and purity. Therefore, for the continuous process it is not obtained a Pareto curve, but a single point, that must be compared to the Pareto curve of the batch. In Fig.3.4, two different cases are shown from a purely qualitative point of view. If the point of the MCSGP lies below the Pareto curve, the MCSGP process can be considered unsuccessful, meaning that it reaches a lower recovery than the batch

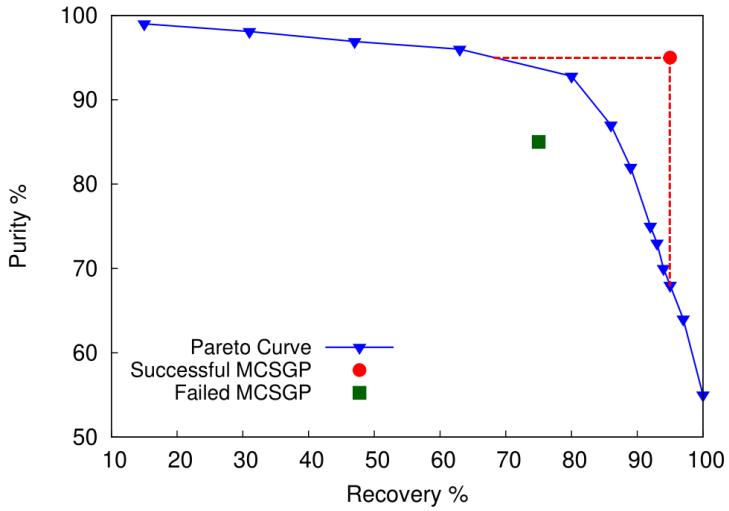

Figure 3.4: Qualitative representation of a Pareto curve related to a hypothetical design batch chromatogram. Reproduced from [99].

at the same purity (see Fig.3.4, green square). On the opposite, if the point lies above the Pareto curve, this means that the recovery of MCSGP has overcome that of the batch. The best performance are represented by points in the upper right corner of the graphic (see Fig.3.4, red point).
If the MCSGP results to be unsuccessful, one or more switching times must be changed. Particularly, Vogg et al. [66] demonstrated that the times defining the product collection window (t_C and t_D) have an influence on the purity of the pools collected, whereas the times t_B and t_E especially affect the recovery.

3.4 Performance parameters

The performance of different processes, in batch and continuous chromatography, can be compared on the basis of some relevant parameters. They are calculated by analyzing the fractions or pools collected during the preparative run with a suitable HPLC method. The areas of target product and impurities are then integrated.
The most important parameter to evaluate in pharmaceutical field is *purity*, since it must satisfy the specifications imposed. It is defined as the ratio of the area of the target peptide peak and the total area contained in that pool or fraction (sum of the areas of peptide and impurities), measured in analytical conditions, expressed in percent:

$$\text{Purity } \% = \frac{\text{Area}_{\text{product}}}{\text{Area}_{\text{total}}} \times 100 \tag{3.1}$$

Recovery or *yield* of the compound of interest must also be considered, and it is a very important aspect when it comes to the purification of high valuable compounds, such as Active Pharmaceutical Ingredients (APIs). It is expressed as the ratio, in percent (%), of the mass of the product recovered in the pool or fraction and the mass of the product injected in the column through the feed.

$$\text{Recovery } \% = \frac{\text{m}_{\text{prod collected}}}{\text{m}_{\text{prod injected}}} \times 100 \tag{3.2}$$

In the MCSGP process, the yield and purity are calculated as a mean of the recoveries and purities of the pools at the steady-state.

Productivity is another fundamental parameter to calculate. It is defined as the amount of target compound collected in a fraction or pool divided by the duration of the preparative method and the total volume of stationary phase. Its unit of measurement is mg/mL/h:

$$\text{Productivity} = \frac{\text{m}_{\text{prod collected}}}{\text{time} \times \text{CV}} \tag{3.3}$$

Therefore, productivity shows how much target compound is purified in the unit of time per column volume (CV). It can happen that MCSGP gives similar results as that of the batch or slightly lower, but this is not often of concern. From an economic point of view, it is preferable to maximize the recovery more than the productivity of the process in case of expensive APIs [100]. For example, the cost of crude glucagon is declared to be around some thousand dollars per gram [101], and thus an increase in the recovery would lead to great economic advantages. Moreover the Eq.3.3 completely neglects the practical benefits coming from the automatization of the process, which is instead a fundamental adavantage.

The last parameter to evaluate is the solvent consumption, that defines the volume of buffer used to obtain a certain amount of target product fulfilling the purity requirements. This value must be minimized. It is expressed as L/g:

$$\text{Solvent consumption} = \frac{\text{V}_{\text{buffer}}}{\text{m}_{\text{prod}}} \tag{3.4}$$

4 Results and Discussion

In this chapter, the results reported in papers submitted or already published at the time of writing this thesis are discussed. In every section a different topic will be considered to avoid repetitions and overlaps. The interested reader is addressed to the reprints of the full papers attached at the end of this thesis.

4.1 Modeling the adsorption behavior of a peptide in gradient and overloading conditions (Papers I, II)

In Papers **I** and **II**, the nonlinear adsorption behavior of an octapeptide called octreotide has been modeled by means of Inverse Method, which has already been described in Section 2.5.

First of all, a chromatogram in gradient elution conditions has been recorded in order to identify a range of ϕ around which the peptide elutes, with ϕ being the fraction of the organic modifier (acetonitrile, ACN) contained in the mobile phase. The values of ϕ considered ranged from 0.23 to 0.28. The retention of biomolecules is strongly affected by the changes in the mobile phase composition; the retention factor decreases exponentially with increasing the percentage of ACN.

For every value of ϕ considered, different isocratic runs were performed at several concentrations of peptide, using both the crude and the pure mixtures. Starting from these chromatograms, it has been possible to determine the adsorption isotherms that regulate the retention of the compound through Inverse Method. Langmuir isotherm has been proven to be the best model to fit the experimental profiles. By comparing the experimental profiles and the simulated profiles, reported in Fig.4.2, it is found that the agreement between them is excellent.

Fig.4.2 shows four different pairs of profiles (experimental and simulated) related to four different concentrations of peptide. The content of organic modifier in these runs were ϕ=0.24. While the isotherm model does not change along the gradient, meaning

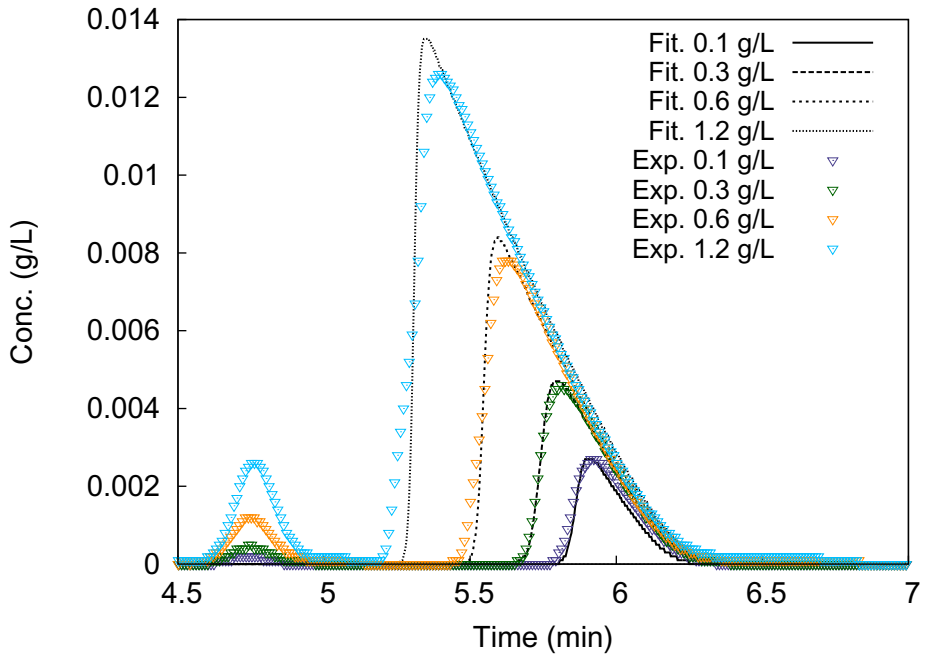

Figure 4.1: Agreement between experimental peak profiles and simulated peak profiles, obtained through Inverse Method, at ϕ=0.24 and considering different concentrations of peptide. Reproduced from [20].

with the mobile phase composition, its parameters do, since they are strictly related to the retention factor. Basically, at different ϕ, the equilibrium adsorption constant b changes, following the Eq.2.12; on the contrary, since the range of ϕ considered is quite narrow, the saturation capacity is expected to be constant.

The values obtained with Inverse Method for the isotherm parameters at different compositions of mobile phase are shown in Table 4.1. Actually, q_s can be considered constant in the range of ϕ taken into consideration, while b is exponentially dependent on ϕ, as can be seen also in Fig.4.2.

Table 4.1: Adsorption isotherm parameters obtained through IM with a Langmuir model at different mobile phase compositions.

ϕ	a	b (L/g)	q_s (g/L)
0.23	5.86	8.14	0.72
0.24	4.21	5.69	0.74
0.25	3.07	4.65	0.66
0.28	1.17	1.86	0.63

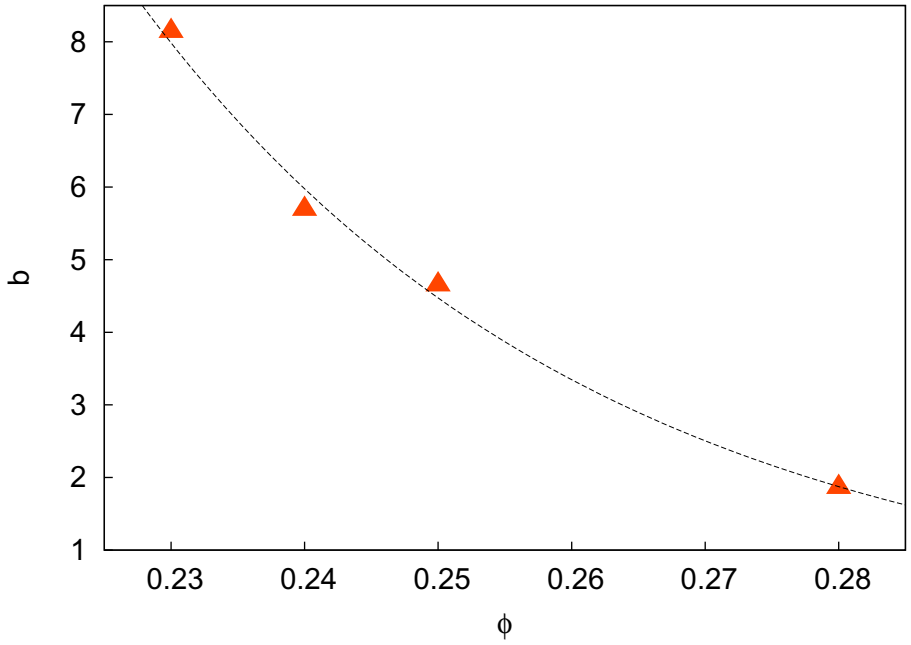

Figure 4.2: Exponential relation between b and ϕ. Reproduced from [20].

The mean value of the four q_s in Table 4.1 results to be 0.69 g/L, and this value is used to simulate the adsorption behavior of the target product in gradient elution condition. From the exponential fitting of b (Eq.2.12), it is obtained a value of S=29 and b_0 =6.3$\times 10^3$ L/g.

At this point, the peptide behavior in overloading and gradient conditions can be modeled by solving the mass balance equation (Eq.2.3), combining it with Eq.2.13 and with the boundary conditions (Eq.2.14). The model obtained proved to be able to predict the overloaded peak profiles under gradient elution conditions. As it can be seen in Fig.4.3, simulated and experimental peak profiles are in very good agreement. This means that the model developed describes satisfactorily the chromatographic system under study. Finally, to test if the model can take into account conditions not used to develop it, two more experiments have been run with a higher injection volume (10 and 20 μL instead of 5 μL). Also in this case, the model developed predicts accurately the peak shape (see Fig.4.4).

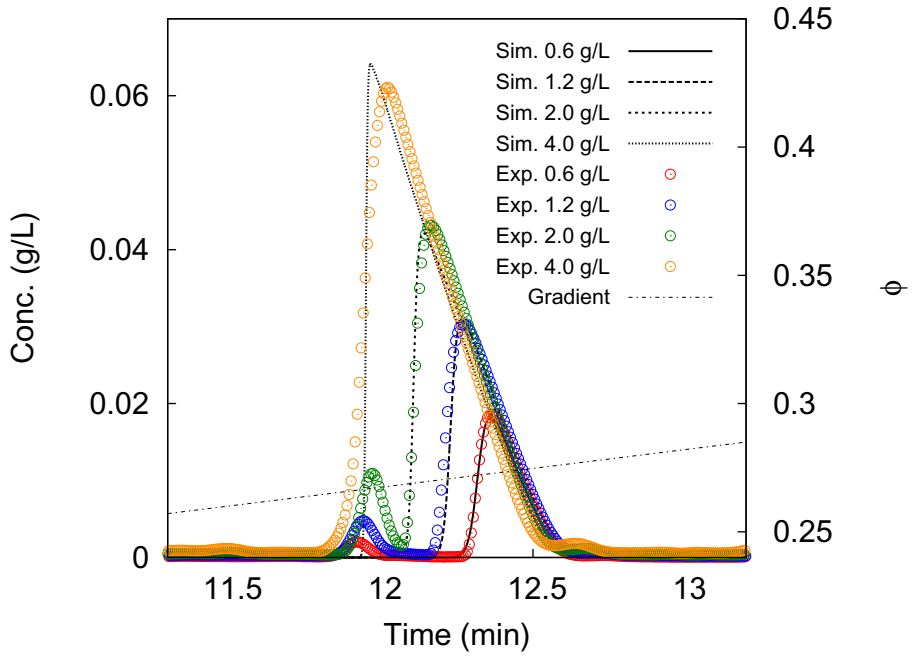

Figure 4.3: Experimental and theoretical peak profiles under gradient elution conditions. As it can be seen, the model is able to predict accurately the adsorption behavior of overloaded peaks. Also the gradient curve is shown. Reproduced from [20].

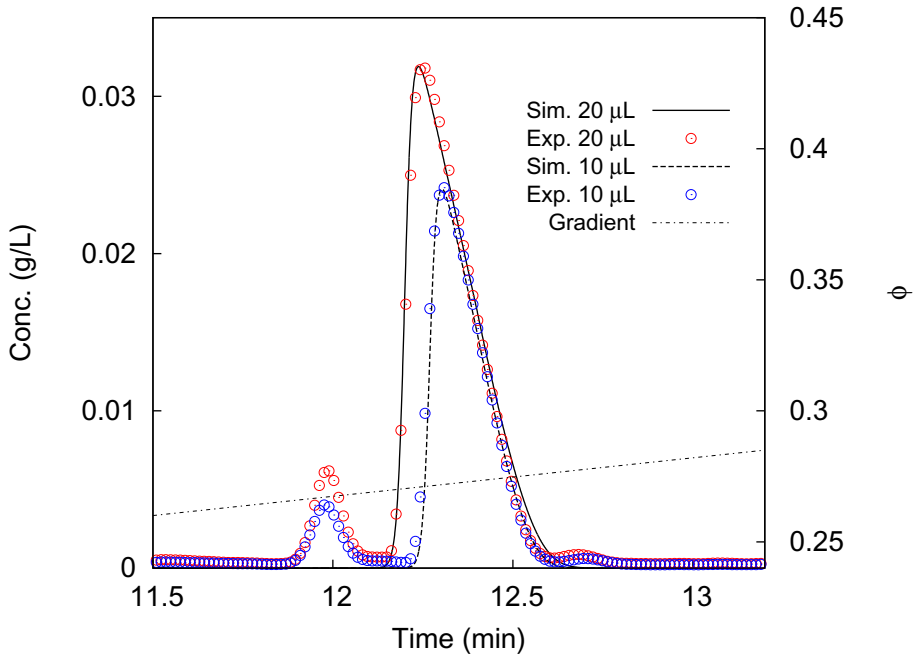

Figure 4.4: Comparison between theoretical and experimental peaks in gradient elution conditions, injecting higher volumes of sample. Also the gradient curve is shown. Reproduced from [20].

4.1.1 Closing remarks

Preparative liquid chromatography, usually under gradient elution conditions, is one of the most employed technique for the purification of large biomolecules, such as peptides. At industrial level, the development of a method is led empirically and on a trial-and-error basis. An approach model-based is to study the adsorption isotherms of the compound of interest, to obtain information about its thermodynamic adsorption equilibria. If the purification is conducted under gradient elution conditions, the adsorption isotherms must be investigated in a range of different compositions of mobile phase, where the elution takes place. The parameters can then be correlated to the content of organic modifier, to develop a model that keeps into account the variation of the isotherm parameters along the gradient.
Different techniques can be used to this aim, but the Inverse Method results to be the most suitable one in the case of biopharmaceuticals. The main advantage of this numerical approach is that it allows to obtain thermodynamic information, and thus to develop a model, even from a limited amount of sample. Therefore, this is particularly convenient in case of high valuable products, which is often the case of APIs.
In this study, the adsorption equilibria of a small peptide (octreotide) have been modeled thanks to the adsorption isotherms acquired in isocratic conditions with Inverse Method. The method developed is consistent and accurate, since it is found that experimental and simulated profiles in gradient elution conditions are in good agreement also when injecting bigger amount of compound.
Developing a model able to predict the peak profiles not only in isocratic but also under gradient conditions could be a great starting point to provide information that may help to optimize large-scale purification, possibly also in continuous chromatography.

4.2 Purification of two bioactive peptides by means of MCSGP process

Within the scope of this thesis, the cutting-edge process called MCSGP has been applied successfully to the case of two peptides synthesized industrially by means of Solid Phase Synthesis. The first one is glucagon (29 amino acids), the study of which has already been published (Paper **III**). For what concerns icatibant (10 amino acids), the study has not been submitted yet, because of patent pending reasons. For the same reason, several experimental details will not be reported explicitly.

4.2.1 The case of a large peptide (Paper III)

Glucagon is a polypeptide made of 29 amino acids. Being it so large, the Solid Phase Synthesis requires a lot of steps, and this leads to a crude mixture containing several impurities. The weight content of glucagon in the crude mixture is 29%, while its chromatographc purity in the feed is 55%.
The purification of the glucagon mixture was first conducted in batch conditions, using as mobile phases two mixtures with different percentages of acetonitrile (ACN) and ammonium acetate 20 mM. The column used in batch was a Daisogel-SP-120-10-C8-Bio 250×4.6 mm, which has a geometrical volume (column volume, CV) of 4.2 mL. The mass of peptide injected for every run corresponds to 1% of loading (meaning 42 mg of peptide per 4.2 mL of column volume). During the gradient elution, fractions are collected periodically and analyzed offline with a suitable HPLC method. From the fraction analysis, the Pareto curve is built, starting from the purest fraction collected and successively adding the adjacent others in order of decreasing purity, as described in Sect.3.3. The batch chromatogram chosen as batch must show at least one portion of the main peak fulfilling the purity requirements imposed; its Pareto curve is used as reference to compare the outcome of the MCSGP runs.
In MCSGP, the mobile phases used are the same as the batch, but the columns employed are different: 2 columns Daisogel-SP-120-10-C8-Bio 150×4.6 mm (same stationary phase as the batch but different length). The total CV of the columns used in MCSGP is 5 mL, a value comparable with the CV of the batch reference. Also, the batch method must be run on one of the columns used in MCSGP: on the basis of this chromatogram the switching times for the MCSGP process are set. This is the so-called *Design Batch Chromatogram*.
The design batch chromatogram obtained for glucagon is shown in Fig.4.5. During the gradient, fractions are collected and analyzed offline, to obtain a profile of con-

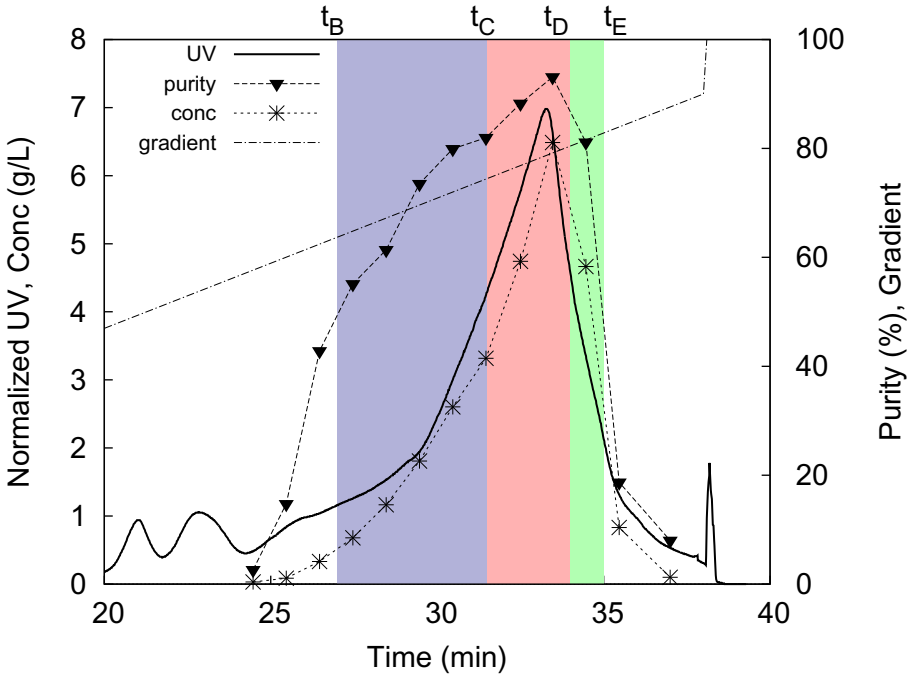

Figure 4.5: Design batch chromatogram related to the purification of glucagon. The UV, concentration and purity profiles are also displayed. Last, the recycling (blue, green) and collection (red) windows with their switching times are shown. Reproduced from [54].

centration and purity along the gradient and to build the Pareto curve. The Pareto curves obtained on both the column in batch conditions are shown in Fig.4.6. The red one refers to the 25 cm column and the blue one to the 15 cm column. The first set of switching times has been chosen starting from the blue Pareto curve, in order to define a collection window with purity satisfing the specifications and with a sufficient recovery. The times determining the beginning of the W/P window and the end of the P/S window are chosen in order not to waste too much target product in the waste streams W and S. The set of times, then, has been fine tuned as described in Sect.3.3, to improve the performance of the MCSGP process.

Table 4.2: Set of switching times used for the MCSGP process applied to the purification of glucagon.

t_A (min)	t_B (min)	t_C (min)	t_D (min)	t_E (min)	V_{inj} (CV)
14.5	27	31.5	33	34	5.7

The set of switching times which gave the best results together with the volume of feed to reinject for each switch are summarized in Table 4.2. 5 cycles have been performed;

after the first switch the system has already reached the steady-state, as it can be seen by overlapping the UV profiles of the cycles (Fig.4.7).
Two pools have been collected per cycle, one at the outlet of every column, and have been analyzed with HPLC. The performance parameters obtained for the pools at steady-state are rather similar to each other; their mean values are taken as outcome of the MCSGP process and are compared to the Pareto curve of the batch on the 25 cm column.
The mean values of recovery and purity at steady-state (87.7% and 89.2% respectively) are reported in Table 4.3 and their relative point is shown in Fig.4.6. The performance of the MCSGP process must be compared with the pool of the batch having a purity similar to the steady-state of the MCSGP. With a purity around 89.2% the recovery increases from 71.2% for the batch to 87.7% for the MCSGP. Therefore, the percentage increase in the the target product recovery is +23% using the MCSGP process.
The productivity of the MCSGP run is calculated on the basis of the mass collected per cycle at steady-state (Eq.3.3); on the other side, for the batch method it is calculated on the basis of the mass having the same purity as the MCSGP pools. Nevertheless, productivity depends also on the total column volume, which is comparable for the two processes, and on the duration of the batch method or of one MCSGP cycle. The steps during which the overlapping regions are recycled require to be diluted inline, and this leads to an increase in the duration of the cycle. Thus, the increase in the recovery of the MCSGP is not sufficient to lead to an increase in productivity as well, which therefore results higher for the batch. Anyway, in case of expensive compounds, which is often the case of APIs, it is economically preferable to obtain a higher yield than a higher productivity, in order not to waste the valuable target product. Moreover, the Eq.3.3 does not keep into account the advantages deriving from the complete automatization of the recycling process.

Table 4.3: Comparison between the performance of the MCSGP process and of the batch run.

	Batch	MCSGP
Purity (%)	89.3	89.2
Recovery (%)	71.2	87.7
Productivity (g/L/h)	9.9	6.1

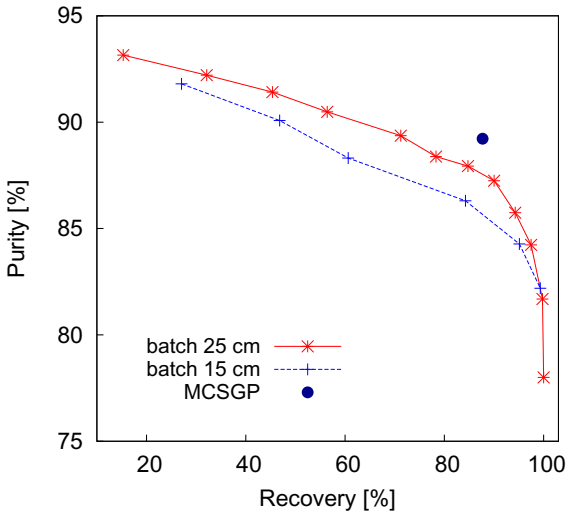

Figure 4.6: Pareto curve of the batch runs obtained on the 25 cm and 15 cm column. Also the performance of the MCSGP run is shown. Reproduced from [54].

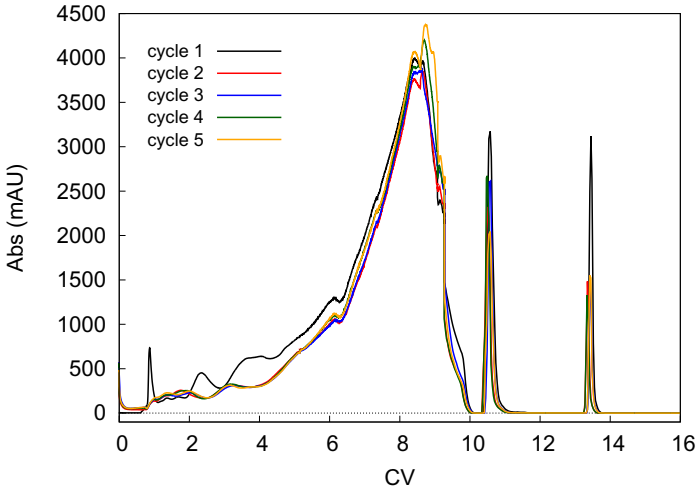

Figure 4.7: UV signals of the 5 cycles of the MCSGP run. From cycle 2 on, the steady-state condition has already been reached. Reproduced from [54].

4.2.2 The case of a small peptide (Paper VII)

The second peptide whose purification has been investigated by means of MCSGP is a decapeptide, icatibant, a selective and specific antagonist of bradykinin B2 receptors, which has recently been considered for its potential improvement of oxygenation in patients affected by COVID-19 disease.
The crude mixture has been obtained industrially through Solid Phase Synthesis. Being the peptide of interest quite short, its synthesis requires a few steps. Therefore, the crude mixture is quite pure: its chromatographic purity is around 88%, while the peptide weight content is 49%.
The purification of icatibant is conducted in reversed-phase preparative chromatography, with a gradient elution program. Also in this case, the column used for the batch and the two columns used for the MCSGP contain the same stationary phase (C18) but differ in their length (250×4.6 mm for the batch, 150×4.6 mm for the columns used in MCSGP). Therefore, the total CV in MCSGP is comparable to the CV in batch.
As usual, the cleaning protocol, gradient slope, loaded amount of feed, etc are kept constant when the method is transferred from batch to continuous. The amount of target product loaded for every run in batch is 1% based on the column volume.

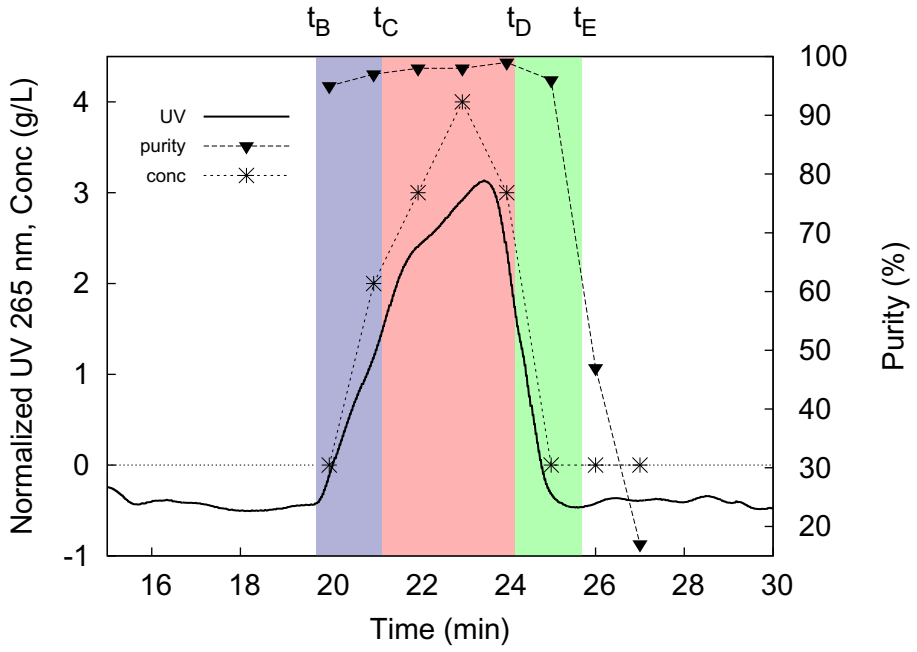

Figure 4.8: Design batch chromatogram of the purification of icatibant. The different recycling (blue, green) and collection (red) regions are represented.

Table 4.4: Set of switching times used for the MCSGP process applied to the purification of glucagon.

t_A (min)	t_B (min)	t_C (min)	t_D (min)	t_E (min)	Mass to reinj/cycle (%)
6.2	19.7	21.2	24.2	25.7	72

The batch method has been performed on the 15 cm column (design batch chromatogram) and on the 25 cm column, the Pareto curve of which has been taken as a reference to compare the performance of the batch and of the MCSGP.
The design batch chromatogram is shown in Fig.4.8. The fractions collected during the gradient in batch have been analyzed with the HPLC; profiles of concentration and purity have been obtained, which are also shown in Fig.4.8. It must be noted that the experimental conditions used for the batch purification allow to achieve a very high purity (greater that 90%) throughout the whole target peak. Also in this case, from the offline analysis, a Pareto curve has been built, in order to decide a first trial set of switching times. The set is reported in Table 4.4, whereas the recycling and collection windows are indicated in color in Fig.4.8. The Pareto curves obtained on both columns are shown in Fig.4.9.
Since in MCSGP it is desirable to keep the mass of target product constant inside the system to reach the steady-state, at every switch a certain amount of fresh feed must be loaded, to replace the amount of peptide leaving the system. Therefore, the mass to reinject at every switch in percentage is equal to the sum of the recovery of the peptide collected in the red region plus the percentage of peptide wasted in the waste streams (blue and green regions). In this case, it results that 72% of the mass injected in the design batch chromatogram must be reinjected during every switch.
In this work, 5 MCSGP cycles have been performed. The first switch of each of them is shown in Fig.4.10. The steady-state is already reached during the second cycle, whose profile is superimposable to all the successive cycles. This assumption is confirmed by the fact that the values of purity and recovery found from the second cycle on are very similar cycle after cycle. Their values, calculated as the mean of purity and recovery of the pools, result to be 99.3% and 95.5% respectively, and they must be compared with the Pareto curve of the 25 cm column (the blue one in Fig.4.9).
As it can be seen in Fig.4.9, the red dot related to MCSGP lies above the Pareto curve, in the upper right corner of the graphic, and this implies that the MCSGP performance overcome the results of the batch, being the total CV of the two processes similar. Differences in the performance must be evaluated at similar purity (99.3% for the MCSGP

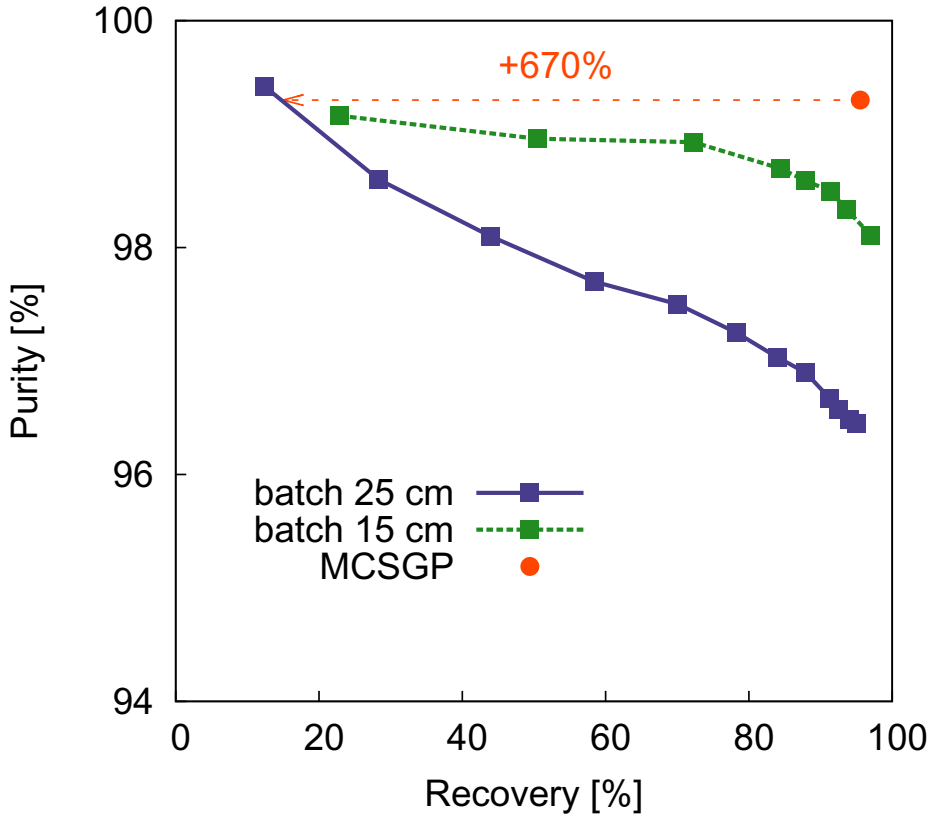

Figure 4.9: Pareto curves obtained for the batch runs with the 25 cm column and 15 cm column and point related to the performance of MCSGP at steady-state.

