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Intensification of purification processes in the production of oligonucleotide and peptide therapeutics

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List of papers

The work of this thesis is based on the following submitted articles and published 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, 1-4, **2020**.

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. **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, 30-34, **2020**.

VI. **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**,** *Ind. and Eng. Chem. Research*, 60(18), 6826-6834, **2021**.

VII. **Downstream processing of therapeutic peptides by means of preparative liquid chromatography,** C. De Luca, G. Lievore, D. Bozza, A. Buratti, A. Cavazzini, A. Ricci, M. Macis, W. Cabri, S. Felletti, M. Catani, *Molecules*, 26(15), 4688, **2021**.

VIII. **Benefits of a Mixed-Mode Stationary Phase to Address the Challenging Purification of an Industrially Relevant Peptide: A Proof-of-Concept Study**, G. Lievore, D. Bozza, M. Catani, A. Cavazzini, T. Chenet, L. Pasti, L. Ferrazzano, W. Cabri, M. Macis, A. Ricci, C. De Luca, S. Felletti, *Separations*, 9(5), 125, **2022**.

IX. **Enrichment and recovery of oligonucleotide impurities by N-Rich twin-column continuous chromatography**, G. Lievore, R. Weldon, M. Catani, A. Cavazzini, T. Müller-Späth, *J. Chromatogr. B*, 1209, **2022**

Published papers not included in this thesis

I. **Is there still room for innovation in chiral stationary phases for liquid chromatography? The fortunate case of the zwitterionic-Teicoplanin**, S. Felletti, C. De Luca, G. Lievore, G. Mazzoccanti, S. Manetto, F. Gasparrini, A. Cavazzini, M. Catani, *LCGC*, 33, 27-32, **2020**.

II. **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, 1737-1745, **2020**.

III. **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, 1737-1745, **2020**.

IV. **Mass transfer kinetics on modern Whlk-01 chiral stationary phases made on fully- and superficially- porous particles**, S. Felletti, M. Catani, G. Mazzoccanti, C. De Luca, G. Lievore, A. Buratti, L. Pasti, F. Gasparrini, A. Cavazzini, *J. Chromatogr. A*, 1637, **2021**.

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Audentes fortuna iuvat
(Vergilius, Aeneis)

Contents

1. Introduction

Over the past decades, significant progress and advancements in several areas of chemical and biological sciences have been shaping the pharmaceutical landscape. Meanwhile the large majority of available therapeutics were synthetic small organic molecules, a steady rise of implementation and commercialization of biologically and biotechnologically derived products began. The terms *Biologics*, embraced by the Food and Drug Administration (FDA) and *Biologicals*, adopted by the European Medicine Agencies (EMA), are employed to target drugs derived from living cells or through biological processes. Under this definition a variety of complex molecules is lumped including proteins, carbohydrates, nucleic acids and gene, cells, blood products, monoclonal antibodies, cellular therapies, and cytokines among other. [1,2] The vaguer definition *biopharmaceuticals*, coined in the 1980s, refers to a pharmaceutical substance inherently biological in nature manufactured using biotechnological methods. [3] This informal term, largely employed by the industry and the scientific community, is intrinsically broader and includes also other compound classes such as peptides and oligonucleotides. These, despite being structurally mimics or strictly related to physiological or natural compounds as their biologics counterpart, are most preferably manufactured via synthetic processes rather than biotechnological ones. [4,5] Despite some denomination incongruities and some internal differences, the whole group of biopharmaceuticals drastically diverges from small organic molecules on size, physicochemical properties, and manufacturing processes. Small molecules sizes usually range between 0,1 and 1kDa whereas the molecular weight of biologics is generally greater than 1 kDa. [6,7] Small molecules structures are generally stable, discrete, raw materials and process-independent, whereby fully characterized in terms of their molecular structure. On the other hand, biologics are extremely complex molecules, with heterogenous structures, and notoriously sensitive to a specific manufacturing process and the raw material used in it. [8] Indeed, when a drug manufacturer requests to introduce a reference product alternative in the market, regulatory agencies do not require a perfect physicochemical overlay of the two molecules but a proof of *high similarity* by extensive characterization of structure and biological functionalities of both the reference product and the proposed biosimilar. [1] Minor variations within specific ranges between the original product and the proposed biosimilar are acceptable, as they are in the batch-to-batch manufacturing of the same molecule, by means of the same process in the same plant. All this is due to the differences between expression systems and the conditions of the applied manufacturing process. [9] The expression “the process defines the product” is therefore quickly explained. All this does not apply to small organic molecules, where batch-to-batch productions and any generic drug must totally reflect the originally approved active pharmaceutical ingredient (API). Biopharmaceutical drugs are generally more sensitive to thermal, light, and chemical degradation demanding significant attention in the intermediate and final product formulation. In this regard, biologics are usually molecules extremely sensitive to transit in the gastrointestinal apparatus and with strong cell penetrability limitations due to their size, charge and hydrophilicity. [10] As a result, they are typically administered parenterally via injection or with other forms of administration among which ophthalmic, transdermal and pulmonary are constantly emerging, to the costs of oral administration. [11,12] Commonly known for their potency and selectivity, biopharmaceuticals have been causing concerns in their long-term use compared to small molecules. Indeed, the safety and efficacy of biopharmaceuticals seems to be severely impaired by the ability of patients to tolerate the drug. Prolonged treatment is associated with serious adverse events including leucopenia, thrombocytopenia, and neuropsychiatric effects, which may necessitate dose reduction or

even cessation of treatment in some patients. In a recent review from Tovey and Lallemand a through excursus about side-effects imputable to biopharmaceuticals is presented. [13] A further critical difference among the two compound classes is the economic impact they have on healthcare systems and drug manufacturers financial balances. Over the last decades, biopharmaceutical therapeutics have been constantly emerging both in absolute unit numbers and, especially, in the overall share of economic market. For instance, in 2017 only 2% of United States prescriptions comprised biologics, yet this small percentage accounted for 37% of net drug spending. [6] The unit market shares see now biopharmaceuticals approaching 10% of the total pharmaceutical sales [14], eventually raising questions about the economical sustainability of biologics drugs use for national healthcare systems and consequently about the likelihood for each patient to access state of the art therapies. In average, a daily dose of biologics costs 22 times more than that of a small molecule. [6] This discrepancy is due to several factors, accentuators of the discrepancy among synthetic and biologic drugs. Firstly, the biologics market is relatively young, with few competitors and composed of many products and processes still under patent protection. On the other hand, the small molecules market is well established with several companies which have been specializing in such developments and productions over the last century. Consequently, a large number of generic products are available for classic drugs, allowing both wider access to therapies and savings in pharmaceutical expenditure. The first biosimilar drug approved, Zarxio (Filgrastim-sndz), was approved only in 2015 [15] and up to date 35 biosimilar products have reached the market against the massive number of 10´000 approved generic drugs. [16] A second factor is the actual complexity and the costs of development and production of biopharmaceuticals. A recent analysis revealed that the average production cost per pack of small molecules was ca. 5$ whereases this value raised to ca. 60$ for biologics. [6] Small molecules are usually synthetized by means of strongly controlled and reproducible chemical reactions followed by the conversion of the active ingredient and selected excipients into pharmaceuticals form suitable for the intended administration route. [17] On the other hand, larger synthetic molecules and biologics manufacturing procedure is generally more complex and commonly divided in two processing phases: upstream (UPS) and downstream (DSP), which will be more thoroughly discussed in the next section.

All mentioned and further downsides are nevertheless counterbalanced by the many advantages resulting from the utilization of biopharmaceuticals. With the advent and the rapid growth of this new class of therapeutics multiple mechanisms to diagnose, prevent, treat, and cure diseases and medical conditions were enabled. [3,7,9] Light has been shed to solve several unmet clinical issues and biopharmaceutical drugs have revolutionized the treatment of a broad spectrum of diseases in nearly all branches of medicine. Among their strengths, their specificity is recognized, targeting almost exclusively the aimed molecules and receptors, therefore strongly decreasing the side effects associated with small-molecules drugs. [18] Their potency allows for reduced doses and have commonly paved the way for improved pharmacokinetics and pharmacodynamic properties, if compared to traditional synthetic drugs. Biopharmaceuticals play a leading role as well in the birth of personalized medicine, i.e., tailored therapies with most accurate responses and highest safety margins. [19] Unlike synthetic drugs, biomolecules exhibit more articulated mechanism of action, and their activity often relies on their conformity based on secondary, tertiary, or quaternary structures. The prevalent rationales behind the development of these innovative therapeutics are either a structural mimic of physiological compounds in light of elucidated physiological mechanisms (e.g., insulins) in order to provide replacement of a patient´s defective biomolecule as well as compensate for its absence due to genetic defects [8,11], or the

immunological stimulation to prevent damages of exogenous pathogens. In this regard, biological drugs in the guise of vaccines have been proving their great potential helping humanity in the fight against SARS-CoV-2, responsible of the ongoing Covid-19 pandemic. [20] The administration of the first-developed mRNA vaccines [1,21] was a turning point in the evolution of the infection´s spread and mortality. Indeed, recent data suggests that more than half a million of human deaths have been averted through the vaccination campaign in the European Economic Area. [22]

Recent trends and projections have been showing how the pharmaceutical R&D and manufacturing sector is shifting from small molecules toward biologic products. A brief excursus on the advantages in adopting such macromolecules is presented, as well as on the rising economic pressure those intrinsically carry with them. Aim of this doctoral path and thesis was the evaluation of technologies capable to increase the throughput of current manufacturing technologies and decrease costs in biopharmaceutical production.

1.1 Biomanufacturing and downstream processing

Given the wide range of molecules falling within the group of biopharmaceuticals, many differences are consequently encountered in the manufacturing process. Figure 1 shows a generic illustration of the various steps taking part to the biomanufacturing process of a biologic drug, e.g., a recombinant protein.

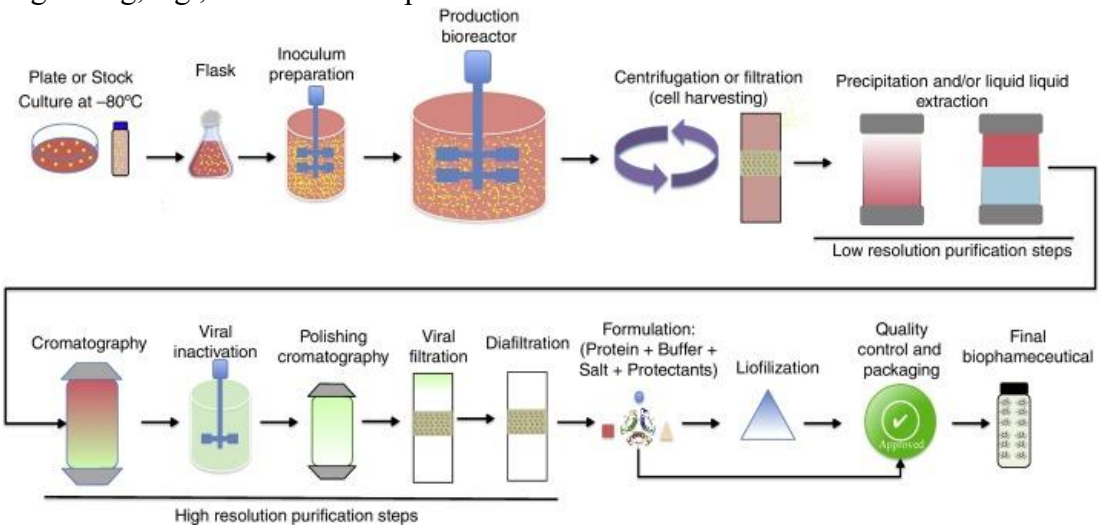

Figure 1 *The biopharmaceutical manufacturing technology flowchart exemplifying the upstream and the downstream bioprocess [10].*

As mentioned earlier, the general biomanufacture flow scheme requires that after protein expression or synthesis, a recovery step during which cells and cell debris are removed, is performed. Finally, the drug substance, which needs to fulfill all critical quality attributes (CQAs), undergoes purification. In Figure 2, the above cited differences between the manufacturing processes of a biologic and large synthetic biopolymer molecules as peptides and oligonucleotides are highlighted.

The aim of large-scale upstream processing is the cultivation of such lines in bioreactors to allow the expression of industrial amounts of recombinant biomolecules. Extremely controlled conditions, such as feeding, temperature, pH, oxygen supply and others are employed to lead the transformation of substrates into desired metabolic products. [26] Once the target molecule has been expressed, the first step in its recovery is harvesting, i.e., removal of cells and cell debris to yield a clarified and filtered fluid, known as the harvested cell culture fluid (HCCF). [27] Following this recovery step, biomanufacturing enters its more delicate part: downstream processing, that is all chemical and physical methodologies

applied to separate and purify target biomolecules. Downstream processing is now routinely found to be the bottleneck in biopharmaceutical manufacturing because its capacity has not kept pace with developments in the upstream production. [28] Indeed, the purification steps are responsible for around 50% of the total costs in an overall bio-manufacture stream. [29] Several techniques as filtration, centrifugation, precipitation, flocculation, or gravity settling have being evaluated and employed.

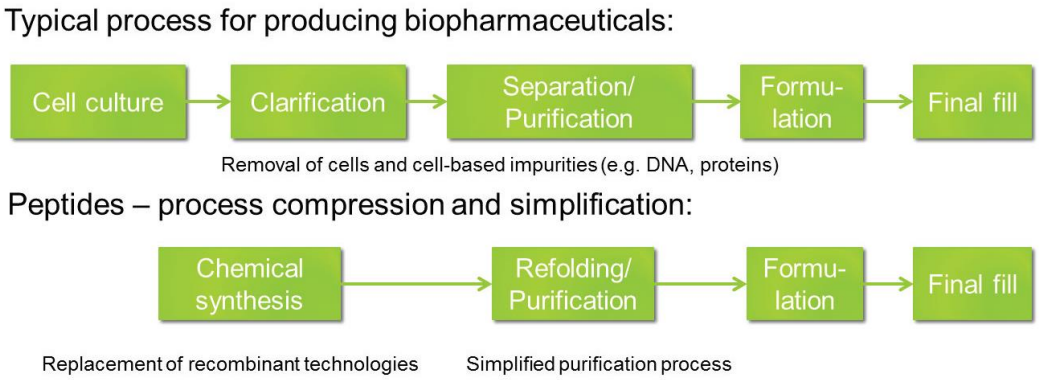

Figure 2 *Comparison of manufacturing processes of a biopharmaceutical produced in cell culture to a chemically synthesized peptide. Large amounts of cell mass and cell components need to be completely removed from the biologically produced therapeutic by employing a complex set of different filtration and separation technologies (e.g chromatography). Chemical peptide and oligos synthesis lack the high level of cell-based impurities which simplifies the purification process. [23]*

However, poor scalability of these procedures, inadequate resolving capacities and lack of reproducibility led to the rise of preparative liquid chromatography as industrial gold standard for downstream processing. [30] Robustness, selectivity, and high resolution of chromatographic methods are among the beneficial characteristics of liquid chromatography. Why are purification steps required when producing a monoclonal antibody or a peptide? To answer this question, it must be noted that despite the continuous improvements in throughput and process yield, upstream processes lead to the expression of the pharmaceutical biomolecule together with several and different kinds of impurities. [4,25] Regulatory agencies, in order to approve a new biotherapeutic molecule, require the manufacturing process to deliver a highly pure compound, whose purity should be assessed thorough a combination of analytical methods. [31]

These impurities may be process-related, i.e., chemical, or biological entities not related structurally to the product but generated due to the nature of the process. For instance, during the cultivation of cell lines, the recovery of the expressed recombinant proteins involves the collateral harvesting of cell media components, substrates, salts, HCPs, DNA or chromatographic media, solvents and buffer components used in purification. Process-related byproducts are commonly removed via affinity chromatography, a biochemical separation technique that relies on a selective and reversible interaction between an analyte and a ligand. The binding specificity between those is exploited for selective absorption of the pharmaceutical entity from a complex mixture, followed by washing steps to remove unbound impurities and the change of factors such as pH, ionic strength, or polarity to cause product elution. [32] An example of such technique is the capture of immunoglobulins, glycosylated proteins, which are processed in stationary phases functionalized with lectin. Lectins are proteins strongly and selectively binding carbohydrate moieties, allowing all other harvested material to flowthrough. In the end, IgG are eluted via temperature or salt concentration mobile phases gradients. [33] This so-called capture step is generally the first chromatographic step in downstream processing.

The second kind of impurities are product-related ones, i.e., compounds structurally similar to the target one which are predominantly high and low molecular weight (HMW, LMW) species of the target product, such as aggregates and fragments for monoclonal antibodies. This second step is named polishing and is meant to boost purity of the harvested captured material and its development differentiates according to the chemical structure of the crude to be processed. As further example, several product-related impurities can be identified in peptides upstream crudes from chemical synthesis: unpaired amino acids, short failed sequences, byproducts caused by deamination, depurination, deprotection failure or adduct formation [4,18,25] are among the possible combinations of undesired species. Likewise, long sequences of amino acids missing or exceeding of one or few monomers to reach the desired polymer length are commonly characterizing such feeds. Respectively, the firsts are referred to as shortmers (n-1; n-2; etc.) and the seconds as longmers (n+1; n+2; etc.). Generally, the removal of such impurities relies again on preparative liquid chromatography. However, because each biopharmaceutical is associated with specific separation challenges, universal generalization is not possible and multiple or even orthogonal chromatographic interaction mechanisms are exploited. For instance, proteins are often polished from their aggregates, fragments as well as from HCPs and leached protein A from the capture step [31,34], by means of ion exchange chromatography (IEC). This is because of their charged status which allow adsorption either on cation exchange (CEX) materials if positively charged or on anion exchange (AEX) resins if negatively charged. Progressive desorption is induced increasing the ionic strength of mobile phase, generally applying a gradient elution protocol. Another technology for protein polishing is Hydrophobic Interaction Chromatography (HIC). Its main advantage is that operating under non-denaturing conditions, such as ambient temperature, aqueous mobile phases, and physiological pH, avoid product folding or denaturation. [34] In HIC, stationary resins particles or base matrix are functionalized with hydrophobic ligands. Protein retention is a result of the interaction between hydrophobic patches on the surface of the protein with these ligands. [35] Retention is promoted using kosmotropic salts, e.g., ammonium sulfate, sodium citrate, potassium phosphate. Those salts interact with water molecules to reduce solvation of protein molecule in solution, decreasing their solubility and exposing their hydrophobic patches to promote binding. Consequently, protein binding occurs at high salt concentration, while elution is obtained progressively decreasing the salt concentration in the aqueous buffer. [35] It is worth mentioning that, despite the added value of this techniques, two major challenges are encountered. First, binding capacities are traditionally limited on HIC, especially in comparison with IEX resins. Then, the use of highly concentrated mobile phases has a negative impact in manufacturing plants being responsible of corrosion for stainless-steel tanks and burdening of disposal costs. [36,37]

Moving towards more hydrophobic molecules such as peptides the technique of choice is generally Reversed Phase chromatography (RPC), which has been proven to be a reliable, efficient, and widely employable technique. In this case, stationary particles of the resin are functionalized with hydrophobic structures as aliphatic chains of different length (C4, C8, C18, etc.) or aromatic rings (phenyl hexyl). Furthermore, in RPC ligand densities are usually higher than in HIC, resulting in greater hydrophobicity. In RPC the hydrophobic regions of the target molecules are adsorbed and only by increasing the organic phase ratio in the mobile phase, bound molecules are displaced from the resin and analyte from its impurities because of the different interaction strengths. Examples of organic modifier employed are acetonitrile, ethanol, methanol, or isopropanol. In this respect, purification scientists are paying growing attention to the selection of greener and easier to dispose solvents in order to meet 4.0 biomanufacturing industry goals. Despite its broad use, RPC purification technique still suffer some limitations: low loadability, high cost and limited selectivity between structurally similar molecules and charged analytes. [38]

To overcome such issues and to allow increased retention strength of bases and cations, Ion-Pair Reversed Phase chromatography was developed. An ion-pair reagent has an ionic end and a nonpolar tail: the non-polar end strongly binds the nonpolar stationary phase while the ionic sample species can be attracted to the immobilized ion-pair reagent, providing chromatographic retention. For instance, acidic ion-pairing agents as trifluoroacetic acid or formic acid, are often added to the mobile phase to pair with basic amino acids, positively charged at acidic pH, finally improving peak shape. [39] Contextually, basic ion-pairing reagents such as tetraethylammonium are inserted in mobile phase to increase selectivity of acidic compounds. [40]

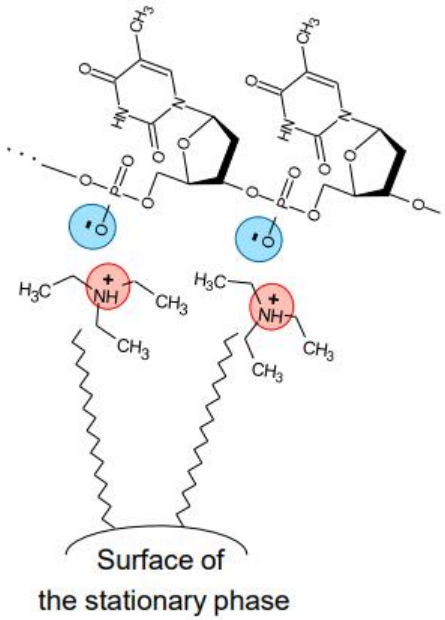

Figure 3 *The Ion-Pairing mechanism. The ion-pairing reagent (a quaternary amine) is employed as a bridge: its lipophilic portion binds to the hydrophobic ligands of the stationary phase and its charged region attracts the conversely charged analyte in the feed mixture, inducing retention.*

It is worth noting that in a classic downstream protocol, afore mentioned techniques are rarely singularly employed. The concept of *orthogonal* chromatography separations, i.e., processes according to two or more types of interactions (e.g., hydrophobic and ionic) has been constantly applied in analytical and industrial scale. [41] The rationale of this mixed purification approaches is to increase yield and productivity of a given separation exploiting multiple analyte anchor sites and to assure the fulfillment of purity constraints of the downstream products. However, the combination of two different chromatographic steps in series causes an increase in the amount of time and eluents needed, hence decreasing productivity. To cope with this aspect, over the years, efforts have been made blending two types of different materials in a single column, leading to the birth of another important technique in separation science: mixed-mode chromatography (MMC) [42, 43].
In literature, many applications of mixed-mode resins applied to biopharmaceuticals both on analytical and preparative scale can be found. Zimmermann A. et al investigated a mixed-mode reversed-phase/weak anion exchanger stationary phase for the analytical isolation of a synthetic oligonucleotide, obtaining higher selectivity than with RP and IP-RP materials. [44]. With respect to preparative chromatographic applications, Voitl et al. demonstrated an increased throughput and purity values achievable in an antibody polishing make use of a weak anion exchange/reversed phase material in comparison with anion exchange and HIC resins. [45]

A published article part of this thesis, which will be deepened further on, is indeed an application of an innovative material for the purification of a synthetic peptide.
Since the beginning of bioprocessing huge achievements have been met and many other alternatives to boost chromatographic purification outcomes, increase resin loading capacities and hence overcoming DSP bottlenecks are nowadays being evaluated. [8,18,35,38,46] Some of those are going to be highlighted in section 1.3.

1.2 Process parameters

Given the huge complexities and variabilities in biomanufacturing, one important statement in the ICH Q8 guidelines is that product final quality should be built in by process design (QbD). [47,48] Quality by design is an approach that aims to ensure the quality of medicines by employing statistical, analytical, and risk-management methodology in the design, development, and manufacturing of medicines. Among quality by design goals, one is to ensure that all sources of variability affecting a process are identified, explained, and managed by appropriate measures. [49] This concept was recently introduced by FDA and EMA aiming to achieve greater understanding of the relationship between critical quality attributes (CQAs) and clinical properties of the product. Therefore, as for all other manufacturing processes, a preparative chromatography method should be thoroughly characterized. In it should be built robustness, reproducibility, and sensitivity, simultaneously delivering high values of throughput.
Both on a lab-scale equipment and a plant infrastructure, the eluate obtained from a preparative chromatography process is collected in fractions. Each discrete pooled unit must undergo offline analytical characterization. Based on the biopharmaceutical nature, various analytical techniques may be applied. The aims of a characterization campaign are to define the structure of the major product and product-related impurities, to relate structure to function, and to quantify target/impurities ratio derived from the process. In addition to fundamental product knowledge, comprehensive characterization allows the future implementation of meaningful comparability protocols that support manufacturing changes. [50] Based on the researched attribute and the nature of the analyte several analytical assays are employed as Enzyme-linked Immunosorbent Assay (ELISA), Polymerase chain reaction (PCR), Western blot, Flow cytometry and many others. Among several techniques, High Pressure Liquid Chromatography (HPLC) coupled to various detectors, certainly represents one of the most widely used technologies to obtain multiple pieces of information from a sample.
Most of the data obtained from analytical chromatography are essential to estimate parameters quantifying and qualifying the performance of a purification process. [51] First and foremost, a purification process is intended to deliver purified material from a crude mixture. Therefore, purity of the pooled material represents a key parameter to be evaluated. The result is expressed in percent and it directly proportional to the integrated chromatographic area of the target product peak and inversely proportional to the sum of all chromatographic peaks area:

$$Purity\ \% = \frac{Area_{product}}{Area_{\ total}} \times 100 \qquad (1.1)$$

When processing valuable materials, it is essential and an economical requisite to obtain as much as possible injected product from the chromatographic unit. Process *yield* is a further parameter, obtained from the ratio of the mass of the product recovered in the pool and the mass of the product injected in the column through the feed.

$$Recovery\ \% = \frac{m_{product\ collected}}{m_{\ product\ injected}} \times 100 \quad (1.2)$$

A third parameter, correlating the amount of target compound recovered with the total duration of the preparative method and the total column volume of the stationary phase is *productivity*. This parameter is used to express the amount of target compound purified in the unit of time per column volume (CV).

$$Productivity\ (mg/mL/h) = \frac{m_{product\ collected}}{time \times CV} \quad (1.3)$$

In order to keep track of the amount of solvent utilized during the manufacture, the solvent consumption factor is introduced. It correlates the volume of buffer needed to deliver a certain mass of target within purity specifications:

$$Solvent\ Consumption\ (L/g) = \frac{V_{buffer}}{m_{\ product\ collected}} \quad (1.4)$$

Finally, an index accounting for the overall process efficiency of the manufacturing process is used: the process mass intensity (PMI). This is given by the ration between the total input mass consumed in the process (buffers, raw material, volume of resin) and the mass of the purified product.

$$PMI\ (g/g) = \frac{m_{process}}{m_{\ recovered}} \quad (1.5)$$

This metric describes the total resource consumption and hence the ecological impact of a manufacturing process. It was firstly described by Budzinski et al. and allows to compare several manufacturing procedures in terms of their environmental impact. [52,53]

1.3 Intensification strategies

A batch chromatographic process is an adsorption-based separation process carried out in single-column mode. A feed mixture, containing both the pure compound and impurities, is injected into the stationary phase, an elution via mobile phases is performed and the product is recovered, and the column purged and re-equilibrated. Only once this is completed, a new cycle of the purification process can be pursued. Currently, batch processes are the gold standard for biopharmaceutical, pharmaceutical, fine chemicals, and food processing industries. However, they do historically suffer of some drawbacks and limitation. For instance, during capture processes, in order to avoid material breakthrough, and therefore expensive product losses, the adsorbent cannot be completely loaded to its static binding capacity, decreasing throughput and productivity [54]. The operation is discontinuous, and the overall process is slowed by equilibration, strip and cleaning in place (CIP) procedures which need to be repeated for each purification. Furthermore, an operator is constantly required to operate the software, to collect the fractions and refill the system with new collecting material. Subsequently, the mentioned fractions need to be analytically characterized for each batch run, thus burdening QC and QA departments. However, probably the most affecting aspect of operating in batch conditions is the yield-purity trade-off: if the impurities present in the feed are structurally similar to the target product, it is common that their chromatographic peaks coelute both in the front or back region of the target peak. This is particularly true for synthetically manufactured biopolymer such as peptide and oligonucleotides, where ternary separations are usually encountered. A graphical representation of this phenomena is shown in Figure 4. Consequently, efforts have been made in developing more performing purification solutions. A first instance, in reality not

affecting the present batch operational pathway, is the improvement of new stationary resin able to outperform existing ones, in other words to increase separation performances and consequently reduce the amount of overlapping materials, delivering wider regions of pure material. In analytical applications, the progressive reduction of particle size in columns (up to the sub-2µm region) thanks to the introduction of ultra-high performance liquid chromatographs (UPLC), able to withstand pressures up to 1200 bar [55], permitted to reach higher efficiencies and separation capacities. However, on a preparative prospective, this approach is not viable. Backpressure limits are tremendously inferior, usually up to 100 bar for lab-scale equipment and up to 50 bar for manufacturing plants. Moreover, in preparative chromatography larger particles are needed to bind as much as possible feed material, with the aim of boosting productivity. As discussed earlier, a valuable alternative is adopting mixed-mode resins and merge orthogonal separation mechanisms in a single column. Crude materials are composed of notably amounts of impurities whose chemical structures may vary in terms of hydrophobicity, carried charge and dimension [39].

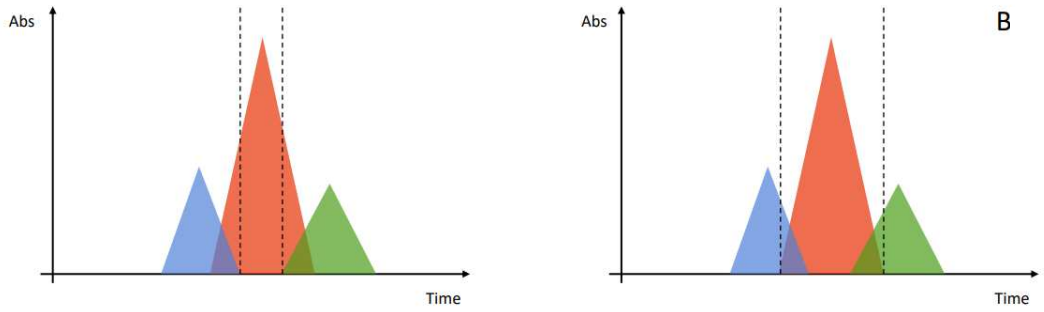

Figure 4 *Yield-Purity trade-off. Weakly adsorbing and strong adsorbing impurities are indicated respectively in blue and green. The target product in red. Grey areas indicates overlapping regions. [39]*

MMC resins use ligands capable of at least two modes of interaction with analytes resulting in interactions combinations enhancing chromatographic selectivity. Furthermore, in some applications remarkably higher loadings were shown to be reached with MMC: for instance, the loading capacity of an IEX/HILIC resin was found to be 10 times higher than that of a RP resin for the same investigated compound [56,57]. Among the latest innovation in this field are Doped Reversed materials: in fact, while most mixed mode resin carry both types of interactions on a single ligand, Doped materials use two separate ligands, each exhibiting its own type of interaction. [58] This distinction allows such materials to have an extreme specific concentration (e.g., 15%) of each ligand unlike most mixed mode materials, where different ligands are generally equally distributed. The basic RP structure of DRP resins generates retention of hydrophobic analytes. However, biopharmaceuticals crudes are usually characterized by analytes and impurities largely differing in their hydrophobic, hydrophilic, and charged regions. Thus, the introduction of doping ion-exchange ligands is exploited to strongly influence final retention strengths on the stationary resin. [59] As rule of thumb, if the ion-exchanger and the analyte bear opposite charges, an increase in retention occurs whereas, if the charge they hold is equivalent, repulsion arises, eventually decreasing the final adsorption intensity. In RP separation, more hydrophobic components (e.g., low-charged peptides) are more strongly bound on a column than less hydrophobic ones (e.g., highly charged peptides) and they do consequently elute later. As introduced by Khalaf et al, considering a three-element separation, it is possible to explain how introducing a repulsive ion-exchanger in a RP resin has a favorable impact in biopolymer polishing applications. Indeed, the elements generally involved in the separation process are a product

(P) of mild hydrophobic features, a more hydrophilic and thus more weakly retained weak impurity (W) and a deeply hydrophobic late-eluting strong impurity (S). The retention of each of those, when repulsive agents are added, is affected as follows: the weak impurity (W), highly charged, is shielded from adsorption by the repulsion between its charge and the doping ion-exchanger, thus eluting earlier. The product (P), partially charged, is only slightly affected by the repulsion and its retention factor decreases marginally. On the other hand, the strongly hydrophobic impurity S is almost not affected by the stationary phase modification and its retention time during the elution is superimposable with the one from the RP- based separation alone. A graphical representation of this phenomenon is available in Figure 5 (right). Chromatographic bands are better resolved and the overlapping peaks regions, the principal reason behind batch chromatography purity-yield tradeoff, theoretically deleted. Hence, a completely pure region of product (P), could be pooled without sacrificing the yield. On the other hand, the authors report how the utilization of doping ion-exchanger of opposite charge respect to the one hold by analytes would have a detrimental impact on the separation outcome. In fact, the weak impurity (W) would be subjected to a boost in retention and, its partially resolved peak in RP conditions, would eventually be adsorbed with homogenous strength compared with product (P) and strong impurity (S).

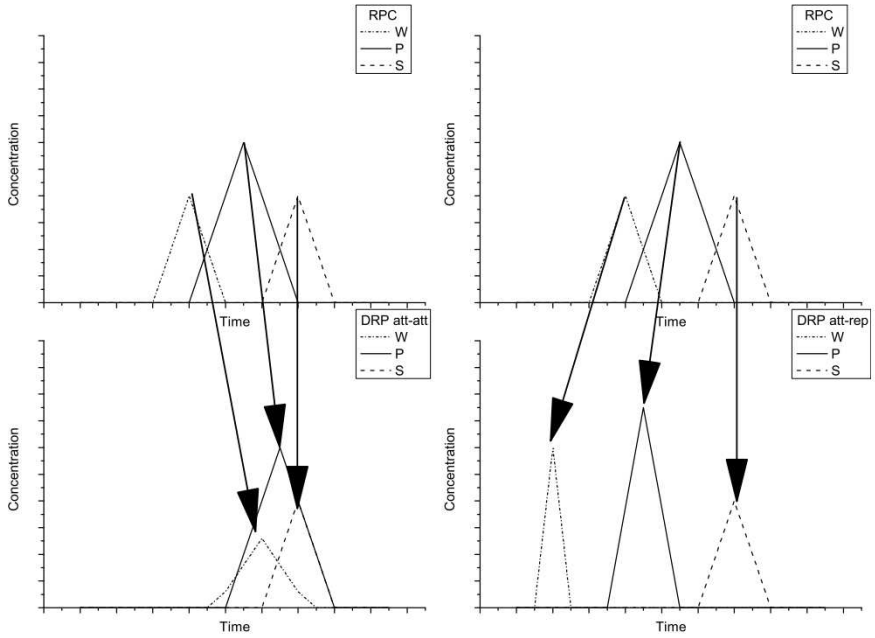

Figure 5 *Schematic representation of chromatograms in RPC (top) and DRP (bottom). The arrows show the effect of the doping IEX groups. Attractive–attractive mode is on the left, whereas attractive–repulsive is on the right. [58]*

Part of the doctoral dissertation is the application of such innovative resins to the polishing of an industrially relevant peptide, Liraglutide.

Implementation of batch processes is accomplished not only via mobile phase and stationary phase optimization, but as well with innovative engineering approaches.

Despite several efforts and attempts that have been made to surmount batch intrinsic drawbacks and the tradeoffs implied in its use, such technology is progressively being recognized as limiting and the purification processes are now shifting towards continuous operation. In chapter 3, a digression on the origin and the latest innovation of continuous chromatography will be presented, whilst in the Result and Discussion section, the research

activity carried out in the field of continuous countercurrent chromatographic processes for the purification of biopharmaceuticals will be described and discussed.

2. Preparative liquid chromatography

As previously introduced, capture and polishing steps in biopharmaceutical manufacturing are mainly carried out via liquid chromatography. Chromatography is a separation process firstly developed by the Russian botanist Tswett in 1903. [60] It is based on the difference between the migration velocities of the different components of a mixture when they are carried by a stream of fluid percolating through a bed of solid particles called column. [61] Between the two phases of this system, an equilibrium is reached for all the components of the mixture. These equilibria are mainly governed by temperature, stationary and mobile phases nature. Thorough knowledge of the events taking place in the separation media allow detailed process characterization and model-based prediction and optimization of those. As a result, growing efforts has been dedicated to the modeling of chromatographic processes. The reason behind is the willingness to reduce process development costs and burden. Indeed, the typical route to establish industrial chromatographic purification processes is a lab-scale experimental investigation on columns of the same nature as the one used in the manufacturing plant but with smaller bed height and particles diameter. This experimental approach is time-consuming and requires large amounts of feed material, commonly limited in quantities and very expensive. Consequently, the adoption of modeling alternatives to avoid such drawbacks and to identify critical process parameter and optimal process design is solidly taking pace.