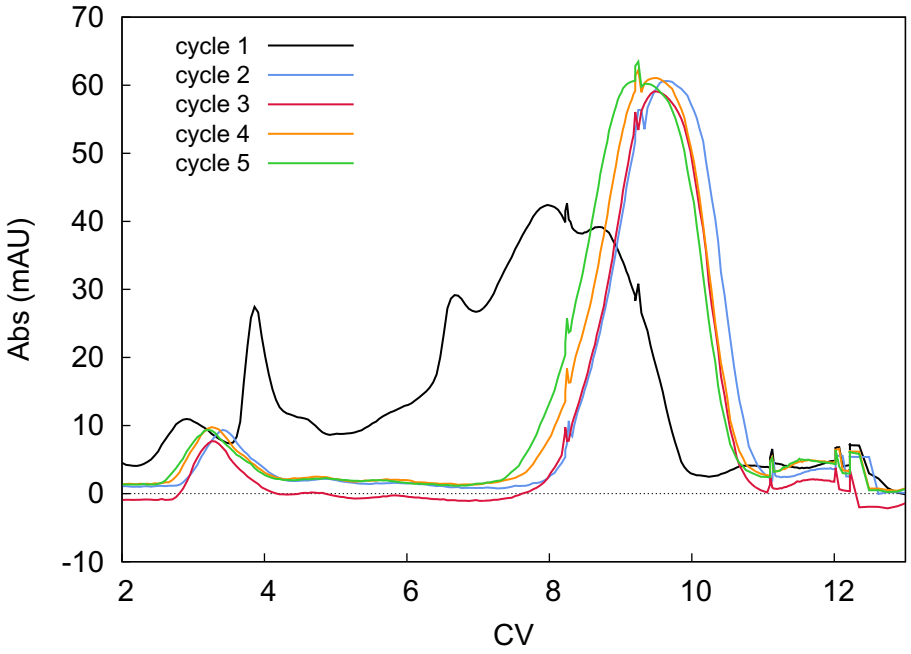

Figure 4.10: UV profiles of the first switch of 5 MCSGP cycles for the purification of icatibant.

Table 4.5: Comparison between the performance of the MCSGP process and of the batch run.

	Batch	MCSGP	Improvement with MCSGP
Purity (%)	99.4	99.3	
Recovery (%)	12.4	95.5	+670%
Productivity (g/L/h)	1.13	7.65	+575%
Solvent consumption (L/g)	25.6	4.5	-81%

and 99.4% for a hypothetical pool in batch conditions). The corresponding recovery in batch is limited to 12.4%, whereas in MCSGP it is 95.5%, and this difference corresponds to a percentage increase equal to +670%.
The impressive gain in recovery achieved has important consequences also on other performance parameters. For the batch method, productivity is calculated considering the peptide mass of a hypothetical pool having the same purity as the MCSGP and the duration of the whole batch method. For MCSGP, the mass considered is twice the mass contained in a pool, which refers to a single switch, and the duration is the one of a cycle. It results an improvement in the productivity of +575% (from 1.13 g/L/h to 7.6 g/L/h).
Eventually, the solvent consumption can be estimated. For the batch method, it results 25.6 L/g, referred to the hypothetical pool as pure as the MCSGP pools. On the other side, the solvent consumption in MCSGP is decreased to 4.5 L/g. This variation corresponds to a net reduction equal to -81%.

4.2.3 Comparison between the two cases

The two cases where MCSGP has been applied in this thesis can be compared. Glucagon chain is three times longer than icatibant (29 amino acids *vs.* 10 amino acids); its synthesis is much more time-consuming and challenging, because it requires several steps. Therefore, this leads to a larger number of product-related impurities which, in turn, worsen the peak overlap in preparative conditions. Furthermore, the experimental conditions of the batch method used in the case of glucagon were kept similar to the ones actually used for its purification at industrial level. Changing the batch method, for example by varing the mobile and stationary phases, could in principle

lead to a better resolution of peaks, that in turn would imply a greater improvement in the performance after the transfer to MCSGP.
As a consequence of these two factors, the improvement in the recovery of glucagon with MCSGP could only be limited to +23%. Anyway, it can be considered a satisfing result: for very expensive product, indeed, even a moderate increase in the recovery can be beneficial from an economic point of view. Clearly, a percentage increase in the recovery as notable as for the case of icatibant lays the foundation to obtain impressive results also for what concerns productivity and solvent consumption.

4.2.4 Closing remarks

Continuous countercurrent techniques represent a concrete and promising alternative to single column purifications. Among them, MCSGP can deal with challenging ternary separations, especially in gradient conditions.
Within the scope of this PhD research project, two MCSGP processes have been developed to purify two peptidic crude mixtures, synthesized industrially, with different degrees of purity. The design of the continuous chromatographic processes is based on a batch method where at least a portion of the main peak satisfies the purity specifications.
The performance of the MCSGP run are strictly correlated to the set of switching times chosen, that define the regions to be collected and to be recycled. All the other experimental conditions have been fixed for the batch run and are preserved in the MCSGP.
The outcomes of the MCSGP processes applied to the peptides of interest are quite different. On the one hand, a good improvement in the recovery has been obtained for glucagon, which anyway is not sufficient to overcome the still better productivity of the batch. However, in case of very expensive products, it is preferable to maximize the recovery, in order to minimize the product wasted.
On the other hand, for what concerns icatibant, whose crude mixture had a much higher purity than the glucagon one, the MCSGP outcome is remarkable in terms of recovery. This improvement has repercussions also in the productivity and solvent consumption.

4.3 Other applications of MCSGP (Papers IV,V)

MCSGP is particularly suitable for difficult ternary separations led in gradient elution conditions. This is often the case of different kinds of pharmaceuticals.

Two classes of biomolecules are currently under investigation because of their potential activity for the treatment or prevention of COVID-19 disease: monoclonal Antibodies (mAbs) and oligonucleotides [7, 8]. The mAbs of interest, anyway, cannot be produced as a pure compound, but rather as a mixture of different isoforms. Obviously, all the isoforms different from the target product must be removed, to ensure a good quality of the product and to meet the market specifications. MCSGP successfully accomplished this task [43, 45, 46].
On the other side, oligonucleotides (ONs) are short nucleic acid chains which have a role in the regulation of gene expression. Nine ONs have already been approved by FDA. They can be obtained through chemical synthesis or recombinant technology, but also in these cases several by-products are produced, and a purification protocol is mandatory. The choice of the separation method relies on the chemical features of the ON; especially, if the purification method is too strong, the ON risks to be denatured and thus a refolding method is required. Liquid chromatography is the technique of choice for the downstream processing of ONs. The literature about this topic is very scarce and strictly limited to single column batch chromatography. Only recently, MCSGP has been successfully employed to separate a mixture of ONs. At a target purity of 92% the yield was increased from 55% to more than 90% [32, 43, 45, 100, 102]. For an in-depth study of the methods of production, analysis and purification of oligonucleotides, the interested reader is referred to Paper**V**.
The very first cases of application of a 6-column or 3-column MCSGP to an industrial sample were related to the separation of Calcitonin, a peptidic hormone made of 32 amino acids, from its impurities [25, 48, 96]. The MCSGP process was designed empirically and a first guess of operating conditions was directly extracted from the experimental single column batch chromatogram. It was found that for the required purity (93%) the MCSGP unit allows to achieve yields close to 100% compared to a yield of 66% obtained for the batch.
About proteins, the case of mono-PEGylated α-Lactalbumin, which needed to be separated from its other PEGylated forms through anion-exchange chromatography, is the most relevant [31]. With MCSGP, at a purity of 93%, the recovery was increased from 56% to 83%, whereas the buffer consumption was decreased of -50%.
Last, the purification of cannabinoids has been investigated with the MCSGP technique. Cannabidiols (CBD), for instance, are a group of cannabinoids, natural compounds extracted from *Cannabis Sativa L.*, which are supposed to have therapeutic properties, including pain relief. Crude CBD extracts from hemp plants contain sev-

eral different natural compounds. However, the regulation forbids the presence of tetrahydrocannabinol (THC) in the CBD mixtures at concentrations higher than 100 ppm, since THC is a psychoactive drug. Traditional preparative purification methods for CBD, such as batch chromatography, are able to reduce the THC content only at the expense of the recovery of CBD. Recently, a CBD mixture has been purified to remove completely the THC through the MCSGP process, isolating the target species with high purity, yield and throughput. Particularly, CBD yield is improved by around 80% at a THC content of 100 ppm, being the CBD purity 99.5% [103].

4.4 Future perspectives about continuous chromatography (Papers IV,VI)

The market of biopharmaceuticals is rapidly growing and new technologies both in the upstream and in the downstream processing are being introduced. The upstream part of the manufacturing marked the most important advances over the years, with the introduction of recombinant technology and perfusion bioreactors working in continuous, especially for what concerns antibodies. On the other side, the introduction of improvements in the downstream processing is slow and challenging. A concerted effort in the downstram is therefore urgent to cover the gap with the upstream. The limitations related to single-column purification processing in the downstream processing of biomolecules can be partially overcome by means of continuous or semi-continuous techniques.

When a capture step is required, which is the case of proteins or mAbs for instance, the Capture-SMB process allows to increase both capacity utilization of the resin and productivity, making it possible to operate also at faster linear velocities than correspondent batch processes.

On the other hand, MCSGP permits to overcome the yield-purity trade-off typical of the polishing steps, by performing an internal recycling of the overlapping regions of the chromatogram where the impurities elute together with the target compound. Many different biopharmaceuticals have been purified by means of this process, especially peptides, proteins and monoclonal antibodies. Recently, also the purification of oligonucleotides and cannabidiols with MCSGP is under investigation. A distinctive advantage of the continuous chromatographic techniques is that the process can be fine-tuned starting from a batch chromatogram and, once the experimental conditions have been set, the separation process can be completely automated for big amounts of feed, limiting the intervention of the operators.

From the industrial perspective, the interest towards continuous chromatography techniques is rapidly growing, not only because of the economic advantages already discussed, but also because they can lead to improvements in the quality and efficacy of pharmaceuticals. Some challenges, anyway, need to be faced. For example, a theoretical model-based approach could be used to optimize the experimental conditions and to fine-tune the process parameters, allowing to save time and resources. Indeed, for the time being, even if preparative chromatography is based on the concepts of nonlinear chromatography, validated theoretical models able to predict the process outcome have not been developed yet.

Continuous chromatographic techniques could become the core of the downstream processing in pharmaceutical industry, but not without significant transformations in the laboratories and in strategy, technology, and operations.

Acknowledgements

Now that my doctoral experience is bringing to an end, it is time to take stock of what I have achieved and to thank some people.
My biggest and most profound thank goes to my supervisor, Prof. Alberto Cavazzini, who has always encouraged me during productive times as well as during tough times, in these three years. Also, he gave me uncountable opportunities from the personal and working viewpoint. He is a very inspiring leader for all of us!
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Of course, my family and long-standing friends need to be acknowledged for always being present during special and hard moments. I have always felt supported and encouraged by you. I am grateful that you are part of my life. Last, to all the people who have been passing through my life and to all that came to stay. I wouldn't be here without all of you.

Alone we may go faster, but together we can go further.

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PAPER I

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Modeling the nonlinear behavior of a bioactive peptide in reversed-phase gradient elution chromatography

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ABSTRACT

The thermodynamic behavior of octreotide, a cyclic octapeptide with important pharmaceutical functions, has been simulated under reversed-phase gradient elution conditions. To this end, adsorption behavior was firstly investigated in isocratic conditions, under a variety of water/acetonitrile + 0.02% (v/v) trifluoroacetic acid (TFA) mixtures as mobile phase by using a Langmuir isotherm. Organic modifier was varied in the range between 23 and 28% (v/v). Adsorption isotherms were determined by means of the so-called Inverse Method (IM) with a minimum amount of peptide. The linear solvent strength (LSS) model was used to find the correlation between isotherm parameters and mobile phase composition. This study contributes to enlarge our knowledge on the chromatographic behavior under nonlinear gradient conditions of peptides. In particular, it focuses on a cyclic octapeptide.

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1. Introduction

Peptides represent a unique class of biochemical compounds. They are of primary importance in human physiology, being able to selectively interact with cells, receptors and other endogenous peptides and to induce specific biological reactions [1,2]. The interest on the use of peptides in pharmaceuticals (e.g., as antitumorals, anticoagulant, anti-hypertensive, antioxidant, antimicrobial drugs), nutraceuticals (for fortification of functional foods) and cosmetics (for skin health and care) is continuously increasing [3–7]. Due to their very high specificity, therapeutic peptides are competitive and advantageous over traditional drugs since they can be effective even at extremely low concentration [7,8]. Moreover, peptides do not accumulate in the human body nor in the environment after they have been excreted, minimizing possible toxic side effects.

From an industrial point of view, therapeutic peptides are produced by two main routes: recombinant synthesis [9] or chemical synthesis strategy [10].

The first one involves the use of suitable microorganisms to produce peptide of interest, through its trascription-transduction machinery [11]. The chemical synthesis approach can be further subdivided into two main strategies: Liquid Phase Peptide Synthesis (LPPS) and Solid Phase Peptide Synthesis (SPPS) [12]. In both cases, the approach involves the use of amino acids ortogonally protected as to enable the specific generation of the desired amino acid sequence through repetitive peptide bond formation. In any case, both recombinant and chemical synthesis do not generally produce target API peptide with an acceptable purity for market requirement. Purification is therefore needed to get the target peptide at the desired degree of purity for therapeutic and pharmaceutical scopes [5,13]. The downstream process (purification and recovery of the target peptide) takes up an important percentage of total manufacturing costs [14].

Preparative liquid chromatography is the most widely used technique for the purification of therapeutic peptides [15–18]. With the purpose of isolating finite amounts of pure compounds, in preparative (or nonlinear) chromatography large volumes of concentrated multicomponent feed are processed at a time. Under overloaded conditions, retention of analytes becomes concentration-dependent, being the adsorption isotherm of the analyte nonlinear. Thus, injected compounds are not eluted from the

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column as a series of Gaussian peaks but chromatograms appear as a complex mixture of tailed bands that may also change shape by increasing sample size. The problems encountered in nonlinear chromatography are extremely complex, not only owing to the effect of nonlinear adsorption isotherms on peak shapes, but also to the dependence of the amount of any component adsorbed on the concentrations of all the species in solution (competitive systems) [19–21]. Even though the theory of nonlinear chromatography has advanced to the point that quantitative predictions are possible, in preparative chromatography, working conditions are usually optimized through trial and error methods, which may cause significant waste of time and compound and thus money. As a matter of fact, when it comes to the separation/purification of (poly)peptides, some general guidelines can be applied to start with. However, the application of these protocols is not a guarantee that the process will be successful. Many aspects in this field require significant experimental and theoretical efforts to improve our understanding of the fundamentals of separation. For instance, the chromatographic behavior of two quasi-identical polypeptides under nonlinear conditions can dramatically change when even a single amino acid differs in their structure. It is well known that the adsorption model for the same peptide can also change not only by changing the mobile phase composition but also depending on the concentration of the peptide itself. But there are no means to predict if and how this will happen. There are, e.g., cases of polypeptides where the curvature of the adsorption isotherm inverts, by moving from one mobile phase modifier to another. Other times, by increasing the concentration of the polypeptide under investigation, the adsorption isotherm, initially Langmuirian, becomes S-shaped. The presence of an inflection point on the isotherm may strongly affect the shape of overloaded peaks. This explains why, in our opinion, it is so important to develop methodologies based on the measurement of adsorption isotherms. The investigation of adsorption behavior and phase equilibria involved in the separation of the target compound using a model-based approach is, therefore, the basis not only to investigate the feasibility of purification process via preparative chromatography but also to possibly provide information (e.g., maximum loading, affinity for the stationary phase) that may help to optimize large-scale purification [16,22–26]. This is particularly important in pharmaceutical manufacturing, where continuous (or semi-continuous) processes could alleviate the trade-off between yield and purity, typical of most batch (single-column) preparative chromatographic separations [27,28]. The first multi-column setup is the so-called simulated moving bed (SMB) process introduced in 1950 for isocratic binary separations of small molecules [19,29–32]. Since then, many different improved versions of continuous processes based on SMB concepts have been proposed to overcome some fundamental issues (process optimization, difficulty to deal with complex mixtures, gradient operation) and technical problems associated to the large number of columns to be operated simultaneously. The most important alternative to SMB is the multi-column counter-current solvent gradient purification (MCSGP) process, which combines linear gradients with the counter-current movement of mobile and stationary phases [33]. Originally realized with at least six columns, the process has modified in order to work with four [34], three columns [35] and more recently only two columns [36]. It has been demonstrated that the outcome of twin column MCSGP processes is easily predictable from batch chromatographic runs [37]. As a consequence, the results of investigation of thermodynamic equilibria influencing the separation in batch conditions can be used during process design to more efficiently move to continuous separations, which are extremely attractive for pharmaceutical industry to replace batch technologies [38–42].

In this work, the adsorption behavior of a therapeutic peptide, octreotide, has been investigated and modeled under reversed-phase liquid chromatography (RP-LC) gradient elution conditions. Octreotide is a cyclic octapeptide belonging to somatostatins [43,44]. Its industrial production can be obtained either with LPPS or SPPS approaches [45] and it is employed in the treatment of hepatocellular carcinoma, cirrhosis of the liver and to contrast some symptomps associated with metastatic carcinoid and Vasoactive Intestinal Peptide (VIP) tumors [46]. Adsorption isotherms of octreotide have been measured on a commercial C_{18} stationary phase by means of the so-called Inverse Method (IM) [19,47–54]. Goal of this work is to demonstrate how the adsorption behavior of octreotide under nonlinear gradient conditions can be predicted with a very low amount of compound and extremely reduced costs with respect to more traditionals techniques of isotherm determination, such as for instance frontal analysis.

2. Theory

2.1. Equilibrium-dispersive model of chromatography

The equilibrium-dispersive (ED) model of chromatography is mostly used to describe nonlinear chromatographic separations for molecules with low molecular weight [19]. This model assumes that mobile and stationary phases are in costant equilibrium and that all the contributions to band broadening (diffusion phenomena and finite rate of mass transfer kinetics) can be lumped into a unique apparent dispersion coefficient, D_a [19]:

$$D_a = \frac{uL}{2N} \tag{1}$$

where u is the mobile phase linear velocity, L the length of the column and N the number of theoretical plates.

The differential mass balance equation describing the accumulation of material in a thin slice of column of thickness ∂z in a ∂t time interval is [19]:

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_a\frac{\partial^2 C}{\partial z^2} \tag{2}$$

where C and q the concentrations of the analyte in the mobile and stationary phases. $F = (1-\epsilon_t)/\epsilon_t$ is the phase ratio and $\epsilon_t = V_0/V_{col}$ the total porosity of the column (with V_0 and V_{col} the thermodynamic void volume and the column voume, respectively).

In order to solve Eq. (2), an isotherm model ($q = f(C)$), expressing q as a function of C, must be chosen.

2.2. Modeling of overloaded profiles under gradient elution chromatography

In gradient elution RP-LC, the volume fraction (ϕ) of the organic modifier in the mobile phase is gradually increased during a chromatographic run. Differently from isocratic conditions, the adsorption isotherm of a species in gradient elution mode is ϕ- (and time-) dependent [53,55,56]. For this reason, it is usually considered that, even if the adsorption isotherm type is not affected by changes in mobile phase composition, its parameters are a function of ϕ [57].

In addition, the Linear Solvent Strength (LSS) model [58–60] is applied to describe the variation of retention factor with the mobile phase composition:

$$\ln k(\phi) = \ln k_0 - S\phi \tag{3}$$

with k_0 the retention factor extrapolated at $\phi = 0$ and S a coefficient characteristic of the system solute-mobile phase.

By considering a simple Langmuir isotherm model (under isocratic elution conditions):

$$q = \frac{aC}{1+bC} \tag{4}$$

where b and $a(= q_s b)$ are the equilibrium and Henry constants of adsorption, respectively (being q_s the saturation capacity), the dependence of isotherm parameters on ϕ could be obtained by combining Eq. (3) and the following relationship between k and a:

$$k = aF \tag{5}$$

It follows that:

$$a(\phi) = a_0\, e^{(-S\phi)} \tag{6}$$

where $a_0\ (= k_0/F)$ is the Henry constant (extrapolated) at $\phi = 0$.

If the range of variation of ϕ is sufficiently narrow, q_s can be considered constant [19,61,62] and, as a consequence, b and ϕ are correlated by the same relation as in Eq. (6):

$$b(\phi) = b_0\, e^{(-S\phi)} \tag{7}$$

where b_0 is the adsorption constant at $\phi = 0$.

By combining Eqs. (4), (6) and (7), the Langmuir isotherm describing the adsorption process under gradient elution conditions can be obtained:

$$q(\phi) = \frac{q_s\, b_0\, e^{(-S\phi)} C}{1 + b_0\, e^{(-S\phi)} C} \tag{8}$$

The mass balance equation (Eq. (2)) can be numerically solved by applying a finite difference method based on the so-called backward-backward scheme [19,63].

Lastly, boundary and initial conditions need to be defined in order to solve the mass balance equation. The Danckwerts-type boundary conditions have been applied [19,64,65] while the gradient in the inlet feed has been simulated as follows:

$$\phi(t,0) = \begin{cases} \phi_0 & 0 \le t \le t_{inj} \\ \phi_0 + \frac{\Delta\phi}{t_g}(t - t_{inj}) & t_{inj} \le t \le t_{inj} + t_g \\ \phi_0 + \Delta\phi & t \ge t_{inj} + t_g \end{cases} \tag{9}$$

where t_{inj} is the length of the rectangular injection profile, ϕ_0 is the initial fraction of organic modifier and t_g is the time of the gradient.

3. Experimental

Column and materials

All solvents were purchased from Sigma–Aldrich (St. Louis, MI, USA). A 150 × 4.6 mm Zorbax Sb-C18 column (5 µm particle size, 80 Å pore size) used to perform separations was from Agilent Technologies (Santa Clara, California, USA). Uracile (Sigma–Aldrich, St. Louis, MI, USA) was injected for the determination of the void volume of the column. Pure and crude (= not purified) mixtures of octreotide were from Fresenius Kabi iPSUM (Villadose, Rovigo, Italy). Crude sample is the product obtained after solid-phase synthesis.

Equipment All the measurements were carried out on an Agilent 1100 Series Capillary LC system equipped with a binary pump system, a column thermostat set at 35 °C and a photodiode array detector. A manually Rheodyne 8125 injecting valve was employed by using different loops to perform detector calibration (500 µ) and injections of overloaded profiles (5, 10, 20 µ). All the experimental profiles were recorded at UV wavelengths of 280 nm, at flow rate of 1 mL/min. Maximum absorbance was below 1000 mAU.

Measurement of overloaded profiles

Overloaded band profiles in both isocratic and gradient elution conditions have been recorded by injecting solutions of peptide with different concentrations: 0.1, 0.3, 0.6, 1.2, 2.0, 4.0 and 6.0 g/L.

Mobile phase A (MP-A) was a solution of 0.02% (v/v) trifluoroacetic acid (TFA) in water, while mobile phase B (MP-B) was 0.02% (v/v) TFA in acetonitrile (ACN). The gradient program was set as follows: (i) the column was firstly equilibrated with 10% (v/v) of MP-B; (ii) in a first linear ramp the percentage of MP-B was increased from 10% to 30% (v/v) over a gradient time, t_{g1}, of 12 min (gradient slope = 1.6% ACN/min); (iii) in a second steeper ramp MP-B was changed from 30% to 90% (v/v) in 3 min, t_{g2} (slope = 20% ACN/min).

Overloaded profiles under isocratic elution conditions were recorded in a range of MP-B from 23% to 28% (v/v). Solubility limit of the peptide in these conditions is 9.0 g/L.

Adsorption isotherm determination

Adsorption isotherms under isocratic elution conditions have been calculated by means of the so-called Inverse Method [19,47,51,66–68]. This method allows the determination of the adsorption isotherm in a few steps, requiring less amount of samples and solvents than other alternative techniques, such as frontal analysis [19,51,68]. The first necessary step is the calibration of the detector. In order to do this, the column has been replaced with a zero-dead-volume connector and 500 µL of each solution of peptide with different concentration have been injected into the system. This operation has been performed for each mobile phase composition. Not surprisingly, differences in detector response were negligible in the very small operative concentration range considered in this work. The maximum absorbance (*Abs*) of each plateau at 280 nm has been recorded and reported in a curve as a function of *C*. Then, (i) experimental profiles at the seven concentrations have been recorded in overloading conditions; (ii) overloaded profiles *Abs vs. t* have been converted into *C vs. t* through the slope of the calibration curve; (iii) an isotherm type and a guess of its initial parameters have been selected; (iv) a system of equations including the mass balance equation and the selected adsorption isotherm have been solved in order to obtain a calculated overloaded profile; (v) the calculated overloaded profile and the experimental *C vs. t* one have been compared; (vi) isotherm parameters have been iteratively changed until the calculated and experimental profiles match as much as possible (the numerical optimization was made by means of the Simplex method, minimizing the sum of the squares of the differences between simulated and experimental profiles) [47,66,69].

4. Results and discussion

Fig. 1 reports an experimental chromatogram recorded under gradient elution conditions by injecting 5 μL of the solution of crude octreotide. The main peak (t_R = 12.5 min) corresponds to

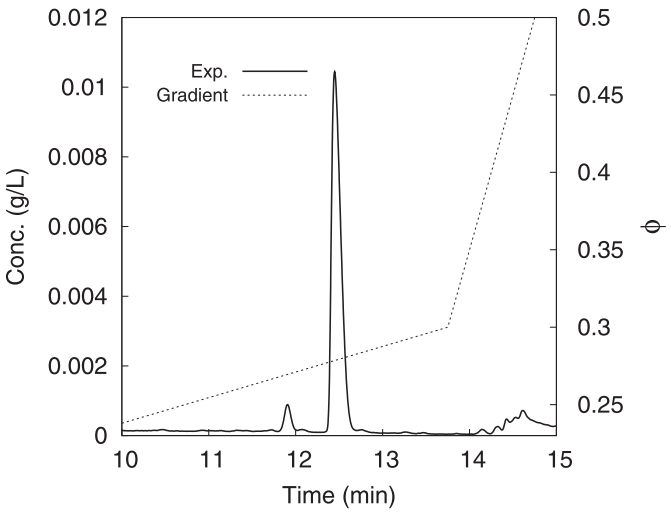

Fig. 1. Experimental gradient elution profile of the crude peptide. Injected concentration: 0.2 g/L; injected volume: 5 µL; wavelength: 280 nm.

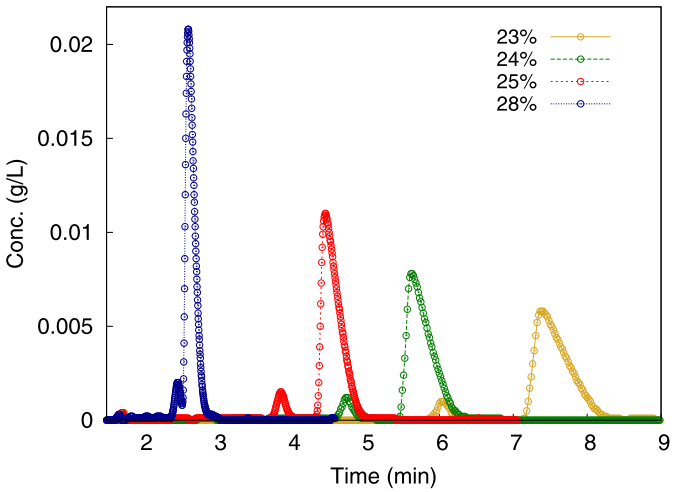

Fig. 2. Comparison between experimental profiles of the crude peptide measured at different volume fractions of organic modifier, ranging from $\phi = 0.23$ to $\phi = 0.28$. Injected concentration = 0.6 g/L; injected volume = 5 μL; wavelength: 280 nm.

the elution of peptide and the smaller one (t_R = 12.0 min) is an impurity from the synthesis.

Taking into account the dwell volume of the system, it was estimated that the elution of peptide takes place approximately around $\phi = 0.25$, therefore a range of ϕ between 0.23 and 0.28 has been chosen for experimental measurements.

Investigation of retention at infinite dilution

The investigation of chromatographic behavior of octreotide at infinite dilution demonstrated that its retention is profoundly affected by changes in the percentage of organic modifier. The dead volume has been determined through an unretained compound, uracil. Indeed, a variation of only roughly 5%, from $\phi = 0.23$ to $\phi = 0.28$, induced a 500% drop of retention factor (from 4.7 to 0.95, respectively, see Fig. 2).

Eq. (3) can be re-arranged according to the displacement model of retention in RP-LC [70]. This model predicts that retention of an hydrophobic molecules from an apolar stationary phase is accompanied by the displacement of a stoichiometric number of solvent molecules adsorbed on the surface [71]:

$$\log k = \log I + Z \times \log\left(\frac{1}{D_0}\right) \quad (10)$$

In this equation, which can be applied in a range where the concentration of organic solvent on the stationary phase is approximately constant, D_0 is the molar concentration of organic modifier, Z the number of molecules of organic solvent displaced by the analyte during retention and I is the value of k when D_0 is 1 M. Fig. 3 shows the variation of log k with $\log(1/D_0)$ for octreotide. From the slope of the linear regression line, the number of displaced molecules has been evaluated. Z resulted to be 8.2 $\pm$ 0.1. This value is significantly large if compared to the molecular weight of the compound, however very close to that obtained for a small polypeptide of comparable molecular mass [63].

Modeling of overloaded profiles under isocratic elution conditions

Adsorption isotherms of both crude and pure peptide solutions at each mobile phase composition have been determined by means of IM. Different adsorption isotherm models have been tested (Langmuir, BiLangmuir, Tóth). Among them, only the Langmuir model was found to satisfactorily fit experimental data. An excellent agreement was found between experimental and calculated peaks of pure and crude solutions of peptide (see Fig. 4). The amount of impurity is so small that it does not compete with peptide for adsorption and its retention time is not influenced by peptide concentration.

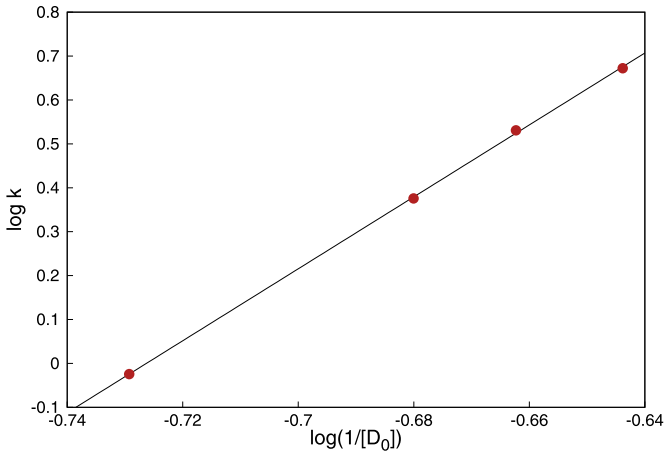

Fig. 3. Dependence of logarithm of retention factor (k) on logarithm of the inverse of ACN concentration (D_0) expressed in terms of molarity. The slope of the linear regression gives an indication of the number of displaced molecules (Z) equal to 8.2 $\pm$ 0.1. $R^2 = 0.999$.