2.1 Linear and nonlinear chromatography

Analytical-scale applications of chromatography, e.g., quantitative and qualitative analysis, are carried out in the so-called *linear chromatography* region, where the equilibrium concentrations of a component in the stationary and the mobile phases are proportional. [62] Thus, the equation describing the analyte equilibrium between stationary and mobile phase is still a linear curve. The peak shape is gaussian and peak height proportional to the mass of each component in the injected sample. In linear chromatography, retention times are independent of the sample composition and of its concentration. On the other hand, *nonlinear chromatography* is mainly encountered in preparative applications: here, in order to obtain high throughputs and productivities, the stationary phase is overloaded. In this case scenario, repartition linearity of analytes between stationary and mobile phase is lost and the equilibrium concentration of a compound in the stationary phase is not any longer proportional to its concentration in the mobile phase. Band profiles, peak shape and retention time of a component will depend on the amount of the compound injected and on the degree of competition for adsorption sites with other analytes present in the mixture. [62] Another important distinction among nonlinear chromatography is between *ideal chromatography* and *nonideal chromatography*. In the first, axial dispersion is unrealistically considered negligible and column efficiency and rate of mass transfer kinetics are considered infinite. Under such conditions, band profiles and peak shapes are exclusively attributed to thermodynamic equilibria. In the second one, closer to reality, the column efficiency is finite and discrete causing non-equilibrium effects to take place because of slow mass transfer kinetics. Hence, in nonideal chromatography together with thermodynamics effects also kinetics influences are considered to describe the chromatographic process and the root causes behind band broadening phenomena. Different models have been proposed to elucidate idealities and mechanisms behind chromatography and few of them are now going to be briefly discussed further.

2.2 Classes of chromatographic models

Chromatographic processes are described by equilibrium theories, employed when the mass-transfer and adsorption-desorption processes are instantaneous (constant equilibrium between the two phases) and by kinetic axioms, adopted when mass-transfer is slow. [63] This is often the case with large biomolecules, mainly encountered in this thesis. Indeed, modeling approaches consider individually or simultaneously several phenomena happening during the repartition of an analyte between stationary and mobile phase. Among those are convection, dispersion, mass transfer pore diffusion and adsorption equilibrium. [64] To enable modeling, some assumptions are introduced [65]:

- The adsorbent bed is homogeneous and packed with spherical particles of identical diameter (thus, size-exclusion effects are overlooked);
- The mobile phase in particles pores is to be considered stationary and not affected by mobile phase movements;
- The eluent is inert and characterized by constant density and viscosity;
- The adsorbent is assumed to be unidimensional, i.e., radially homogeneous;
- The process is isothermal and the column is operated under constant conditions (pressure, temperature, flowrate);

An aspect characterizing each chromatographic separation is that the mass of each component injected, included mobile phases, travelling and exiting the column is constant. In other words, the subtraction to the amount of a components entering the column of the amount leaving it is zero. The introduction of the Mass Balance Equation (MBE) enables a mathematical description of this. A differential mass balance equation accounting for accumulation (first two terms, respectively regarding accumulation on mobile and stationary phase), convection (third term) and dispersion (first term on the right) is written as

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_{L,i}\frac{\partial^2 C}{\partial z^2} \tag{2.1.1}$$

where C and q are the concentrations of the analyte in mobile and stationary phases, F is the phase ratio expressed as $F = (1-\varepsilon)/\varepsilon$, being ε the total porosity and D_L the axial dispersion coefficient. u represents the local flow velocity whereas z is the considered column thickness and t the time interval of reference. [63] To solve the MBE and thus predict the band shape, it is pivotal to understand the correlation of the concerned compound between mobile and stationary phases. Several chromatographic models elucidating those mechanisms have been proposed [62] and few of them will be briefly introduced in the next sections.

2.2.1 The ideal model

The ideal model assumes infinite column efficiency, no axial dispersion ($D_{L,i}$ nil) and local equilibrium between stationary and mobile phases. In this ideal conditions, mass transfer kinetics and axial dispersion are not involved in determining band profiles. Considering no axial dispersion contribution, the mass balance equation is now given as

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = 0 \tag{2.1.2}$$

In the ideal model, individual band profiles are influenced solely by the nonlinear thermodynamics of equilibrium. It can be applied to gather information about the thermodynamic behavior of a chromatographic column under highest performance. [66]

2.2.1 The Equilibrium-Dispersive model

The equilibrium–dispersive model accounts for a finite extent of axial dispersion and a finite rate of mass transfer kinetics between the two phases of the chromatographic system. The contribution of axial dispersion and finite mass transfer kinetic is lumped in a coefficient, D_a, i.e., the apparent axial dispersion term

$$D_a = \frac{uL}{2N} \tag{2.1.3}$$

This is possible because, considered consistent high efficiencies, the mass transfer resistance is sufficiently low to be simply accounted as an additional contribution to the mentioned coefficient. [61] Indeed, this model is not suitable for characterizing protein or large-size molecules, where mass transfer kinetics is generally too slow. Consequently, in the ED model the mass balance equation is expressed as follows

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_a\frac{\partial^2 C}{\partial z^2} \tag{2.1.4}$$

2.2.2 The General Rate model

Eventually, has to be mentioned the general rate model because it is the most detailed and comprehensive model when a mechanistic model is chosen to characterize a chromatographic process. It accounts for convective mass transfer of the solutes in the bulk phase, the diffusive mass transfer of solutes both in the film surrounding the beads and, in their pores, and for adsorption phenomena of analyte within pores surface. Therefore, this model considers separately the stagnant mobile phase, inside the pores and the percolating mobile phase, flowing between the particles, and describes both by a specific mass balance equation. [66]

The mass balance equation for the bulk mobile phase in the interstitial volume is

$$\frac{\partial C}{\partial t} + u_h\frac{\partial C}{\partial z} + \frac{3}{r_p}\frac{F_e N_0}{z} = D_L\frac{\partial^2 C}{\partial z^2} \tag{2.1.5}$$

Where u_h is the interstitial velocity of the mobile phase, r_p the average radius of the stationary phase particles and N_0 is the mass flux of the analyte from the mobile phase to the external surface of the stationary phase. F_e is correlated with the interstitial porosity. [63] On the other hand, the mass balance equation describing the diffusion of analyte within the pores of the resin particles is

$$D_p\left(\frac{\partial^2 C_p}{\partial r^2} + \frac{2}{r}\frac{\partial c_p}{\partial r}\right) = \varepsilon_p\frac{\delta C_p}{\partial t} + (1+\varepsilon_p)\frac{\partial C_s}{\partial t} \tag{2.1.6}$$

where C_p is the concentration of the analyte within the pores, r is the radial distance in the spherical particle, D_p is the pore diffusivity coefficient, ε_p is the internal porosity of the stationary phase and C_p and C_s respectively the analyte concentration inside the pores and adsorbed on the stationary phase.

2.3 Adsorption equilibria

As discussed earlier, the resolution of the mass balance equations derived from the different chromatographic models requires fundamental understanding of adsorption equilibrium. [62] Adsorption isotherms are functions correlating concentration of adsorbed analyte in the stationary phase at equilibrium with the mobile phase, given constant temperature and

pressure. These thermodynamic features are due to the nature of the compound and to the nature of interactions it establishes with the beads. The correlation is usually linear in the low concentration range and enters the nonlinear region when high concentrations are employed, and saturation is reached. [35] Multiple adsorption models have been employed to describe all the possible interactions between the analytes and the mobile and stationary phases. The differences are due to the kind of relations between adsorbate and adsorbent material, the homogeneity or heterogeneity of the latter and the composition of the feed material which may lead to competition behaviors in site adsorption. Their comprehension is fundamental to model preparative or nonlinear processes; hence some of the most encountered isotherm models are going to be briefly described and a section will be dedicated to the determination techniques of such models.

2.3.1 Linear isotherm

Linear isotherms are used to describe analytical applications of liquid chromatography, i.e., when relatively small amounts of molecules are injected into the chromatographic system. In such cases, no competition is considered to take place between analytes for the adsorption on the stationary phase's free adsorption sites. This model follows a linear relationship between q, the amount of solute adsorbed on the surface, C, the amount of solute dissolved in the mobile phases, and a, the Henry´s adsorption constant:

$$q = aC \tag{2.1.7}$$

where a, as well known as the slope isotherm, is related to the retention factor k' of the analyte and the phase ratio F as follows:

$$a = \frac{k'}{F} \tag{2.1.8}$$

This adsorption model is useful for the description of analytical applications, however it is not accurate and suitable for the description of preparative separation, where competition phenomena among analytes and mobile phase for the adsorption on the resins may appear.

2.3.2 Langmuir isotherm

This model assumes energetic homogeneity of the resin, i.e., the stationary phase is exclusively characterized by one type of adsorption site, and monolayer adsorption, i.e., no further form of adsorbate-adsorbate interaction is occurring. Graphically, it is characterized by a plateau that indicates the reaching of the saturation capacity, meaning that no further adsorption sites are available for binding. The Langmuir isotherm is described by the following equation:

$$q = \frac{aC}{1+bC} = \frac{q_s bC}{1+bC} \tag{2.1.9}$$

where, b is the equilibrium constant and q_s the saturation capacity, i.e., the maximum concentration of analyte that can be adsorbed on the stationary phase. If no further analyte is dissolved in the mobile phase, C nil, the Langmuir isotherm is reduced to a linear isotherm $q = q_s bC$, where $q_s b$ corresponds to the Henry´s adsorption constant a. Among several available models, Langmuir is certainly one of the most frequently employed to describe, fit, and model the adsorption of compounds in liquid chromatography. It has been proven valid in many applications with biopharmaceutical compounds such as peptides and oligonucleotides. [35]

2.3.2 Bilangmuir isotherm

In the Langmuir model we assumed homogenous adsorption surfaces. However, several stationary phases are composed both intentionally (mixed-mode resins) and non-intentionally (free silanols not properly endcapped) of nonhomogenous surfaces, therefore characterized by two different adsorption sites. The Bilangmuir model equation considers the possibility of either similar or different interaction mechanisms of the given analyte with the two available adsorption sites. It is written as:

$$q = \frac{q_{s,1} b_1 C}{1+b_1 C} + \frac{q_s b_2 C}{1+b_2 C} \quad (2.2.1)$$

where $q_{s,1}$ and $q_{s,1}$ are the saturation capacities of site 1 and 2, while b_1 and b_2 represent the equilibrium constants of the two sites. This model has been mainly employed to describe and model enantiomers separation on chiral stationary phases (CSP). However, it has also been reported in describing the behaviors of biopharmaceuticals entities: for example, two peptides, bradykinin and kallidin, on a reversed-phase resins [67] and a protein, albumin, on weak anion exchange resins [68], were found following Bilangmuir isotherms principles.

2.3.3 Determination of adsorption isotherms

In the previous sections, the importance of a detailed knowledge of thermodynamics phenomena in establishing a successful preparative chromatographic purification has been introduced. It is particularly relevant if we consider that operating at high concentrations, adsorption dynamics and band profiles rely on the feed composition and on the amount injected. When establishing a downstream procedure, priority should be given to the investigation of the adsorption equilibria between feed and the chosen stationary resin. From this study, optimal parameters can be obtained before moving to the experimental evaluations allowing consistent savings of consumables and valuable materials, such as feed, solvents, and resins. Initially, the determination of adsorption isotherms started using static methods, i.e., the analysis of solutions at the equilibrium state. Static methods involve known volumes or masses of adsorbent material equilibrated with equally known volumes of solutions at a given solute concentration. Following the adsorption process, the liquid media concentration is measured consequently determining the amount of adsorbed compound per unit (mass or volume) of adsorbent material. [69] Such procedure, has multiple drawbacks. Firstly, it is carried out outside a chromatographic column leading to possible inconsistencies when shifting to chromatographic systems. Secondly, several experiments and huge amounts of material are necessary to derive the whole isotherm curve. To overcome such issues, scientists developed multiple chromatographic methods to investigate equilibrium isotherms. Among those, the most recent and employed ones are Frontal Analysis (FA), the Perturbation Method (PM) and the Inverse Method (IM). In *Frontal Analysis*, a feed solution of known concentration is used to equilibrate the chromatographic column. Successively, increasingly concentrated solution is injected at the column inlet, observing and recording breakthrough curves. [70] From such curves, it is possible to extrapolate certain values resolving the equation clarifying the amount of material adsorbed in the stationary particles and the amount still dissolved in the mobile phases. Such values are V_R and V_0, respectively the retention volume of the shock and the column volume, as graphically described in Figure 6.

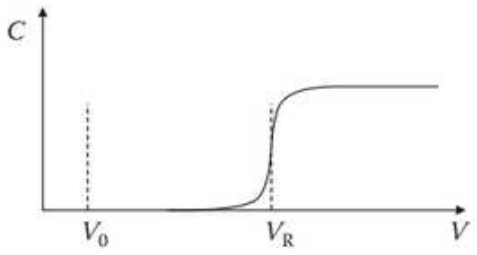

Figure 6 *Breakthrough curve from a Frontal Analysis experiment and key values for isotherm determination*

The aforementioned formula is given as follows

$$q = \frac{(V_R - V_0)C}{V_{ads}} \tag{2.2.2}$$

Eventually, a technique is presented that is frequently used to individuate thermodynamic parameters which has also been successfully applied in a publication part of this thesis: the *Inverse Method (IM)*. Such methodology consists of the numerical estimation of the adsorption isotherm parameters and their optimization by comparing true experimental nonlinear chromatographic profiles with corresponding profiles obtained by solving a previously designed chromatographic model. The steps part of this mathematical optimization are: (i) the choice of an adsorption isotherm mode; (ii) the calculation of an overloaded profile integrating the mass balance equation via the selected isotherm; (iii) the comparison of the calculated profile with an experimental one followed by the isotherm parameters optimization in order to achieve an exact overly of the two profiles. This method allows to obtain relevant data without utilizing large amounts of material. In fact, only detector calibration and few preparative runs are needed to tune the chromatographic method and to assess if the initial selection of the isotherm to use was correctly done. Such advantages are of extreme interest if the target of the optimization and of the subsequent separation is an expensive biopharmaceutical or a compound available in low quantities. The IM has already been successfully applied to a wide range of compounds. [61, 69, 70] A valuable example was its application from Marchetti et al. in the modeling of the separation of a synthetic peptide (Nociceptin) on a reversed-phase column. [71]

2.4.4 Linear Solvent Strength model

Chromatographic methods employed to separate biopharmaceuticals, both at the analytical and preparative scale, are generally based on gradient elutions, i.e., chromatographic runs where the composition of the organic modifier (for RP-HPLC) or of the salt concentration (for IEX) is progressively increased. This is applied to exploit the strong dependance on the modifier that analytes characterizing complex mixtures have. When studying adsorption isotherms, this must be considered. Indeed, while in isocratic elution the adsorption isotherm is constant along the entire column, in gradient elution it changes based on the developing organic modifier concentration. With the Linear Solvent Strength Model, Snyder et al., have proved how isotherm parameters and a solute retention factor (k) are a function of φ, the modifier concentration in the mobile phases [72]. Thus, under such conditions, the variation of the retention factor is described as:

$$\ln k(\varphi) = \ln k_0 - S\varphi \tag{2.2.3}$$

where k_0 is the retention factor at $\varphi = 0$ and S a constant typical of the specific solute-mobile phase composition. Translating this new correlation to the previously introduced Langmuir isotherm (Eq. 2.1.9), the relationship between isotherm parameters and φ is

extrapolated. Initially the correlation between the Henry constant a (Eq. 2.1.8) with the retention factor k, can be related to the φ amount and written as:

$$\mathrm{a}(\varphi) = a_0 e^{(-S\varphi)} \tag{2.2.4}$$

considering that a_0 indicates the Henry′s constant at $\varphi = 0$. Furthermore, under the assumption that the saturation capacity, q_s, does not change in the range of the mobile phase employed during the gradient, also the dependance of the equilibrium constant b on φ can be established:

$$b(\varphi) = b_0 e^{(-S\varphi)} \tag{2.2.5}$$

considering that b_0 indicates the equilibrium constant at $\varphi = 0$. Combining this latter information with the Langmuir equation, we can derive how adsorption on the stationary phase is influenced by the modifier composition:

$$q(\varphi) = q_s \frac{b_0 e^{(-S\varphi)} C}{1 + b_0 e^{(-S\varphi)} C} \tag{2.2.6}$$

Such information is pivotal to the understanding of the outcomes in gradient elution chromatography and essential in modeling such processes.

3. Continuous chromatography for ternary separations

Limits and drawback of batch chromatography, the necessity to reduce overall downstream costs and the need for more efficient technologies led to intensive efforts in the field of chemical engineering in order to develop new and more sustainable manufacturing solutions. Frequently, partially pure side fractions originated in single column processes are subject to re-chromatography, i.e., the same or similar chromatographic unit operation is carried out using the side fractions as load material. Through this operation, a fraction of the product can be recovered pure, however the separation is more difficult as the load material has a higher content of impurities than the regular feed material. Re-chromatography also has a series of operational disadvantages including regulatory limitations, side fraction storage and handling, side fraction stability and quality control. In continuous chromatography processes recycling of impure portions is automatically accomplished using the same resin and solvents as in batch chromatography, without requiring further separation optimization. The shift to continuous processes permits enhanced results in resin and buffer consumption, in the recovery of overlapping partially pure regions and in process parameter final outcomes of productivity, yield, purity and process mass intensity (PMI). [73] Already at the beginning of 1950´s the first solutions continuous processes appeared and only few years later the countercurrent principle was finally applied also to the field of chromatographic separations. Among the first innovations, it is continuous annular chromatography (CAC) which consists of an annular bed of stationary phase which rotates around its axis, subjected to a continuous crosscurrent flow of the mobile phase. With the feed being supplied continuously at a fixed point, a separation is obtained in bed length and angular coordinates. [35] Nevertheless, it was rapidly found that the application of a countercurrent movement between the mobile phase and the chromatographic media optimizes mass transfer efficiency, finally increasing the adsorbent material utilization. [74] Thus, the application of such phenomenon to a continuous process would have furtherly boosted separation efficiencies. In a first demonstration, a system delivering an actual countercurrent flow of the stationary phase material and a liquid was named True Moving Bed (TMB) process. From the beginning, it was clear that friction and pressures caused by the real movement of the beads, could have not permitted a scalability of this invention. Still, the countercurrent contact can be reached avoiding an actual movement of the resin beads. To do so, the stationary phase is packed in smaller columns which are then connected to different part of the process by periodically switching inlets and outlets of the columns via valves. In other words, instead of moving the solid with respect to fixed inlet and outlet lines, it is easier to move inlet and outlet lines with respect to fixed chromatographic beads. [75] This technology, based on a simulated movement, was named after its mechanism: Simulated Moving Bed (SMB). The first application and the original concept of SMB were introduced in 1961 for hydrocarbon separations by Broughton et al. [76] Over the last decades, its use has extensively increased for further application in several manufacturing areas such petrochemicals, pharmaceuticals, and chemicals. [77] On the other hand, the transition of the SMB technology to the field of biopharmaceuticals has been hampered by an intrinsic process-related limitation: it did not support linear gradient elution. Since nearly all biopolymer chromatographic purifications are ternary separations characterized by early eluting impurities, the target product, and late eluting impurities, they require the application of linear gradients (the progressive increase of the modifier concentration in the mobile phase over time) to vary the equilibrium distribution function during the elution and eventually gain sufficient resolution between all the compounds. The first attempt to introduce a gradient in SMB processes was suggested by Clavier et al. [78] They proposed the application of gradients in SMB processes using

supercritical eluent and exploiting different pressure levels and varying mobile phases densities to induce gradients. In a more large-scale applicable solution, a method implementing a gradient in elution condition by altering the composition of the solvent was proposed by Antos et al. [79] However, despite continuous in nature this technique did not yet consider the countercurrent movement of the two phases, essential to optimize mass transfer efficiencies. In 2002, Abel et al introduced the gradient SMB unit where the sections of the unit, in the raffinate and extract zone, operated isocratically but with a different modifier concentration. [80] Neither of the cited solutions allowed both continuous processes, countercurrent movement of the two phases, contemporaneously with the application of a linear solvent gradient. In these terms, a groundbreaking event was the invention in 2007 of the multicolumn countercurrent solvent gradient purification (MCSGP) process. [81, 82]

3.1 Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)

The application of countercurrent continuous separation to the field of biopharmaceutical manufacturing was made possible especially by the introduction of the MCSGP technology. This is based on countercurrent movement of the two phases and on a linear gradient application, allowing pure fraction collection and internal recycling of contaminated product fractions, e.g., bands of product overlapping with impurities. With such technology, the trade-off between purity and yield can be finally alleviated also in complex ternary separations. [83] Through the internal recycling, this technology is able to deliver the target compound with high yield and purity simultaneously, finally saving expensive amounts of material and avoiding reprocessing operations. In its first embodiment, MCSGP was operated with six columns. The hardware complexity led to the simplification of the process reducing the columns first to four and successively to three units. [84] In its most recent version, the technology is enabled through two twin columns, drastically reducing tubing and valves, thus alleviating the overall system costs, complexity, and footprint. [85]

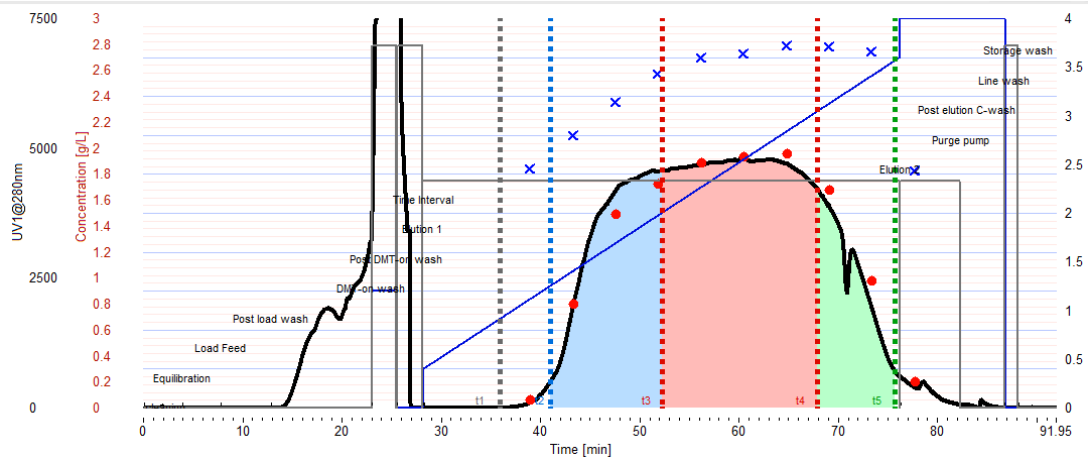

Figure 7 *Design batch chromatogram characteristic times employed for recycling and collecting portions determination [ChromIQ® -MCSGP Wizard]*

The continuous polishing is based on the same buffers, resin, and chromatographic conditions of the benchmark batch method. Indeed, as starting point to design a MCSGP process the selection of a *design batch chromatogram* is required. From this latter are derived basic parameters as flow rates, resin loading and gradient slope. Furthermore, the recycling sections are defined with the aid of batch offline analytics, precisely elucidating the overlapping regions retention times. As showed in Figure 7, the design batch chromatogram is divided into 4 zones using fine section borders ($t_1 - t_5$), which correspond to the

switching times that compose the MCSGP elution protocol. In particular, t_1 corresponds to the modifier gradient starting time and is employed by the wizard to set dilution parameters, t_2 is the trigger point where the depletion of weak impurities is completed and recycling of W/P overlapping regions start, t_3 determines the beginning of product collection which continues up to t_4, where overlapping regions P/S are inline diluted to the downstream column. Finally, t_5 determines the end of the recycling window and the beginning of the stripping procedure. During the process, the two columns alternate between interconnected and batch states. During interconnected states internal recycling reduces product loss and allows to achieve high yields, whilst batch phases allow to obtain product at high purity or elute product-free waste streams. A complete cycle of a twin-column MCSGP process includes two switches, each characterized by four identical pairs of tasks both in an interconnected and in a batch way (I1, B1, I2, B2). Those are briefly described below and graphically illustrated in Figure 8 thereafter.

- In Phase I1, the overlapping region W/P is eluted from the upstream column and internally recycled into the downstream column. The stream is inline diluted with a solvent with low eluotropic strength (generally Mobile Phase A).
- In Phase B1, pure target product P is eluted and collected from the first column, while in the second column fresh feed is injected.
- In Phase I2, the remaining partially pure region of P/S is eluted from the first column and inline diluted to the downstream resin.
- In Phase B2, the first column is purged to get rid of the strongly adsorbing impurities whereas in the second one is applied the gradient allowing the elution of weak impurities but not of W/P overlapping regions.

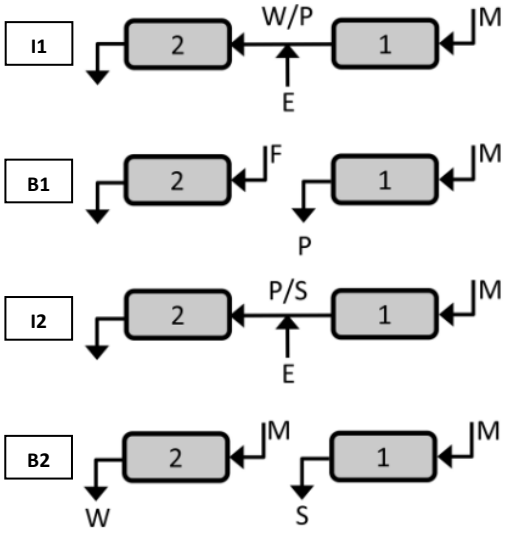

Figure 8 *Twin-column MCSGP process in interconnected (I1,I2) and batchwise (B1,B2) configuration. Figure caption: M (mobile phase), F (feed), P (product), W/P (weak/product), P/S (product/strong). [87]*

The Inline dilution flow rates assure that the entrance of the recycled material on the downstream column happens at low modifier concentrations, to ensure binding. The only difference between the two switches is in the column position. In the first switch, column 1 is downstream of column 2 and vice versa. This order is due to a start-up method normally applied to accelerate the onset and progression of steady state conditions. In fact, in a standard procedure of the MCSGP purification a first method ("StartUp") responsible for the loading of an increased quantity of raw material compared to the batch process is applied.

Subsequently, the real continuous process takes place with phase I. The process is run in a cyclic manner and reaches a cyclic steady state, generally in 2 to 3 switches, in which the amount of product collected in each cycle is equal to the amount of feed loaded (batch phases). Chromatographic efficiency and the eluate quality is kept constant cycle to cycle. However, the MCSGP process may suffer solvent/buffer variations, conductivity/pH changes, differences in column packing, and temperature oscillation. It is pivotal to tightly control such parameters and thus avoid peak profile shifts and associated disruption of product quality. Such variations may be particularly impacting in case of numerous cycles, in which a slight change in retention time of the product peak may result in the interruption of the cyclic state and loss of purified material. A dynamic process control was proposed to overcome the negative impacts of such oscillations and keep the MCSGP process at its set point even if process parameter changes occur. [88] This dynamic control makes use of the UV signals and triggers recycling or collection only when predetermined absorption thresholds are reached. Nowadays, MCSGP is well established in the industrial environment and has been applied to several molecule classes, including monoclonal antibodies [89, 90], immunoglobulins [91], peptides [51, 85] and oligonucleotides [92, 93].

3.2 N-Rich

The advantages introduced by continuous chromatography can be employed in multiple ways. So far, the attention has been paid on the intensification of processes for the manufacture of biopharmaceuticals. However, the twin-column technology finds application also in the isolation and concentration of impurities and by-products. As discussed in the "Biomanufacturing and downstream processing" section, despite growing performances in the upstream procedures, in the production of a biopharmaceutical compound many impurities and side-products are collaterally created. In order to guarantee high safety standards, a mandatory aspect of the pre-clinical drug development of biopharmaceuticals is the isolation and characterization of impurities for structural, biochemical, and toxicological elucidation, and cell or animal-based safety assay. [95] Maximal thresholds for reporting, qualification, and identification of impurities in biopharmaceutical drugs are regulated by the ICH Q3A (R2) guidelines. [96] On the other hand, for certain compounds, as oligonucleotides, regulatory agencies have yet to provide definitive values and manufacturing firms rely on white papers or generalist approaches. [97] Even though in certain scenarios it is more straightforward to directly synthetize the impurity of interest, the collection of side products is normally done by means of chromatography. The standard approach is their pooling via analytical scale or preparative batch-wise separation. However, in such scenario the operator faces a tradeoff between productivity and purity: employing HPLC isolation, only a tiny amount of material is collected per run and extensive processing times are thus required, whereas the use of preparative scale batch chromatography often delivers appropriate quantities of material but with highly unresolved profiles and thus insufficient purity values. The N-rich process, an automatic countercurrent continuous twin-column technique, alleviates the described trade-off: the desired impurities are recycled and selectively enriched, whilst interfering substances are depleted, in a cyclic fashion. N-Rich can be set up to target a single compound, or a region of the chromatogram containing several compounds of interest. A N-Rich design procedure (Figure 9) is generally based on standard gradient purification methods (batch) and is composed of four main steps:

- The first method (*Startup*) begins by loading feed material onto the first column and performing a linear gradient elution.
- During the second method (*Enrichment*), a region of the chromatogram containing target impurities is transferred from the first column, with in-line dilution, and re-

adsorbed to the second column. Non-target material is either discarded or collected in a separate pool. In the meantime, fresh feed is loaded onto the second column in addition to the recycled target. This step leads to an enrichment of the target molecules relative to other compounds in the mixture. The process step is repeated in a cyclic fashion between the two columns, progressively increasing the concentration of the target impurities.

- Phase three (*Depletion*) is a single switch without addition of new feed. This step depletes non-target compounds while internally recycling the accumulated target impurities before the final elution step. The depletion step greatly improves the final purity obtained for impurities that are closely eluting with the main compound peak.
- Finally, in the fourth phase (*Elution*), the enriched target material is eluted with a shallow gradient over two columns in-series and the target material is collected performing a fine fractionation. This strategy maximizes the resolution of the enriched compounds, and the pure target material is recovered at a higher concentration than with batch methodology.

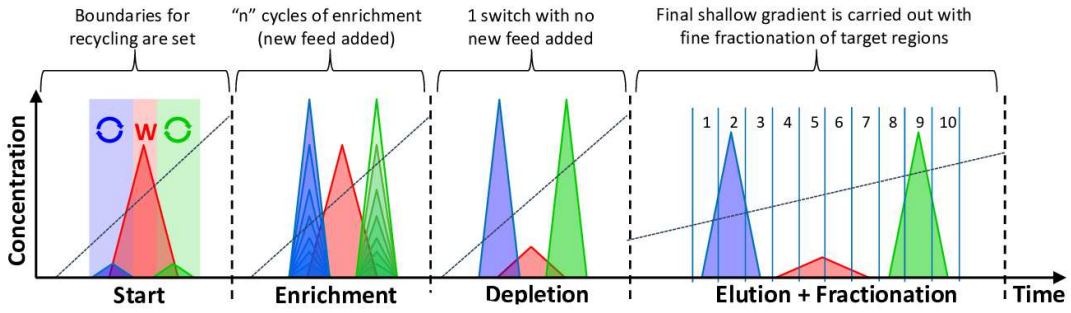

Figure 9 *N-Rich flowchart scheme [86]*

N-Rich enables the use of semi-preparative scale resins with high loading capacity boosting productivity. At the same time, it permits to achieve high resolution exploiting displacement effects and thanks to the countercurrent movement of resins and mobile phases. The continuous process delivers fractions of higher concentration compared to batch chromatography, decreasing the overall solvent consumption and the up-concentration steps. Furthermore, the automatization of the recycling and elution processes requires fewer operator activities, e.g., pooling, analyzing, merging, and reprocessing fractions, and consequently reduced operational footprint and qualified personnel time consuming. [86] Such technology was successfully applied to the enrichment of monoclonal antibody isoforms [98] and peptide impurities [99]. Part of this thesis was the implementation and tuning of an N-Rich procedure for the enrichment and collection of a broad range of impurities present in a 24-mer double-stranded RNA synthetic crude.

4. Result and Discussion

In this chapter, the results obtained in published papers are presented. The chapter is organized in different sections, each summarizing the results obtained in a specific research topic. For further details, the reader is referred to reprints of the full papers appended at the end of the thesis

4.1 Adsorption thermodynamic determination and nonlinear gradient elution modeling of a therapeutic peptide (Paper I, II)

The comprehension of thermodynamic mechanism responsible for adsorption of biomolecules on the stationary phase is of pivotal importance in the transition from an experimental chromatographic purification development to a simulated approach based on mechanistic modeling. The advantage of such transition, considered in the "Preparative liquid chromatography" section, is primarily the reduction of invested time and costs during method development. In Paper I and Paper II, thermodynamic adsorption parameters of Octreotide, a therapeutic peptide constituted of 8 amino acids, were determined, and subsequently used to simulate the chromatographic profile under preparative (nonlinear) purification conditions. For such polishing application, gradient elution is normally employed, as the retention of biomolecules and of their structurally related impurities are strongly dependent on the mobile phase concentration of the organic modifier. The adsorption isotherm type of a molecule in gradient elution is constant but its parameters are φ (organic modifier content) dependent. In order to accomplish these elucidations avoiding the consumption of large quantities of peptide, the Inverse Method technique (see 2.4.3 "Determination of adsorption isotherms") was employed. The workflow initially required the determination of isotherm parameters, followed by their application in the resolution of the selected chromatographic model to finally provide an accurate simulation of overloaded gradient elutions peaks.

Initially, an appropriate chromatographic model was selected. The choice fell on the equilibrium-dispersive (ED) model, commonly used in describing preparative separations for low molecular weight analytes. To solve the associated mass balance equation (Eq. 2.1.4), a Langmurian isotherm model was designated. The selection of the isotherm type was estimated from on the peak shape of the preparative chromatogram, described by an initial shock and followed by a peak tailing. Furthermore, for the separation carried out under gradient conditions, the Linear Solvent Strength (LSS) model, relating retention factor variation with the mobile phase composition, was considered. Secondly, on the column selected for the project, a Zorbax Sb-C18 column (150×4.6 mm, CV=2.49 mL) with particle size of 5µm, we recorded a gradient elution chromatogram at the UV wavelength of 280 nm. This allowed to identify the organic modifier (φ) range of elution, 0.23-0.28. Following detector calibration, for each of these φ value, isocratic conditions runs were performed injecting samples (crude and raw peptide) of different concentration (0.1, 0.3, 0.6, 1.2, 2.0, 4.0 and 6.0 g/L).

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

Table 1 *Adsorption isotherm parameters obtained at different φ through Inverse Method.*

From the obtained data, precise isotherm parameters were extrapolated (see. Table 1) via the Inverse Method by selecting the correct isotherm model (Langmurian) and fitting experimental records.
The simulations obtained relating the found isotherm to the chromatographic model perfectly matched the experimentally obtained curves, as reported in Figure 10.

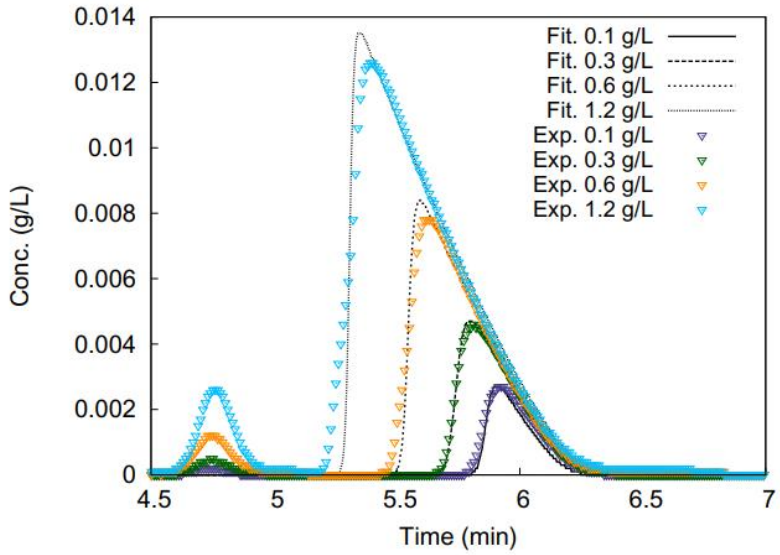

Figure 10 *Comparison between experimental and simulated peaks obtained with IM at multiple concentrations of crude material, performing isocratic elution (φ=0.24).*

The extracted values of a, b and q_0 of the Langmuir isotherm, were consequently used to solve Equation 2.2.6, describing overloaded separation under gradient elution conditions. The outcome of the simulation is put in comparison with the registered experimental signal in Figure 11. A very good agreement is obtained also for higher concentrations and thus loadings.