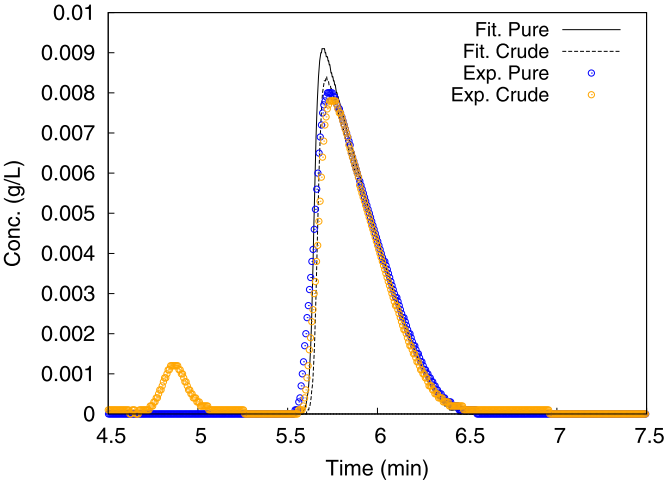

Fig. 4. Comparison between experimental peaks of crude (orange circles) and pure (blue circles) peptide and their corresponding calculated profiles. Dotted and solid lines corresponds to the fitting profiles of the crude and the pure peptide, respectively. $\phi = 0.24$, injected concentration = 0.6 g/L; injected volume = 5 µL; wavelength: 280 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Adsorption isotherm parameters obtained through IM with a Langmuir model at different mobile phase compositions.

ϕ	a	b (L/g)	q_s (g/L)
0.23	5.86	8.14	0.72
0.24	4.21	5.69	0.74
0.25	3.07	4.65	0.66
0.28	1.17	1.86	0.63

Fig. 5 compares some experimental and simulated overloaded profiles of the crude peptide recorded at $\phi = 0.24$ and various loading concentrations. Some small discrepancies in the front part of the peaks, especially for the two highest concentrations, could be due to the presence of kinetic phenomena that are neglected by the ED model. However, the rear parts of experimental and calculated profiles excellently match even at high concentrations.

The best isotherm parameters obtained at the different isocratic conditions investigated in this work are reported in Table 1. As it

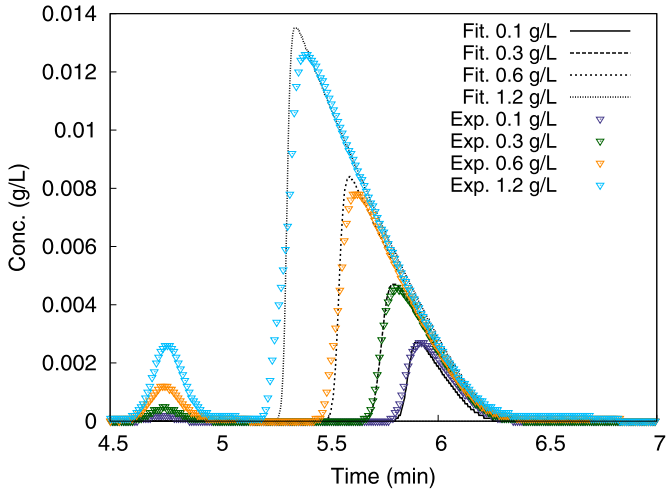

Fig. 5. Comparison between experimental and simulated peaks obtained with IM (Langmuir adsorption isotherm) for four different concentrations of the crude peptide in isocratic conditions (ϕ=0.24). Injected volume: 5 µL; wavelength: 280 nm.

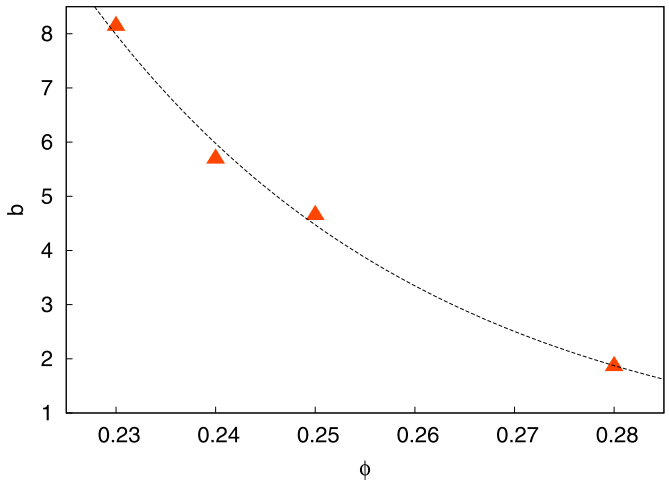

Fig. 6. Dependence of the equilibrium adsorption constant (b) on the fraction of organic modifier (ϕ) according to Eq. (7) (R^2=0.998).

can be evinced, q_s values are in a very good agreement, supporting the hypothesis of a small variation of q_s if this range of ϕ is significantly small. Moreover, the variation of b with ϕ follows the trend described by Eq. (7) (see Fig. 6). By fitting experimental data with an exponential equation, values of 29 and 6.3 $\times$ 10^3 L/g have been calculated for S and b_0, respectively. For the saturation capacity q_s the average value of 0.69 g/L was taken in the simulation of gradient elution experiments.

Modeling of overloaded profiles under gradient elution conditions

Substituting the above value of b_0, S and q_s in Eq. (8), the equilibrium-dispersive model with the feed conditions (Eq. (9)) can be solved to simulate gradient elution runs. As it can be observed from Fig. 7, where calculated profiles (solid lines) and experimental ones (coloured circles) are compared, a very good agreement between theoretical and experimental profiles has been obtained even at high concentrations.

In order to test model reliability and potential to predict conditions not considered in its development and parameter tuning, two more experimental runs at increasing loading volume have been considered, that is 10 and 20 µL. The match between experimental and predicted peaks was satisfactory (see Fig. 8). This means not only that the simple Langmuir model (based on the assumption that the adsorption surface is energetically homogeneous) is

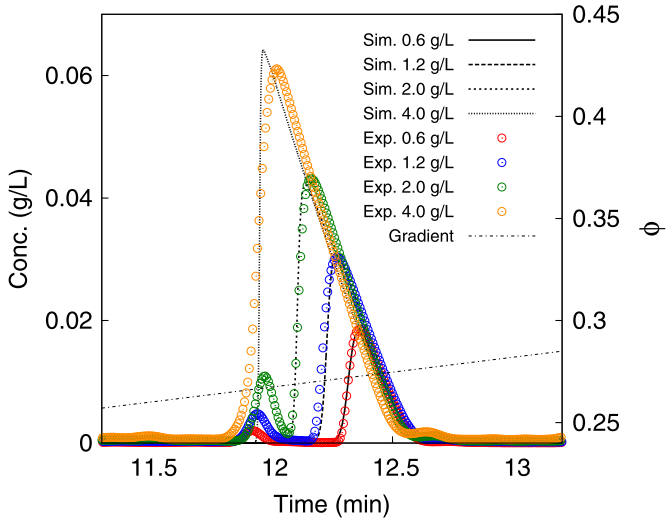

Fig. 7. Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption model) of four different concentrations of crude peptide. Injected volume: 5 µL; wavelength: 280 nm.

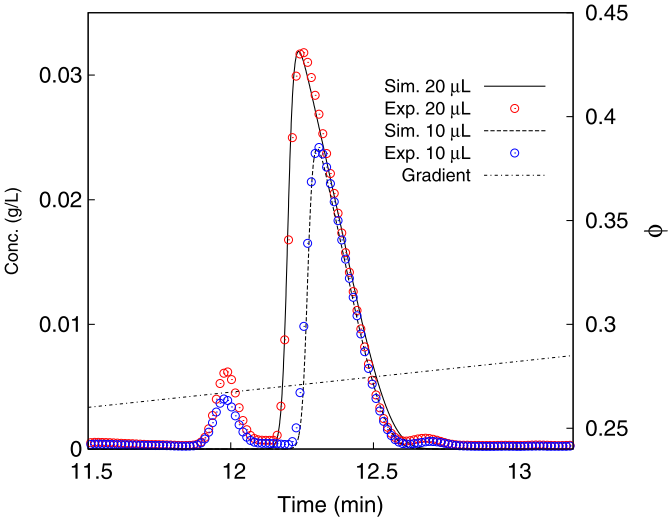

Fig. 8. Comparison between experimental and simulated peaks in gradient elution conditions. Injected concentrations: 0.5 g/L. Injected volume: 10 µL (blue) and 20 µL (red); wavelength: 280 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adequate to describe the adsorption mechanism of this peptide on this stationary phase but also, most importantly, that very small amount (μg) of peptide are sufficient to gather information on its adsorption equilibria and to model the separation under nonlinear gradient conditions.

5. Conclusions

Gradient preparative RP-LC is one of the most widely used technique for the purification of synthesized peptides. A reasonable approach to develop a purification method via preparative HPLC is based on the prior investigation of thermodynamic equilibria regulating retention of peptides on the stationary phase. This is practically translated into the calculation of their adsorption isotherms under different mobile phase compositions in a range of ϕ where elution takes place, in order to find the relationship between isotherm parameters and variation of organic modifier in the mobile phase.

Most of the times, the amount of available peptide is reduced or its cost is elevated. When this is the case, modern techniques of isotherm determination, based on theoretical hypotheses on the adsorption model and the simulation of peaks under overloaded conditions, can be efficiently employed to achieve the relevant information.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Chiara De Luca: Investigation, Visualization, Writing - original draft. **Simona Felletti:** Investigation, Visualization, Writing - original draft. **Marco Macis:** Resources, Supervision. **Walter Cabri:** Resources, Supervision. **Giulio Lievore:** Investigation, Validation. **Tatiana Chenet:** Data curation. **Luisa Pasti:** Formal analysis. **Massimo Morbidelli:** Supervision. **Alberto Cavazzini:** Supervision, Funding acquisition, Project administration, Resources, Writing - review & editing. **Martina Catani:** Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Antonio Ricci:** Resources, Supervision, Funding acquisition.

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PAPER II

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Short Communication

Determination of the Thermodynamic Behavior of a Therapeutic Peptide in Overloading Conditions in Gradient Elution Chromatography

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ABSTRACT

The aim of this work is to model the adsorption behavior of a cyclic octapeptide, octreotide, in reversed-phase gradient elution liquid chromatography. Adsorption isotherms of the peptide were firstly determined in isocratic conditions, using different peptide concentrations and compositions of the mobile phase (mixtures with different percentage of organic modifier, ranging from 23 to 28% v/v). Inverse Method (IM) was applied to determine the parameters describing the isotherm equation for every composition of mobile phase considered. Then, the isotherm parameters have been correlated to the amount of organic modifier in the mobile phase, through the Linear Solvent Strength model (LSS). In the end, it was possible to predict the chromatographic behavior of the cyclic octapeptide in overloading gradient conditions, the knowledge of which can be useful when scaling the method in preparative conditions.

Keywords: Peptide; Inverse method; Adsorption isotherm; Langmuir isotherm; Nonlinear chromatography

INTRODUCTION

Peptides are a particular class of biomolecules largely employed in pharmaceuticals, nutraceuticals and cosmetics. Not only do they act specifically towards a particular target receptor, making them effective also at very low concentration, but also, they do not accumulate in the human body, and this contributes to avoid dangerous side effects [1-4]. Usually, peptides can be produced through recombinant synthesis or through Liquid-phase or Solid-Phase synthesis [5,6]. Anyway, none of the production methods leads to the single target product, but rather to a wide range of impurities. As a consequence, one or more purification steps are required to reach the purity requirements imposed by regulatory agencies [4]. The main technique used for the purification of peptides is liquid chromatography in preparative conditions, which means that large volumes of feed with high concentration are processed in a single run. Since the amount of product injected into the column in preparative chromatography can be very large, the adsorption isotherm of the compound is nonlinear. Preparative conditions also imply that the retention of analytes is concentration-dependent and moreover chromatographic peaks are not gaussian but show a strong asymmetry such as fronting or tailing. Separation problems related to complex mixtures in overloading conditions are challenging also because the amount of a component adsorbed on the stationary phase depends on the concentration of all the other species in solution.

Usually the operating conditions of the purification process are determined using trial and error strategies, with consequent waste of product and time. The knowledge of thermodynamic equilibrium of the target peptide can give information on the maximum loading and on the affinity of the product for the stationary phase. This can be a help in the design of the separation process, also for large-scale purification and for processes employing continuous chromatography technology [7-12].

The adsorption isotherm of a compound is traditionally determined using Frontal Analysis, a technique which employs large amounts of product. The technique used in this study,

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called Inverse Method, permits to obtain the isotherm parameters injecting only low amounts of the compound of interest, allowing saving material and time [13-16]. The procedure followed to apply the Inverse Method is reported in bibliography [17-20] and it has been described in detail by the authors in a previous paper [21].

In this work, Inverse Method has been applied to obtain the adsorption isotherm of octreotide, a cyclic octapeptide, at different isocratic conditions in reversed-phase chromatography. The correlation of isotherm parameters with the composition of the mobile phase, determined through Linear Solvent Strength (LSS) model, has been used to predict the adsorption behavior of the peptide under overloading and gradient conditions. The prediction of the peak profile in gradient conditions could be exploited during the design of the purification process in preparative conditions.

MATERIALS AND METHODS

The adsorption was studied on a commercial column, a 150 × 4.6 mm Zorbax SB-C18 column, with 5 µm particle size e 80 Å pore size. The synthetic crude of octreotide was obtained by means of Solid Phase Synthesis by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy), which also provided the pure octreotide to be used as a standard.

The measurements were performed using an Agilent 1100 Series Capillary LC system equipped with a photodiode array detector, the wavelength of which was set at 280 nm. Different loops have been used for the calibration of the detector (500 µL) and for the measurements (5, 10, 20 µL).

Solutions with different octreotide concentrations (0.1, 0.3, 0.6, 1.2, 2.0, 4.0 and 6.0 g/L) were prepared to study the overloaded band profiles of the compound of interest both in isocratic and in gradient conditions. The mobile phases used were 0.02% trifluoroacetic acid (TFA) in water (MP-A) and in acetonitrile (MP-B), respectively. The isocratic conditions examined were in a range from 23 to 28% (v/v) of MP-B.

Adsorption isotherms were determined through Inverse Method, which allows obtaining thermodynamic information in few steps using very low amounts of compound. The procedure followed for the Inverse Method and other experimental conditions employed are reported [21].

RESULTS AND DISCUSSION

Firstly, a gradient method has been performed on a solution of crude octreotide to find a range of ϕ (fraction of organic modifier) where the peptide elutes. Taking into account the dwell volume of the system, it has been estimated that the peptide elutes around ϕ=0.25. As a consequence, a range of ϕ between 0.23 and 0.28 has been considered for the isotherm determination.

At infinite dilution, it is possible to affirm that the retention factor is dramatically affected by the amount of organic modifier in the mobile phase, as it can be seen from Figure 1. A variation in the mobile phase from ϕ=0.23 to ϕ=0.28 causes a 5 times reduction in retention factor (from 4.7 to 0.95).

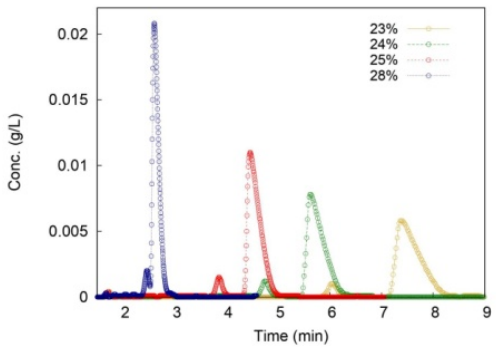

Figure 1: Variation of the retention with the fraction % (v/v) of organic modifier. Comparison between experimental peak profiles of a crude peptide sample at different ϕ, from 0.23 to 0.28. Sample conc=0.6 g/L; Vinj=5 µ L. Reproduced with permissions from study by De Luca et al. [21].

Inverse Method has been applied to determine the adsorption isotherm parameters at each composition of mobile phase considered, for both the crude and the pure octreotide samples. Langmuir, Bilangmuir and Tòth isotherm equations have been tested, but only the Langmuir isotherm appropriately fitted experimental peak profiles. The Langmuir isotherm equation is written as (Equation 1):

$$q = \frac{q_s b C}{1 + bC} \qquad (1)$$

where q_s is the adsorption saturation capacity and b is the adsorption equilibrium constant; the product $q_s \times b$ is the Henry constant (a). By changing the mobile phase composition, the adsorption isotherm model does not change, but its parameters q_s and b do. Anyway, if the range of concentrations of organic modifier is narrow, q_s is likely to be constant; therefore, only b changes during the gradient, which means that b is a function of ϕ. The reader is addressed to De Luca et al. [21] for a theoretical discussion of the formulas employed.

To make a comparison between the peak profiles of different crude peptide concentrations at a given composition of mobile phase Figure 2 is reported, which shows both the experimental and the simulated overloaded peaks. The equilibrium-dispersive model, which is the model chosen in this study for the calculations of the Inverse Method, neglects kinetic phenomena which possibly occur in fact. This could be the reason for small differences between the measured and the experimental peak profiles, especially in the front part and for higher concentrations. On the other side, the agreement in the rear part is perfect at every concentration. The agreement between experimental and theoretical peak profiles has been found to be excellent also for the pure octreotide samples.

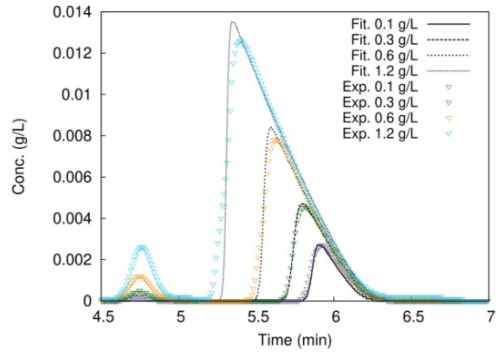

Figure 2: Agreement between calculated and experimental profiles at four different peptide concentrations in isocratic conditions. The black lines are the theoretical profiles obtained through Inverse Method using a Langmuir isotherm model, whereas the colored circles represent the experimental chromatograms. Vinj=5 µL; **ϕ**=0.24. Reproduced with permissions from [21].

The Inverse Method calculations give back a value for q_s and b for every isocratic condition; q_s and b do not change by varying the peptide concentration at a given **ϕ**. From Table 1 it can be noted that, as expected, in the narrow range of **ϕ** considered q_s values are very similar, around 0.69 g/L. On the contrary, b varies with **ϕ** following an exponential trend:

$$b(\phi) = b_0 e^{(-S\phi)} \qquad (2)$$

Where the parameter S is a coefficient characteristic of the system solute-mobile phase and b_0 is the adsorption constant extrapolated at **ϕ**=0. These parameters are found to be 29 and 6.3×10^3 L/g respectively; they are employed to predict the overloaded peak profiles when the elution takes place not in isocratic but in gradient conditions. The Langmuir isotherm is modified to keep into account that b is not constant anymore during the elution, but it changes while changing the amount of organic modifier during the gradient, as described in Equation 3:

$$q = \frac{q_s C b_0 e^{(-S\phi)}}{1 + C b_0 e^{(-S\phi)}} \qquad (3)$$

The equilibrium-dispersive model can be solved using the Langmuir isotherm modified (Equation 3) and the values of q_s, b_0 and S previously found. The outcome of the calculations is a simulated peak profile in gradient conditions, which is to be compared to the experimental peak also obtained in gradient conditions.

As it can be noted from Figure 3, the agreement between the theoretical profiles and the experimental peak is very good at each concentration. In the case of Figure 3, the injection volume is only 5 µL; other measurements and calculations with higher injection volumes (10, 20 µL) have been performed to test the reliability of the model.

It was found that even at higher loading, the match between the predicted and the experimental peaks is still very satisfactory. Thus, the model developed permits to predict the thermodynamic behavior of the peptide in overloading gradient conditions using only small amounts of product, in the order of µg.

Table 1: Parameters obtained at different **ϕ** by fitting the experimental peak profiles using Inverse Method with a Langmuir isotherm model.

ϕ	a	b (L/g)	q_s (g/L)
0.23	5.86	8.14	0.72
0.24	4.21	5.69	0.74
0.25	3.07	4.65	0.66
0.28	1.17	1.86	0.63

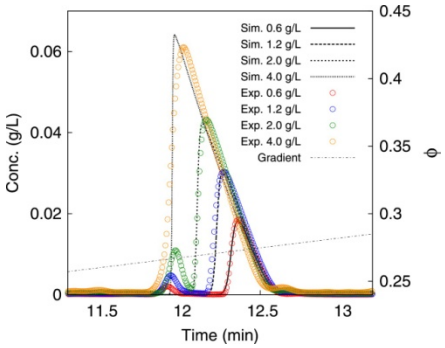

Figure 3: Comparison between experimental and simulated peaks in gradient conditions for four different crude peptide concentrations. The calculated peaks have been obtained using the model developed in this work and the Langmuir isotherm equation modified Vinj=5 µL reproduced with permissions from [21].

CONCLUSION

In this study, a model able to predict the peak profile in overloaded gradient conditions has been developed. The first step is the calculation of the adsorption isotherm under different isocratic conditions, using the Inverse Method. Then the variation of the adsorption isotherm parameters is correlated to the fraction of organic modifier in the mobile phase and, thus, to the gradient. The Inverse Method allows obtaining relevant thermodynamic information using just small amounts of product (some micrograms). The investigation of the thermodynamic equilibria involved in the retention of the peptide in RP-LC is a good starting point to develop a purification method through preparative chromatography in gradient conditions, avoiding the trial-and-error strategy. This would be particularly convenient especially for valuable compounds.

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PAPER III

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From batch to continuous chromatographic purification of a therapeutic peptide through multicolumn countercurrent solvent gradient purification

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ABSTRACT

A twin-column Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process has been developed for the purification of a therapeutic peptide, glucagon, from a crude synthetic mixture. This semi-continuous process uses two identical columns operating either in interconnected or in batch mode, thus enabling the internal recycle of the portions of the eluting stream which do not comply with purity specifications. Because of this feature, which actually results in the simulated countercurrent movement of the stationary phase with respect to the mobile one, the yield-purity trade-off typical of traditional batch preparative chromatography can be alleviated. Moreover, the purification process can be completely automatized.

Aim of this work is to present a simple procedure for the development of the MCSGP process based on a single batch experiment, in the case of a therapeutic peptide of industrial relevance. This allowed to recover roughly 90% of the injected glucagon in a purified pool with a purity of about 90%.

A comparison between the performance of the MCSGP process and the classical single column batch process indicates that percentage increase in the recovery of target product is +23% when transferring the method from batch conditions to MCSGP, with an unchanged purity of around 89%. This improvement comes at the expenses of a reduction of about 38% in productivity.

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1. Introduction

Over the last decades, the interest towards small proteins and peptides in the pharmaceutical field has dramatically increased. This trend has been driven by the introduction of novel synthetic strategies, based on amino acid modifications and incorporation of other moieties, that allow to modulate not only the pharmacokinetic properties of these biomolecules but also their specificity towards a particular target. As a result, peptides currently find many applications as antimicrobials, antioxidants, anti-hypertensives, anticoagulants, but also as appetite regulators and functional foods, to name but a few [1–4].

The industrial production of therapeutic peptides is mainly carried out by means of solid phase synthesis. This technique consists in the addition of one amino acid at a time at the end of the growing chain tied to an insoluble resin, in a cycle of deprotection-wash-coupling-wash. However, since it is not possible to perform an intermediate purification, many impurities (for example diastereoisomeric products, incomplete protected sequences, wrong amino acid insertions, oxidations, reductions, etc.) coming from incomplete or side-reactions can be generated [5,6]. The target peptide needs to be isolated from these impurities in order to meet the strict purity specifications required for pharmaceuticals, therefore one or more purification steps are necessary. This point of the downstream process is often the bottleneck in terms of time and costs in the whole production of synthetic peptides [7–

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10]. Liquid chromatography on a preparative scale is the most employed method for the industrial purification of peptides [5,6,11–15]. However, the presence of impurities chemically similar to the target represents a critical issue during the purification process since their chromatographic peaks can overlap both in the front and in the tail of the peak of the target [16]. As a consequence, batch processes generally lead to a severe trade-off between purity and yield. Indeed, by enlarging the product pool window and including the overlapping regions, purity is reduced but yield can be improved; on the contrary, if a very narrow product window is taken purity increases at the expense of yield. This problem is intrinsic of single-column batch preparative chromatography [6,16].

The yield-purity trade-off can be overcome by employing continuous (or semi-continuous) countercurrent purification techniques [16,17], where two (or more) identical columns, properly connected through several switching valves, are operated both in batch and in interconnected mode, alternatively. As in other countercurrent techniques, the movement of the stationary phase in the opposite direction as that of the mobile one is not real but it is simulated through the switching valves, that change the connection between the inlets and the outlets of the columns and hence the path accessible to the mobile phase. This leads to considerable advantages in terms of yield of purification [18]. Moreover, the continuous internal recycling of partially unresolved component into the system allows to completely automatize the purification process [19,20]. Among the continuous techniques, Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is particularly suitable for the purification of complex mixtures containing product-related impurities co-eluting with the target [6]. Indeed, MCSGP can be applied to ternary separations, such as the case of a target that almost coelutes with more weakly adsorbed (from here on called W) and more strongly adsorbed (from here on called S) impurities, in the front and in the tail, respectively [7,21]. Moreover, the great advantage of MCSGP over other continuous purification techniques (such as Simulated Moving Bed) is that it can handle separation problems where a gradient of modifier is required [9,22–25]. This is the case of purification of large biomolecules, whose retention is strongly affected by the organic modifier concentration [24,26,27]. In particular, when a large number of product-related impurities is present, shallow gradients should be used in order to increase the selectivity of the separation [28].

In its first arrangement, MCSGP was realised with 6 columns [29,30]. Later on, this unit has been more and more simplified to three [31] and finally to only two columns [6], which clearly results in a decreased complexity from the point of view of tubing and valves.

Briefly, the operating principle of twin-column MCSGP is the following: two identical columns (with the same stationary phase as that of the design batch) carry out four different tasks in parallel but shifted of half a cycle, as illustrated schematically in Fig. 1. In particular, the portion of the peak satisfying the purity requirement is collected from the upstream column during every cycle (zone 6); on the contrary, the unresolved side portions of the peak (front and tail), that are contaminated with impurites but contain a large amount of peptide, are recycled into the downstream column (zones 5 and 7). Then the columns exchange position. In this way, it is possible to avoid the product loss that would occur if the overlapping fractions were discarded. Note that in order to inject the same quantity of peptide cycle after cycle, a certain amount of fresh feed needs to be injected into the downstream column receiving the overlapping fractions [6,14]. All the operations will be further described in the Theory section.

In this work, the MCSGP process is applied to the purification of a synthetic crude mixture of glucagon, a linear polypeptide hormone consisting of 29 amino acidic residues (MW = 3485 Da) excreted by the pancreatic α-cells. This is the principal hyperglycemic hormone, acting as a counterbalance to insulin.

The objective of this work is to illustrate, using an industrially relevant purification process, how to quickly design a MCSGP process from a single column chromatogram, without any specific need of process performance optimization. A comparison between the performances of batch (single column) and MCSGP (two columns) processes is also presented.

2. Theory

2.1. MCSGP principles

The starting point to design an MCSGP process is the definition of a design batch chromatogram, through which parameters such as loading, gradient slope and regeneration procedure are defined. This chromatogram must be optimized in order to meet purity requirements at least in some portion of the main peak. Generally, a single column process is made up of four parts: equilibration, load, elution and stripping (that is, washing with high organic modifier concentration to remove whatever chemical is still inside the column). In turn, the elution of the feed can be divided in 5 steps: elution of W, overlapping region containing W/P, window where the product P is pure, overlapping region containing P/S, elution of S. The overlapping windows are undoubtedly contaminated but contain a large quantity of target product; therefore, they cannot be wasted but need to be recovered, in order to obtain a satisfactory process yield. In batch processes, this is performed through an external recycle, with waste of time and risk of errors made by the operator. The MCSGP technique allows for the automation of this step, which is performed inside the unit continuously, with no interruption in time.

When the process is transferred from batch to continuous chromatography, the columns work alternately either in batch or in interconnected manner. Let's consider Fig. 1, which represents schematically the case where column-1 is in the upstream position and column-2 is in the downstream position; this means that what comes out from column-1 can be recycled in column-2. The gradient program starts in column-1 and W impurities begin to elute (zone 4); this stream is wasted. In the meanwhile, column-2 undergoes the stripping and equilibration phases (zone 8), to get ready to receive the feed. During this step the columns work in batch mode. As soon as the overlapping region W/P starts eluting from column-1, the valves exchange configuration and the eluate enters column-2 (zones 5 and 1 respectively). This is the recycling of W/P which happens in interconnected mode. Then during the Product Elution Window (PEW), the pure peptide eluting from column-1 is collected (zone 6), while column-2 is loaded with fresh feed to compensate the loss of product leaving column-1 (zone 2, batch mode). Next, P/S window is recycled into column-2 (zone 3) after leaving column-1 (zone 7, interconnected mode). At this point, the gradient can be performed in column-2, which has been completely loaded, and column-1 is stripped to remove S. At this point, the columns exchange position (column-1 is now in downstream position and vice versa) and this represents the end of the switch; after 2 switches, when the columns come back to the initial configuration, a cycle is completed [12,32,33]. Hence, each column is loaded and eluted once per cycle. It must be highlighted that the loading step is subdivided in three steps: uptake of W/P, injection of fresh feed, uptake of P/S. If all the operating conditions, such as load amount, gradient slope, etc., are kept constant from batch to MCSGP, then the only degrees of freedom characterizing the continuous process are the values of the characteristic elution times or elution column volumes, which define the five zones of the elution [14]. Particularly, the elution of W starts at the beginning of the gradient, in correspondence with the time t_A, and it

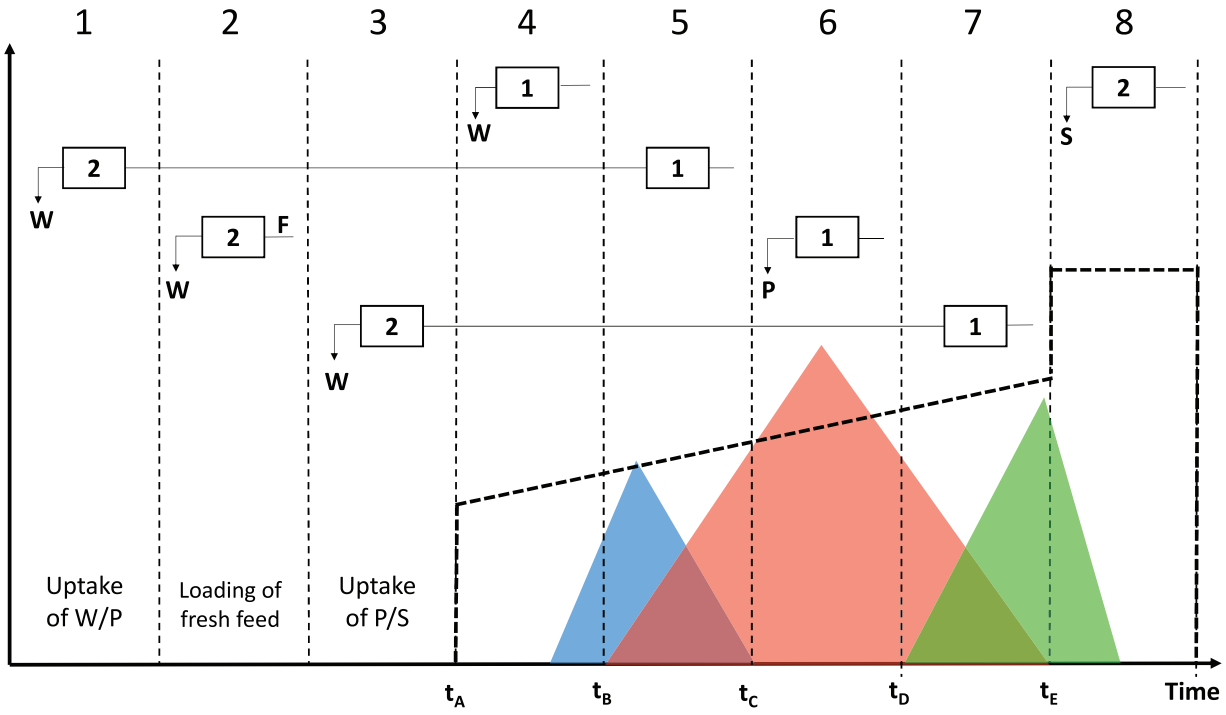

Fig. 1. Schematic illustration of a batch chromatogram and the corresponding tasks within a switch where column-1 is in the upstream position. The blue (green) peak corresponds to the weak (strong) impurities, while the red peak is the target product. Five intervals are identified where different fractions are eluting: W (t_A to t_B), overlapping of W and P (t_B to t_C), P (t_C to t_D), overlapping of P and S (t_D to t_E) and S, during the stripping. t_B, t_C, t_D and t_E are the characteristic switching times of the MCSGP process. t_A is the time where the gradient starts. Note that the loading of the target in column-2 happens in three steps: during the recycling of W/P, the loading of fresh feed and the recycling of P/S. The linear gradient of the modifier is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ends at the time t_B, where W/P begins to elute from the upstream to the downstream column. The PEW extends from t_C to t_D, at the beginning of the elution of P/S, which on the other side ends at t_E. Each of these characteristic times, if multiplied for the elution flow rate, leads to a characteristic elution volume (CV_B, CV_C, CV_D, CV_E). t_A, on the other hand, indicates the time where the gradient starts (see Fig. 1).