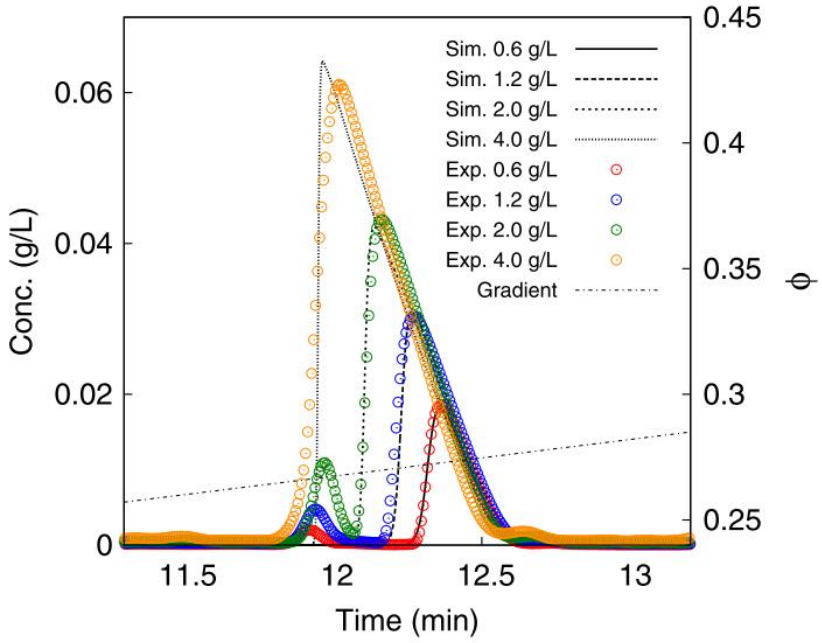

Figure 11 *Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption model) of four different concentrations of crude peptide.*

4.1.1 Closing remarks

The transition to simulation-based development of peptides purification alleviates some of the burdens of the experimental development, eventually decreasing the resources employed and making the process more cost-effective and sustainable.

In these studies, a model able to predict the peak profile in overloaded gradient conditions was developed via the Inverse Method, thus with very little consumption of feed material (in the magnitude of micrograms). The first step required the estimation of the adsorption isotherm under isocratic conditions. Thereafter, thermodynamics parameters were correlated to the variation of the organic modifier in the mobile phase, according to the linear solvent strength model. This further step is essential to properly predict the experimental outcome, since the standard approach to isolate peptides is via gradient elution. The development of complex preparative chromatographic methods through simulations, thus avoiding the lab-based trial-and-error approach, is of great interest for biomanufacturing companies and a rapidly rising research topic. With these manuscripts we contributed to this field with an actual therapeutic specie, matching the behavior of a real industrial SPPS crude via simulation. A further step in this direction is the modeling of purity regions in the chromatogram, which allows a better fine tuning of the product collection and thus of the process parameters. This was not considered in these applications and would require a better understanding and modeling of the adsorption features of the other substances characterizing the crude (i.e., the impurities).

4.2 Doped Reversed Phase materials for implementing single column chromatography outcomes (Paper VII, VIII)

Single column chromatography operated in overloading conditions is the technique commonly used to address complex separations in downstream and polishing processes. Batch chromatography, despite being among the most effective and reliable tools in the purification of biomolecules, still suffers some limitation and tradeoffs. A detailed overview of the opportunities and challenges associated with the use of batch chromatography, is part of this thesis and presented in **Paper VII**. Within the scope of the thesis, an innovative stationary phase, named Doped Reversed Phase, has been tested and applied to the difficult polishing process of a large peptide, Liraglutide. The results are presented in **Paper VIII**.

4.2.1 Development of a Liraglutide polishing strategy via Doped Reverse Phase material (Paper VIII)

Liraglutide is a 31 amino acids polypeptide, analog of the physiologically secreted hormone GLP-1, used in the therapy of type-2 diabetes and obesity. Fresenius Kabi iPsum (Villadose, Italy), a biopharmaceutical company with which our group actively collaborates, provided the feed material used in this study which was synthetized via solid-phase synthesis. During the upstream step, aiming to address pharmacokinetic hindrances and to improve pharmacodynamic properties, the original structure is derivatized with a fatty acid side chain and a glutamyl spacer. Such modifications have a detrimental effect on the polishing steps, which are normally carried out by means of reversed phase chromatography (RPC) for peptides, because of their strong hydrophobicity. Indeed, the introduction of an aliphatic fat chain drastically increases the hydrophobic interaction strength of the crude on the hydrophobic chains of the resin. Consequently, irreversible adsorption phenomena and a reduction in chromatographic selectivity between product and impurities were observed operating in RPC conditions. Furthermore, to achieve satisfactory purity values of the final product the original polishing protocol required two orthogonal methods, i.e., two separation techniques determining compounds discretization via different type of interaction. To

address both the problems at once, we developed a purification protocol using Doped Reversed Phase material as stationary resin. DRPs are a peculiar class of *mixed-mode* resins. Traditional mixed mode materials bear two functional groups with different adsorption chemistry on the same ligand in a rough ratio of 1:1. The resins employed in this study, in detail described in section "1.3 Intensification strategies" are reversed-phase resin doped with a small, determined, and constant amounts of ion-exchanger, generally comprised between 5-15%. Referred to experimental conditions and to the charge carried by the analytes, the ion-exchangers can work either in attractive or repulsive ways. Since in our contest the hydrophobic interaction between the analytes and the RPC resin was already strong, ion exchangers inducing the analyte to be repulsed, thus, inducing a final decrease in total retention were selected. Liraglutide possesses an isoelectric point (pI) of 4.9 and its solubility is pH-dependent: it is freely soluble at pH above 8.5 but precipitates in more acidic conditions. Thus, the operating conditions were accordingly adjusted, and the crude was dissolved in the preparative mobile phase A (MP-A): triethylamine phosphate buffer 25mM, pH 8.5 (corrected with orthophosphoric acid 85%).

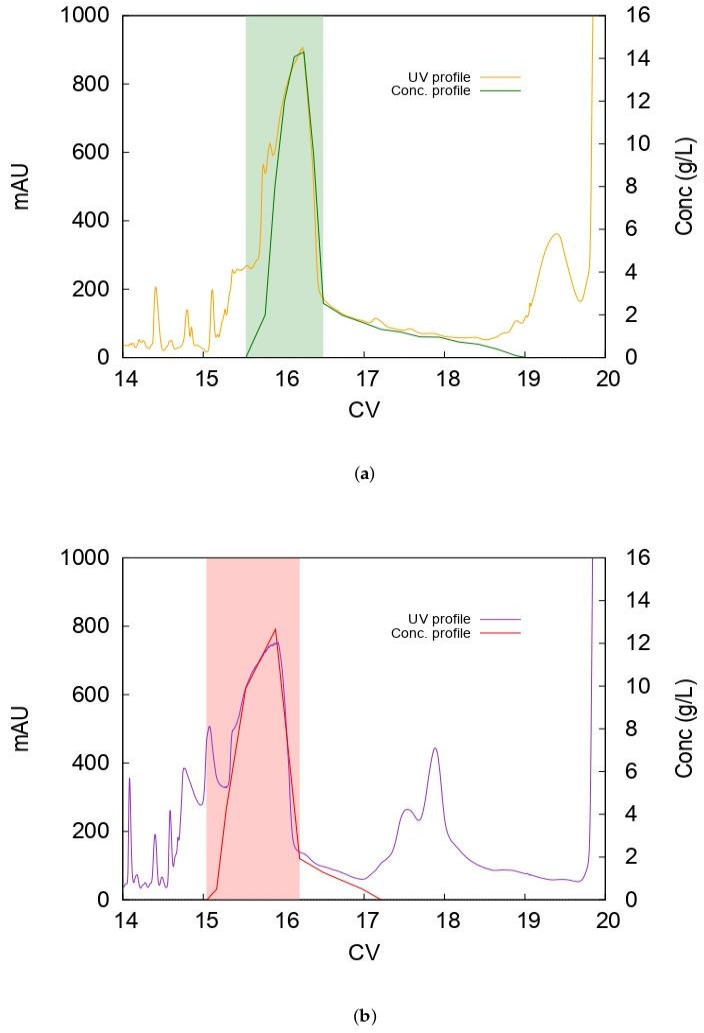

Figure 12 *UV profile and concentration profile of Liraglutide with (a) the C8 column and (b) the DRP column. The colored regions represent the pooled windows. Reproduced from [100]*

Eventually, the feed was negatively charged and, hence, the ion exchanger material selected were sulfonate groups (CIEX ligands), in a 10% ratio, aiming to obtain repulsion between the elements. The experimental work aimed to compare a similar chromatographic protocol

operated with the standard RP stationary phase and the DRP one. The evaluation was based on fraction collection of the eluate (1 fraction every 1mL), each of which underwent offline analytical characterization to determine purity, peptide concentration, and recovery by means of the formula listed in "1.2 Process parameters".
In Figure 12, the overlays of the UV lines and their relative product concentration obtained in both the scenario using the RP (octyl carbon chain – C8) resin and the DRP (90% C8 – 10% sulfonate groups) are illustrated. From the observation of the profile concentration curve, it is clearly visible that in *(a)* the peptide concentration profile is very broad and tailed, probably due to strong adsorption of the lipophilic portion of Liraglutide to the hydrophobic chains of the resin. Conversely, the middle of the concentration curve is remarkably reduced in *(b),* and we ascribe this attribute to the decreased retention of the crude materials, partially repulsed by the charged groups constituents part of the doped resin. In particular, by a first rough contrast, it can be observed how the whole peak, with the doped column, elutes in about 2 CVs, whereas, with the C8 column ca. 3.5 CVs are required to completely elute the product peak. Furthermore, beside being narrower, the product peak elutes earlier if processed with the mixed-mode column, confirming the general impact of the repulsing ionic charges on retention. For both the separations, a Pareto curve, diagram relating purity and recovery, was plotted (Figure 13). Generally, such relation varies inversely: the purest fraction collected contains a marginal amount of peptides, whereas when the collecting window is broadened, higher recoveries are obtained at the cost of purity losses.

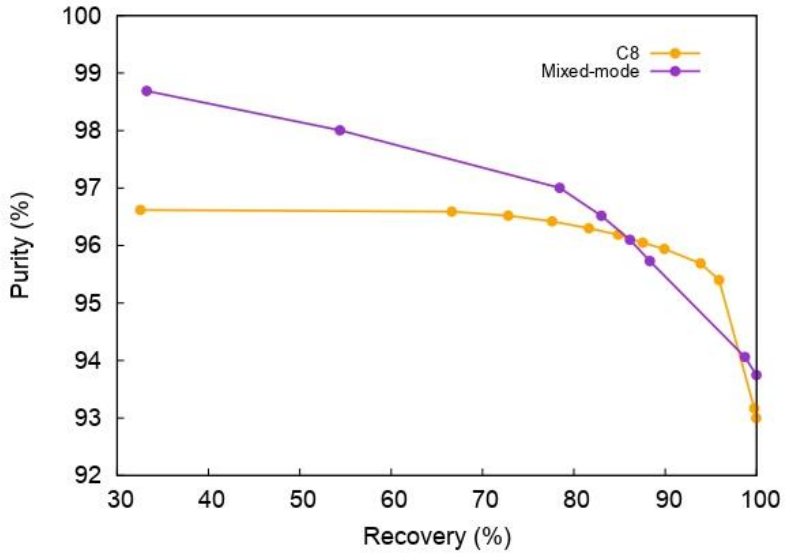

Figure 13 *Pareto plots of the two experiments using RP and DRP columns*

A distinct increase in purity values is achieved by separating the crude with the mixed-mode column. At the same time, it is also noted that such an advantage is lost at recovery values above ca. 85%, probably due to the collateral reduction in retention of strong adsorbing impurities, eventually co-eluting with the tail of the target product. It is worth noting, however, that the purification protocol was not adapted according to this event and that it simply resulted in a reproduction of the existing one. It can confidently be stated that with a more accurate fine-tuning of the elution gradient when applied to the doped column, an even more effective outcome would have possibly been achieved. Unfortunately, this activity was not part of the project goals. Nevertheless, positive outcomes are evident: in a scenario where a purity constraint of 97% was set, the operator using a classic reversed phase column would have needed to completely reprocess the first-dimension purified material, probably with a column characterized by a different separation mechanism. The doped material, on the other

hand, could deliver up to 81.2% of the peptide within specifications. Only the residual 18.8% would thereafter require reprocessing. This is even more evident for higher purity constraints. In addition, it must be noted that, due to the reduced portion of peptide tailing, the total volume of material collected would be fewer and at higher concentrations. This has a beneficial impact on the following downstream steps, such as ion-exchange chromatography or lyophilization.

4.2.2 Closing remarks

Nowadays, batch chromatography is the gold standard procedure employed to address complex mixture purification. Versatility, robustness, and reproducibility make of batch processes the easy choice when compounds such as biomolecules are to be purified. Despite this, single column technologies suffer of several hindering limitations. First among them, the tradeoff between purity and yield which causes large portion of eluate to not match purity specification and consequently require re-processing or disposal. All this at high costs. Pushed by companies needs to optimize downstream processing and regulatory agencies willingness to guarantee more uniformity between manufactured lots, new technologies are quickly and steadily emerging. However, plenty of room is still available in batch engineering optimization and mobile/stationary phases chemistry development. An experimental work reported in this thesis treated an innovative resin, which showed attributes able to partially alleviate the tradeoff and the limitation of single column RPC..

4.3 Twin-column continuous countercurrent technologies and their applications (Paper III, IV, V, IX)

Within the scope of this thesis, two twin-columns continuous countercurrent chromatographic processes were applied both for the purification of biopharmaceutical compounds and for the isolation and concentration of crude´s impurities. In the dedicated chapter ("Continuous chromatography for ternary separation"), the cutting-edge technologies Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) and N-Rich were illustrated. In **Paper IV** and **Paper V**, we deepened into the continuous chromatography scenario, and we provided an introduction to rationales, mechanisms, achievements and possible applications of the MCSGP process. From an experimental perspective, in **Paper III** and **Paper VI** we described the successful applications of the above-mentioned technology to two polypeptides (Glucagon and Icatibant), whereas in **Paper IX** the process development procedure using the N-Rich technique to automatically isolate and magnify several impurities of an oligonucleotide synthesis crude is presented.

4.3.1 The transition from batch chromatography to MCSGP for the polishing of two bioactive peptides

In Paper IV and Paper V, two examples illustrating the scale-up from single column batch chromatography to twin-column continuous chromatography for the polishing of polypeptides are presented. Initially, we evaluated the case of glucagon, a 29 amino acids therapeutic peptide, employed in the diabetes treatment. Crude solution of glucagon was synthesized via Solid Phase Synthesis by Fresenius Kabi iPsum (Villadose, Italy) and was described by a chromatographic purity of 55%. HPLC analysis of feed material and of all pooled fractions were conducted according to the USP method for glucagon.

Initially, multiple batch experiments varying gradient composition or slope were assessed using a 25cm column (Daisogel-SP-120-10-C8-Bio i.D. 4.6 mm) in order to define the most performing batch conditions. Such evaluation is based on the interpretation of a Pareto curve, which correlates the amount of peptide collected in a fraction (recovery) and its purity. The utilized mobile phases were MPA: ammonium acetate 20 mM in water/ACN (80/20 %v/v);

MPB: ammonium acetate 20 mM in water/ACN (60/40 %v/v). The use of mixed mobile phases is crucial to guarantee smooth and proper functioning of the chromatographic system pumps and reduces the impact of minor inconsistencies during buffers preparation. The selected conditions for the 25cm column purification were subsequently applied to a 15cm-lenght column of the same resin, used to establish the *design batch chromatogram*, starting point of any MCSGP process design. The shift to 15 cm columns has two rationales: in principle, to obtain a fair comparison when productivity values of the process are calculated for both single column and continuous chromatography, the overall column volumes of resin employed during the two processes should be comparable. Ideally, the length of the columns used in MCSGP should be the half of the one used in batch chromatography. Secondly, the two columns carrying out the twin-column continuous experiment, ought to have constant geometrical dimensions. In our experimental set-up a tradeoff was necessary due to the unavailability of two packed hardware of 12.5 cm. In Figure 14, the outcome of the design batch chromatogram together with the purity values and the target product concentration profile, both obtained through fraction analysis is reported.
This information allowed a targeted choice of both the recycling and the product collection windows. This latter (in red) determines the portion of eluate completely within purity specifications and not requiring further processing.

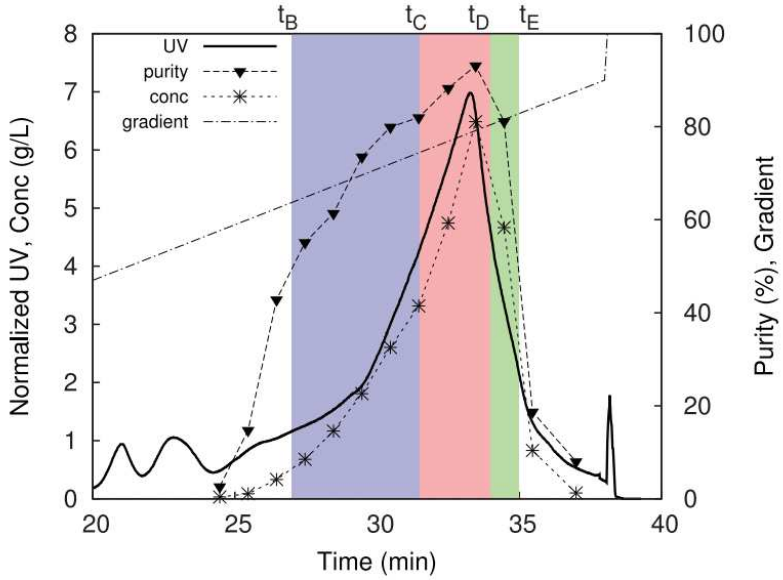

Figure 14 *Design batch chromatogram, offline analytics obtained target concentration and purity values are essential prerequisite to establish pure product P collection windows, and the recycling zones during an MCSGP experiment. [85]*

On the other hand, the portion undergoing recycling are characterized by material only partially purified, that on a classic batch chromatography procedure would be discarded or separately stored for discontinuous reprocessing. With the aid of the software (ChromIQ) operating the preparative system CUBE, specific valve-switching times were designated. As graphically outlined, a blue zone ($t_b - t_c$) containing target product and weakly adsorbing impurities (W/P) and a green zone ($t_d - t_e$) characterized by product and strongly adsorbing impurities (P/S) were noted. During each switch, these zones are transferred to the downstream column following appropriate inline dilution with MPA to diminish the organic solvent content and thus secure retention. By doing this, the 43% of a standard batch loading (32% W/P and 11% P/S) is transferred from the first to the second column and accounted for when determining the following loading step. Indeed, the target loading (g/L) must be

kept constant among switches to achieve a cyclic stationary state and eventually obtain pools of the homogenous quality and features. In Figure 15, we showed the attainment of a constant overlay of the UV signals from cycle 2 on, synonym of a reached stationary state.