It is noteworthy that the overlapping regions need to be diluted with a compensation buffer before being loaded to the next column. Indeed, these regions elute during the gradient and this implies that the concentration of modifier is continuously increasing during this period. Dilution is therefore necessary to allow their adsorption on the stationary phase. Usually, the first recycle stream (W/P) is diluted so as to reach the modifier concentration value at the beginning of the elution of W/P, which allows the product to adsorb and the weak impurities to start moving. On the other hand, the P/S recycling stream is diluted to the modifier concentration value at the beginning of the gradient, in order to adsorb both the product and the strong impurities.

2.2. Performance parameters

In order to quantify the performance of a purification process, either in batch or in continuous, some suitable parameters need to be introduced. These are calculated from the HPLC chromatogram of a fraction (in the case of a batch) or of a pool (in the case of MCSGP). The most important parameter is the purity of the target compound, which must satisfy the strict requirements imposed. It is defined as the ratio of the area of the target peak in the product pool (or fraction) to the total area in the product pool or fraction (sum of the areas of the target and impurities), measured in analytical conditions:

$$\text{Purity} = \frac{\text{area}_{\text{target}}}{\text{area}_{\text{tot}}} \times 100 \quad (1)$$

Process recovery, otherwise called yield, is the ratio between the mass of the target recovered within purity specifications ($m_{target\ recovered}$) and the mass loaded in the feed ($m_{target\ loaded}$):

$$\text{Recovery} = \frac{\text{m}_{\text{target recovered}}}{\text{m}_{\text{target loaded}}} \times 100 \quad (2)$$

Finally, productivity is defined as the mass of the target recovered within purity specifications, divided by the corresponding operation time (t_{run}) and the total volume of stationary phase, measured in CVs (i.e., the geometrical volume of the column):

$$\text{Productivity} = \frac{\text{m}_{\text{target recovered}}}{\text{t}_{\text{run}} \times \text{ CV}} \quad (3)$$

In case of an MCSGP run, these parameters are calculated per cycle, for example t_{run} is the duration of a cycle (which, in the case of batch processes, represents the total duration of a purification run). It must be also taken into account that in MCSGP two columns are used, therefore the total volume of the stationary phase, that is the CV value in the denominator of Eq. (3), is twice the volume of one single column. Moreover, in MCSGP, the performance parameters are typically computed as the mean of the values obtained for all the cycles that are at the steady-state, which in practice is defined as the condition reached when the UV profiles of two consecutive cycles can be completely superimposed.

By fractionating the chromatogram eluting from a single column, it is possible to estimate the performance parameters corresponding to different sizes of the overall target collection fraction. Thus, starting from the purest fraction (which also exhibits the lowest recovery) and successively including neighboring fractions, the values for purity and yield corresponding to various hypothetical batch pool can be measured. As stated above, the broader

the pool, the lower the purity, and moreover the higher the recovery. These pairs of values when reported on a recovery versus purity plot describe a so called pareto curve, which characterises the performance of this specific single column process, by defining for each purity value what is the maximum recovery that can be achieved. For the MCSGP process instead, for each operation (that is, for each set of characteristic times) a single pair of purity and recovery values is obtained. This is calculated as the average parameters of the pools at the steady-state. With reference to the same plot mentioned above, the point belonging to the Pareto of the specific MCSGP process considered can be compared to the Pareto corresponding to the single column process. It is clear that points closer to the upper-right corner of the plot correspond to better process performances, meaning that at the same purity the recovery is higher.

3. Experimental section

3.1. The crude mixture

Glucagon has been synthesized by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy) through Solid Phase Synthesis. The peptide constitutes 30% on weigh of the crude mixture, and has a chromatographic purity of 55%. In this research, only the first step of the purification process was investigated, where the purity requirement is rather low, around 85–90%. A second step is then needed to achieve the required purity specifications.

The crude mixture was dissolved in a solution of 20% acetonitrile (ACN) and 0.01% trifluoroacetic acid (TFA), having a pH=1.8. The feed was prepared at 1 g/l and stored for no more than two days, in order to avoid gelation, which is very common for glucagon, particularly at high concentration or under vigorous stirring [34].

3.2. Preparative separations

The columns used were three Daisogel-SP-120-10-C8-Bio, functionalised with C8 chains. The pore size was 120 Å, the particle size 10 µm and the internal diameter 4.6 mm. The column used for the batch was 25 cm long and the two columns for MCSGP were each 15 cm long. The columns are completely identical apart for the length.

To perform both the single column and the MCSGP runs, a ContiChrom CUBE Combined instrument has been used (ChromaCon/YMC, Zurich, Switzerland) equipped with two UV detectors and a Foxy R1 fraction collector. The wavelength was set at 280 nm.

The buffers used for the linear gradient were two mixtures of aqueous and organic solvents. In particular, mobile phase A (MP-A) was 80% ammonium acetate 20 mM in water and 20% ACN, whereas mobile phase B (MP-B) was 60% ammonium acetate 20 mM in water and 40% ACN. The duration of every step is expressed in eluted column volumes, CVs. The feed is loaded at 3 mL/min with a concentration of 10 mg/mL$_{column}$, corresponding for the batch to 10 CVs of feed with a concentration of 1 g/L. On the contrary, only a certain percentage of these 10 CVs is injected in each switch in the MCSGP, as decided for each operation from time to time, based on the time windows chosen. Basically, the amount of fresh feed to be injected for each switch in MCSGP corresponds to the difference between the mass loaded in batch and the mass of the target product contained in the recycling windows. The mass to inject is then equal to the target product wasted in W + wasted in S + collected in the window P in the batch process (see Fig. 2).

After the loading, 2 CVs are used to wash the column with 35% MP-B, at 2.5 mL/min. Now the column can undergo the gradient,

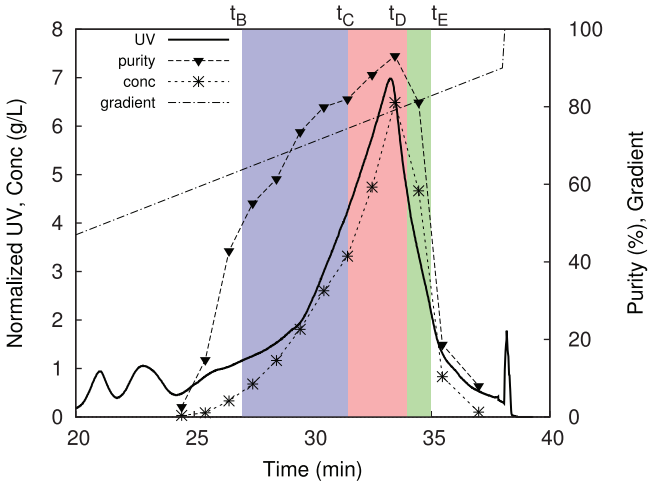

Fig. 2. Scheme of the design batch chromatogram together with the linear gradient of the modifier concentration, the purity profile and target product concentration profile. The values have been obtained by analyzing the fractions collected at every minute at the outlet of the 15 cm column. Moreover, the intervals for the recycling of W/P (blue) and P/S (green) and the collection of the target product (red) chosen for designing the MCSGP process and the relative switching times (in the top of the graphic) are highligthed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which lasts for 11 CVs and goes from 35 to 90% of MP-B, at a rather low flow rate (1.2 mL/min). At the end of the elution, a stripping step is required to wash the column and remove all the strongly adsorbed impurities; for this, a buffer made of 90% ACN and 10% ammonium acetate 20 mM is used for 3 CVs at 3 mL/min.

An MCSGP process is basically composed of three steps: i) firstly, there is a so-called "Start-Up" step, where the same quantity of feed as in the batch run is loaded on the first column; ii) then the cycles start; and iii) finally a so-called "Shut-Down" is performed, where no feed is injected, and the columns are cleaned to end the process.

As compensation buffer, MP-A, containing a very low concentration of ACN, was used for intercolumn online dilution.

Fractions were collected every 60 s for the batch process, while for MCSGP one pool was collected for every switch (two pools per cycle). It was observed that steady-state conditions were usually achieved after two or three switches; the whole process was run for 5 cycles (10 switches) in total.

3.3. Analytics

Every fraction, pool and feed have been analysed in HPLC on an Agilent 1100 (Agilent, Santa Clara, CA, USA) according to the method reported by USP. A 150 × 3.0 mm ACE 3 C_{18} column packed with 3 µm particles was used. Mobile phases were a phosphate buffer solution at pH = 2.7 (MP_A) and a mixture of water/acetonitrile 60:40%(v/v) (MP_B). Gradient program run from 39 to 88% MP_B in 4 min, followed by 1 min of isocratic at 88% MP_B. Then initial conditions (39% MP_B) were restored in 1 min. Detection wavelenght was set at 214 nm. Injection volume was 15 μL. Calibration was performed using samples with known concentration of pure peptide, ranging from 0.05 to 2 g/L.

4. Results and discussion

4.1. Design batch chromatogram

As a preliminary study, several batch experiments have been run with different gradient conditions, that is, different slope and

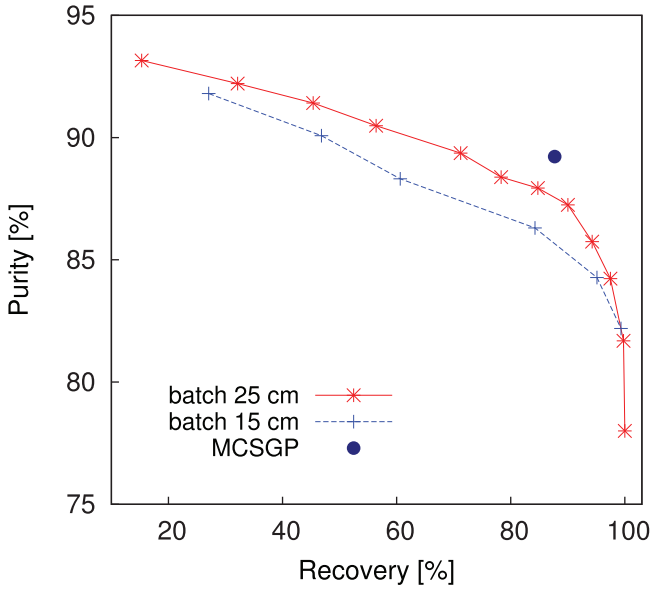

Fig. 3. Pareto curves corresponding to two batch runs using a 15cm column (blue crosses) and a 25cm column (red stars). The single point refers to the MCSGP operation. Note that the productivity values are different for the different operating conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Switching times and injection volume per switch for the MCSGP process.

t_B (min)	t_C (min)	t_D (min)	t_E (min)	V_{inj} (CV)
27	31.5	33	34	5.7

initial and final modifier concentrations, using the 25 cm long column. Among these, the conditions showing the least steep Pareto curve (i.e., where the purity decreases less dramatically with increasing recovery) has been chosen as the design batch chromatogram. In the case under examination, the gradient described in Section 3.2 has been found. This leads to the chromatogram shown in Fig. 2, where the values of purity and target product concentration in the various fractions are indicated, together with the UV signal. In the purest fraction, meaning with the narrowest collection window, the purity is around 93%, while, on the other hand, the recovery is only 15%. By enlarging this window, purity decreases and yield improves, as it is illustrated on the Pareto curve in Fig. 3. The same method has also been run on the short column (15 cm). As expected, the Pareto curve of the longer column lies above that of the 15 cm one, meaning that, for the same recovery values, higher purities can be obtained on the 25 cm column. Note that the performance obtained in batch conditions on the 25 cm long column is used as reference for a fair comparison between batch and continuous runs.

4.2. MCSGP

The MCSGP method has been set up starting from the design batch chromatogram run on the 15 cm column, which has been used to select the times t_B to t_E and then to define the recycling and collection windows. Several MCSGP runs have been performed with different sets of switching times, while all the other parameters have been kept constant. The choice of these times strongly affects the MCSGP process and even a difference of half a minute can change the outcome of purification. The best set of operating times is reported in Table 1 while the corresponding collection and recycling windows are illustrated in Fig. 2. These points correspond

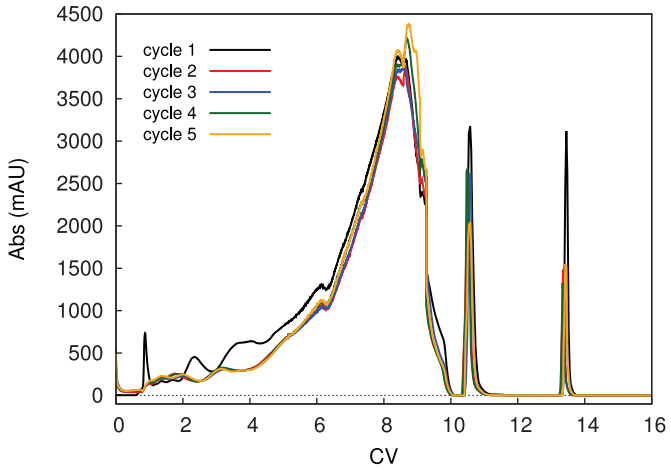

Fig. 4. Overlap of the UV signals measured at the outlet of one column of the MCSGP unit during 5 different cycles. The sharp peaks on the right correspond to the strip and re-equilibration of the column after the overlapping fraction P/S has been recycled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

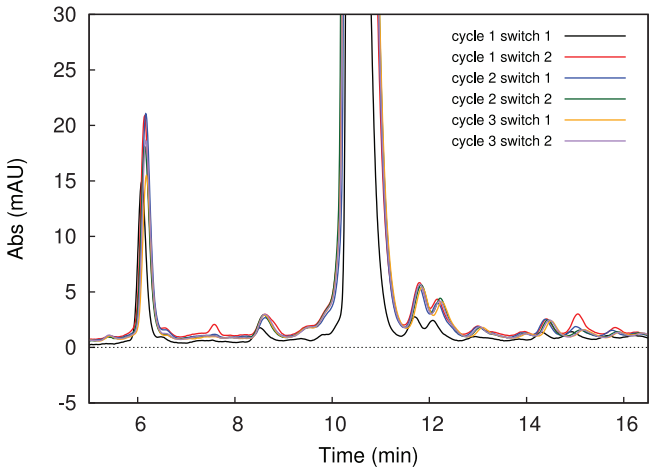

Fig. 5. Analytical HPLC chromatograms of the product fractions collected during the first six switches after the start-up of the MCSGP unit. It appears that, except for switch 1 in the first cycle, all the other chromatograms overlap, indicating that steady-state conditions have been achieved. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to an hypothetical batch pool (betwen t_C = 31.5 min and t_D = 33 min) on the 15 cm column with a purity of 89% and recovery of 53% (meaning that 53% of the injected peptide is recovered in the target product collection window). The remaining mass of the injected peptide is distributed in the other fractions as follows: 32% in W/P, 11% in P/S and 4% is wasted in weak and strong.

In order to keep the loading constant, 57% of the batch loading has been reinjected at every switch in MCSGP, taking into account the fact that 43% (32% + 11%) of the peptide is already present into the downstream column after the recycling of W/P and P/S. After one cycle, steady-state conditions were already reached. The profiles of the 5 cycles during MCSGP are shown in Fig. 4. As it can be seen, the first cycle is rather different from the others, while the remaining four are well overlapped, suggesting that steady-state conditions are already achieved after the first cycle. Also the analytical chromatograms of the first three cycles (six switches) are compared in Fig. 5. Also here it is possible to note that only the first switch differs significantly from the others.

With these operating conditions, the average target product purity in the MCSGP pools resulted to be 89.2% with a recovery of

Table 2
Performance of the batch and the MCSGP purification of glucagon using a 25 cm column and two 15 cm columns, respectively.

	Batch (25 cm)	MCSGP (2 × 15 cm)
Purity (%)	89.3	89.2
Recovery (%)	71.2	87.7
Productivity (g/L/h)	9.9	6.1

87.7% (see Table 2). For a fair comparison of process performance, the Pareto curve has been measured also using a 25 cm column, with a volume (CV = 4.2 mL) similar to the total volume of the two 15 cm columns used in the MCSGP unit (CV = 2 * 2.5 = 5 mL). Of course, the same stationary phase was chosen for the comparison. For a similar purity as that of the MCSGP, a recovery value of 71.2% has been obtained in batch. This means that MCSGP, with this particular set of switching times, allows to increase the recovery of about 16% (from 71.2% to 87.7%). This difference corresponds to a percentage increase in the target product recovery of about +23% in the MCSGP with respect to the batch process. It is important to point out that when the target product is very expensive (as it is the case of glucagon) any improvement in recovery leads to economic benefits when the target product is particularly expensive.

Another relevant parameter in evaluating the performance of a purification process is productivity. In order to compare the two processes, we considered for the batch process, at the numerator, the mass of the fraction of the design chromatogram having the same purity as the MCSGP operation. The duration of the batch run was 39 min while the duration of a cycle in MCSGP was 64 min. Accordingly, as reported in Table 2, the value of the productivity is lower for MCSGP by about 38%. This is due to the fact that MCSGP requires the use of lower flow rates during the interconnected steps than that used for the batch for the entire elution period. However, in case of very expensive products, such as glucagon or other biotherapeutics, it is preferable to maximize the recovery, in order to diminish the amount of waste product, with respect of productivity. In any case, the increase in recovery more than compensates the loss in productivity.

5. Conclusions

In this work, a simple procedure to design an MCSGP process for the purification of an industrial synthetic glucagon crude mixture starting from a single batch column experiment has been presented. The MCSGP performance strictly depends on the set of switching times that are chosen to define the collection and recycling windows, whereas all the other experimental parameters (mobile phases, gradient slope and duration, loading, etc.) are the same as those used in the single column run. At the same purity of 89%, the gain in the target molecule recovery was found to be +23% in the MCSGP in comparison with the value found for the batch process.

This increase in yield was not sufficient to compensate the still better productivity of the batch, calculated exclusively as the gram of purified peptide collected in the pool per run and per mL of stationary phase. However, in terms of overall process performance, this loss is more than compensated by the increase in recovery. Moreover, it must be noted that once the operational conditions for the MCSGP process have been defined, the purification process can be completely automatized and very large amount of feed can be continuously purified cyle after cycle, with no human intervention.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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PAPER IV

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Modern trends in downstream processing of biotherapeutics through continuous chromatography: The potential of Multicolumn Countercurrent Solvent Gradient Purification

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ABSTRACT

Single-column (batch) preparative chromatography is the technique of choice for purification of biotherapeutics but it is often characterized by an intrinsic limitation in terms of yield-purity trade-off, especially for separations containing a larger number of product-related impurities. This drawback can be alleviated by employing multicolumn continuous chromatography. Among the different methods working in continuous mode, in this paper we will focus in particular on Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) which has been specifically designed for challenging separations of target biomolecules from their product-related impurities. The improvements come from the automatic internal recycling of the impure fractions inside the chromatographic system, which results in an increased yield without compromising the purity of the pool. In this article, steps of the manufacturing process of biopharmaceuticals will be described, as well as the advantages of continuous chromatography over batch processes, by particularly focusing on MCSGP.

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1. Introduction

Since the 1980s, biopharmaceuticals have emerged as an innovative class of therapeutics, due to their highly specific activity, a feature that cannot be imitated by traditional drugs. Indeed, they show high specificity towards the target receptors, which makes them very effective even at low concentrations [1,2]. Moreover, most of them are also present in the human body, therefore their side effects are reduced if compared to other chemical drugs. In the last months, their potential has been even more rekindled due to the fact that many of the therapeutics currently under testing for the treatment or prevention of COVID-19 disease are based on biopharmaceuticals (especially monoclonal antibodies or oligonucleotides) [3–5].

In the last years, manufacturing of biopharmaceuticals has been intensively improved. The method chosen to obtain the biomolecule of interest represents the upstream step of the manufacturing process [6,7]. For instance, recombinant technology is the main method to obtain monoclonal antibodies, hormones and blood factors. In this context, continuous bioreactors (e.g., perfusion bioreactors) are getting ever more popular, at the point that they have started to replace traditional batch processes. Alternatively, biopharmaceuticals can be extracted from their natural source or they can be chemically synthesized. The latter strategy, anyway, can be applied only to produce short biopolymeric chains, e.g. polypeptides. These recent innovations in the upstream of biopharmaceuticals have not been followed by similar enhancement in the downstream process, at the point that the latter currently represents a bottleneck in the whole production of biotherapeutics [8–10]. The term downstream in general indicates both the recovery and the purification of a product from a complex mixture [11]. The purification methods of choice must distinguish between molecules that often show only slight variations in size, hydrophobicity or charge. The most versatile, selective and flexible technique to satisfy this need is liquid chromatography. Usually, more than one chromatographic step is required to satisfy the specifications imposed to reach the market [12,13]. Traditionally, these chromatographic purification

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processes are conducted in batch conditions, often using a single chromatographic column [14].

In general, at least two different purification steps are usually necessary to isolate the target product with the required purity. The first part of the purification process is the removal of process-related impurities, i.e. species that are not chemically similar to the target molecule [6]. They usually include nucleic acids, host cell proteins, lipids, components of the cell culture media, salts, etc. which derive from the manufacturing process. Very often, affinity chromatography in batch conditions is the technique employed, in a bind-and-elute mode [15]. This procedure is called capture step and it consists in loading a large amount of feed into the column until its breakthrough. The product specifically binds to the stationary phase, whereas all the other different species flow through the column and can be discarded. For instance, *Staphylococcus* Protein A-based stationary phase is largely employed for the purification of monoclonal Antibodies (mAbs), since it allows binding mAbs specifically but reversibly [16]. During this phase, it is important to ensure the recovery of the maximum amount of the target, whereas it is not necessary to satisfy strict purity requirements.

After the capture step, one or more polishing steps are required in order to satisfy the rigorous purity requirements for pharmaceuticals. In order to do that, the product must be separated also from product-related impurities, which are, instead, very often similar to the target molecule (e.g., truncated, deamidated species, etc.) [17]. Most of the time, this is a very challenging task. Affinity chromatography cannot be applied at this stage because of the similarity between the target product and the impurities. Therefore reversed-phase, ion-exchange and hydrophobic interaction chromatography are rather preferred as methods of choice [6]. In order to improve the resolution of the peaks, it is advisable to work in gradient conditions, since the retention of biomolecules is largely dependent on the composition of the mobile phase (e.g., on the salt concentration or on the percentage of organic modifier) [9,18–20].

In preparative chromatography, the similarity between the target and its impurities often result in peaks overlapping, where the target product is intermediate between weakly and strongly adsorbing impurities [21]. Consequently, collecting a considerable amount of pure product is almost impossible. In fact, a widening of the collection window results in an improved yield at expenses of a reduced purity and vice versa. This translates in a yield-purity trade-off, a limit peculiar to batch chromatography [22].

In this frame, multicolumn continuous chromatographic approaches have become increasingly appealing in the field of high value biological products [15], due to the possibility of partially overcoming this limitation. In general, multicolumn continuous chromatography leads to several advantages, especially increased recovery and better resin utilization, but this comes at the expense of the hardware complexity [23].

This paper focuses on Multicolumn Countercurrent Solvent Gradient Purification (MCSGP), one of the most recently developed countercurrent multicolumn techniques specifically designed for challenging separations where many product-related impurities are present. Its operating principles will be discussed and its advantages over traditional single-column techniques will be presented. Method transfer from batch to continuous will be also illustrated, together with a synthetic overview of most interesting applications of MCSGP. In doing this, an effort has been done to describe the process from the viewpoint of analytical chemists (more than that of chemical engineers) in order to make the technology more familiar to this community.

2. Relevant parameters for purification processes

Before describing the fundamentals of batch and continuous processes, some relevant parameters need to be defined. They are usually evaluated by analyzing the eluted fractions by means of a proper analytical high-performance liquid chromatography (HPLC) method.

Purity is the first parameter that is essential for pharmaceutical scopes. It is defined as the ratio between the area of the product peak and the total area of the HPLC chromatogram: purity is calculated as the mean of the purities of the pools at the steady state.

$$\text{Purity } \% = \frac{A_{product}}{A_{total}} \times 100 \quad (1)$$

Also, recovery (or yield) of the target at the end of the process needs to be carefully evaluated. This is particularly important when very expensive Active Pharmaceutical Ingredients (APIs) are purified. It is defined as the mass fraction of the product recovered in the eluted stream with respect to the mass of the product dissolved in the feed injected into the column.

$$\text{Recovery}\% = \frac{m_{\text{prod collected}}}{m_{\text{prod injected}}} \times 100 \quad (2)$$

Moreover, also productivity can be defined; it is expressed as the mass of target product collected in the eluent stream per total volume of stationary phase and per time. Thus, this parameter indicates how much product is produced per minute and per column volume (V_{col}):

$$\text{Productivity}(\text{mg} / \text{mL} / \text{h}) = \frac{m_{\text{prodcollected}}}{V_{col} \times \text{time}} \quad (3)$$

where V_{col} is calculated as the geometrical volume of the column (in case of multicolumn processes the geometrical volume of all the columns must be considered), whereas the time considered is the duration of a run in batch conditions or a cycle in MCSGP (see later on).

3. Limits of batch chromatography

The outcome of the separation (i.e. resolution of the main peak from the impurities) has a high impact on the performance of the whole process.

As mentioned before, it frequently happens that batch purifications, especially when many product-related impurities are present, are affected by a yield-purity trade-off. This situation is schematically represented in Fig. 1. If the overlapping regions are completely discarded, the purity in the pool will be elevated. However, a considerable amount of product still underlies the overlapping portions of the peak. If the collection window is broadened, yield will increase but at the same time purity will decrease since portions of the peak contaminated with impurities are collected. This trade-off is a limit intrinsic to batch chromatography. The difficulty of reaching a good purity and a good yield at the same time makes traditional batch chromatography often impractical [24].

One could think of decreasing the loading of the feed or the gradient slope, but this would lead to longer times and in turn to higher solvent consumption and lower productivity. Alternatively, more efficient columns can be used but smaller particles would lead to higher backpressures. Therefore, none of these options can effectively be a solution to the problem [25,26].

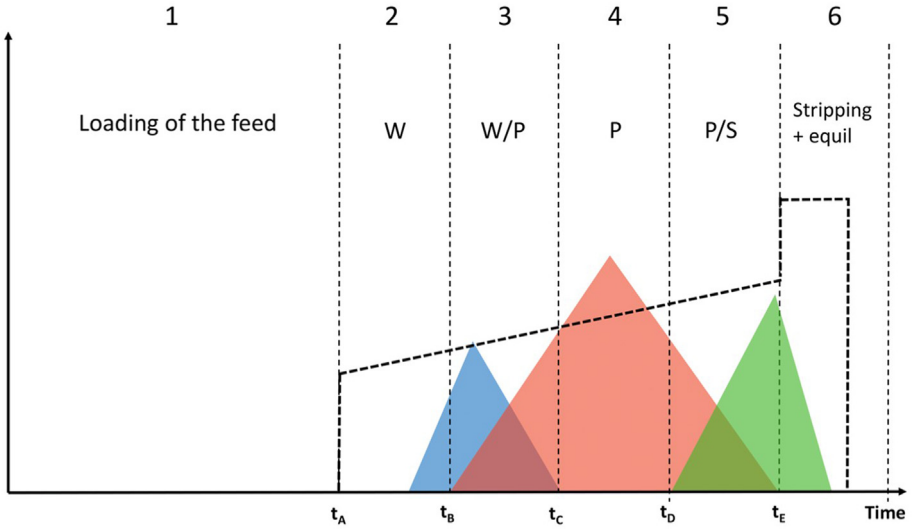

Fig. 1. Schematic representation of a batch chromatogram.

4. Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)

An appealing possibility to overcome the limit of batch chromatography described above is to replace the single column process with a continuous (or semi-continuous) countercurrent chromatographic process, where the chromatographic system is continuously fed with the crude mixture. To realize the continuous (or semi-continuous) mode, the instrument must be equipped with two or more (identical) columns connected through a series of valves. The term countercurrent refers to a class of chromatographic processes in which the stationary and the mobile phase move into two opposite directions. The movement of the stationary phase is not real but simulated through the switching of the inlet and outlet valves of the columns [6,16].

The use of continuous chromatography operations has considerable advantages not only in terms of recovery of the product (as it will be illustrated in the following) but also in terms of automatization of the purification process.

The first continuous countercurrent chromatography setup was Simulated Moving Bed (SMB), introduced in 1950 to separate two different components under isocratic conditions [27,28]. Since then, many improved versions of the technique have been proposed, but essentially SMB has been limited to the separation of binary mixtures. A dozen years ago, some researchers connected two SMB units in series to purify ternary mixtures [6,29]. In the first SMB process, one compound can be separated from the two remaining species, which enter into the second unit to be further separated. An advantage of this setup compared to MCSGP is that the chromatographic conditions (column, mobile phase, etc.) can be chosen independently for the two units. This, e.g., can improve resolution. On the opposite, not only the experimental setup (connecting tubings, valves, etc.) is much more complex in SMB than in MCSGP but also SMB separations are limited to only isocratic operations.

Recently, two appealing alternatives to SMB have been introduced that can be applied to both capture and polishing steps. Indeed, in the first case, captureSMB can be efficiently used to isolate the target product from its impurities exploiting affinity chromatography interactions. For the sake of space, this technique will not be described in this paper, therefore the interested reader is addressed to other recent papers on the subject [6,23,30–35].

On the other hand, in this work we will focus in particular on the description of MCSGP, a countercurrent technique that can be used for the polishing step. It is practically based on the same principles of SMB, but it allows to manage ternary separations (i.e. separations of target products from co-eluting impurities in the front and in the rear part of the target peak). Moreover, it allows to work under linear gradient conditions which is extremely advantageous when dealing with biomolecules [36,37]. In its first setup, MCSGP was based on the use of six identical columns [19,38]; later on, the equipment has been more and more simplified until arriving at the final version with only two columns [22,25], which is characterized by a reduced complexity in tubing, valves and connections.

4.1. Starting point: the design batch chromatogram

In order to understand the principles and the great potential of MCSGP, let us consider again the batch chromatogram schematically represented in Fig. 1. It represents the case of a *center-cut* or *ternary* separation carried out under gradient elution conditions, where the main compound elutes between weak and strong impurities, and their peaks partially overlap [6,39,40]. As it can be observed, it is divided in different zones.

- Zone 1: the column (previously equilibrated with the eluent) is loaded with some fresh feed. Once the analyte is adsorbed onto the stationary phase, the modifier gradient can start (at time t_A).
- Zone 2: weakly adsorbing impurities (from now on called W), which are less retained than the target product, start eluting from the column.
- Zone 3: product (P) starts eluting from the column, but the weakly adsorbing impurities are still eluting. Since W and P are not well resolved, their peaks overlap. The product in this zone obviously does not fulfill the purity requirement, because it is contaminated by species W, but at the same time it cannot be wasted and needs to be recovered to obtain a good process yield.
- Zone 4: the target compound does not coelute with any other species and hence purity fulfills the requirement for pharmaceutical scopes.
- Zone 5: this is another overlapping region where the target compound coelutes with the strongly adsorbing impurities (called S).

- Zone 6: the column is stripped with a high percentage of organic modifier, to remove S impurities, and then it is equilibrated again with the eluent composition at the beginning of the gradient.

Fractions of the eluate are periodically collected during the gradient and then analyzed by means of HPLC to obtain a purity profile (zones 2–5).

As reported in Fig. 1, the zones defining the recycling and collection windows in the batch process are delimited by some *characteristic times*, which are necessary to transfer a chromatographic method from batch to the MCSGP process, as it will be explained in the following.

Also, it must be highlighted that the letter W (or S) does not refer to a single weakly (or strongly) adsorbing species, but rather to a group of impurities which have a similar chromatographic behavior.

The chromatogram obtained in batch is then used to design the MCSGP process, thus it is called *design batch chromatogram*. It must be calculated on one of the two columns that will be employed for MCSGP.

4.2. Operating principles of MCSGP

Conversely to preparative batch chromatography process, the MCSGP technique, for its intrinsic features, allows to obtain the target product with high purity and high yield at the same time. The main factor enabling an improved performance of MCSGP compared to that of the batch process is the automatic internal recycling of the partially purified side fractions. When working in batch chromatography, the side portions of the main peak, containing both W (or S) and a remarkable amount of P, are discarded from the main collection window but most of the time they are manually reprocessed into the system by the operator, with risk of error and waste of time [41]. In twin-column MCSGP, on the contrary, the recycle is accomplished automatically between the two columns, with no need of intervention by the operator. The two identical columns work either in series (interconnected mode) or in parallel (batch mode), depending on the position of the inlet and outlet column valves [42,43]. When transferring a method from batch to continuous chromatography, this method is performed on both the columns, but shifted of half a cycle [22].

Figs. 2 and 3 represent a case where column-1 is in the upstream position and column-2 is in the downstream position; this means that the recycling regions eluting from column-1 are loaded in column-2:

- Firstly, column-1 is loaded with fresh feed, as in the batch process. When the gradient starts, the first group of analytes to elute is W; this fraction does not contain P (zone 4) and thus it is discarded. At this stage, the columns are disconnected.
- Then, valves switch position and the columns get interconnected. This means that W/P, the overlapping region of W and P, is directly loaded from column-1 (zone 5) into column-2 (zone 1). Inline dilution is applied to ensure that W/P is re-adsorbed on column-2.
- The columns work again in batch mode and a window where product purity satisfies the requirements imposed is recovered from column-1 (zone 6). At the same time, column-2 is loaded with some fresh feed (zone 2).
- After that, the columns get interconnected again to allow the recycling of P/S region from column-1 (zone 7) into column-2 (zone 3). Inline dilution is applied to ensure that P/S is re-adsorbed on column-2.
- Now that column-2 has been fully loaded, it can undergo the solvent gradient: W impurities start eluting (zone 4); on the other side, column-1 is being stripped to remove S and it is also equilibrated (zone 8).

At this point, the columns have switched position. When they exchange position again and return to the initial configurations, a cycle is completed. Thus, one cycle is composed of two switches. Generally, after few switches, the chromatographic system reaches the steady-state, which is demonstrated by the fact that the UV profiles are completely superimposable cycle after cycle. The reader should note that UV profiles are detected at the outlet of the column, before the eluent stream is sent to the waste, to the fractionator or to the other column. Under steady-state conditions, then, very close values of purity and recovery are obtained for every collected pool. Therefore, after the steady state has been reached, the number of cycles to be performed for the entire purification process depends essentially on the amount of fresh feed that must be purified. In order to better understand the meaning of steady-state, an example is reported in Fig. 4. This picture shows the

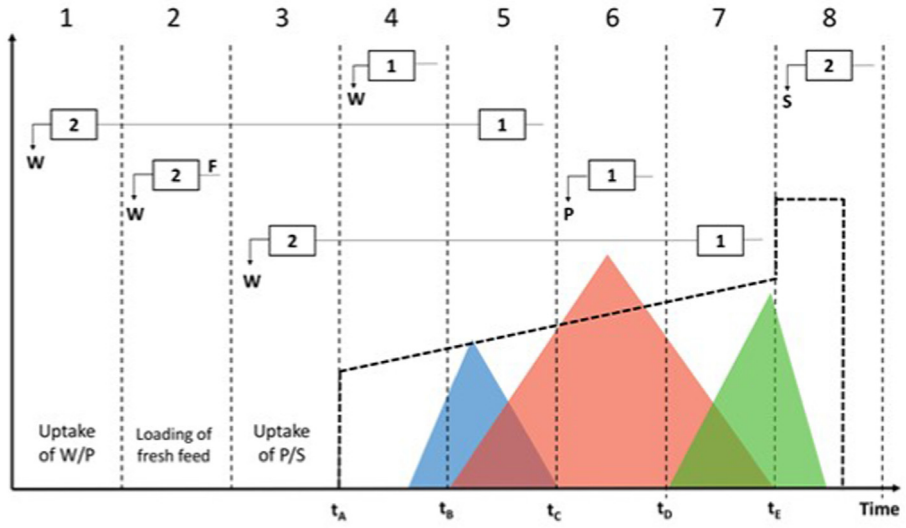

Fig. 2. Schematic representation of a single switch chromatogram in MCSGP. Reproduced with permissions from Ref. [44].

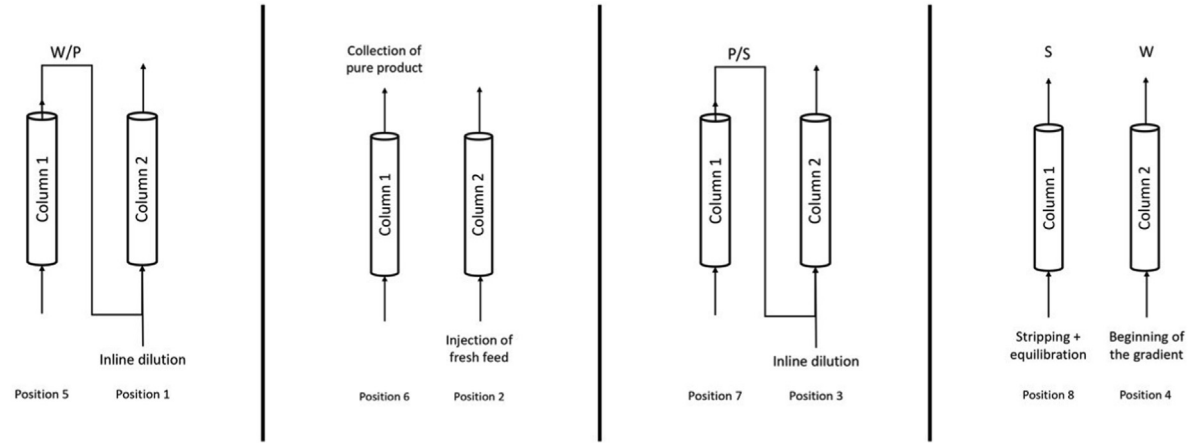

Fig. 3. Schematic representation of the path of the eluent stream during the first switch of an MCSGP cycle. The flow direction depends on the position of the inlet and outlet columns valves.

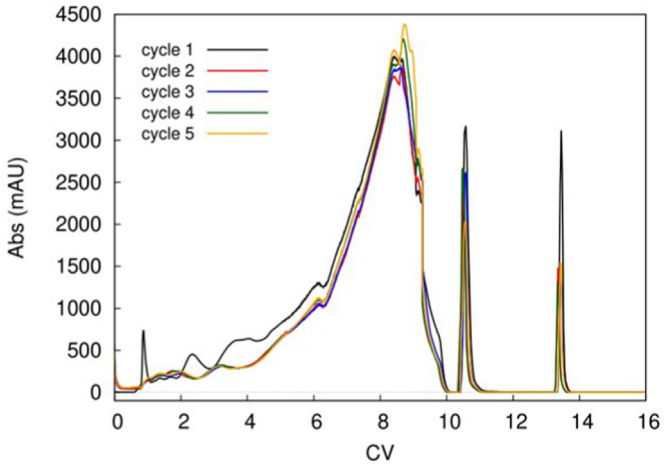

Fig. 4. Chromatograms (overlapped) of the first switch of five cycles of a MCSGP run for the purification of crude mixture of glucagon. Sharp peaks on the right correspond to the stripping and equilibration of the column. Reproduced with permission from Ref. [41]. CV: column volume (mL).

elution profiles of the first switch of five MCSGP cycles (these experiments were performed by some of the authors of this review in a former study). The biopharmaceutical of interest in that case was a crude synthetic mixture of a therapeutic peptide (Glucagon) [44]. As it can be noted, only the first cycle shows a different UV profile with respect to the others, meaning that cycles from 2 to 5 have reached steady-state conditions.

The characteristic times of the design batch chromatogram in Fig. 1 correspond to the switching of the inlet and outlet valves of the columns in MCSGP (see Fig. 2), that regulates the path accessible to the eluent stream. Fig. 3 shows in detail the path followed by the mobile phase during the disconnected and interconnected steps. t_B indicates the moment where the overlap of W/P starts flowing out, then the product elutes from t_C to t_D, and finally the overlap of P/S elutes until the time t_E. t_A represents the moment where the solvent gradient starts.

An important aspect to be considered is that the overlapping regions contain a higher percentage of modifier than at the beginning of the gradient. Therefore, when they are recycled, they need to be diluted with an inline dilution stream, so that the product can be adsorbed on the stationary phase. The fraction containing W/P is diluted to reach the modifier concentration that can be found at t_B, so that the product adsorbs on the stationary phase while the weak impurities start moving along the column. The window containing P/S is diluted to reach the percentage of organic modifier at the beginning of the gradient (t_A), because both the product and the strong impurities must be retained.

The amount of fresh feed which is injected switch after switch (zone 2 of Fig. 2) is calculated in order to maintain the mass of target compound constant into the system. Therefore, the mass of P to be loaded at every switch is the difference between the quantity of target product loaded in the batch run and the amount of target product which is recycled within the overlapping regions (zone 1 and 3).

4.3. Transfer of a batch method to MCSGP

The first thing to do in order to transfer a batch method to MCSGP is to calculate a Pareto curve reporting purity as a function of yield for the batch method (see Fig. 5). This is practically done by analyzing through HPLC the fractions of eluate stream collected from the batch column. Purity and yield of the target in each fraction are thus calculated. The result is a purity profile along the

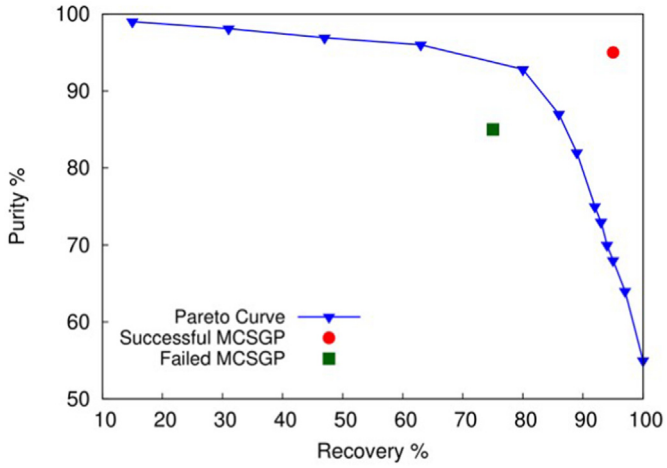

Fig. 5. Blue triangles: Pareto curve of a hypothetical design batch chromatogram. Red and green points: performance of two hypothetical MCSGP processes (red: successful; green: unsuccessful).

gradient which serves to decide which portion of the peak fulfills the purity requirements. This part of the chromatogram will represent the product elution window. Starting from the purest fraction, one needs to imagine pooling it with the neighboring fractions, adding one fraction at a time in order of decreasing purity. By enlarging the pooling window, purity decreases whereas recovery increases. The values of purity and recovery found for every hypothetical pool are then plotted to define the Pareto curve.

In order to make a fair comparison between batch and MCSGP, the Pareto curve should be calculated not only for one of the two columns used in MCSGP but also for a longer column, with a comparable V_{col} to the total V_{col} of MCSGP. This column will serve as a reference batch. The difference between the reference batch and the design batch is that the first is needed to compare the performance of the processes (at comparable V_{col}), whereas the second one is necessary to set the switching times for MCSGP.

During the transfer of a batch method to a MCSGP process, all the operating conditions are kept constant, such as loading of the feed per column, gradient slope and duration of every step of the method. Thus, the only variables that can be changed to modulate the performance of the MCSGP process are the switching times.

The first trial is usually done by choosing the values of t_C and t_D corresponding to a certain hypothetical pool in which purity fulfills the requirements and recovery is sufficiently high. t_B and t_E must be set in order to minimize the amount of product eluting in the waste windows. Fig. 2 represents an ideal case where no product is wasted in zones 4 and 8, however in other cases it is preferable to waste a small amount of very impure product in these zones rather than risking the accumulation of impurities in the system during recycling. Since the values of purity and recovery for each cycle are constant after the steady state has been reached, in MCSGP one obtains a point and not a Pareto curve. If the point of the MCSGP lies below the Pareto curve, the MCSGP process can be considered unsuccessful, meaning that it reaches a lower recovery than the batch at the same purity. On the opposite, if the point lies above the Pareto curve, this means that the recovery of MCSGP has overcome that of the batch. From a practical viewpoint it must be said that points on the top right corner of the graphic are an indication of a successful MCSGP. This concept can be better visualized by looking at Fig. 5. This graphic shows from a purely qualitative point of view a Pareto curve related to a batch chromatogram where the purest fraction (99% purity) corresponds to only 15% of recovery, whereas if yield were 100%, purity would decrease to 55%. If the MCSGP is unsuccessful, the set of switching times must be changed. Particularly, it has been proven that the times t_B and t_E greatly influence recovery. On the other side, the times t_C and t_D especially impact on purity since they define the product elution window [26].

The last parameter to consider when comparing purification processes is productivity. In some cases, MCSGP gives similar results as that of the batch [25] or slightly lower [44], but this is only partially a concerning point. Indeed, when dealing with very costly biotherapeutics, it is preferable, from an economic viewpoint, to maximize the recovery of the product rather than productivity of the process. Just to make an example, the cost of raw glucagon is declared to be around some thousand dollars per gram [45]. It is evident that an increase in recovery is reflected in a great economic advantage. Moreover, the typical definition of productivity given in Eq. (3), usually considered when comparing the processes, does not consider the economic advantage coming from the automatization of the process, which is, instead, a very important point.

4.4. Applications of MCSGP

The main field where MCSGP has been successfully applied is the purification of biomolecules (such as protein, antibodies and peptides), where a wide variety of mobile and stationary phases were tested.

The interest towards antibodies, especially mAbs, as therapeutics is increasing, and thus their demand. At the same time, mAbs are produced as a mixture of different isomers, which must be separated to ensure a good quality of the product and meet the market specifications. The MCSGP process has been proven to be a successful method for this scope and for this class of biomolecules [16,46–48].

MCSGP process allowed to reach a higher yield and better productivity than the batch also in the case of a mono-PEGylated protein, the α-Lactalbumin. The mixture of proteins with different degrees of PEGylation was separated using anion exchange chromatography [17].

Beside proteins, also mixtures of peptides have been purified through MCSGP process. The very first cases of application of a 6-column or 3-column MCSGP to an industrial sample were related to the separation of Calcitonin, a peptidic hormone made of 32 amino acids, from its impurities [19,21,49]. Lately, some of the authors of this review have successfully investigated the purification of an industrial mixture of Glucagon (29-amino acids peptide) using a 2-column MCSGP equipment. In that case, the yield was 23% higher than the batch, with a purity of nearly 90% [44].

Another class of biotherapeutics for which the MCSGP has been proven to be a good purification strategy is that of oligonucleotides. This technique applied to a mixture of oligonucleotides allowed to increase the mass recovered by 50% at a target purity of 92% [50].

MCSGP can be applied also in the case of cannabinoids identification and purification. Cannabidiols (CBD), for instance, are a group of cannabinoids, natural compounds extracted from *Cannabis Sativa* L. CBD is under investigation for their therapeutic properties; anyway, the regulation imposes strict limitations for the concentration of tetrahydrocannabinol (THC) in the CBD mixtures, since THC is a psychoactive substance. MCSGP has been successfully applied to obtain a THC-free product [51].

Table 1 reports a comparison between the performance obtained in batch and in MCSGP for the purification of different target molecules. As can be noted, MCSGP results to be a successful process when it comes to tricky ternary separations of expensive biomolecules and biopharmaceuticals, especially if their batch purification shows a strong yield-purity trade-off. In those cases, MCSGP can lead to an increase in yield and consequently to a benefit also with respect to the economics of production [41].

Table 1
Comparison between the performance of batch and MCSGP processes for different purification cases found in literature.

Compound	Batch			MCSGP			Ref.
	Purity %	Recovery %	Productivity (g/L/h)	Purity %	Recovery %	Productivity (g/L/h)	
Oligonucleotide	91.6%	55.7%	11.9	91.9%	91.2%	5.89	[50]
Cannabidiol	THC < 100 ppm	52%	8	>99.5% (THC < 100 ppm)	94%	60	[51]
Peptide (glucagon)	89.3%	71.2%	9.9	89.2%	88%	6.1	[44]
Peptide	98.7%	19.3%	3	98.7%	94.3%	28	[22]
Monoclonal antibody	92%	85%	1.8	92%	94%	2.6	[25]

5. Conclusions and outlook

Thanks to nonstop technological improvements, continuous, or semi-continuous (periodic), countercurrent preparative liquid chromatography has nowadays reached the stage of mature technology. These techniques are increasingly gaining importance from the industrial viewpoint and they are considered a promising candidate that can revolutionize the purification of biomolecule at a manufacturing level. The current increasing interest towards continuous purification processes is primarily driven by the improved quality, which directly translate into drug safety and efficacy, of the final products in addition to economic advantages, related to the high automation degree and improved yields. This is particularly so when the goal is to maximize product-recovery rather than process-productivity, that is the case of, e.g., highly molecularly active molecules. Many of today and tomorrow targeted therapeutics belong to this class. From a wider perspective, thus, the technology has the potential to act as driver for the shift to precision medicine [52].

There are, however, still many challenges to overcome. From a theoretical viewpoint, there is room for studies focusing on the modeling of the process [9,53–61]. Even though this relies essentially on the well-known theory of nonlinear chromatography, robust, validated models able to simulate the process in all its stages are not yet available. This will favor the optimization of purification conditions and, in the meantime, it will improve the confidence to use the technology. The availability of robust and reliable models will also favor the introduction of automation and digitalization. Using model-based algorithms, derived also from machine learning techniques, it is possible to control the operation of these units in terms of both rejecting disturbances, so as to keep the product under specifications, and keeping optimal operating conditions in terms of minimal production costs, i.e., productivity and buffer consumption [62]. The application of model predictive control techniques appears perfectly suitable for this purpose as already done in the frame of the chiral SMB continuous process [63].

Although, as seen above, MCSGP can be applied very conveniently down-stream to batch or fed-batch bioreactors, we believe that it will play a major role also in the establishment of continuous and integrated processes for the manufacturing of therapeutic proteins [64]. The most important pharma regulatory Agencies look positively at these developments and are active in the definition of Quality Aspects (QA) and ad-hoc regulatory actions for continuous manufacturing [65,66]. The time is therefore ripe for change.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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PAPER V

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Oligonucleotides: Current Trends and Innovative Applications in the Synthesis, Characterization, and Purification

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Oligonucleotides (ONs) are gaining increasing importance as a promising novel class of biopharmaceuticals. Thanks to their fundamental role in gene regulation, they can be used to develop custom-made drugs (also called N-to-1) able to act on the gene expression at pre-translational level. With recent approvals of ON-based therapeutics by the Food and Drug Administration (FDA), a growing demand for high-quality chemically modified ONs is emerging and their market is expected to impressively prosper in the near future. To satisfy this growing market demand, a scalable and economically sustainable ON production is needed. In this paper, the state of the art of the whole ON production process is illustrated with the aim of highlighting the most promising routes toward the auspicated market-size production. In particular, the most recent advancements in both the upstream stage, mainly based on solid-phase synthesis and recombinant technology, and the downstream one, focusing on chromatographic techniques, are reviewed. Since ON production is projected to expand to the large scale, automatized multicolumn countercurrent technologies will reasonably be required soon to replace the current ones based on batch single-column operations. This consideration is supported by a recent cutting-edge application of continuous chromatography for the ON purification.

1. Introduction

Nucleic acids play a fundamental role in all living organisms, being the main actors in duplication and protein formation processes. These events occur thousands of times every day in the human organism, with hundreds of errors committed by the cells at different levels. Fortunately, cells have efficient control systems, able to protect the organism from these errors (i.e., mutation) and from their effects.[1] However, even these control systems sometimes undergo failure. When this happens, a degenerative process may arise, with the consequent appearance of a disease.[2] Such pathologies are manifold and often severe such as cancer, muscular dystrophy and cystic fibrosis, as well as cardiovascular and neurological diseases.

Different pharmaceuticals have been developed over the years to deal with these pathologies. These are mainly divided in two classes: small molecules and macromolecular drugs.[3] The former are often characterized by poor selectivity and specificity, which macromolecular drugs can significantly improve, thus paving the way to high therapeutic indexes with reduced dosages and adverse effects.[4] Among this class, biologics such as peptides and antibodies (e.g., monoclonal, transgenic, recombinant, polyclonal) have tremendously grown in interest in the last few years, as indicated by the steep increase in the number of products that entered clinical trials.[5] However, these therapeutics have some limitations especially related to their poor pharmacokinetics, with only the 20% of the dose eventually interacting with the target.[6] Moreover, these compounds act on the cellular process after the protein translation and this may be a limitation especially for the treatment of degenerative processes.[7] On the other hand, the possibility of developing drugs that influence the cellular processes at an earlier point in time could bring significant advantages.[8] For example, silencing a gene instead of continuously treating the proteins it is responsible for could provide a deeper and more effective treatment of the degenerative processes. This new perspective, based on gene therapies,

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is made possible by the maturation in the genome sequencing techniques.[9–11] Genome sequencing is indeed useful not only for the diagnosis of rare diseases, but also for the development of customized therapeutics, within the so-called N-of-1 approach.[12,13]

These new frontiers in custom-made drugs able to act at a pretranslational level provide the main motivation for the increasing interest and efforts in the development of oligonucleotides (ONs) as possible therapeutic agents.[14] ONs are short nucleic acid chains, which are made unique by their peculiar potential in the regulation of gene expression. Indeed, an increasing number of synthetic ONs is being developed for a wide range of applications and medical indications, with a few of them in the final stages of human clinical trials and Food and Drug Administration (FDA) approval.

To date, nine ON-based therapeutics, listed in **Table 1**, have been approved by FDA,[15–19] and more than 180 ON-based clinical trials are reported on ClinicalTrials.gov, 37 of which are indicated as Active/Recruiting.

These achievements, and particularly the encouraging results obtained by antisense oligonucleotides (AONs) in clinics, have triggered an increasing demand for high-quality ON-based therapeutics.

In most cases, ON-based drug candidates include AONs, small interfering RNA (siRNA), antagomirs, microRNA (miRNA), decoys, aptamers, and CpG ONs. They differ in their chemistry, chemical structure and mechanism of action so that a few classifications have been suggested. A frequently used one is based on their target: AONs, siRNA, antagomirs, and miRNA target RNA, while decoys, aptamers, and CpG oligonucleotides act on proteins.

Generally, ONs are oligomers whose synthesis and purification are characterized by several difficulties. Primarily, unmodified ONs may undergo degradation by nucleases when introduced into biological systems and show unfavorable cellular uptake and biodistribution.[20,21] Therefore, chemical modifications, for example, the phosphorothioate backbone and sugar modifications, are often required to provide nuclease stability.[22] Over the years, many of these issues have been addressed by establishing suitable reaction pathways and methods for the preparative scale ON synthesis have been developed. This enabled to reduce significantly the production costs compared to the first pioneering attempts. Significant advances in synthetic technologies, mainly based on the development of optimized reagents, have allowed improved yields and purities of the ON-based produced drugs. For example, the replacement of the natural phosphate internucleotide with the phosphorothioate linkage (often referred to as first generation backbone modification) and several sugar modifications (referred to as second generation oligonucleotide modifications) have provided enhanced pharmacokinetic and pharmacodynamic properties.[23,24] However, effective delivery of ONs to their intracellular sites of action remains a major challenge.[25,26] Numerous drugs containing modified nucleotides are currently under development, but not all of them are suitable for clinical applications. This mainly depends on the potency and toxicity of ONs on one side, and on the availability of large-scale synthetic protocols on the other. Among all modified nucleotides, to the best of our knowledge, only seven chemistries are involved in the ONs currently employed in clinical or preclinical tests.[27] These are schematically summarized in **Figure 1**. The first antisense drug (Fomivirsen) approved by FDA was a 21 nucleotides (nt) phosphorothioate DNA (PS DNA). Today, the majority of therapeutic ONs incorporate the PS backbone modification to improve biological stability and cellular uptake.[28,29] PS is usually used in combination with second-generation sugar modifications. The most common sugar-modified therapeutic ONs are 2′-*O*-modified RNAs, such as 2′-*O*-methyl (2′-*O*-Me) or 2′-*O*-methoxyethyl (2′-*O*-MOE) RNA. Compared to normal DNA or RNA, they improve binding affinity and stability toward endonuclease digestion.[30,31] FDA has approved four 2′-*O*-MOE RNA drugs. Among these, Mipomersen and Inotersen are 20-nt gapmer with 2′-*O*-MOE RNA in the flanking sequences surrounding the core DNA region, combined with PS linkages throughout the ON. On the other hand, Nusinersen and Milasen are 18- and 22-nt AONs respectively, uniformly modified as PS 2′-*O*-MOE. 2′-*O*-Me and 2′-F RNA are two other sugar modifications included in the therapeutic FDA-approved aptamer Macugen: all the purine ribose sugars are 2′-*O*-Me modified and the pyrimidine ribose sugars are all 2′-fluorinated. The substitution of 2′ oxygen with fluoride in ribose nucleoside increases the binding affinity of 2′-

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Table 1. FDA approvals of ON-based therapeutics per year, as of 20th December 2019.[15–19].

Name (Brand Name)	ON Class	Chemistry	Clinical application	FDA approval
Fomivirsen (Vitravene)	21 nt AON	PS DNA	CMV retinitis	1998
Pegaptanib (Macugen)	27 nt RNA Aptamer	2′-O-Me-RNA/2′-F-RNA	Age-related macular degeneration	2004
Mipomersen (Kynamro)	20 nt Gapmer AON	PS 2′-O-MOE RNA/PS DNA	Familial hypercholesterolemia	2013
Eteplirsen (Exondys 51)	30 nt Splice-switch AON	PMO	Duchenne muscular dystrophy	2016
Defibrotide (Defitelio)	ssDNA Aptamer	DNA	Hepatic veno-occlusive disease	2016
Nusinersen (Spinraza)	18 nt Splice-switch AON	PS 2′-*O*-MOE RNA	Spinal muscular atrophy	2016
Patisiran (Onpattro)	siRNA	2′-*O*-MOE RNA	Polyneuropathy	2018
Inotersen (Tegsedi)	20 nt Gapmer AON	PS 2′-*O*-MOE RNA/PS DNA	Polyneuropathy	2018
Milasen	22 nt Splice-switch AON	PS 2′-*O*-MOE RNA	Batten disease	2019

F RNA more than the 2′-*O*-Me or 2′-*O*-MOE.[32] Another phosphate modification in clinical trials is the N3′-P5′ thiophosphoramidate (NPS) linkage in which the 3′ bridging oxygen is replaced with an NH group offering good affinity for the RNA target and improved nuclease resistance.[33] Imetelstat, a 13-nt ON based on NPS chemistry, is currently in phase II/III for myelofibrosis and myelodysplastic syndromes.[34] Spiegelmers (L-RNA) are a new class of ON therapeutics built from non-natural L-nucleotides: the mirror-image configuration confers enhanced plasma stability and immunological passivity. From the list of Spiegelmers, three are currently in several active clinical trials.[35] Several locked nucleic acids (LNA) are employed in various stages of clinical or preclinical studies.[36] LNA includes bicyclic ribose analogue nucleotides producing a dramatic increase in binding affinity: up to +7 °C per modification when binding RNA.[37,38] A large number of additional promising analogues of LNA have been synthesized with similar hybridization properties as LNA, but greater nuclease stability.[39] Tricycle-deoxyribonucleic acid (tc-DNA) is another promising constrained nucleotide for therapeutic splice-switching applications due to their preferred localization to the nucleus[40] and the treatment of Duchenne muscular dystrophy (DMD) in particular.[41] Two families of modified therapeutic ONs are based on structures that diverge completely from the natural nucleic acids. These are phosphorodiamidate morpholino oligomers (PMO) and peptide nucleic acid (PNA), both of them showing increased bonding affinity, in part because of their neutral character. PMO have shown significant application as splice-switching ONs, in particular Eteplirsen has been FDA approved for DMD treatment.[42] To date, PNA has not attained clinical trials, nevertheless, recent studies report the therapeutic potential of PNA as antibacterial compounds and anticancer drugs.[43]

Due to this growing attention paid to ONs and the increasing number of clinical trials in which they are involved, in this review paper we describe the state of the art of ON production. In particular, we critically discuss both the upstream, based on solid-phase synthesis or on recombinant technology, and the downstream part, largely based on the use of chromatography. Considering the first positive results as therapeutic agents, it is reasonable to envision that in the near future relatively large production facilities will be needed. This will probably require the use of more efficient continuous technologies, replacing the current ones based on batch operation (e.g., ref. [44]). Indeed, this situation reminds the recent development of monoclonal antibodies (mAb) as therapeutic agents. In this case, in the first 15 years after the approval of the first mAb, orthoclone OKT3 in 1986, only 15 mAbs received approval for clinical use and more than 50% of them were subsequently removed from the market after a few years.[45] The turning point for the burst in the number of mAbs admitted to the clinical trials was the realization of efficient production and purification processes. This, combined with the successful therapeutic activity of mAbs in the treatment of many pathologies, motivated the intensification of the research activity in this class of therapeutics.[45] Accordingly, one of the objectives of this review is illustrating how continuous chromatography, and in particular multicolumn solvent gradient processes (MCSGPs) can play a significant role in the transformation of ONs manufacturing, which would enable a sustainable scalability of their production to satisfy the market demands.

2. Synthesis of ON-Based Therapeutics

2.1. Solid-Phase Synthesis

The clinical success of ONs is mostly due to the possibility of assembling different sequences of a wide range of chemically modified ONs by a single fully automated solid-phase synthesis platform. The key steps of this synthetic approach based on the phosphoramidite chemistry, that is, deprotection, coupling, sulfurization/oxidation and capping, as schematically sketched in **Figure 2**, are common to most of the chemically modified therapeutic ONs. These include 2′-*O*-methoxy (2′-*O*-Me), 2′-*O*-(2-methoxyethyl) (2′-*O*-MOE) and 2′-F oligoribonucleotides, LNA, tc-DNA and Spiegelmers,[35] in both phosphorothioate (PS) and phosphodiester (PO) linkage (Figure 1A). Nucleoside phosphoramidites are the key components during assembly of ON-based therapeutics discussed earlier, considering that most of the atoms making up ONs is contributed by the phosphoramidites. A general structure of phosphoramidite monomers is shown in **Figure 3**A.