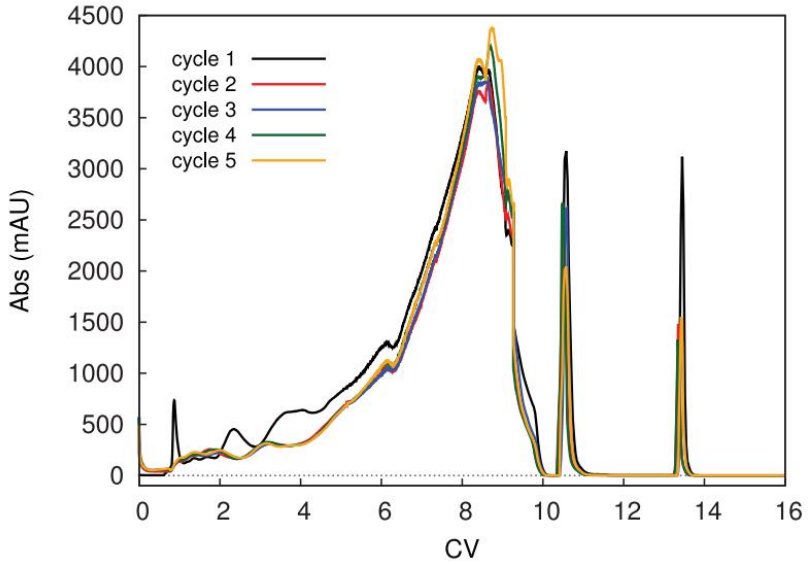

Figure 15 *Overlay of the UV signals measured by UV1 during 5 different cycles. [85]*

Once the preparative experiments and the analytical evaluations were completed, it was possible to compare the different processing strategies. As reported in Table 2, the pools purities were analogues by operating with batch or continuous chromatography. In addition, the analytical chromatograms of the target product (P) eluate obtained in each different cycle showed matching UV profiles and reproducible selectivity between peaks. The great advantage of operating continuously was the possibility to increase the product yield of 16.5%, from 71.2% (batch) to 87.7% (MCSGP). Such increase allows significant amounts of target material to be recovered and delivered at high purity values in a continuous manner, avoiding expensive losses of pharmaceutical compound and non-efficient batchwise reprocessing of fractions partially containing the target peptide.

	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

Table 2 *Comparison of the two processes performance.*

The undesired reduction in productivity is mainly caused by two factors: firstly, the overall amount of resin employed in the continuous process is inaccurately 5 cm higher of that of the batch run. Another relevant aspect is the considerable required time for the execution of the inline dilution streams to adjust the organic ratio of the recycled material. In this regard, the reader should be aware that with the recently implemented operating software new capabilities allow the inline dilution flow rate fine-tuning. These rates can now be increased up to the reaching of the pressure constraints, speeding up the overall transferring and inline dilution activity.

Eventually, for the two batch experiments Pareto curves were plotted and put in comparison with the MCSGP operating point (Fig. 16). As expected, the batch run carried out on the

25cm column allowed for higher outcomes in term of purity across the whole range of yield. In fact, a longer bed height is capable of boosting separation capabilities and material loadings when other factors such pressure constraints do not hinder the scale-up. The blue dot in the graphs represents the continuous chromatography result obtained with the developed method. Given a constant value of purity, the relative reachable yield is increased as previously described.

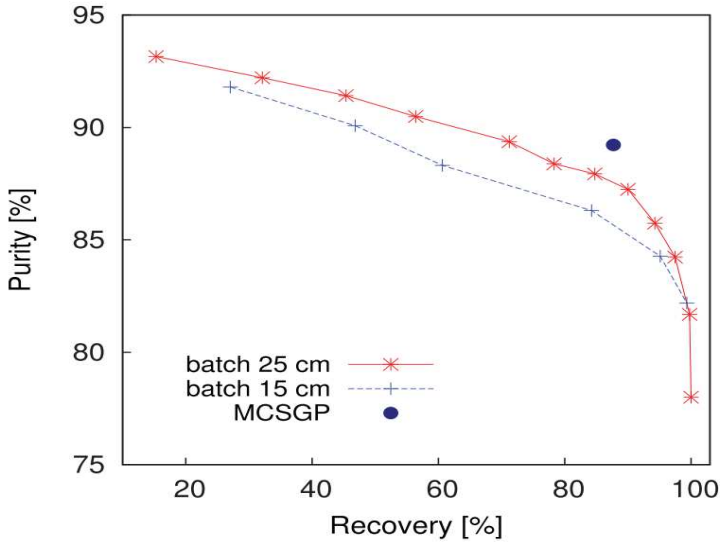

Figure 16 *Pareto curves of the two batch runs. MCSGP is only represented via the single blue dot, representing the experimental operational point.*

In Paper VI, the outcomes, and capabilities of the MCSGP technology as polishing tool were tested and evaluated on a second peptide, Icatibant. Such molecule, a ten amino acids peptidomimetic antagonist of bradykinin B2-receptor finds its primary application in the symptomatic treatment of acute attacks of hereditary angioedema. The solid phase synthetized crude material was again obtained from Fresenius Kabi iPsum (Villadose, Italy). The analytical method delivered a chromatographic purity of the crude of ~88%. To be such a high value of feed purity a single-step purification procedure was shown sufficient to reach the purity specification of 99%. Reverse Phase was selected as operational condition, with Daisogel-SP-120-10-ODS-BIO being selected as stationary phase. The column size for the batch run was 250 mm × 4.6 mm, whereas the dimensions of the two columns used in the MCSGP process were 150 mm × 4.6 mm. Thus, the Column Volume dimension of the batch run was of 4.2 mL against the overall (considering both columns) dimension for MCSGP was 5 mL. The final increase in column bed height for the continuous set up, is counterbalanced by the decrease of process productivity, considering Equation 1.3. Mixed mobile phases were A) triethylamine phosphate buffer TEAP 20 mM/ACN 90/10 and B) TEAP 20 mM/ACN 50/50. Mobile phase A was also employed as inline dilution agent, during interconnected phases of MCSGP. The column load was kept constant across the two experiments and kept fixed at 10 g/L_{resin}. After column equilibration for 2 CVs with 12% MP-B, feeding is applied followed by a gradient elution in 18 CVs from 12% to 37% MP-B. In conclusion, a regeneration and re-equilibration protocol was applied. The acquired batch chromatogram served both as benchmark and as *design batch chromatogram*. Similarly, as described for the example in paper IV, the offline analytics permitted to distinguish pure and partially pure zones of the preparative chromatogram. The regions were Icatibant and weak or strong impurities coeluted, were identified within the chromatogram and the relative triggering times were employed to establish the recycling windows and the

inline dilution rates for the MCSGP run. The separation and the transfer of material between the two columns, i.e., cycle, was operated continuously for five times. The superimposed UV signals recorded at the outlet of the first column during the first switch of each cycle are presented in Figure 17.

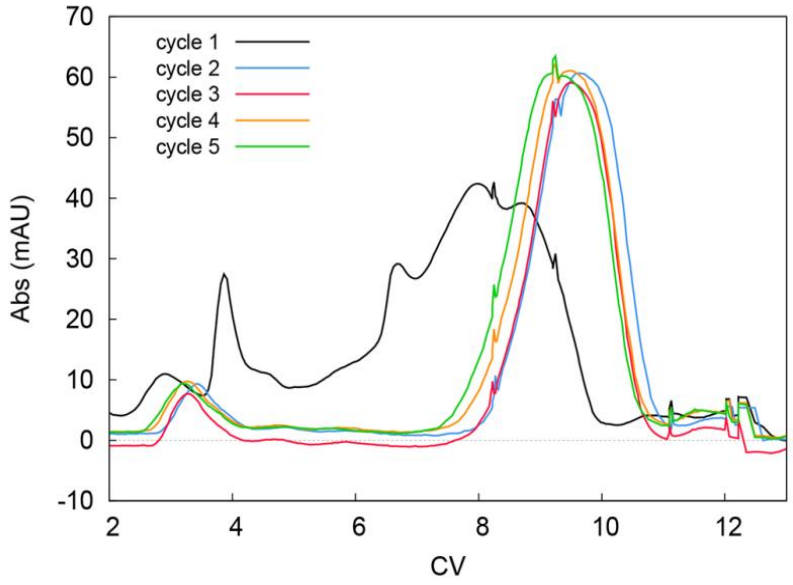

Figure 17 *UV signals superimposition of the five cycles operated.*

The first switch signal appears quite different from the following signals. The reason behind this is the sum of several factors, rather than a singular one. First, the ratio of components in the column is constantly changing during the cycles and reaches a nearly steady proportion just after some switches. In addition to that, the recycling of certain regions of components to the downstream column requires the additional flowrates of inline dilution streams which, based on the selected starting point of the gradient, may slightly alter the composition of the mobile phase across the gradient. Furthermore, more complex aspects such as displacement effects or organic solvent stagnation in pores may be responsible for this initial mismatch. However, the degree of establishment of the cyclic steady state is evaluated from the UV signals output starting from the second cycles. A closer match is indeed observable across the remaining cycles, and this was further confirmed by the chromatographic analysis of the collected pools. The eluate quality and concentration were constant and comparable, eventually confirming the maintained separation capacity of the resins across the multiple steps. The robustness and effectiveness of the multicolumn experiment was confirmed by the process comparison. In Table 3, the values reported in "1.2 Process Parameters", are reported and used to describe the two different strategies.

	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 3 *Performance parameters comparison of the two processes*

Both processes delivered pools meeting the purity requirements. However, the MCSGP run, yet not optimized, dramatically boosted the overall recovery of material and the productivity. This means, that if with a single column set-up, only the 12.4% of the injected material was recovered with a purity above 99%, the remarkable amount of 95.5% was on the opposite pooled via MCSGP. Such a difference is as well appreciable from the pareto curve showed in Figure 18.

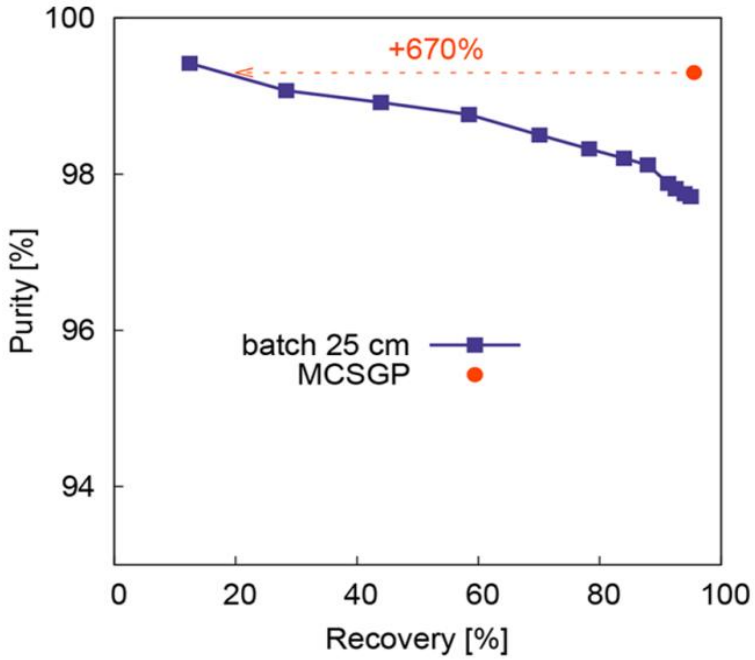

Figure 18 *Pareto curve of the batch run (25cm) and, in orange, the point representing the MCSGP performance at steady state.*

The reader should note that the reason why the productivity increase is slightly inferior compared with the mass recovery increase is because of the higher consumption of stationary resins in MCSGP (total column length comparison batch=25cm and MCSGP=30cm). Finally, the solvent consumption per gram of material produced at a certain purity is ca. five time less when operating the continuous run. With the aimed transition of the pharmaceutical industry to more sustainable and green processes, the reduction of mobile phase consumption is of great interest. Furthermore, saving of consumables during production has a beneficial impact on the manufacturing economics. In conclusion, a truly important aspect not accounted for previously in Table 3 are the advantages gained with the automatization of a process, among which reduction of human intervention, chances of errors and dead times, and improving consistency of product quality over manufacturing batches.

4.3.2 Automatic isolation and enrichment of oligonucleotide impurities by means of the N-Rich technique

In Paper IX the capabilities of continuous chromatography were furtherly assessed, but this time our attention shifted from target components to impurities and byproducts. Since a mandatory aspect of the pre-clinical drug development of biopharmaceutical drugs is the isolation and characterization of impurities, there is a need for effective and efficient methods to obtain sufficient amounts (in the range of milligrams) at high purities to conclude the required structural, biochemical, and toxicological elucidations. The current impurities seclusion scenario is characterized by tedious, inefficient, and time-consuming approaches. In fact, both batch chromatography and analytical-scale separation suffer from strong limitations: the high quantity recovery of impurities with batch chromatography (nonlinear

loading) comes at the cost of insufficient levels of purity of those. On the other hand, the high purities obtainable with analytical chromatography are counterbalanced by negligible mass recoveries per each run that would require months to obtain few micrograms. These tradeoffs may be alleviated by continuous chromatography, in specific by the twin-column countercurrent process N-Rich, thoroughly presented in section "3.2 N-Rich". In this paper we developed a batch strategy and a N-Rich protocol to identify the most productive strategy leading to the collection of high quantities at reasonable purities of a wide range of byproducts part of a 24-mer double-stranded RNA therapeutic oligonucleotide. Analytical chromatography, carried out on an Acquity UPLC Oligonucleotide BEH C18 (2.1 mm i.D. × 50 mm, 1.7 µm) with mobile phases buffered with HFIP and TFA, proved a feed purity of 75.6%. Thus, a quarter of the employed feed was composed of byproducts, equally distributed as weak and strong adsorbing species. The first step was the development of a batch protocol, which served both the purposes of representing the single column benchmark and the reference method for the continuous chromatography setup. Column, mobile phases, and protocol can be found in the paper attached at the end of this thesis.

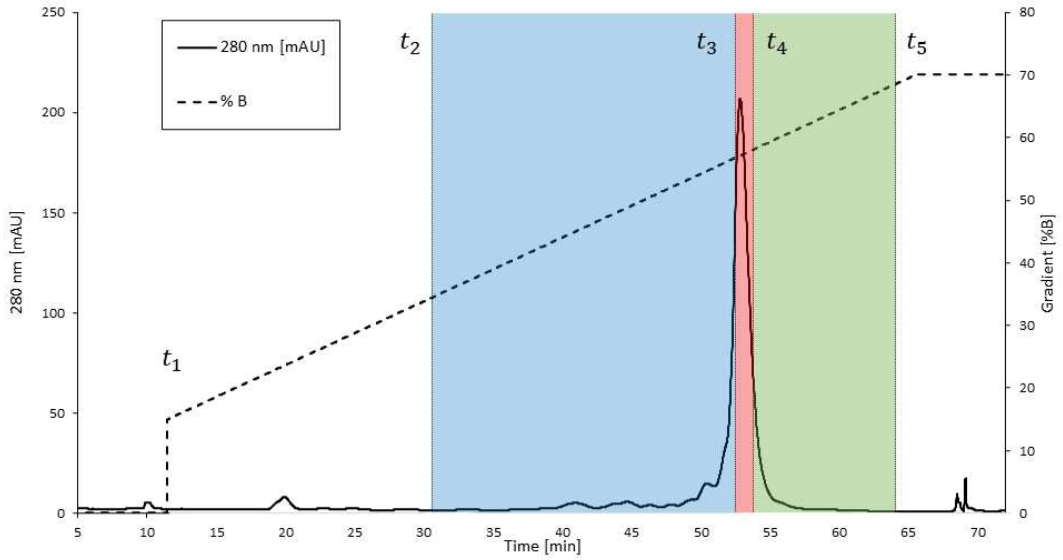

Figure 19 *Batch chromatogram. The colored regions are determined with the selection of section borders (t_1-t_5): in blue the weakly adsorbing impurities region, in red the region containing the pure product and in green the strongly adsorbing impurities area.*

In Figure 19, the batch chromatogram obtained is shown. The resin was loaded up to 0.25 g/L of target material, to maintain a certain degree of selectivity. Fractionation was performed during gradient elution and fractions were analyzed by offline UHPLC. By combining the batch chromatogram with the analytical results it was possible to identify 4 zones using five section borders ($t_1 - t_5$). Specifically, these section borders correspond to valves switching times ruling the N-Rich elution protocol. In particular, t_1 corresponds to the modifier gradient starting time and it is employed by the ChromIQ software to set dilution parameters, t_2 is the trigger point where the elution of the weakly adsorbing impurities begins that are internally recycled to the second column. The window defined by t_3 and t_4 corresponds to a region of nearly pure main compound, which underwent depletion at each switch. Eventually, in the t_4 to t_5 interval, strongly adsorbing impurities are eluted and inline diluted before entering the second column. Precisely, the switching times were selected as follows: t_1 = 14.8 min, t_2= 31.3 min, t_3 = 52.8 min, t_4 = 54.3 min and t_5 = 64.3 min. Once section borders for impurity recycling and product removal are configured, the

wizard automatically determines the load per switch, the in-line dilution factors for the recycling phases, and the gradient start and end concentrations of each section to be operated by the CUBE system pumps. Moreover, we made use of a threshold intensity value which triggered product removal, via a UV-based dynamic process control system. This feature is introduced so that any shift in product retention time due to column aging, buffer variabilities, and other possible inconsistencies over multiple cycles, would be compensated for and cause no impact on process performance. The success of this feature and of the overall recycling activity is graphically conveyed by Figure 20, with the superimpose of the progressive switches.