In the phosphoramidite approach, the synthesis is performed using solid supports where the chain grows in the 3′ to 5′ direction.[46] In the past, controlled-pore glass (CPG) was

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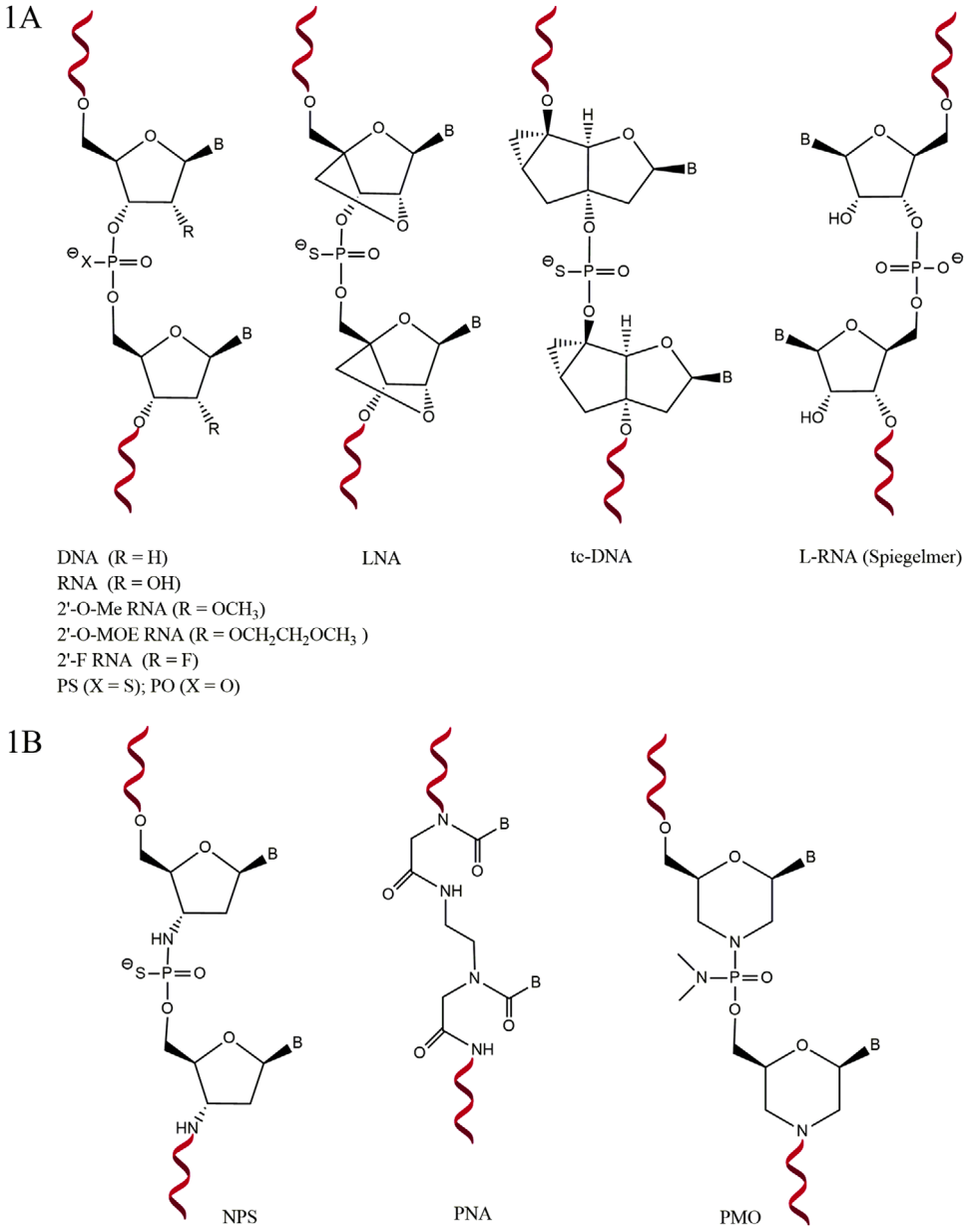

Figure 1. Chemical structures present in ONs currently employed in clinical or preclinical tests: A) ONs synthesized via the phosphoramidite approach; B) ONs synthesized through other approaches: N3′-P5′ phosphoramidates (NPS), peptide nucleic acid (PNA), and morpholino (PMO).

the solid support of choice. Today, due to the large-scale needed in the production of therapeutic ONs, high-loading polymeric solid supports are generally preferred. Among the latest generation of polystyrene supports, it is worth mentioning Primer Support 5G from GE Healthcare that can be loaded up to 350 μmol g^{-1}, allowing ON synthesis at scales up to 750 mmol per reaction. Deprotection (or deblocking) is the first step of the synthesis cycle and it is accomplished in acidic conditions (3-10% dichloroacetic acid in toluene) with the removal of the protective group (4,4′-dimethoxytrityl group, DMT) from the support-bound nucleoside. The correct exposure time to acidic conditions during this step is important in determining the final crude ON purity and yield. Overexposure results in depurination (adenosine primarily),[47] whereas too short detritylation times lead to incomplete deprotection with a consequent increasing fraction of ON sequences that lack in a single nucleotide (the so-called $n-1$ impurities).[48–50]

Next, as shown in Figure 2, the ONs are assembled through the coupling step of activated phosphoramidites. Normally, for large-scale synthesis, 1.5–2.0 equivalent excess of activated phosphoramidite is required to obtain a coupling efficiency larger than 99% for each cycle. The activation of amidites is typically achieved using either a 1*H*-tetrazole-type (usually 5-(benzylthio)-1*H*-tetrazole (BTT) or 5-(ethylthio)-1*H*-tetrazole), or an imidazole-type (usually 4,5-dicyanoimidazole (DCI)) activator.[51] Sulfurization or oxidation follows the coupling. In this step, the reactive

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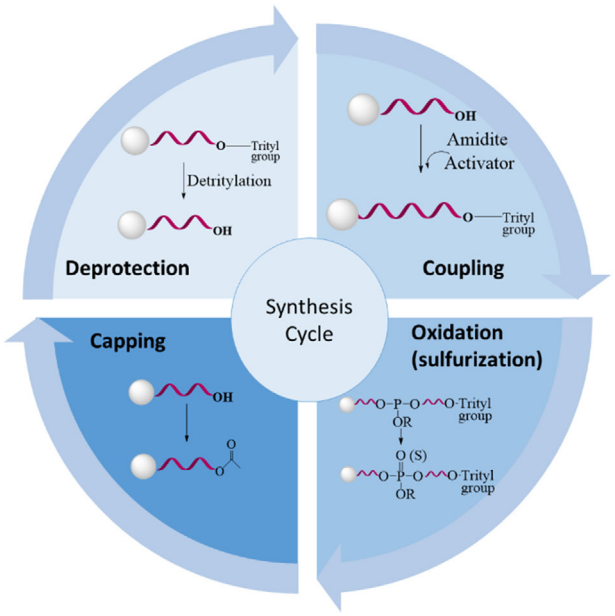

Figure 2. The four steps of the synthesis cycle using the phosphoramidite approach.

phosphite triesters are converted into more stable PS or PO triesters. The sulfurization reaction is fast and efficient (PS conversion greater than 99.9%) with freshly prepared phenylacetyl disulphide (PADS), but impurity formation has been detected with aged PADS solutions.[52] The final step in each synthesis cycle, referred to as capping, is used to silence by acetylation any terminal nucleoside that failed to react, with the aim of minimizing the $n-1$ impurities. Despite the ON synthesis by the phosphoramidite approach has experienced a decisive improvement in the last years to satisfy the ON market requirements, several by-products are still associated with this synthetic route.[53,54] To improve the separation of the desired ON from capped failure sequences, the ON is generally synthesized with its 5′-DMT protective group in place. Once the synthesis is complete, the support-bound ON is generally processed to remove base and phosphorothioate/phosphodiester protecting groups, and to cleave ON from the solid support. This is achieved through a two-step basic treatment, which avoids the addition of acrylonitrile to the $N-3$ position of thymine.[55]

Phosphorothioate is probably the most used backbone modification. Usually, PS substituted therapeutic ONs are random mixture of stereoisomers (up to 2^n diastereoisomers) because PADS sulfurization creates in the achiral phosphodiester a chiral phosphorothioate center with no stereochemical control. A scalable solid-phase synthetic process that yields therapeutic AONs having high stereochemical and chemical purity has been recently realized using the stereopure phosphoramidites shown in Figure 3A.[56] The influence of the stereochemistry of PS modifications on the pharmacological properties of ONs has also been investigated.[57]

In addition to the widely used phosphoramidite approach, several alternative oligonucleotide chemistries have been developed such as PMO, PNA and N3′-P5′ thiophosphoramidates ONs (NPS), as schematically sketched in Figure 1B. The respective activated monomers are shown in Figure 3B. Phosphorodiamidate morpholino ONs (PMO) contain modified morpholine rings, sewed together with phosphorodiamidate linkages and standard nucleobases. They are prepared on a solid-support using a two-step cycle process that consists of deprotection (detritylation) and successive coupling of the chlorophosphoramidate-activated monomers.[58] PNA is a modified oligonucleotide with a peptidic backbone instead of a phosphoribosyl backbone. Solid-phase synthesis is performed employing the 9-fluorenylmethoxycarbonyl (FMOC) chemistry.[59] Two different kinds of solid support can be used: a peptide amide linker (PAL) resin or a xanthen alkonic acid (XAL) resin. A typical synthetic cycle consists of three steps (deprotection, coupling and capping) where the coupling occurs between an activated acidic monomer and the growing PNA chain. N3′-P5′ thiophosphoramidate ONs (NPS), present each 3′-oxygen replaced by a 3′-amine group in the sugar. The solid-phase synthesis is performed in the 5′ to 3′ direction using activated 3′-tritylamino-5′-*O*-phosphoramidite monomers.[60]

2.2. Recombinant Technology

It is out of doubt that nowadays the most established technology for the production of ONs, independently from their chain length, is the solid-phase synthesis.[61] On the other hand, this technique loses progressively its efficiency as the nucleic acid chain length increases.[62,63] Therefore, the long DNA and RNA chains are mostly produced biologically. Now, some authors argue that also synthetic ONs are very different from the ones produced naturally by the cells.[15,64] As an example, RNAs transcribed from the genome of cells do not typically contain any modification in the ribonucleotides and have only few post-transcriptional modifications (such as methylation, acetylation and hydroxylation).[64] This is in contrast to synthetic ones, which contain a panoply of different modifications. These alterations have some advantages, such as increasing half-life of the drug, however they can lead to immunogenic responses. On the contrary, natural post-transcriptional alterations, being more similar to cellular RNA molecules, can depress immune response.[64]

This justifies the interest in the biological production of therapeutic ONs as well, aside to the solid-phase synthesis. However, it is worth mentioning that the direct expression of the intended ON in microorganisms has only been achieved with very low yield, due to the abundant presence of RNases that attack and degrade heterogeneous RNAs present in the cells.[15] This has motivated researchers to find valuable alternative strategies to produce ONs through recombinant technologies. These include, for example, t-RNA scaffolds, in which the recombinant RNA to be produced is masked as a natural RNA of the cell, thus enabling its expression in fast-growing bacteria such as *Escherichia coli*.[65,66] An alternative strategy is the stabilization of the RNA to be produced through the use of p19, a protein expressed by the plant viruses *Tombusviruses* that possesses a strong affinity and selective binding to siRNAs. The stabilization offered by p19 has been exploited for the production of siRNAs in *E. coli*. These natural siRNAs showed exceptionally high efficiency toward the knockdown of the target gene when used to transfect mammalian cells.[67] Other approaches make use of rRNA[68] or optimal ncRNA[15] scaffolds. These techniques have been

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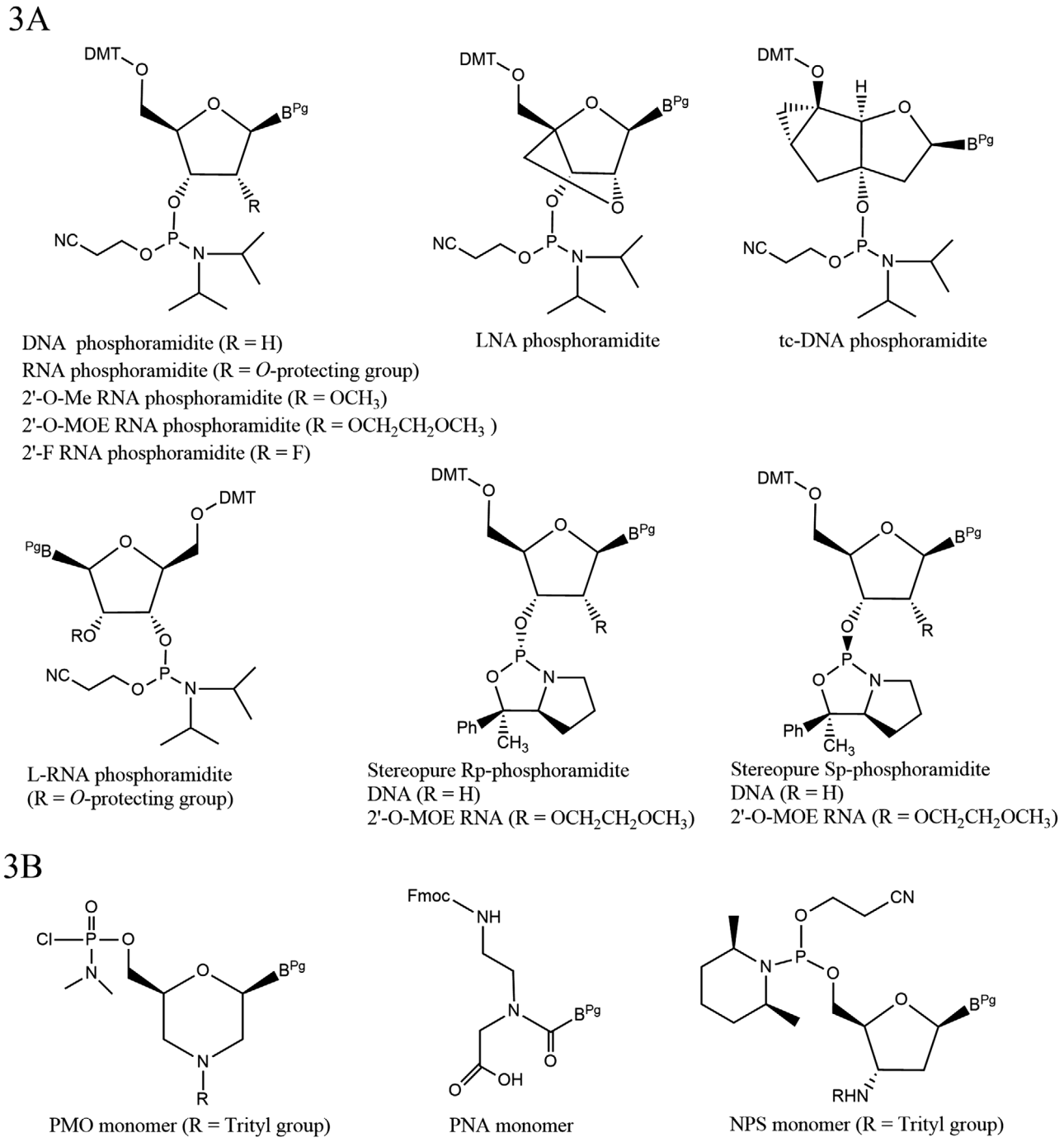

Figure 3. Chemical structures of monomers as starting material: A) nucleoside phosphoramidites; B) PMO, PNA, and NPS monomer.

implemented with some degree of success,[69] even in diverse bacteria,[70] leading to significant yields and reducing production costs,[64] very significant for the case of one of the most expensive drug classes on the market.[71] This is due to the lower costs associated with fermentation, when compared to synthetic synthesis via nucleoside phosphoramidites oligomerization.[64] In addition, Pereira et al.[70] produced pre-miR-29b via fermentation using *Rhodovulum sulfidophilum*, which was able to excrete to extracellular medium the therapeutic ON, leading to a concentration of 182 μg L^{-1} in the extracellular media after 40 h of fermentation. The obtained product is mostly free from the attack of RNAases and endotoxins, thus improving the recovery yield.

Another interesting study by Ho et al.,[69] reports a novel strategy for the production of ncRNAs based on a more stable ncRNA carrier, which improves the expression yield of the oligonucleotide of interest up to 80% of the total RNA. In addition, the t-RNA scaffold method developed by Ponchon et al.[66] has been shown to produce RNA at the scale of milligrams per

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liter of culture—a rather interesting level for biopharmaceutical production. Furthermore, since the scale-up procedures for fermentation bioreactors are well established,[72] these processes have the potential to be readily scaled at the commercial scale.

From a more visionary perspective, it is worth pointing out that the bio-production of ONs would be very similar to that of therapeutic proteins.[64] Nowadays, the vast majority of proteins for clinical applications are recombinant proteins, produced via fermentation due to the assurance of correct folding and post-translational modifications and are not synthetized in vitro.[64] In this context, the adoption of continuous processes, and in particular perfusion bioreactors as the production unit, is getting more and more popular with respect to the classical batch technologies. Perfusion bioreactors are continuous bioreactors that differ from other configurations (e.g., chemostats) due to the presence of a cell-retention device. This distinguishing element enables the achievement of high cell densities in the bioreactor. In turn, the high cell density, conjugated with a continuous set-up, marked a decisive augmentation in the protein productivity.[73] Hence, perfusion bioreactors provide a key step in the intensified and continuous integrated manufacturing of therapeutic proteins and in particular of mAbs.[74] We expect also for the synthesis of ONs a similar technological evolution, as soon as these drugs will achieve significant success in relevant therapeutic indications.

3. Analytical Characterization of Oligonucleotides

The use of ONs as therapeutics requires an accurate quality control during the entire production process. As discussed earlier, many impurities may be formed during their synthesis, including the so-called shortmers ($n-1$; $n-2$; etc.), longmers ($n+1$; $n+2$; etc.) and other by-products caused by deamidation, depurination, deprotection failure or adduct formation.[75] Therefore, sensitive and selective analytical methods, able to separate the target molecules from impurities, are needed for the assessment of purity of both crudes and final active pharmaceutical ingredients (APIs). Different analytical techniques have been applied in the years for the analysis of the produced ONs. Among the others, it is worth citing capillary gel electrophoresis (CGE),[76–83] ideal-filter capillary electrophoresis (IFCE),[84–86] hybridization-based enzyme-linked immunosorbent assay (ELISA)[87] and ion-mobility spectrometry (IMS).[88] However, high performance Liquid Chromatography (LC) is currently the technique of choice for this, mainly due to its sensitivity and resolution.[89]

Since ONs can bear an elevated number of negative charges depending on their length, the most effective elution modes are ion-pair reversed-phase liquid chromatography, anion exchange LC, hydrophilic interaction liquid chromatography, and mixed-mode LC.[90] Detection of ONs is usually performed through a UV detector, typically at 260 nm, where they exhibit very strong absorbance by their heterocyclic ring. The use of fluorescence detection has also been reported.[91,92] Obviously, LC interfaced to mass spectrometry (MS) is employed for detection of trace impurities, as well as for sequence determination and structure characterization.[93]

In the following sections, the most recent results and applications in the field of analytical chromatography are discussed. For the sake of convenience, the information has been organized based on the elution modes discussed earlier. For a more complete treatment of the chromatographic techniques employed for the characterization of ONs, the reader is also referred to other recent reviews on the subject.[75,90,93–95]

3.1. Ion Pair Reversed-Phase Liquid Chromatography

Ion pair reversed-phase liquid chromatography (IP-RPLC) represents the most widely used technique for the analysis of ONs.[75] Suitable counterions, typically quaternary alkyl ammonium compounds, are added in the mobile phase. The exact mechanism of ion-pairing has been subject of debate for several decades. Essentially, there are two possible theories. The first one hypothesizes that the formation of ion-pairs between charged analytes and counterions occurs in the mobile phase. Then, neutralized ion-pairs are adsorbed on the hydrophobic stationary phase. Since the sequence of nucleobases present in the structure of ONs determines their hydrophobicity, it also governs their chromatographic retention. For DNA-based ONs, this increases in the following order: C < G << A < T.[96,97] Based on a second theory, alkyl counterions are firstly adsorbed on the stationary phase. This creates a sort of dynamically charged surface, which provides the stationary phase with an ion-exchanger character, thus allowing the separation of analytes based on their charge. Most likely, the separation of ONs under IP-RPLC is due to a combination of these mechanisms,[90] where both hydrophobicity and charge play a role.

The first ion-pairing reagent used for the separation of ONs has been triethylammonium acetate (TEAA).[98] Li and co-workers report the separation of a siRNA comprising a 21-nucleotide sense strand with one PS stereocenter and of a 23-nucleotide antisense strand containing two PS stereocenters by using TEAA in the mobile phase. siRNA duplex diastereomers were efficiently separated on cyano columns, while bridged ethylene hybrid (BEH) C18 and BEH phenyl columns gave better results for the separation of related single-stranded siRNA stereoisomers. Resolution of diastereoisomers improved by increasing the temperature over that required for annealing siRNA.[99] Interestingly, it was shown that the use of shallower gradients and lower triethylammonium concentration increased the stereoselectivity of PS pentameric oligonucleotide diastereoisomers, separated on a XBridge C18 column.[100] Among structural factors affecting selectivity of this class of ONs, it is worth mentioning the type of nucleobase, on the one side, and the position of modified linkage, on the other. Indeed, it has been reported that diastereoselectivity is higher for cytosine-based pentamers (compared to thymine-based ones) and when substitution occurs in the center of the pentamer, instead of at its end.[100] Other alkylammonium compounds have been investigated, including tributylammonium acetate (TBAA), hexylammonium acetate (HAA) and triethylammonium bicarbonate. Moreover, the combination of triethylamine (TEA) with hexafluoroisopropanol (HFIP) is particularly suitable for LC-MS separations since HFIP not only stabilizes pH but also enhances the MS signal.[90,101–103] Thanks to its volatility (much higher than that of, e.g., acetate), HFIP can be efficiently evaporated from droplet surfaces at the electrospray ionization (ESI) source. Due to its unique

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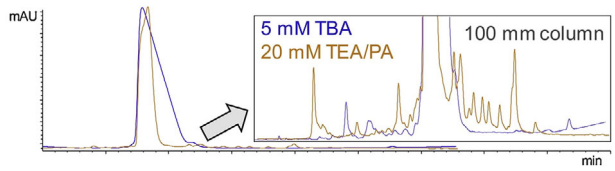

Figure 4. Chromatograms showing the IP-RPLC separation of a phosphate diester oligonucleotide using large (blue, tributylamine) and small (orange, mixture of triethylamine and propylamine) amines. Column: Waters XBridge C18 OST column (2.1 × 100 mm, 1.7 µm), 250 µL min^{-1}. Reproduced with permission from.[105] Copyright 2019, Elsevier B.V.

ion-pairing properties, which allow to improve resolution and selectivity of separation, TEA/HFIP is the preferred mobile phase for the separation of ONs also with other kinds of detection (especially, UV or fluorescence).

Alternative types of alkylamines have been investigated as mobile phase modifiers in combination with HFIP. Even if the chromatographic selectivity can be very similar, the concentration of alkylammonium ions can have a strong impact on the separation of different ONs, including DNA and RNA. It was demonstrated by transmission electron microscopy and dynamic light scattering experiments that when the alkylamine concentration is larger than 20 mM, alkylammonium ions form micelles in the mobile phase.[104] Under these conditions, therefore, micellar chromatography controls the retention mechanisms. When, on the contrary, this concentration is lower than 20 mM, the ions remain more strongly bound to the stationary phase. Accordingly, the retention mechanism is that typical of ion-pair chromatography.

Alkylammonium ions (acetate as counterions) have been recently considered for the LC-MS separation of product-related ON impurities, in particular positional isomers. The use of small alkylamines such as propylamine and isopropylamine provided sharper peaks and better resolution of impurities in the analysis of phosphate diesters, over the more commonly employed TEA, TBAA and HAA (see **Figure 4**).[105] However, when used to separate the closed-related impurities of PS ONs, small alkylamines lead to severe peak broadening. The replacement of the phosphodiester linkage with a PS moiety is a common strategy used to increase the hydrolytic stability of the ONs, but this modification introduces an additional chiral phosphorus center in the molecule that multiplies the number of stereoisomers. It is important to point out that separation of PS close-related impurities can be very challenging also in this mode of chromatography and that desulfurization of thiophosphate linkages into their phosphate diester form is often required.[105]

Several attempts have also been made to replace HFIP with other fluoroalcohols. Basiri et al. found that the MS signal of PS ONs can be enhanced when a combination of *N,N*-dimethylcyclohexylamine (DMCHA) and 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP) is used as mobile phase.[106] The enhancement was significantly stronger than with any combination of HFIP and ion-pairing reagents. It is also important to point out that HFIP and HFMIP produce significantly different mass spectra, due to different charge-state distribution-patterns, for ONs.[107] In addition, HFMIP is characterized by more pronounced desalting ability with respect to HFIP, which usually allows to obtain mass spectra that are simpler to interpret. From the analysis of different ON samples, by using different combinations of alkylamine and fluoroalcohols, Liu et al. concluded that the use of hexylamine with either HFIP or HFMIP is more suitable for small ONs, while tripropylamine in combination with HFMIP has to be preferred for large synthetic ONs.[107]

In the vast majority of cases, C18 stationary phases are employed for the separation of ONs in IP-RPLC.[90] Recently, other adsorbents have also been considered. These include C18 with polar end-capped groups,[108] pentafluorophenyl-based stationary phases,[102] as well as styrene-divinylbenzene[109] and phenyl-based resins.[110] In particular, π–π interactions, typical of phenyl-based stationary phases, have been demonstrated to increase retention of ONs.[110] In order to reduce mass transfer and improve the separation of ONs, columns packed with particles of very small diameter (below 2 µm) or core-shell particles have been used. Separations of ONs are indeed characterized by very low mass transfer. As an example, core-shell particles have been recently employed for the rapid determination of high-resolution fingerprints of single-stranded and double-stranded RNA.[111] On the other hand, the effect of particles pore-size on mass transfer has not been systematically investigated so far. Monolithic columns are also attractive for the separation of ONs.[109,112,113] Interestingly, Qiao et al. have demonstrated that hairpins are always less retained than random coils on monoliths, while no retention was observable with particle-packed columns.[112] This has been attributed to the fact that hairpins are more easily deformed into particle-packed columns, leading to more complex retention behaviors.

3.2. Ion-Exchange Liquid Chromatography

Anion-exchange liquid chromatography (AEXLC) with UV or fluorescence detection represents a very useful technique for the separation of ONs, which bear several negative charges. AEXLC stationary phases possess positive charged groups on their surface (e.g., quaternary ammonium groups). Negatively charged ONs are retained through electrostatic interactions. Separation is controlled by modifying the amount of competitive anions present in the mobile phase. Typical mobile phases consist of NaCl in phosphate buffer or tris(hydroxymethyl)aminomethane (Tris). Longer ONs (with a larger number of charged sites) are more retained on AEXLC stationary phases than shorter ones. Therefore, AEXLC is very efficient for the separation of $N - x$ deletions. On the opposite, other chromatographic modes must be employed for the resolution of ONs with very similar structures.[90] Monoliths have also been tested as support for AEXLC. A methacrylate-based monolithic column, for instance, was shown to be suitable for the separation of both single- and double-stranded DNA molecules with a mobile phase made of NaCl in Tris-HCl buffer (20 mM).[114]

3.3. Hydrophilic Interaction Liquid Chromatography

The first attempt of separating ONs in hydrophilic interaction liquid chromatography (HILIC) conditions dates back to 1990,

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when Alpert successfully separated a mixture of homo-ONs.[115] He proved that retention was influenced by the buffer concentration in the mobile phase. Elution was obtained by using shallow salt gradients. This technique, after about 20 years when it was considered only sporadically,[116–120] has been recently reconsidered as an effective tool for the separation of charged ONs. HILIC offers in fact a viable alternative to IP-RPLC, which often exhibits low sensitivity when coupled to MS, due to signal suppression in the presence of ion-pairing reagents. Studzińska et al. systematically investigated the influence of different factors, including mobile phase salt type and concentration, on the separation of ONs in HILIC-ESI/MS.[121] They found that retention of PS ONs is affected by the number of nucleotides in the sequence and the percentage of nitrogenous bases. Moreover, the elution strength was higher when ammonium formate was used, compared to ammonium acetate. HILIC allowed to reach significantly lower limit of quantifications compared to IP-RPLC. On the other hand, if the objective is to resolve very complex multi-component mixtures, IP-RPLC remains the method of choice.

Interesting examples of fast separations of unmodified ONs (less than 10-min analysis time) were reported in HILIC by using a Zorbax column, with 10 mM TEAA buffer and acetonitrile as mobile phase.[122] The same approach allowed also to resolve sequence isomers and chemically modified ONs such as phosphorylated, phosphorothioated, and fluorescently labelled species. Fast separations of deoxynucleic acid oligomers, modified and unmodified oligoribonucleotides, and PS DNA ONs have been performed on diol columns by using ammonium acetate buffer and acetonitrile as mobile phase.[123]

Finally, as mentioned before, the very efficient desalting occurring under HILIC conditions allows to obtain simpler mass spectra than in IP-RPLC.[122] Therefore, HILIC represents a sensitive and robust alternative to IP-RPLC with significant potential for both qualitative and quantitative analysis.

3.4. Mixed-Mode Chromatography

Since ON mixtures generally contain a wide variety of impurities, a single separation mode often is not sufficient to separate all of them. For example, separation of $N - x$ deletions, which represent the most abundant impurities produced from the failed addition of nucleotides during the synthesis, can be efficiently achieved by means of AEXLC. On the other hand, AEXLC is not suitable to detect depurinations, base substitutions, PS diastereoisomers and other species with subtle changes on their sequence. In these cases, the complementary IP-RPLC mode is preferred. The recently developed mixed-mode stationary phases combine reversed-phase and ion-exchange properties in the same column, representing a very attractive alternative for the separation of ONs.[124] The mobile phase is pivotal in determining the behavior of these columns. Indeed, depending on the composition of the mobile phase either hydrophobic interactions or ionic-exchange prevail. In the case of Scherzo mixed-mode columns, for instance, hydrophobic interactions have been demonstrated to be dominant when the mobile phase is made of ammonium acetate or triethylammonium acetate. On the other hand, if operated under NaCl or NaBr salt gradients, these columns exhibited a completely different retention trend for $N - x$ deletions and also allowed for the resolution of isomeric ONs.[125] pH of the mobile phase also plays an important role to control retention and selectivity. For example, in the case of alkylamide stationary phases, as reported in ref. [126], when pH is buffered at 6 or 7, the negative charges on the stationary phase are not efficiently shielded by the salts. ONs are therefore repelled from the surface and retention is dominated by hydrophobic interactions. On the contrary, when pH is around 5 or below, electrostatic interactions are predominant. Very recently, Lämmerhofer's group synthesized two novel mixed-mode stationary phases, by means of a stable polymer coating strategy, that allowed limiting the bleeding phenomena often observed with prior brush-type RP/weak anion exchanger (WAX) congener with bifunctional siloxane bonded ligand. In a second version, the residual thiols on the polymeric RP/WAX were oxidized to sulfonic acids to obtain a RP/WAX/strong cation exchanger (RP/WAX/SCX) stationary phase. These groups act as surface anchored counterions, shifting the still net positive surface charge to lower ζ-potentials. This modification allowed accelerating the separation of negatively charged ONs, with a reduction of the total analysis time by about 50%.[127]

4. Preparative Chromatography for the Purification of ONs

In order to promote the commercialization of ONs as therapeutics, they need to be produced in sufficient amount firstly for their bioactivity to be characterized, then for preclinical and clinical trials and, eventually, for the transition to the market.[128] Efficient technologies for the preparative purification of significant amounts (mg to g) of target ONs from production impurities (such as shorter nucleotidic chains, aggregates, small abortive transcripts, etc.) are therefore urgently required. The choice of the purification method relies on the chemical features of ONs, especially their modifications and applications. For instance, it is very well known the importance of the tertiary structure of these molecules in influencing their pharmaceutical activity. If the purification method is excessively harsh, denaturation of ONs can occur, requiring a refolding protocol in order to recover the original structure and functional activity.

Two are the reference techniques for the downstream processing of ONs, namely preparative chromatography and polyacrylamide-gel electrophoresis (PAGE). Our anticipation is that, thanks to their larger versatility, greater flexibility, ease of automation, scaling-up and continuous production, LC applications will become the preferred choice for the preparation of therapeutic ONs. Therefore, PAGE-based purification methods will not be discussed any further in this review. The interested reader can refer to specific reviews and papers on this subject.[128–130]

In most cases, ON purifications via LC are based on the methods already discussed before for analytical purposes, including ion-exchange liquid chromatography (IEXLC), reversed-phase liquid chromatography, and IP-RPLC. On the other hand, only a few examples of size-based separations via Size Exclusion Chromatography (SEC) have been reported. Here the problem is that usually the required purity cannot be met in a single step and

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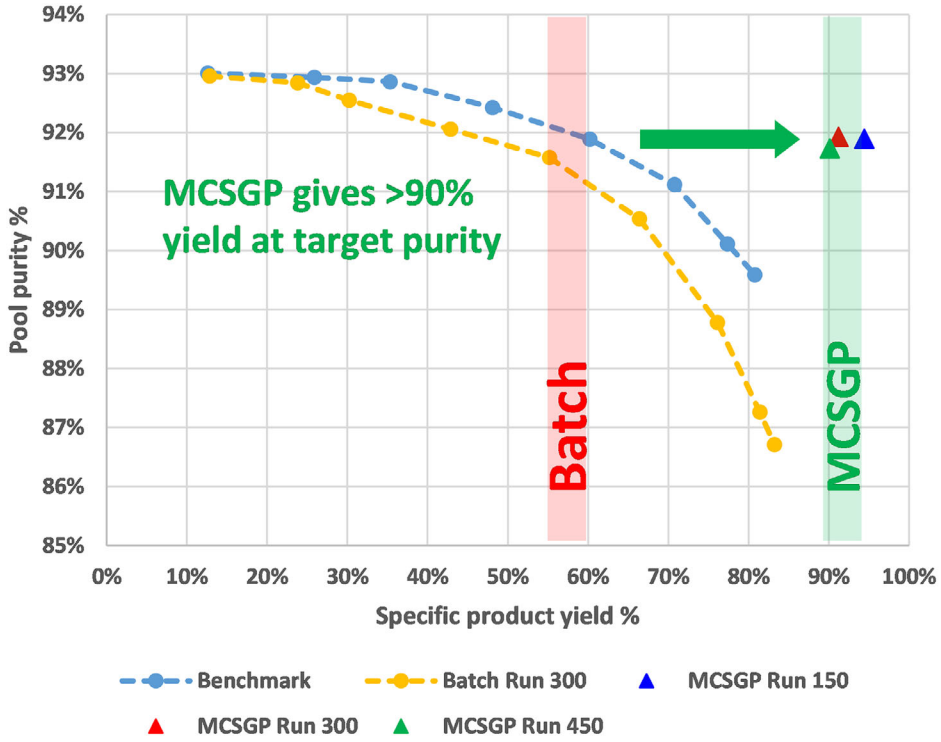

Figure 5. Pareto curve of the MCSGP (triangles) and the single-column batch reference run (circles). The data points of batch chromatography represent product pools of different sizes. MCSGP data points correspond to product pools of complete runs (5 cycles each). Reproduced with permission from.[144] Copyright 2019, ChromaCon.

therefore other purification strategies (e.g., extraction, other LC separations, etc.) need to be performed after SEC.[130,131]

4.1. Preparative Ion Exchange Liquid Chromatography

As discussed earlier, ONs very often carry charges on their chains, so that ion-exchange chromatography is one of the preferred techniques for their purification. Stationary phases usually bring tertiary or quaternary ammonium groups, and are used in gradient mode using salt as a modifier. ONs are separated based on the charge of PO groups or other substituents along the chain. Usually, the PO backbone carries a negative charge so that the full-length ON is the last to elute.[132,133] Both weak and strong anion exchangers have been considered for the purification of ONs. Small abortive transcripts show a small negative charge, while larger transcripts exhibit a larger negative charge which increases with their size. Accordingly, plasmid DNA template has the largest negative charge while T7-RNA Polymerase has neutral isoelectric point (pI). These species have been successfully separated on a weak anion exchange resin, that is, diethyl amino ethyl (DEAE)-sepharose, with the following retention times: small abortive transcripts < target oligonucleotide < plasmid DNA. This method allowed the direct purification of the crude mixture, without the typical preliminary extraction step with phenol.[134] On the other hand, DEAE-sepharose has been proven to be unsuitable for separating RNAs with different 3′ ends. Among strong anion-exchangers, it is worth mentioning the MonoQ stationary phase.[135] It has been found that this stationary phase is able to separate ONs depending on their lengths, without the preliminary extraction step. Separation of ribonucleoside tri-phosphate (rNTP), short abortive transcripts and target RNA on IEXLC has also been investigated. However, the adopted high ionic strength and pH values can have a denaturing effect on ON structures, so that weak anion-exchange is to be preferred if the native RNA structure is to be preserved.

4.2. Preparative Reversed-Phase Liquid Chromatography

Reversed-phase liquid chromatography (RP-LC) has also been considered for the purification of ONs. Since in RP-LC, the adsorption is strongly related to the percentage of organic modifier in the mobile phase good resolutions are obtained only with shallow gradients, at the cost of significantly longer process time. This technique is particularly suitable for ONs modified with hydrophobic groups or with fluorescent dyes. The drawback is that this notoriously leads to the biomolecule denaturation, thus compromising its bioactivity.[136–138]

4.3. Preparative Ion Pair Reversed-Phase Liquid Chromatography

In the case of preparative IP-RPLC, usually an alkyl amine is added to the mobile phase as ion pair reagent. The involved complex adsorption mechanism depends on the charge of the ONs, the length of the alkyl substituents and the percentage of organic modifier in the mobile phase.[97,139] Longer alkyl chains in the

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ion-pairing buffer lead to longer retention times.[138,139] This technique is particularly suitable for the separation of RNA chains with only slight differences in the sequence and with length no larger than 60-mers.

5. From Batch to Continuous Chromatography

As we discussed earlier, the literature on the preparative purification of ONs is still very scarce, and in addition it is strictly limited to single column (batch) chromatography. In this operation, it is well known that, in order to achieve the desired purity of the target molecule, it is necessary to properly narrow the collection window. This typically implies low recovery yields that in the case of ONs may be as low as 40%, and in many cases even lower. To avoid wasting the target product, the overlapping regions in the eluting stream (where the target component is still present but with an excessive amount of impurities) are manually recycled and reprocessed. This is a very labor-intensive activity decreasing tremendously the productivity of the process, which in turn becomes inacceptable for commercial standards.

This problem, referred to as the purity-yield trade-off, is an intrinsic feature of single column batch operations. The alleviation of this trade-off can be obtained with the adoption of multicolumn or continuous chromatography (e.g., ref. [44]). By simultaneously operating a series of (identical) chromatographic columns properly connected through several switching valves, this technique permits not only the continuous and automatic operation of the unit, but also to mimic the countercurrent movement of the stationary phase in the opposite direction to that of the eluent stream. This leads to significant improvements in the efficiency of the process, which can be quantified in terms of a significant recovery increase, with the same purity, with respect to single-column operations.

In particular, for the purification of mixtures of ONs, the so-called Multicolumn Solvent Gradient Purification (MCSGP) process, based on only two twin columns, seems particularly suitable.[140–143] This technique, previously used for the purification of peptides, proteins and monoclonal antibodies, has been recently used for the purification of a typical mixture of ONs.[144] As mentioned earlier, it was found that, at fixed purity (92% in this case), MCSGP allowed to increase the yield from 55% to more than 90% with respect to single column operation, thus alleviating its characteristic purity-yield trade-off expressed by the Pareto curves reported in **Figure 5**. This peculiar yield increase in MCSGP is due to automated internal recycling of the product-containing impure side-fractions. This recycling prevents the wasting of the desired product without compromising the pool purity. On the other hand, the automation of the procedure makes it effortless. Therefore, MCSGP represents a scalable process solution for ON purification with equipment available for both process development on bench scale and pilot or commercial scale under good manufacturing practice (GMP) standards.

6. Conclusion

The FDA approval of the first ON Fomivirsen in 1998 marked the raise of a new class of drugs holding promise in the treatment of severe diseases that were previously considered untreatable.

However, after more than 20 years of research and of good preclinical results confirming the expectations, only nine products eventually reached the market. This is principally imputable to poor delivery, uptake and bioavailability of ONs in vivo. On the other hand, a relevant aspect that may explain the delay in the FDA approval for several ON chemistries is the lack of economical and efficient production protocols of ONs with desired features and purity.

Indeed, the upstream marked the most important advances over the years. Nowadays, the phosphoramidite-based solid-phase synthesis is a reliable strategy for the preparation of chemically modified ONs with reasonable productivities. An automated synthesizer is now commonly used on 500–1000 mmol scale for kilo-scale production of therapeutic ONs. In addition, the evolution of continuous bioreactors, driven by the increasing demand of peptides and mAbs, is nowadays leading to the reconsideration of the synthesis of ONs through recombinant technologies. These show the advantage of a reduced immune response, even though careful synthetic protocols need to be designed to minimize the degradation operated by nucleases. As a matter of fact, it can be concluded that different chemistries are required for different technologies, such as splice-switching, aptamer, siRNA, and miRNA.

A more delicate aspect in the ON production is the downstream processing. Only a few reports exist on the preparative purification of ONs and these are limited to the use of single column (batch) chromatography. The characteristic purity-yield tradeoff of this batch configuration evidently affects the whole process. A concerted effort in this field is therefore urgently required to cover the gap with the upstream. In particular, we envision that the application of multicolumn countercurrent chromatography can be an important breakthrough in the field as preliminary experiments using the MCSGP technology suggest.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biopharmaceuticals, chromatography, continuous chromatography, drugs, gene therapy, multicolumn solvent gradient process, oligonucleotides, solid-phase

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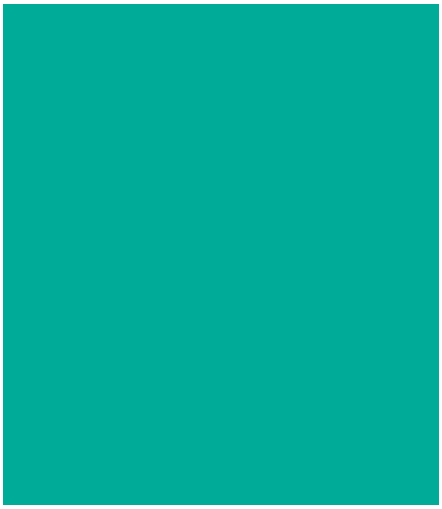
PAPER VI

Boosting the Purification Process of Biopharmaceuticals by Means of Continuous Chromatography



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Many biopharmaceuticals are currently purified by means of two or more successive single-column (batch) chromatographic steps. The first one is usually a *capture* step, which is used to remove non-product-related impurities, such as host-cell proteins and DNA. The second step is referred to as the *polishing* step, which removes product-related impurities, such as fragments and aggregates. However, single-column processes suffer some intrinsic limitations. Indeed, in the capture step, the trade-off between capacity utilization and productivity can be very relevant, while polishing processes are characterized by yield-purity trade-off. These limitations can be alleviated through continuous, or semi-continuous, countercurrent purification techniques. These processes display superior purification performance, allowing for the automated internal product recycling in the system composed of multiple identical columns, either interconnected or operated in parallel. In this paper, the advantages of capture simulated moving bed (captureSMB) for the capture step and multicolumn countercurrent solvent gradient purification (MCSGP) for polishing purposes will be illustrated.

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Biopharmaceuticals have rapidly grown in popularity among the medical community in recent years, as a result of unprecedented advancements in biologics and human genetics. Due to their high affinity toward a specific molecule or receptor, biomolecule-based therapeutics have been proven to have very high efficacy even at low concentrations. Moreover, endogenous (or endogenous-like) biomolecules are better tolerated by human bodies than traditional therapeutics, preventing or diminishing the occurrence of side effects after their administration. For these reasons, biological drugs for the treatment of already existing and emerging diseases represent the basis for tomorrow's medicine.

The sudden outbreak of the COVID-19 pandemic disease caused by the new coronavirus 2019-nCoV (now officially designated as severe acute respiratory syndrome-related coronavirus, SARS-CoV-2), has led to an urgent demand for novel therapies for the treatment of clinically advanced conditions. Several options can be taken into consideration for the treatment or prevention of COVID-19, mostly based on the use of biopharmaceuticals, including vaccines, monoclonal antibodies (mAbs), oligonucleotide-based therapies, peptides, interferon therapies and small-molecule drugs (1–4). Particularly relevant is the case of the mAb tocilizumab, under clinical evaluation for its ability to prevent the inflammatory process responsible for the worsening of pneumonia and pulmonary distress in patients affected by COVID-19 (4).

The industrial production of biopharmaceuticals has rapidly progressed in the last few years. However, the recent developments in cell culture and fermentation processes (such as for the production of mAbs) and solid-phase synthesis (for the production of peptides and oligonucleotides, for example) have not been matched by equivalent improvements in purification (downstream) processes, which often represent the bottleneck, in terms of both cost and time, in the entire production process (5).

Preparative liquid chromatography is the preferred method of choice to achieve the purified target at an acceptable degree of purity for therapeutics (6,7). Most of the modern downstream processes need at least two single-column purifications. The first one is usually called *capture* step, which serves to remove all non-product-related impurities, such as host-cell proteins and DNA. Successive *polishing* steps are then used to obtain the target at the desired degree of purity, by removing all product-related impurities. These are species, produced during the synthesis, with very similar chemical characteristics to the target compound (such as, truncated or deamidated species and aggregates, for example). The removal of these impurities via chromatography is very challenging, because their chromatographic behavior is often similar to that of the target. This situation very often leads to overlapping regions in the chromatogram where target and impurities are coeluted. The collection of these regions improves the yield of the separation at the expense of the overall purity. On the other hand, the discharge of these regions saves the overall purity at expenses of the process yield. These considerations are at the basis of the well-known purity-yield trade-off, affecting the performance of elution chromatography.

Among the strategies that can enhance the downstream process, multicolumn countercurrent continuous, or semi-continuous, chromatographic techniques seem to be particularly suitable. One of the greatest advantages of continuous techniques is that the purification process can be completely automated, with no human intervention, with a considerable saving of time. These approaches involve the use of two (or more) "identical" columns of the same dimensions and stationary phase, connected through a series of valves. This system allows not only the internal product recycling of the overlapping regions for enhanced product–impurity separation, but also to simulate the apparent opposite movement of the stationary phase with respect to the mobile one, from where the term *countercurrent* is derived to refer to these techniques.

The countercurrent separation of two compounds can be explained through the simple graphic represented in Figure 1. Let us imagine that a slower turtle and a faster rabbit are moving in the direction of the blue arrow (right). Suddenly, they fall onto a conveyor belt moving in the opposite direction (left). Depending on the relative velocities of the turtle and the rabbit (compared to that of conveyor belt that can be properly varied), the slow turtle will be transported to the left of the conveyor belt, while the fast rabbit will continue its run to the right. At the end, the two animals will be separated at the opposite sides of the conveyor belt. In this representation, the turtle is the strongly adsorbed compound (slower velocity into the column), while the rabbit is the weakly adsorbed one (moving faster). The blue arrow represents the direction of the mobile phase. Finally, the conveyor belt represents the countercurrent movement of the stationary phase.

The first countercurrent multicolumns setup was simulated moving bed (SMB) applied for the first time more than 60 years ago for the separation of binary mixtures (8–11). Since then, the SMB concept has been modified and improved, particularly in the direction of reducing the number of columns connected together. This paper focuses, in particular, on two of the most recent improved versions of the traditional SMB concept, captureSMB and multicolumn countercurrent solvent gradient purification (MCSGP). Their advantages over traditional single-column techniques for the purification of therapeutic biomolecules are illustrated.

CaptureSMB

The capture step usually deals with very large volumes of feed coming from the upstream process containing a large number of non-product-related impurities. An affinity resin is used to selectively capture the target molecule. All the other impurities will not bind to the stationary phase, and, therefore, they can be easily removed.

Let us consider a typical case where capture processes are employed—the purification of mAbs with Protein A stationary phase (12). In batch chromatography, the feed is injected into the column by adjusting the loading on the base of the dynamic binding capacity (DBC) value, which can be experimentally evaluated by a breakthrough curve (see Figure 2). A 1% DBC (the capacity at 1% of the breakthrough curve) is taken as reference limit to indicate the saturation of all available affinity sites on the stationary phase. By loading the column beyond this limit, there would be a loss of the target, which would not bind to the stationary phase. Therefore, in batch processes, the column is usually loaded up to 80–90% of 1% DBC, with a 10–20% margin in order to avoid any target-compound loss. After the loading, the target is eluted from the column and the resin is washed and regenerated.

Even if very high yield and purity can be obtained by means of batch purifications, there is an intrinsic trade-off between capacity utilization and productivity. Capacity utilization (*CU*) is defined as the ratio between the loading (*L*) and the maximum saturation capacity of the stationary phase (Q_{sat}), which also corresponds to the static binding capacity (SBC):

$$CU\% = \frac{L}{Q_{sat}} \times 100 \quad [1]$$

Productivity (for an *n*-column process) is defined as:

$$Prod\ \% = \frac{m_{target\ recovered}}{t_{run} \times n \times V_{col}} \times 100 \quad [2]$$

where $m_{target\ recovered}$ is the mass of the target collected at the end of the run, t_{run} is the duration of a run and V_{col} is the geometrical volume of the column. For a batch process, n = 1. Productivity is expressed in g/L/h.

To explain the trade-off of batch capture processes, it must be considered that capacity utilization can be increased by changing the DBC value. Indeed, higher DBC values can be obtained by steepening the breakthrough curve. This can be achieved by decreasing the loading flow velocity. However, lowering the loading flow velocity negatively impacts productivity, which will be unavoidably decreased (besides, buffer consumption increases).

This trade-off can be alleviated by employing multicolumn countercurrent processes (7). One of the most modern approaches for the capture step in semi-continuous mode is captureSMB. In its simplest version, two identical columns (packed with Protein A resin in the case of mAb purification) are connected through a series of valves. It is a quite complex process that can be

Table I: Equation for capacity utilization calculation in batch and captureSMB processes. Capital letters refers to areas shown in Fig. 2.

CU% batch	CU% captureSMB
$\frac{A}{A+B+D} \times 100$	$\frac{A+B}{A+B+D} \times 100$

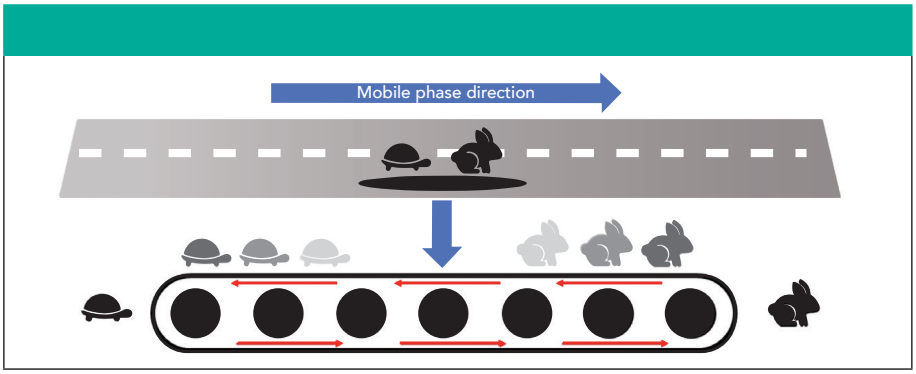

Figure 1: Schematic representation of the countercurrent mechanism; see text for details. Shadowed images of the turtle and the rabbit serve to simulate their movements. Modified with permission from reference (7).

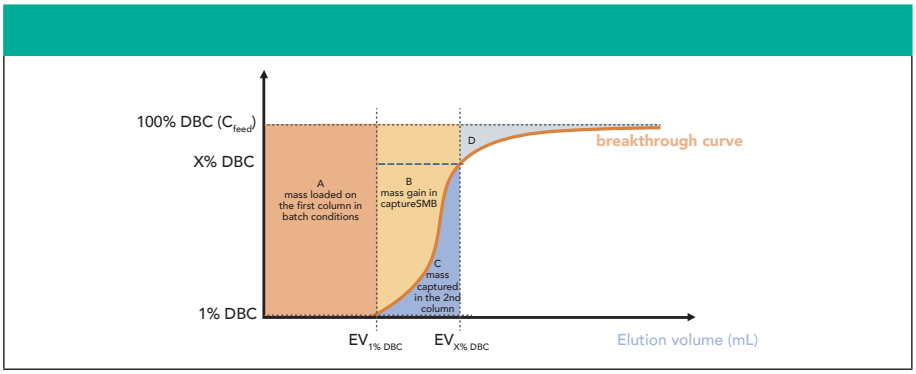

Figure 2: Schematic representation of a breakthrough curve. Area *A* represents the mass that can be loaded in the first column in batch conditions to reach 1% DBC. In twin-column captureSMB, the mass loaded on the first column is given by *A* + *B* while mass *C* is captured on the second column. The maximum saturation capacity of the stationary phase is given by the sum of masses *A* + *B* + *D*. $EV_{1\%\ DBC}$ and $EV_{X\%\ DBC}$ are the elution volumes at 1% and X% DBC, respectively.

briefly summarized in the steps represented in Figure 3. Interested readers are referred to references (13–15) for a comprehensive description.

As it can be seen from Figure 3, there are moments when columns are sequentially loaded and washed (so-called interconnected steps), and others where columns are not connected to each other (batch steps). During batch steps, one column is washed, eluted, and regenerated, while loading is continued on the other. A full cycle is completed when the two columns turn back in their initial position. What is worth mentioning is that captureSMB makes it possible to drastically increase capacity utilization. A schematic representation is given in Figure 2, where a hypothetical breakthrough curve is represented. In batch chromatography, only the mass represented by area A is loaded on the column. This corresponds to the mass that can be loaded before 1% DBC.

In twin-column captureSMB, the loading can be increased. Therefore, the first column is loaded up to a X% DBC (usually 70% DBC), containing the mass corresponding to A + B in Figure 2, while mass in area C (breaking through from the first column) will be captured in the second column. The total *A* + *B* + *D* area corresponds to the maximum saturation capacity, Q_{sat}. Thus, according to this scheme, capacity utilization for the two processes can be expressed, as reported in Table I.

As an example, captureSMB showed an increase of +26% in productivity and +11% in capacity utilization at a linear velocity of 150 cm/h for the purification of an IgG1 antibody on Amsphere JWT-203 protein A resin (16). The outcome was even better at 600 cm/h, with increases of +35% and +41% for productivity and capacity utilization, respectively. These results indicate a further advantage of captureSMB over batch processes, that is the possibility of operating at higher linear velocities since loadings are performed at much higher values than 1% DBC.

Another example is reported in (17), where mAb fragments have been purified in captureSMB by using a Capto L resin. Here, results showed a clear advantage of captureSMB over the correspondent batch process by achieving a +60% increase in loading, a +93% higher productivity, and a -54% in buffer consumption.

Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)

Differently from the capture step, polishing is needed to remove all product-related impurities, including, but not limited to, isoforms, truncates, aggregates, and deamidates. These impurities are usually produced during the synthesis, and they usually have very similar chromatographic characteristics to those of the target. The presence of product-related impurities can generate several peak overlapping regions in the chromatogram, where slightly weaker, W, and slightly stronger, S, adsorbing impurities are co-eluted with the front and the rear part of the peak of the target product, P (see Figure 4). In these cases, batch purifications are most likely governed by a yield-purity trade-off. This means that, in order to obtain a pool with acceptable purity for pharmaceutical standards, the collection window need to be narrowed at the cost of yield (and vice versa). To avoid wasting considerable amounts of target product, the overlapping regions (where the target component is still present but with an excessive amount of impurities) are manually recycled and reprocessed. This is a very labor-intensive activity that tremendously impacts on the productivity of the process.

The yield-purity trade-off can be alleviated by employing multicolumn coun-

tercurrent techniques. Among these, the multicolumn countercurrent solvent gradient purification (MCSGP) is a semi-continuous process suitable for the challenging purification of complex mixtures, that also permits the use gradient elution (18–21). This is particularly interesting for the separation of large biomolecules, whose retention is strongly affected by the organic modifier concentration (20,22–24). In Figure 3, the principles of MCSGP, in the case of a ternary separations, are schematically depicted. As in captureSMB, also in MCSGP two (or more) identical columns are used.

Differently from captureSMB, where recycling occurs during loading, in MCSGP instead recycling takes place during elution (see Figure 5). Indeed, the feed is loaded on the first column, the overlapping regions (W/P and P/S) are recycled on the second column while the purest fraction of product (P) is collected from the first one. Then the second column is fed with fresh feed, in order to keep the loading constant, and the elution starts now from the second column to the first one. One cycle ends when the two columns turn back in their initial position. The process runs in a cyclic way, and a steady state is reached where purity and recovery do not change cycle after cycle. This mechanism partially overcomes the yield-purity trade-off usually faced in batch separations. Indeed, the recycling of overlapping regions can increase the yield of the collected product while maintaining purity that is at least equivalent to that of a batch process (product purity strictly depends on the pooling criteria). The interested reader can find a detailed description of the process in references (12,25,26–28).

MCSGP has been successfully applied to the purification of many classes of biomolecules. Different chromatographic media can be used in MCSGP, ranging from reversed-phase columns for the purification of peptides (25) to ion-exchange for the purification of oligonucleotides (29) or mAb charge variants (21,30).

It is worth mentioning that even a small increase in yield can be very advantageous when dealing with very expensive biopharmaceuticals. For example, references (21,30) report the purification of charged variants of mAbs with MCSGP on an ion-exchange column. An increase in yield of +56% and +74% was observed for the purification of bevacizumab (used for the treatment of many cancer diseases) and trastuzumab (used for the treatment of breast cancer), respectively, by maintaining purity constant with respect to batch purifications (30). Also, the purification process of oligonucleotides can be boosted through MCSGP. Indeed, the yield in the purification of a mixture of oligonucleotides on HiScreen Q Sepharose FFcolumns was increased from 60% to 91% by moving from batch to MCSGP, maintaining the purity at 92% (29). Recently, some of the authors of this paper have applied the MCSGP process to the purification of

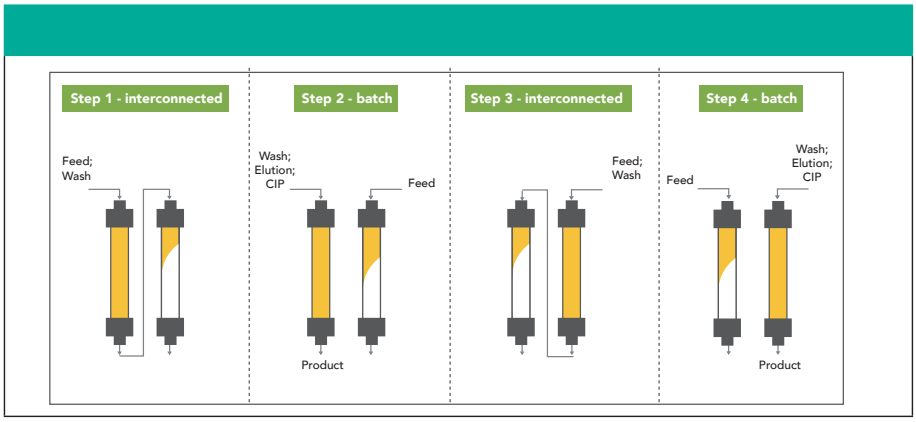

Figure 3: Schematic representation of twin-column captureSMB process. CIP stands for cleaning-in-place.

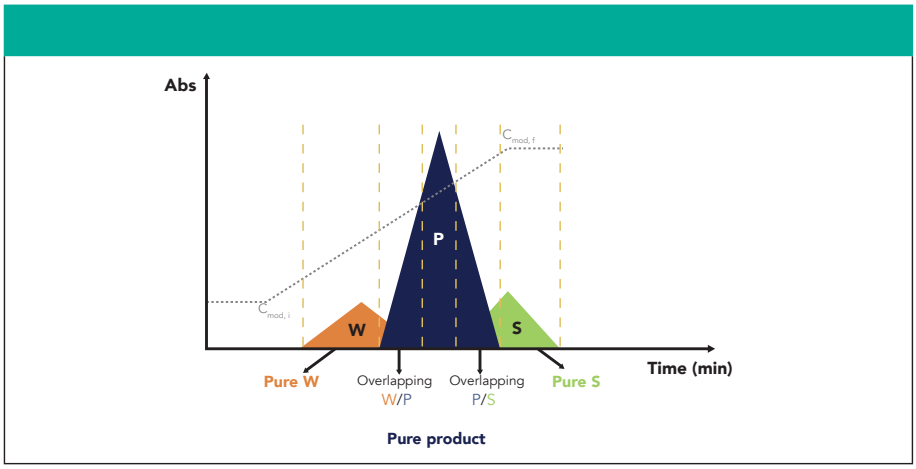

Figure 4: Schematic illustration of a ternary separation where the chromatographic peak of the target product (P) partially overlaps with those of two product related impurities. Here W refers to weakly adsorbing impurities and S to strongly adsorbed ones. Dotted grey line represents a hypothetical gradient of the modifier from an initial concentration $C_{mod, I}$ to a final concentration $C_{mod, f}$.

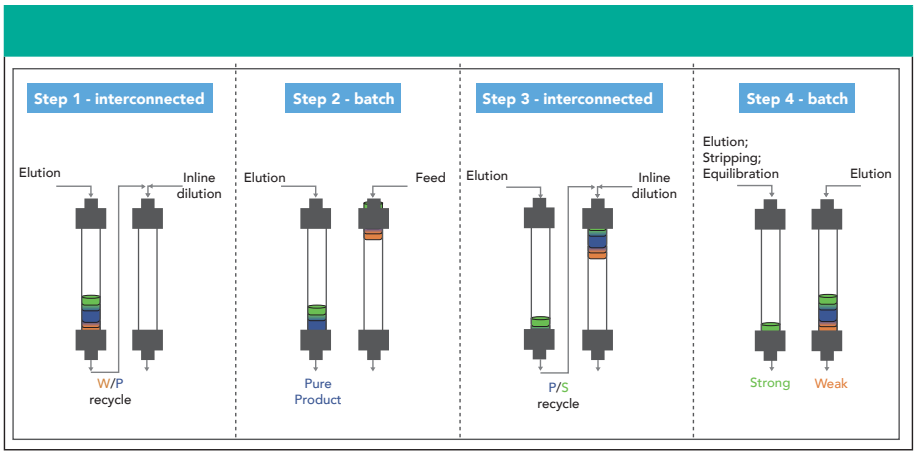

Figure 5: Schematic illustration of a twin-column MCSGP process.

a therapeutic peptide from solid-phase synthesis on a C8 stationary phase (25), allowing for a +23% yield compared to the batch process, with an unchanged purity of 89%.

Conclusions and Future Perspectives

Continuous, or semi-continuous, countercurrent techniques make it possible to partially overcome common limitations of current single-column purification strategies that often represent a bottleneck of the whole production process. CaptureSMB makes it possible to increase both capacity utilization of the resin and productivity for the capture process, making it possible to operate also at faster linear velocities than correspondent batch processes. This technique is particularly suitable for the purification of mAbs on Protein A stationary phases, but it can be used with any other affinity system (for example, protein-ligand). On the other hand, MCSGP permits to alleviate the yield-purity trade-off typical of polishing batch processes by allowing for the internal recycling of overlapping regions of the chromatogram where the target is still present in a considerable amount but polluted with impurities. This technique has been successfully applied for the purification of peptides, oligonucleotides, and charge variants of mAbs, but it can be used for any other class of biomolecules.

The greatest advantage of these techniques is that, once the experimental conditions have been optimized, the purification process can be completely automated. Therefore, no human intervention is required to process large quantities of material.

Thanks to these advantages, multicolumn countercurrent techniques represent a convenient alternative over traditional batch purification processes for the ongoing development of novel therapeutics, vaccines, and monoclonal antibody therapies for the treatment of many diseases, including pandemic COVID-19.

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PAPER VII

Process Intensification for the Purification of Peptidomimetics: the case of Icatibant through Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)

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Abstract

Biopharmaceuticals are subjected to very strict purity requirements to be marketed. At the same time, peptides and other biomolecules are industrially synthesized through techniques (e.g., solid phase synthesis) leading often to the formation of many impurities with molecular characteristics very similar to the target product. Therefore, the purification of these mixtures via preparative chromatography can be very challenging. This typically involves ternary or central-cut separations, characterized by chromatograms where the central peak, corresponding to the target product, exhibits significant overlapping on both sides with impurities slightly more or less adsorbable. In single-column (batch) preparative chromatography, this leads to a typical yield-purity trade-off, meaning that high purity can be obtained at the cost of low yield and vice-versa, with obvious consequences on the overall production costs. This study demonstrates how this limitation can be alleviated using the continuous countercurrent operating mode, conducted on a multicolumn system, as a tool for process intensification.

In particular, the Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process has been applied to the purification of an industrial crude mixture of icatibant, a peptidomimetic

antagonist of bradykinin B2-receptors, which has been recently also considered for the treatment of patients affected by COVID-19 disease. It is shown that MCSGP allows conjugating process simplicity (using only two columns) with a significant improvement in process performance compared to the corresponding batch process. This includes all process performance parameters: yield, productivity and buffer consumption for a given purity specification of icatibant.

Keywords

Continuous chromatography; MCSGP; peptide purification; icatibant; biopharmaceuticals; yield-purity trade-off

1. Introduction

Protein-based drugs have become an important class of therapeutics since 1980s and are increasingly being considered for several therapeutic indications [1, 2]. Peptides and peptidomimetics (small protein-like chains designed to mimic a peptide) constitute a relevant fraction of these biopharmaceutics [3, 4, 5, 6]. They are constituted by a relatively short sequence of amino acids and therefore can be conveniently synthesized by solid phase synthesis, a procedure introduced for the first time by Merrifield [7]. This technique implies the cyclic repetition of the sequential deprotection-wash-coupling-wash steps, each allowing for the introduction of a new amino acid. The first unit of the chain is bound to an insoluble solid resin, which makes it possible to recover the peptide by means of simple filtration. Of course, the longer the peptidic chain, the greater the number of steps involved in the synthesis, and the higher the risk of obtaining undesired products due to secondary reactions [8]. The toxic effects of such impurities are obviously unknown and therefore they must be removed from the crude peptide solution to fulfill the strict purity requirements imposed by regulatory Agencies [9, 10, 11, 12]. These impurities are usually referred to as “product-related impurities” [13, 14], and are very similar to the target product, differing, for example, just for one amino acid or one chiral center.

Most protein-based therapeutics are currently industrially purified by means of one or more successive and orthogonal single-column (batch) preparative reverse phase (usually C8 or C18) chromatographic steps [15, 16, 17, 18]. However, due to the high chemical similarity of the impurities to the target peptide, their chromatographic behavior – which is not based on specific

binding- is very similar to that of the target [3, 4]. Accordingly, these purification processes are very challenging and constitute a significant fraction of the entire production cost.

The complexity of the crude produced by solid phase synthesis, containing many product-related impurities, requires the use of gradient elution, based on the dependence of the biomolecule retention on a reverse phase over the percentage of organic modifier contained in the mobile phase [19]. Typically, for such similar product-related impurities, both the front and the tail of the target peak overlap with peaks of adjacent impurities, which are slightly more weakly and slightly more strongly bound than the target compound, respectively. This means that, on the sides of the window where the target product elutes with high purity, there are two windows where the target and the impurities coelute. This situation, which worsens when increasing the feed loading, constitutes the difficulty of this purification process. One could choose to collect the whole target peak, including the overlapping regions, obtaining high yield but scarce purity. Alternatively, it is possible to collect only the central part of the target peak and discard the overlapping regions, thus leading to high purity but low recovery. This is usually referred to as the "purity-yield trade-off", which dominates the performance of batch purification processes [20, 21]. These constitute the core of the downstream portion of the protein manufacturing process, and in general provide the bottleneck of the commercial units in the entire biopharmaceutical industry [22].

This trade-off can be alleviated by process intensification, which in this case involves the use of multicolumn continuous chromatographic processes. In a *batch* process, the loading of the feed is a discontinuous operation: after the feed (crude) has been loaded into the column for a certain time, the loading is stopped, and the gradient elution is started. On the other hand, in a *continuous* (or cyclic) process the feed is continuously (or following a time cycle) loaded into the purification unit, which is constituted by two or more columns. Multicolumn processes typically exploit the concept of *countercurrent* chromatography: thanks to a system of valves connecting the columns, the movement of the stationary phase in the opposite direction than the mobile phase is in fact simulated [2, 23]. Such countercurrent movement increases the interphase mass transfer rates, thus making the process more efficient. In addition, the overlapping regions, eluting from one column and containing both product and impurities, are automatically recycled inside the other column. On the contrary, in batch chromatography, the overlapping windows are either discarded (with a considerable waste of product) or manually reprocessed in the next batch run [24, 25], with significant losses in productivity. In addition, automation of the recycling operations avoids waste of time and risks of errors connected with manual operation.