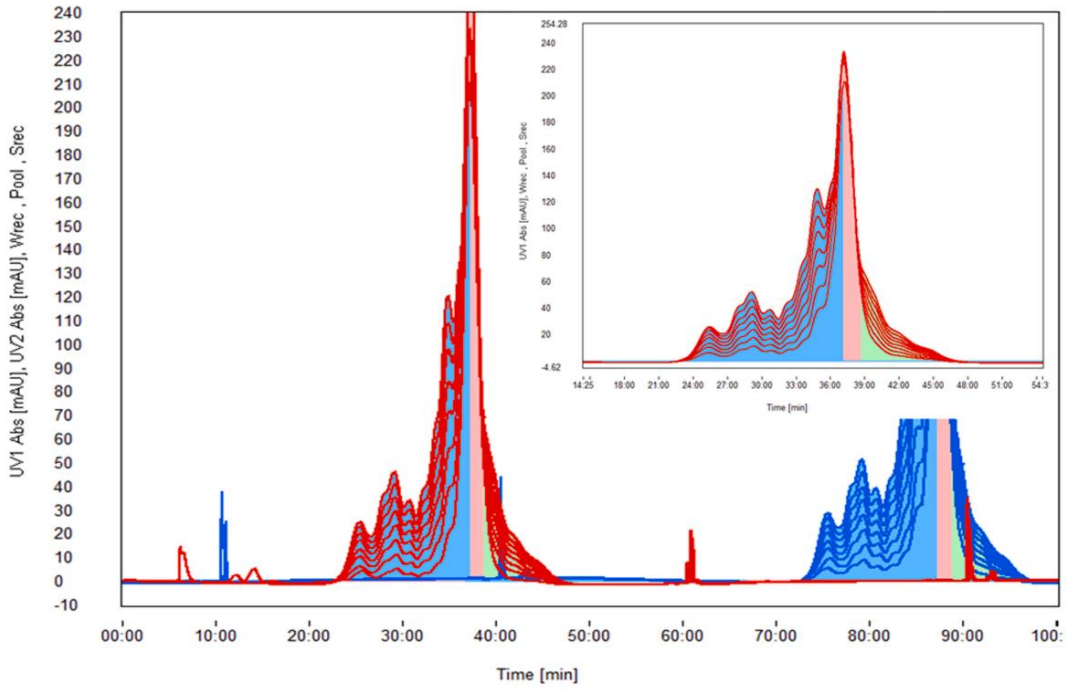

Figure 20 *Accumulation phase in N-Rich: A progressive and gradual increase of the concentration of the target compounds from cycle to cycle is demonstrated. Interfering substances are instead constantly depleted and reduced in the overall percentage.*

Indeed, a nearly perfect match in elution profiles and progressive growth of all impurities chromatographic profile in the recycling portions was achieved. Meanwhile, the main compound region, devoid of critical impurities, was successfully removed at each switch and consequently resulted at the same intensity values of a single column batch run. After successfully completing the "Accumulation" step, the "Depletion" method was applied. This method corresponds to a switch without loading new feed and thus leads to further depletion of any excess non-target compounds that would otherwise contaminate the desired target impurities. Consequently, a major increase of the target compounds purities and their relative enrichment was obtained.

Eventually, the accumulated compounds were subjected to a conclusive fractionation, in which the pumps operated at halved mobile phase flowrate (100cm/h) performed an elution gradient from 20% to 60% MP-B in 25 CVs. Such a shallow and targeted gradient aimed to boost selectivity and increase the degree of separation between enriched impurities. Fractions were collected and their analytical characterization allowed to identify those containing impurities of a sufficiently high quality and to establish a comparison between the batch and continuous experiment outcomes. An analytical evaluation of the N-Rich outcome is shown in Figure 21 where the analytical chromatogram of the feed material is

presented (A) followed by its overlay with the chromatograms obtained injecting equal amount of material evaluating several fractions obtained during the final elution phase containing the enriched impurities (B). In this latter graph, singular peaks of high intensity, each representing an isolated target impurity, can be identified both on the weakly and strongly adsorbing regions of the chromatogram. This graphical comparison clarifies the enrichment capabilities of the continuous chromatography approach. The throughput of linear isotherm range HPLC separation (Fig. 21-A), with minor substances barely reaching detection limits, is outperformed by the N-Rich technology which progressively accumulated targeted substances and allowed their final separation and collection.

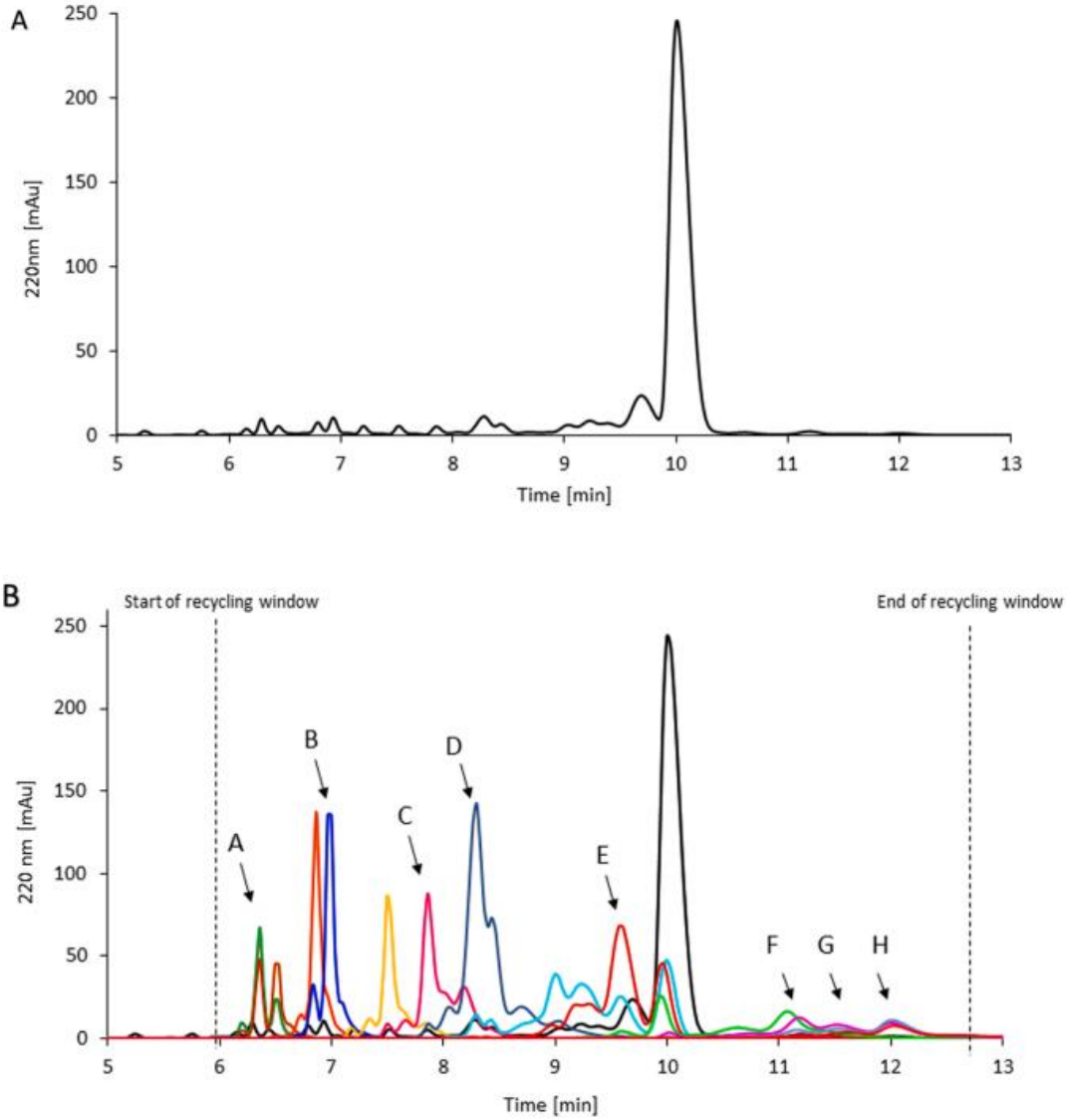

Figure 21 *A) Analytical chromatogram of the 24-mer oligonucleotide feed B) Overlay of the feed analytical chromatograms (black) and of the enriched impurities chromatograms.*

For the numerical comparison eight impurities isolated both batch-wise and towards continuous chromatography were chosen. Table 4 indicates their retention time and relative values obtained from purity and mass recovery assessments. For each listed compound, identified by its retention time, the highest purity values obtained via HPLC fraction analysis were selected as representative of batch or N-Rich isolation capabilities. Once selected the fractions containing the highest value of purity of each of the eight compounds, we then proceeded estimating their concentration, i.e., the mass of material dissolved in the pooled

eluate. Overall, N-Rich has proved advanced capabilities in alleviating the purity-productivity tradeoff of batch chromatography. Singular components were obtained through this technique with values of purity and productivities up to 15-fold and 20-fold times higher, respectively. Finally, we also proposed a productivity scenario for the collection of 1 mg of the previously named impurity D (r_t= 8.2 min) in which we estimated that N-Rich would necessitate 8 days of processing time, with batch chromatography and HPLC requiring in turn 10 to 87 days of processing time.

Compound	Retention time (min)	Batch		N-Rich	
		Purity (%)	Concentration (μg/mL)	Purity (%)	Concentration (μg/mL)
A	6.3	25.2	1.85	60.8	6.58
B	7.2	11.9	0.46	59.9	6.81
C	7.7	20.9	1.81	59.8	17.02
D	8.2	12.9	1.11	53.7	21.57
E	9.5	21.1	15.61	49.3	19.38
F	11.3	3.6	1.27	49.5	5.74
G	11.6	1.5	0.15	22.8	2.48
H	12.2	4.6	0.46	38.3	3.69

Table 4 *Purity and concentration performance of batch and N-Rich processes carried out on the CUBE system with the same stationary resin and mobile phase buffers.*

Additionally, the purity values obtainable for the same compound would correspond to the illustrated one in Tab 4., thus with analyte obtained via batch four time less pure than with continuous chromatography. In such scenario, we omitted purities for the HPLC-wise isolation since it was not possible to collect the compound in question (or any other) with a sufficient concentration for re-analysis.

4.3.3 Closing remarks

Given the challenges, which the biopharmaceutical industry is facing, biomanufacturing needs to be inevitably intensified. Especially, downstream processing needs to close the gap with the upstream scenario and become more effective and resource conscious.

Continuous countercurrent techniques are recognized as valid alternatives to batch purifications, but still suffer resistances for their complexity. The twin columns techniques covered in this thesis, allow to alleviate common single column chromatography tradeoffs while decreasing hardware complexities compared to other periodic countercurrent (PCC) systems. In our experimental activity, we proved MCSGP able to consistently deliver purified material within high quality specifications and to avoid material waste (yield reduction) thus boosting process productivity in the purification of two therapeutic peptides. Such technology exhibited full automatization, robustness and cost-effectiveness, pivotal points of the next 4.0 Biopharmaceutical Industry.

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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.

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

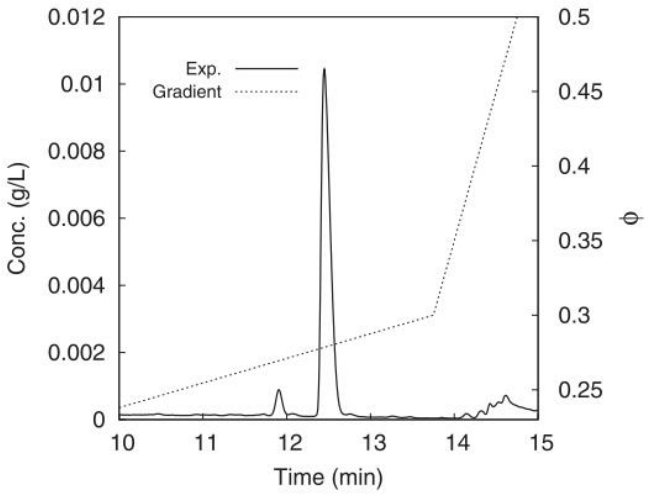

Fig. 1. Experimental gradient elution profile of the crude peptide. Injected concentration: 0.2 g/L; injected volume: 5 µL; wavelength: 280 nm.

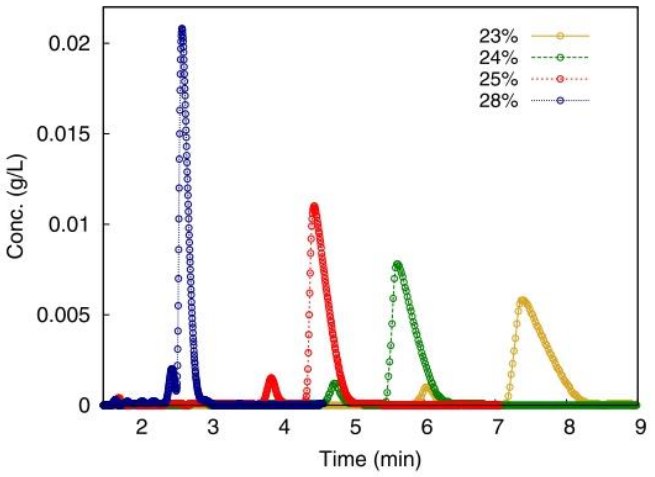

Fig. 2. Comparison between experimental profiles of the crude peptide measured at different volume fractions of organic modifier, ranging from $\phi = 0.23$ to $\phi = 0.28$. Injected concentration = 0.6 g/L; injected volume = 5 μL; wavelength: 280 nm.

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.

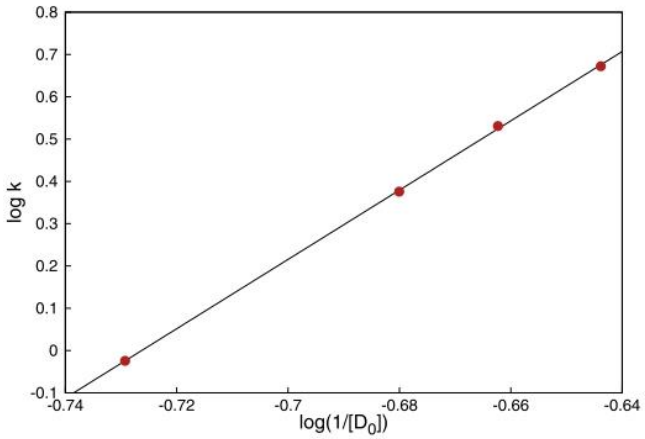

Fig. 3. Dependence of logarithm of retention factor (k) on logarithm of the inverse of ACN concentration (D_0) expressed in terms of molarity. The slope of the linear regression gives an indication of the number of displaced molecules (Z) equal to 8.2 $\pm$ 0.1. R^2 = 0.999.

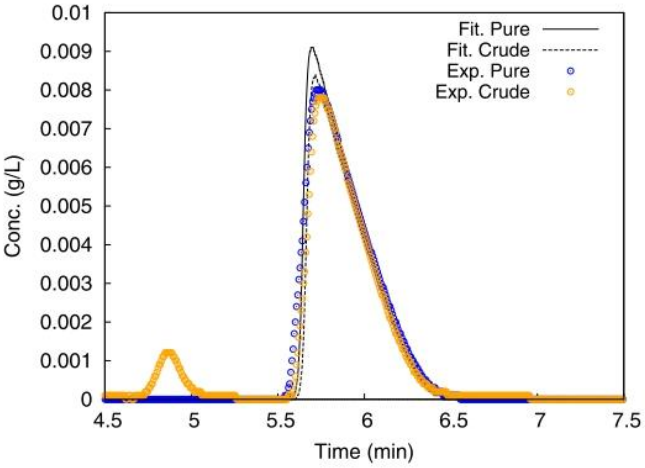

Fig. 4. Comparison between experimental peaks of crude (orange circles) and pure (blue circles) peptide and their corresponding calculated profiles. Dotted and solid lines corresponds to the fitting profiles of the crude and the pure peptide, respectively. $\phi = 0.24$, injected concentration = 0.6 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

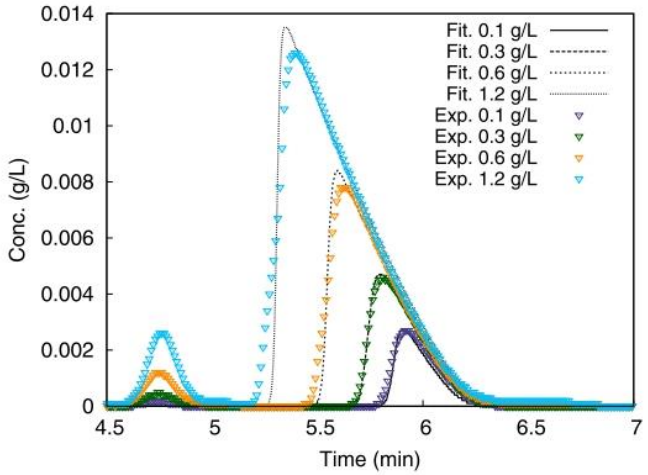

Fig. 5. Comparison between experimental and simulated peaks obtained with IM (Langmuir adsorption isotherm) for four different concentrations of the crude peptide in isocratic conditions (ϕ=0.24). Injected volume: 5 µL; wavelength: 280 nm.

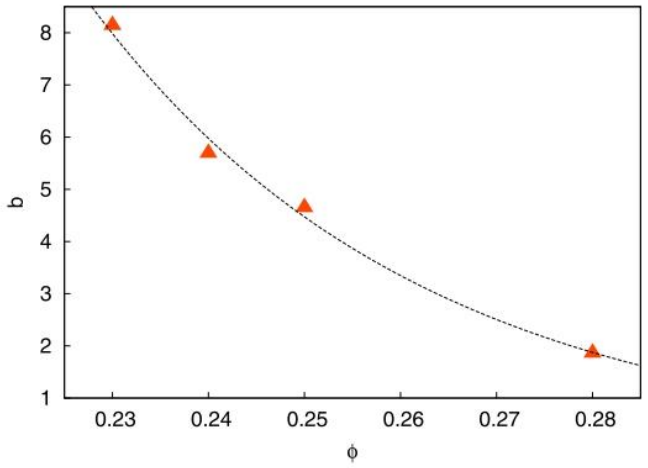

Fig. 6. Dependence of the equilibrium adsorption constant (b) on the fraction of organic modifier (ϕ) according to Eq. (7) (R^2=0.998).