In this paper, we consider, among all possible multicolumn processes [2], the twin-column Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process, which requires only two columns and, in our opinion, provides the best compromise between process simplicity and efficiency. In particular, it has been shown that MCSGP allows alleviating the purity-yield trade-off typical of the batch processes, for example providing much higher yields for the same purity values [26, 27, 28, 29, 30].

The MCSGP process has been developed about fifteen years ago [31]. In its more complex set-up, it was implemented with eight columns, but later it has been simplified to use only two columns [32]. MCSGP has been successfully employed for different applications, all related to challenging separation of complex mixtures, such as monoclonal antibodies, peptides and proteins, cannabinoids, oligonucleotides [33, 23, 34, 35, 36, 37, 38, 20, 39, 40] [41]. Differently from other continuous techniques such as Simulated Moving Bed [42, 43], MCSGP can be applied to *center-cut* or ternary separations operated in the gradient mode, such as in the case of reverse chromatography for peptide purification, where a modifier gradient is required [2, 44, 45].

In this work, the MCSGP process has been applied to the purification of a crude mixture of icatibant, a small peptidomimetics of ten amino acids, industrially synthesized through solid phase synthesis. Icatibant is a selective and specific antagonist of bradykinin B2 receptors commonly used in the treatment of symptoms of hereditary angioedema [46, 47, 48]. In addition, recent studies have revealed the potential of icatibant towards the improvement of oxygenation in patients affected by COVID-19 at early stage [49, 50]. The quantitative improvement of recovery, productivity, and solvent consumption is discussed in comparison with the corresponding batch process.

2. The MCSGP Process

2.1 Operation

The twin-column MCSGP process makes use of two identical columns working alternatively in batch and in interconnected mode, accomplishing the same eight tasks of the chromatographic method, as shown in Figure 1 and discussed later in detail, but shifted by half a cycle (four tasks). The valves switch position to connect the two columns when the overlapping regions start eluting from the first one. This is equivalent to an external recycle when operating with a single column, but it is performed internally and automatically in the MCSGP process. Such interconnected mode allows loading the overlapping windows directly into the second column, in order not to waste the target

product. On the contrary, the columns work in batch (disconnected) mode when the window containing the product with the required purity is eluting from the first column. In this step of the process, the second column is being loaded with fresh feed. The two columns are disconnected during the elution of the regions containing only impurities to be discarded.

The operation of the MCSGP process can be better described with reference to the schematic batch chromatogram shown in Figure 1. It is essential that a window, even very narrow, of the chromatogram exists, where the target product (P) fulfills the purity specifications. In Figure 1, five regions can be identified along the gradient: zones 4 to 8, starting at times t_A through t_E, respectively. In the first one (zone 4), the impurities more weakly adsorbed than the target product (from now on called W) elute. Zone 5 represents the region where the tail of W impurities overlaps with the target peak (this zone will be referred to as W/P). Here, a large amount of the target peptide elutes, but with an insufficient purity. However, this part of the chromatogram cannot be simply wasted but it must be recycled to obtain a good process yield. In the third region (zone 6), the product stream eluting from the column is within specifications and therefore it can be collected. In zone 7, the tail of the product peak overlaps with the front of the strongly adsorbed impurities (S), defining the P/S zone. This part, also, is to be recycled in order not to spoil the process yield. Finally, the column is stripped to remove the S impurities (zone 8). These windows are defined by the five-time values t_A to t_E, referred to as *switching times*, which are the key design parameters to define the performance of the MCSGP, as elaborated more in detail in the following.

In the scheme represented in Figure 1, column-1 is in “upstream” position and therefore is the first to receive the gradient eluting buffer. At first, similarly to a batch run, the W impurities elute and are discarded (zone 4), since this region does not contain the target product. At the same time, stripping and equilibration are performed in column-2, which is in “downstream” position (zone 8). During this step, the columns work in batch mode, thus they are not interconnected. Next, when the first overlapping region (W/P) elutes from column-1 (zone 5), the two columns are interconnected, so that this stream is fed to column-2 (zone 1), so that at the end of this step, column-2 is partially loaded. After this interconnection step, the region containing the pure product elutes from column-1 and is collected (zone 6). At the same time, some fresh feed is loaded into column-2 (zone 2). In this step the two columns work again in batch mode. When the second overlapping region starts eluting from column-1 (zone 7), the valves switch position again to connect the two columns so that the eluate from column-1 is fed to column-2 (zone 3). At this point, the same amount of product which is injected in a single-column batch experiment has been loaded in

column-2. This step is the end of the first switch, meaning that at this point the columns exchange position: in column-2 (which is now the upstream column) the gradient elution starts, while column-1 (which is now the downstream column) is stripped and equilibrated. The cycle is completed after two switches, namely when column-1 is again in the upstream position.

As mentioned above, the five steps constituting a switch are defined by the five-time values t_A to t_E, which correspond to the moments where the valves switch position to connect or disconnect the columns. In particular, t_A defines the beginning of the gradient elution, t_B represents the time at which the target product starts eluting from the column, but it is contaminated with W, and therefore it is recycled to the downstream column from t_B to t_C. The window delimited by t_C and t_D corresponds to the elution window of the product within specifications. After t_D and until t_E the overlapping region P/S is recycled to the downstream column. These are the process parameters to be defined to design a MCSGP operation.

2.2 Design

The discussion above suggests a simple procedure for the design of the MCSGP operation. The first step is the selection of stationary phase, mobile phase, elution gradient, loading and all the parameters which define a batch, single column operation. Here the objective is to identify convenient conditions to obtain a reasonable separation, corresponding to a chromatogram like the one shown in Figure 1, with the only condition of the existence of a pooling window where the product is within purity specifications. This can be taken as a starting point for the design of the MCSGP operation. In particular, all the experimental conditions are kept identical while moving from batch to MCSGP, such as stationary and mobile phases, stripping and equilibration protocols, elution gradient slope, amount of peptide loaded per column and so on. The only parameters which are still to be defined to fully characterize the process are the five switching times t_A to t_E, which consequently are the ones univocally defining the outcome of the MCSGP run [3, 51, 4].

Another important aspect to be considered in designing the MCSGP operation, is the inline dilution of the stream leaving the upstream column before it enters the downstream one to reach again binding conditions. The overlapping portions of the peak elute in fact along the gradient, so the concentration of organic modifier in this eluting stream is much higher than in the feed stream. Therefore, if it were not diluted with a compensation buffer, the target product would not bind to the stationary phase and would breakthrough from the second column right away. The compensation buffer used for the inline dilution is generally the mobile phase containing the lower

percentage of organic modifier. In this work, the used compensation buffer contains only 10% of acetonitrile (MP-A) compared to the buffer MP-B which contains 50% ACN, as discussed in detail in Section 3. In particular, the W/P eluting stream is diluted until the organic modifier concentration corresponding to the time value t_B is reached, so that only the target product adsorbs. On the other hand, the P/S eluting stream is diluted until the organic modifier concentration corresponding to that at t_A is reached, so that both the target product and the impurities S are adsorbed.

2.3 Process Performance Parameters

The fractions or pools collected during batch or MCSGP operation are analyzed offline using a suitable HPLC analytical protocol, described later in detail. The obtained results are used to estimate the parameters that quantify the performance of the processes. Since every pharmaceutical must respect very strict purity specifications, purity is the first parameter to be considered. It is expressed as a percentage and corresponds to the ratio between the chromatographic area of the target product peak and the sum of the areas of all the peaks in the HPLC chromatogram:

$$\text{Purity } \% = \frac{\text{A}_{\text{product}}}{\text{A}_{\text{total}}} \times 100$$

Yield or recovery, on the other side, is the percentage ratio between the mass of target product collected in the product pool and the mass loaded into the column:

$$\text{Recovery } \% = \frac{\text{m}_{\text{prod collected}}}{\text{m}_{\text{prod injected}}} \times 100$$

The third parameter to be considered is productivity, which represents the amount of target peptide within purity specifications recovered in the product pool per unit time and column volume:

$$\text{Productivity (mg/mL/h)} = \frac{\text{m}_{\text{prod collected}}}{\text{V}_{\text{col}} \times \text{time}}$$

Note that the operation time is the total duration of the run for the batch, while it is the duration of a single cycle for the MCSGP. Moreover, the column volume refers to the single column in batch and to the total volume of the two columns in MCSGP. Finally, the solvent consumption indicates

the volume of buffer used to obtain a certain mass of target product within purity specifications, during a batch run or a cycle in MCSGP:

$$\text{Solvent Consumption (L/g)} = \frac{V_{buffer}}{m_{prod}}$$

The purity-yield trade-off in batch can be visualized thanks to the so-called Pareto curve. During the gradient in batch conditions, fractions are periodically collected. Every fraction is analyzed by means of HPLC to estimate the mass of the target product and of the impurities. Starting with the purest fraction and then proceeding by adding all adjacent fractions, we compute purity and recovery for all cumulative fractions. These correspond to various hypothetical pools, each one characterized by a different pair of purity and recovery values. These are then reported on the same diagram leading to a curve of optimal points showing how the recovery can only be increased by tolerating a lower purity and vice-versa. This corresponds to the Pareto curve of the process, and each point is characterized by better performance, in either purity or recovery, with respect to any other operating condition on the same curve. On the other hand, for the MCSGP process, a single pair of values of purity and recovery is measured per switch, referring to the performance parameters of the eluate during the pooling window. The points measured at different switches ideally coincide when the two columns have the same efficiency and once cyclic steady state conditions are reached. The point related to MCSGP operation can be compared to the Pareto curve corresponding to batch operation: if it lies above the curve, it means that for the same purity specification, the recovery reached by the MCSGP is higher than that corresponding to batch operation.

3. Materials and methods

3.1 Feed

The crude mixture (feed) of icatibant (target product) was produced by solid phase synthesis by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy). The target product content in the solid peptide crude mixture is 49% wt. The feed is prepared by dissolving the crude mixture (a white powder) in a solution with a composition of 3% Acetonitrile and 97% ammonium acetate 50 mM, to reach a final concentration of icatibant of 2.5 g/L. The pH is adjusted to about 6.8 with NH_4OH. After one hour agitation, the feed is filtered with 0.45 μm filters. The chromatographic purity of icatibant in the

crude mixture was assessed to be about 88% in the feed, using the HPLC method described in section 3.3.

3.2 Process Operating Conditions

Purification of icatibant has been performed on a Contichrom CUBE combined (Chromacon YMC, Zurich, Switzerland) equipped with two detectors set at 265 nm, placed at the outlet of the two columns. The eluent is collected in pools with a Foxy R1 fraction collector.

Since the feed shows a quite high purity, a single step purification procedure is sufficient to reach the purity specification of 99%. The separation was performed on a stationary phase which is identical for the columns of both the batch and MCSGP processes, namely a Daisogel-SP-120-10-ODS-BIO, with a pore size of 120 Å and a particle size of 10 µm. The column size for the batch run was 250×4.6 mm (L×I.D.), whereas the dimension of the two columns used in the MCSGP process was 150×4.6 mm. Thus, the geometrical volume or Column Volume (CV) of the batch column is 4.2 mL while that of a single MCSGP column is 2.5 mL. A mixture of triethylamine phosphate buffer TEAP 20 mM : ACN 90:10 (referred to as MP-A) and a mixture of TEAP 20 mM : ACN 50:50 (referred to as MP-B) were used as mobile phases in the gradient elution. MP-A is also used in the in-line dilution of the overlapping fraction during the interconnected phase of the MCSGP operation.

The operating conditions for the batch and the MCSGP process are as follows. First, in both cases, the column is equilibrated with 2 CV (meaning a volume of buffer corresponding to two column volumes) of 12% MP-B at 4 mL/min. Then the feed is loaded to obtain a concentration of 10 mg/mL_{column}, corresponding for the batch run to 4 CVs of feed with 2.5 g/L of target product, with a flow rate of 3 mL/min. On the other hand, in the MCSGP process, only a fraction of this volume of fresh feed is loaded for each switch, depending on the values of the selected switching times t_B to t_E. The reason is that at every switch the downstream column is partially loaded with the stream coming from the upstream column during the interconnected phase. Therefore, since it is desirable to keep the loaded mass of the target product constant from switch to switch, the fresh feed loaded for each switch is calculated as the difference between the mass of target product loaded in the design batch operation and that already loaded during the interconnected phase. After the feed has been loaded, the column is washed with 2 CVs at 12% MP-B and then the gradient starts. During the gradient elution, the percentage of MP-B increases from 12 to 37% during 18 CVs. During this step, the flow rate is kept low (1.5 mL/min), so as to improve the separation. In the batch run, fractions are collected during the gradient every 60 seconds, while in the MCSGP operation two pools are

collected during each cycle (one pool from each column). In the end, the stationary phase is stripped with 100% MP-B to remove any impurity that could still be in the column.

During the single column operation, the system is loaded with the crude, then eluted and finally washed and equilibrated. In the MCSGP process, on the other hand, the crude is cyclically loaded while three streams are collected: the target product which is pooled once per switch, and the heavy (strong) and the light (weak) impurities. However, at the very beginning the unit has to go through a transient operation before reaching the cyclic steady-state operating conditions [33]. This is done through a specific start-up procedure, whereby the first column is loaded to the same extent as in the batch operation, while starting from the second cycle the loading procedure described above is adopted. In this work, the unit has been operated for five cycles before executing the "Shut-Down" procedure, where no more crude is fed to the unit to fully clean both columns.

3.3 Off-line analytics

An Agilent 1100 HPLC (Agilent, Santa Clara, USA) equipped with a Diode Array Detector has been used for the offline analysis of all the collected samples: feed and outlet streams. The column was a 250×4.6 mm Kromasil 5-100-C18, with a particle size of 5 µm and a pore size of 100Å. The wavelength chosen was 226 nm, while the injected volume was 2 µL. The calibration curve was obtained using pure samples of the target product with a concentration ranging from 0.1 to 2 g/L. In the analytical gradient chromatography runs two buffers were used: MP-a (0.01% Trifluoroacetic Acid (TFA) in water) and MP-b (0.01% TFA in ACN). The percentage of MP-b changed from 20 to 50% in 33 minutes along the gradient; next, it was increased to 84% in 3 minutes, and after 4 minutes of stripping it was decreased to the initial value.

4. Results and discussion

4.1 The Batch process

As already discussed in Section 3.1, the chromatographic purity of the icatibant produced via solid phase synthesis (which is about 88%) has been estimated through the percentage area of the target peak in the analytical HPLC chromatogram, reported in Figure 2. Icatibant elutes at 10.9 minutes, while all the other minor peaks are impurities that need to be removed. In particular, the species eluting before the target product are the ones hereinafter referred to as W. At the opposite, the species adsorbing more strongly to the stationary phase and hence eluting later than the product are collectively referred to as S.

Since the purity specification for icatibant is 99%, a chromatographic purification is required for reaching the market requirements. The performance of a traditional batch purification was first evaluated in the case of the icatibant crude mixture. A 4.2 mL resin was used and loaded with 10 mg/mL_{resin} of protein. The column volume was chosen in order to be close to the cumulative column volume used in the MCSGP process, which is equal to 5 mL (2.5 mL per column), and then provide a better term of comparison for the two processes. The batch chromatogram obtained for the gradient elution as described in Section 3.2 is shown in Figure 3. Since in the central window the target product is within purity specifications, this chromatogram has been taken as the *design batch chromatogram* and used as the basis to develop the MCSGP process. Fractions were collected every minute and the target product concentration and purity measured offline as described above. Concentration and purity profiles have been superimposed to the normalized online UV signal in Figure 3. It is seen that in a rather large fraction of the chromatogram the target product purity is larger than 90%.

Multiple batch process conditions can be evaluated from this experimental run by progressively enlarging the pooling window. If this from one end led to an obvious increase in the product yield, from the other inevitably determined a reduction in the pool purity. This purity-yield trade-off typical of a batch purification is clearly shown in the Pareto curve reported in Figure 4.

4.2 The MCSGP process

As discussed above, the starting point to design the MCSGP process is the design batch chromatogram obtained with the 4.2 mL column and shown in Figure 3. Based on its Pareto curve, different combinations of switching times (t_B, t_C, t_D and t_E) can be chosen, which lead to different MCSGP performances. The time values selected in this work are the following: t_A=6.2 min; t_B=19.7 min; t_C=21.2 min; t_D=24.2 min; t_E=25.7 min, as evidenced by the colored regions in the same figure. This corresponds to a hypothetical collection fraction (red area between t_C and t_D) with 98.7% purity and 67% recovery. About 5% of the target product is lost in the regions $t < t_B$ and $t > t_E$ (Figure 3), whereas the overlapping regions W/P ($t_B<t<t_C$, blue region in Figure 3) and P/S ($t_D<t<t_E$, green region in Figure 3) contains 11% and 17% of the target product, respectively. Now let's imagine transferring this chromatogram from batch to continuous. The overlapping regions, which would be kept within the unit during the interconnected phase, contain overall 28% of the target product introduced in the unit with the feed. On the other hand, the remaining target product would leave the unit either as the recovered product P within specifications (67% of the mass) or in the waste streams (5% of

mass mentioned above). Since it is desirable, cycle by cycle, to load the same amount of peptide as the batch, and since 72% (67% in P + 5% in W and S) of the target compound would leave the system, then 72% of the target compound injected in batch should be loaded at every switch. By doing this, the mass leaving the system would be completely replaced by the mass injected during the switch. In this work, the MCSGP unit has been operated for five cycles. The UV signals recorded at the outlet of the first column during the first switch of each cycle are superimposed and shown in Figure 5. It is seen that the signal corresponding to the first cycle is quite different than the others, which on the contrary are almost overlapping, confirming that cyclic steady state was reached already during the second cycle. This conclusion is confirmed by the purity and recovery values measured for the five cycles, the average values of which have been computed as 99.3% and 95.5%, respectively (see Figure 4).

4.3 Comparison between batch and MCSGP performance

In order to make a fair comparison between the results obtained with the batch and the MCSGP units, the total column volume of the two processes must be comparable. In the batch operation a 25 cm long column has been used, corresponding to a total CV of 4.2 mL, while the two 15 cm long columns used in the MCSGP have an overall CV equal to 5 mL (=2×2.5 mL).

The performance obtained with the MCSGP operation described above is compared to the Pareto curve corresponding to the batch operation in terms of purity and recovery in Figure 4. As reported in Table 1, it appears that the steady state values of purity and recovery, corresponding to 99.3% and 95.5%, respectively, are clearly superior to all possible batch operations. In particular, with the batch unit, the recovery for a hypothetical pool with a purity similar to that given by the MCSGP unit would be only 12.4%. This means that the MCSGP allowed for a +670% increase in the target product recovery, with respect to the batch.

In order to complete the comparison between batch and continuous operation we have to consider also the process productivity, which indicates the amount of purified icatibant per unit time and unit column volume. It was found that also productivity improves, going from 1.13 g/L/h (batch) to 7.6 g/L/h (MCSGP), corresponding to a +575% increase. For the batch run, the mass considered is that of a hypothetical pool having the same purity as that of the MCSGP, with a duration of 67 minutes. For the MCSGP, the mass considered is twice that contained in an MCSGP pool (which refers to a single switch), with a cycle duration of 50 minutes. Finally, also the solvent consumption plays a relevant role on the final production cost and needs to be considered. For the batch process,

again considering a hypothetical pool having the same purity as the MCSGP process, the buffer used per mass of purified target product is equal to 25.6 L/g. On the contrary, in the MCSGP steady state operation this value decreased to 4.5 L/g. This corresponds to a reduction of the overall buffer consumption of 81%. The improvement of the performance parameters obtained with the MCSGP process are synthesized in Table 1.

It is worth noting that the comparison presented here is not exhaustive since neither one of the two processes has been rigorously and independently optimized. The selected operating conditions for the batch process have been selected through an empirical procedure, based on our experience and we are confident that the conditions identified are quite reasonable, at least with the considered stationary and mobile phases used, which are typical for an industrial production environment. These have then been extrapolated to the MCSGP unit, without attempting their optimization specifically for continuous operation.

The considerations above indicate that quite some effort has still to be made to improve the design of continuous units in order to fully exploit their potential. On the other hand, the results reported in this work for a case of industrial relevance indicate very clearly that the potential of continuous technology in the purification of peptides, and in general of therapeutic proteins, is indeed very large.

5. Conclusion

Continuous countercurrent operations enable a significant intensification of the classical batch chromatographic purification of therapeutic proteins. In particular, when considering the center-cut purification of complex mixtures using gradient chromatography, the twin-column MCSGP process is an appealing strategy to alleviate the typical purity-yield trade-off of single-column batch operations.

In this work a comparison between these two operating modes is illustrated in the case of the purification of a crude mixture of icatibant produced through solid phase synthesis. A quite significant process intensification has been observed leading to large improvements in all process performance parameters: target product recovery, productivity, and buffer consumption for a product within purity specifications, that is 99.3%. The purification of icatibant from this synthetic crude is particularly difficult due to the presence of many impurities exhibiting a very similar chromatographic behavior to the target product. This is the situation where the potential of continuous technologies like MCSG is best exploited. This is readily seen in terms of process yield,

where more than 87% of the loaded target product is lost during purification in batch operation, against the less than 5% with the MCSGP unit. This is also seen in terms of process intensification, where the productivity increase from 1.13 to 7.6 g/l/h implies a reduction of almost 7 times in the unit volume or duration of operation for producing the same amount of purified target product.

It is worth noting that the performance parameters values considered above do not consider the obvious advantages coming naturally when increasing the degree of process automation, which is obviously achieved with a continuous unit operating at cyclic steady-state conditions in time, compared to one operated discontinuously batch after batch. This involves obvious aspects related to reducing human intervention, chances of errors and dead times, and, probably most important for biopharmaceuticals, improving consistency of product quality. This is a very important aspect to be considered since full automatization of the production processes selectively respond to one of the challenges of Industry 4.0.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

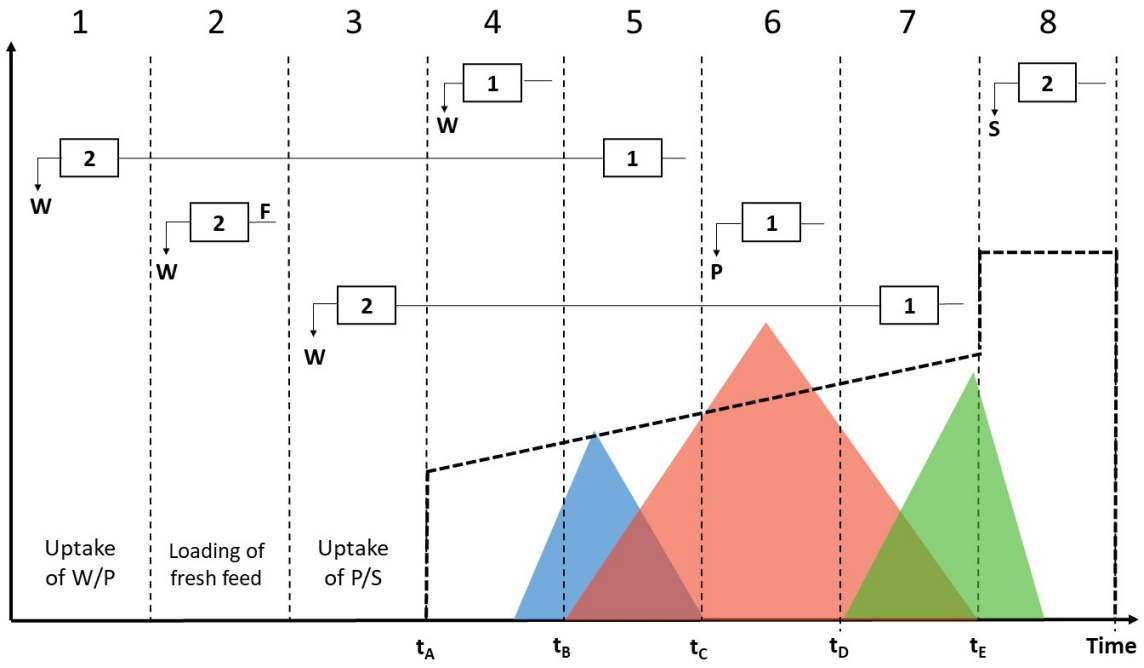

Figure 1

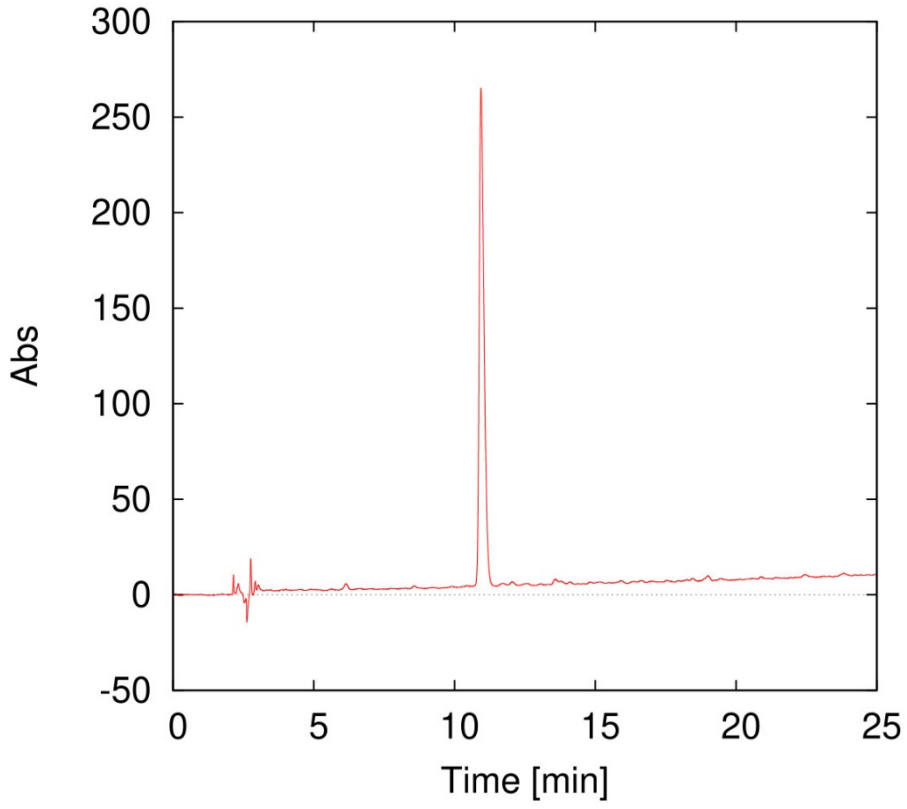

Figure 2

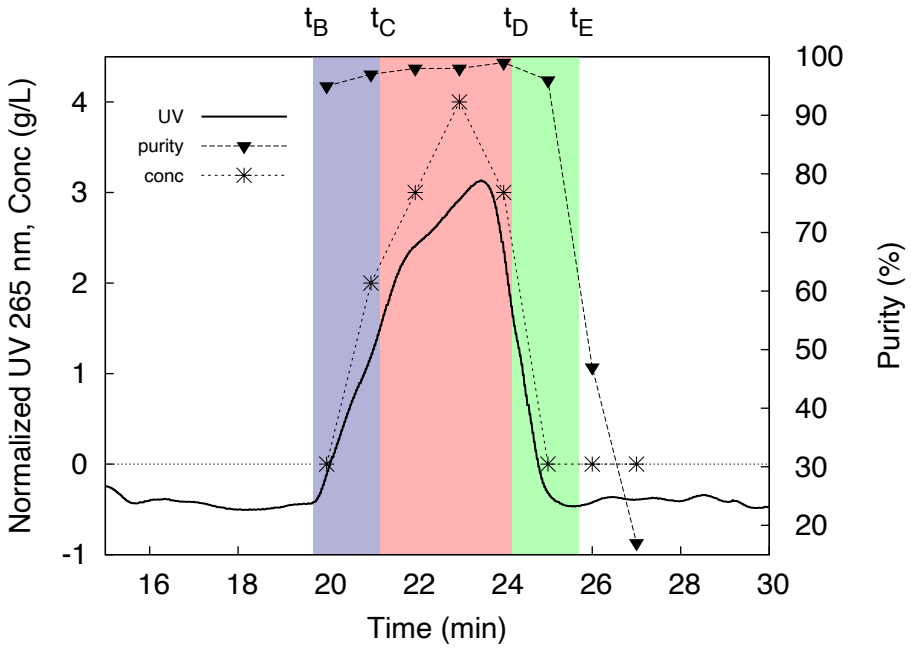

Figure 3

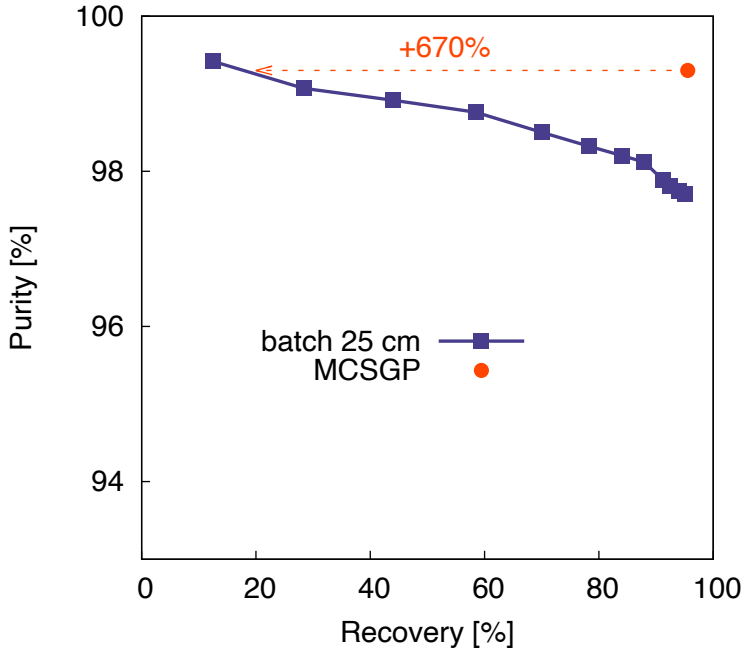

Figure 4

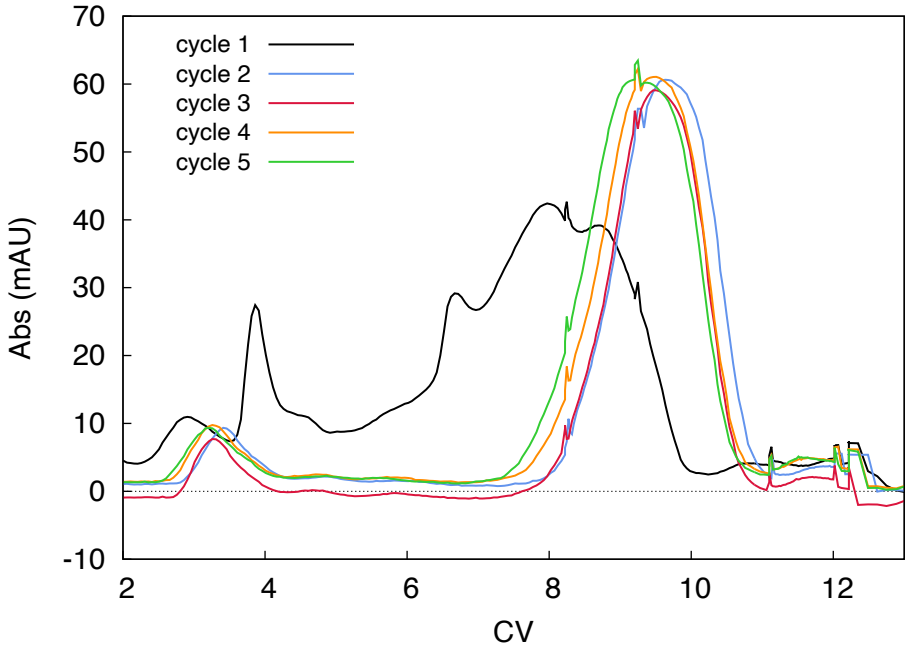

Figure 5

Figure captions

Figure 1: Schematic representation of a batch chromatogram and its different characteristic regions. The blue peak denotes weakly adsorbed impurities (W), the red one the target product peak (P) and the green one represents the strongly adsorbed impurities (S). Reproduced with permission from [3].

Figure 2: Analytical HPLC chromatogram of the icatibant feed.

Figure 3: UV (at 265 nm), purity and concentration values in the column outlet stream as a function of time for the design batch chromatogram.

Figure 4: Pareto curve obtained for the batch run with the 25 cm column and the point representing the MCSGP performance at steady-state.

Figure 5: UV in the stream leaving the upstream column during the first switch of five MCSGP cycles.

Table

	Total Column Volume (mL)	Purity %	Recovery %	Productivity (g/L/h)	Solvent consumption (L/g)
Batch run	4.2	99.4	12.4	1.13	25.6
MCSGP run	5	99.3	95.5	7.65	4.5
Process gain (MCSGP vs. batch)	-	-	+670%	+575%	-81%

Table 1

Table caption

Table 1: Comparison of the performance parameters of the batch and MCSGP processes and summary of performance improvements by using MCSGP.

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