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

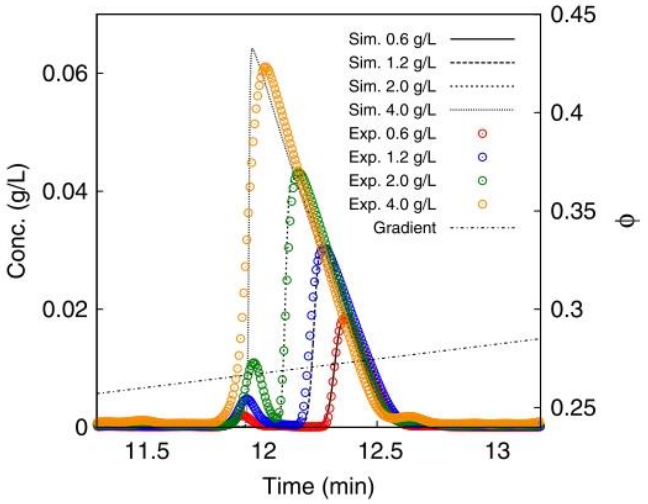

Fig. 7. Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption model) of four different concentrations of crude peptide. Injected volume: 5 µL; wavelength: 280 nm.

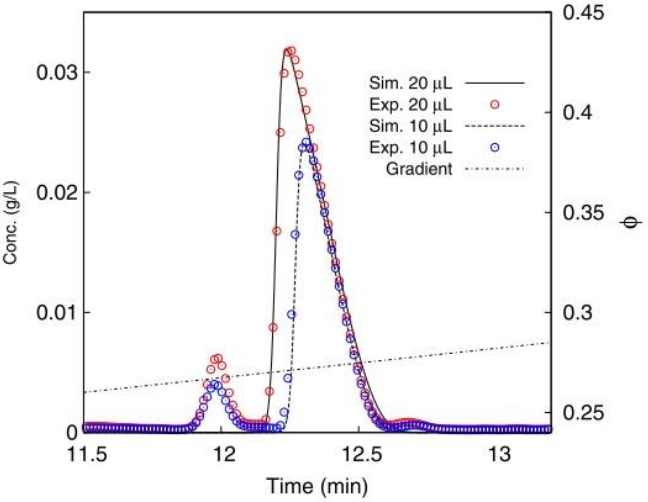

Fig. 8. Comparison between experimental and simulated peaks in gradient elution conditions. Injected concentrations: 0.5 g/L. Injected volume: 10 µL (blue) and 20 µL (red); wavelength: 280 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version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

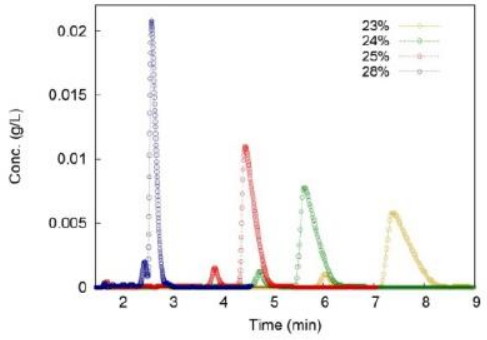

Figure 1: Variation of the retention with the fraction % (v/v) of organic modifier. Comparison between experimental peak profiles of a crude peptide sample at different ϕ, from 0.23 to 0.28. Sample conc=0.6 g/L; Vinj=5 µL. Reproduced with permissions from study by De Luca et al. [21].

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.

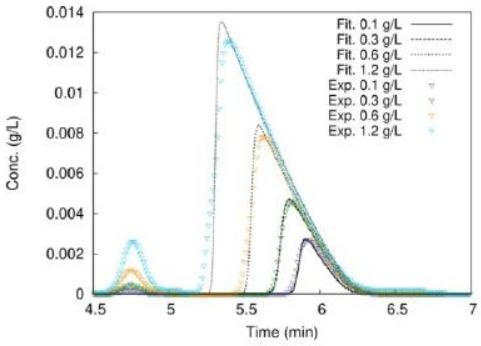

Figure 2: Agreement between calculated and experimental profiles at four different peptide concentrations in isocratic conditions. The black lines are the theoretical profiles obtained through Inverse Method using a Langmuir isotherm model, whereas the colored circles represent the experimental chromatograms. 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

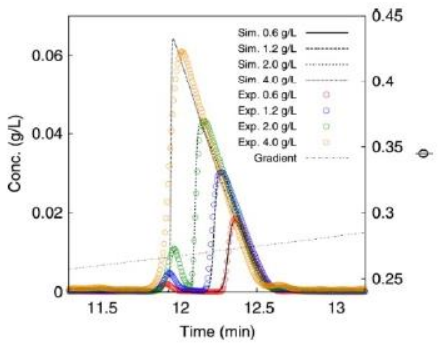

Figure 3: Comparison between experimental and simulated peaks in gradient conditions for four different crude peptide concentrations. The calculated peaks have been obtained using the model developed in this work and the Langmuir isotherm equation modified Vinj=5 µL reproduced with permissions from [21].

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.

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

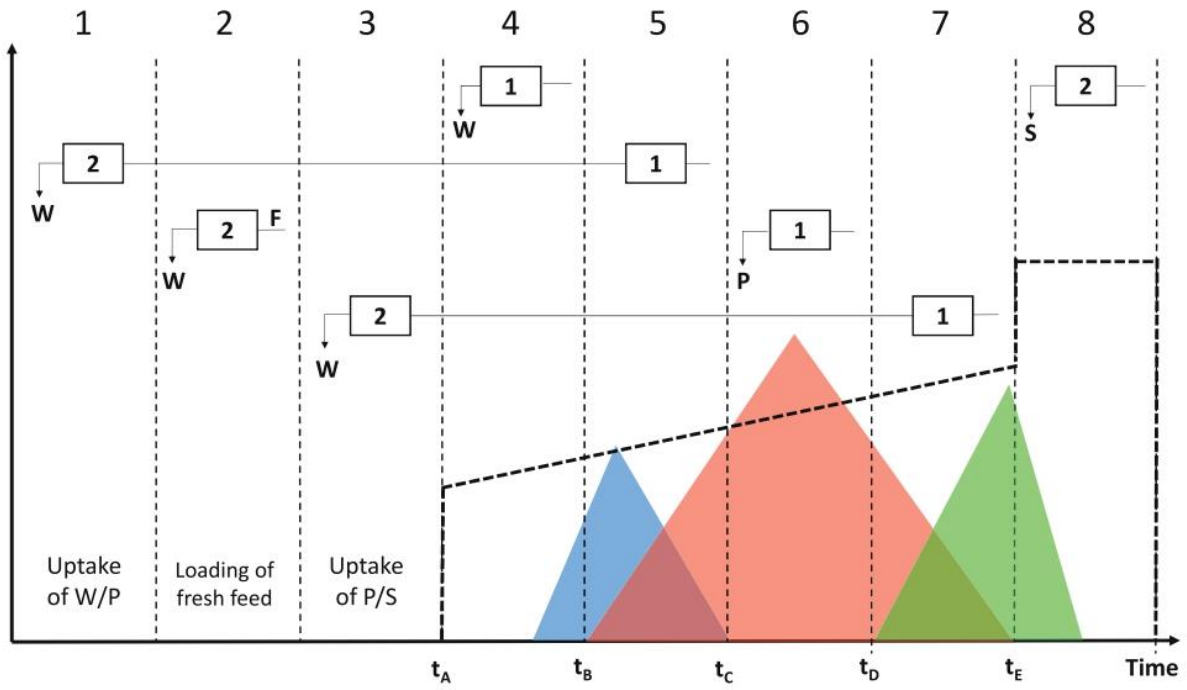

Fig. 1. Schematic illustration of a batch chromatogram and the corresponding tasks within a switch where column-1 is in the upstream position. The blue (green) peak corresponds to the weak (strong) impurities, while the red peak is the target product. Five intervals are identified where different fractions 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,

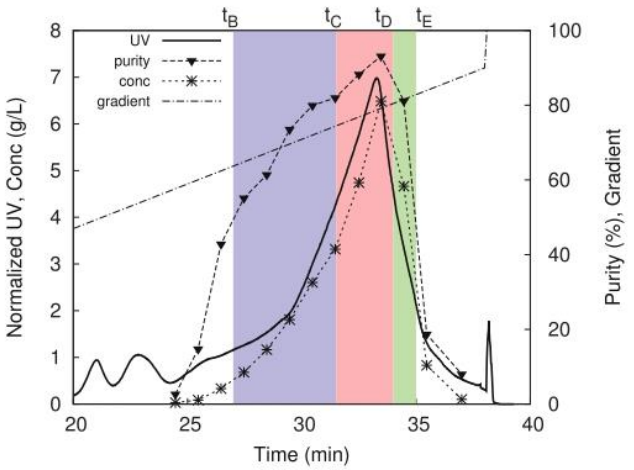

Fig. 2. Scheme of the design batch chromatogram together with the linear gradient of the modifier concentration, the purity profile and target product concentration profile. The values have been obtained by analyzing the fractions collected at every minute at the outlet of the 15 cm column. Moreover, 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

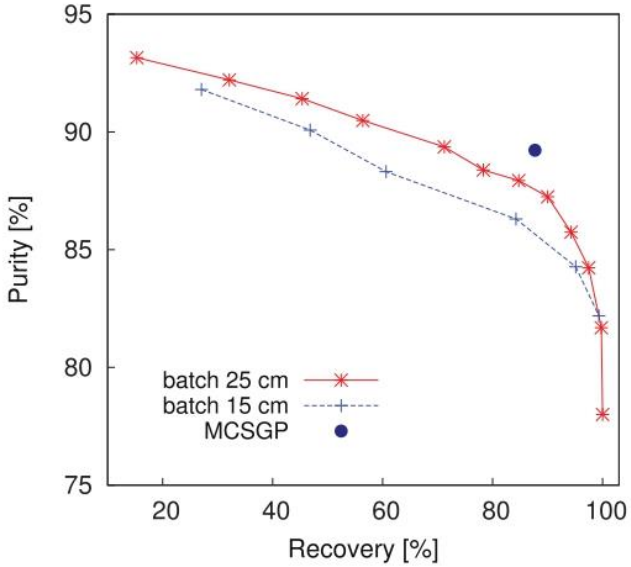

Fig. 3. Pareto curves corresponding to two batch runs using a 15cm column (blue crosses) and a 25cm column (red stars). The single point refers to the MCSGP operation. Note that the productivity values are different for the different operating conditions. (For interpretation of the references to colour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

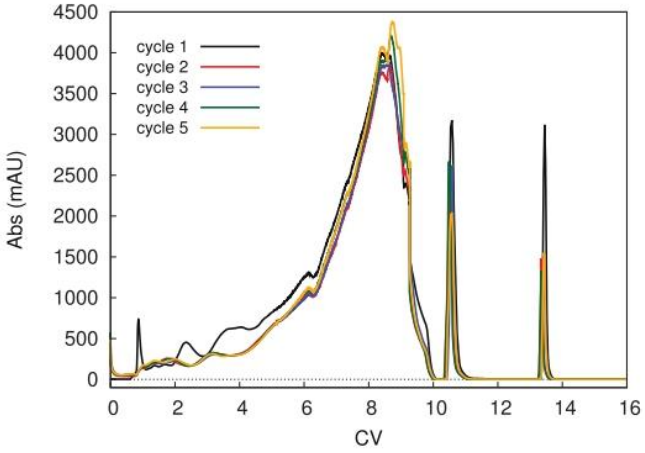

Fig. 4. Overlap of the UV signals measured at the outlet of one column of the MCSGP unit during 5 different cycles. The sharp peaks on the right correspond to the strip and re-equilibration of the column after the overlapping fraction P/S has been recycled. (For interpretation of the references to colour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

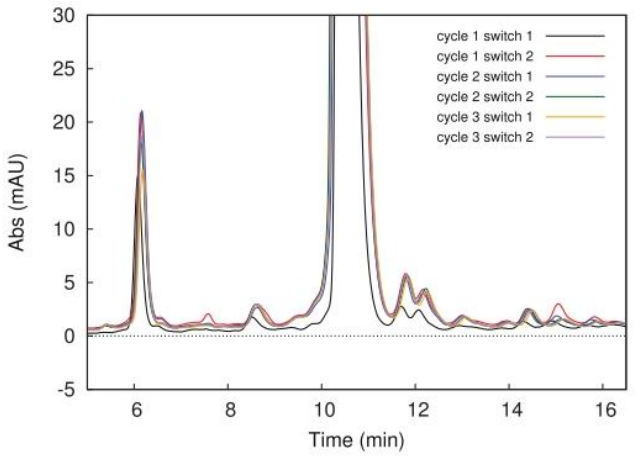

Fig. 5. Analytical HPLC chromatograms of the product fractions collected during the first six switches after the start-up of the MCSGP unit. It appears that, except for switch 1 in the first cycle, all the other chromatograms overlap, indicating that steady-state conditions have been achieved. (For interpretation 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.

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.

$$\text{Purity } \% = \frac{A_{\text{product}}}{A_{\text{total}}} \times 100 \tag{1}$$

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.

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

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}):

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

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].

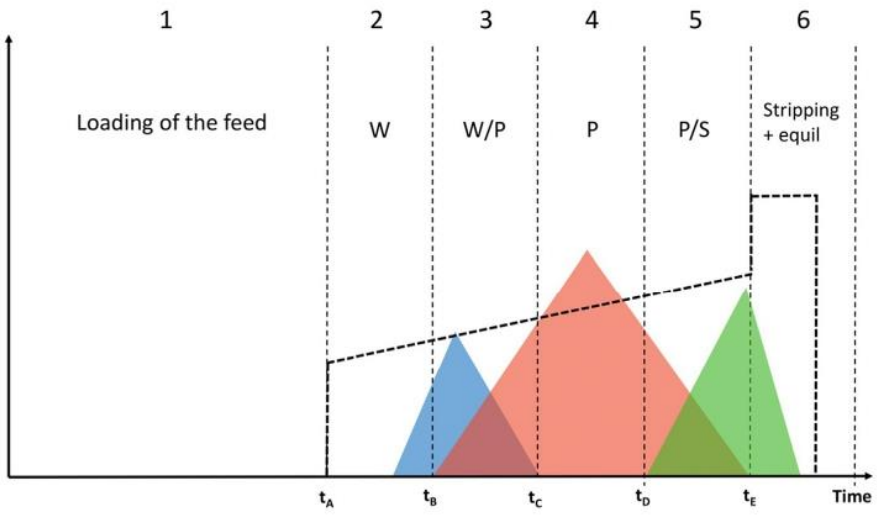

Fig. 1. Schematic representation of a batch chromatogram.

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

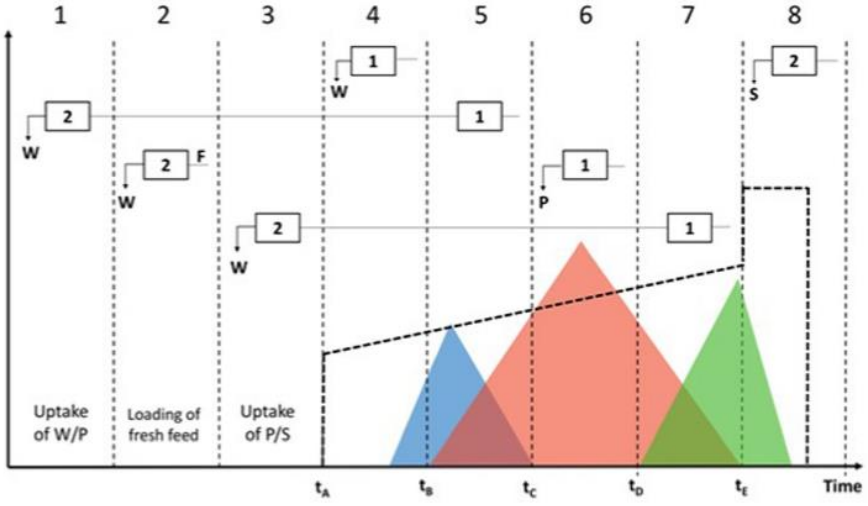

Fig. 2. Schematic representation of a single switch chromatogram in MCSGP. Reproduced with permissions from Ref. [44].

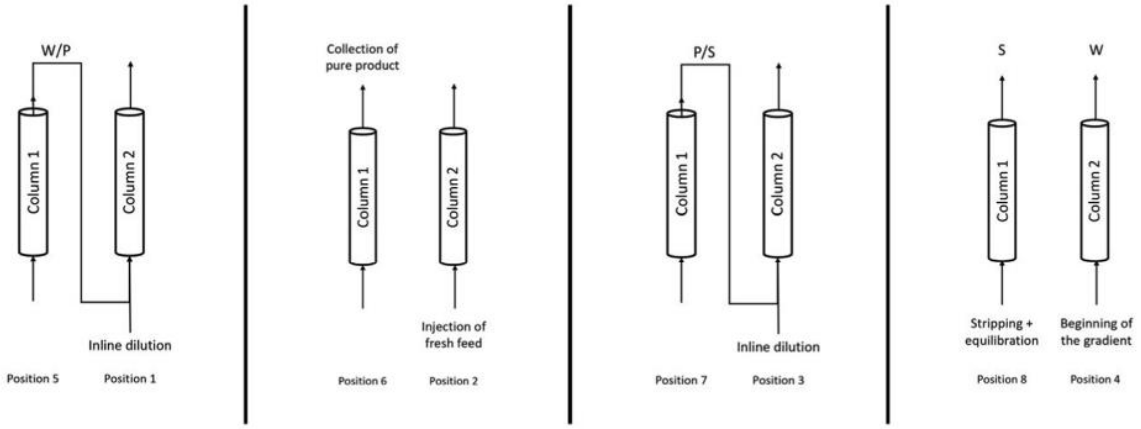

Fig. 3. Schematic representation of the path of the eluent stream during the first switch of an MCSGP cycle. The flow direction depends on the position of the inlet and outlet columns valves.

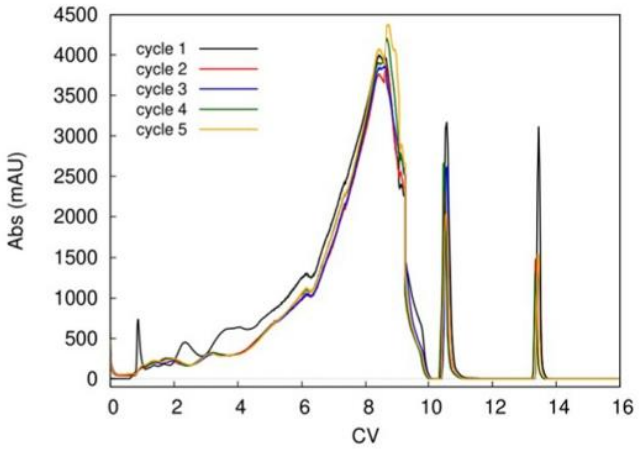

Fig. 4. Chromatograms (overlapped) of the first switch of five cycles of a MCSGP run for the purification of crude mixture of glucagon. Sharp peaks on the right correspond to the stripping and equilibration of the column. Reproduced with permission from Ref. [41]. CV: column volume (mL).

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

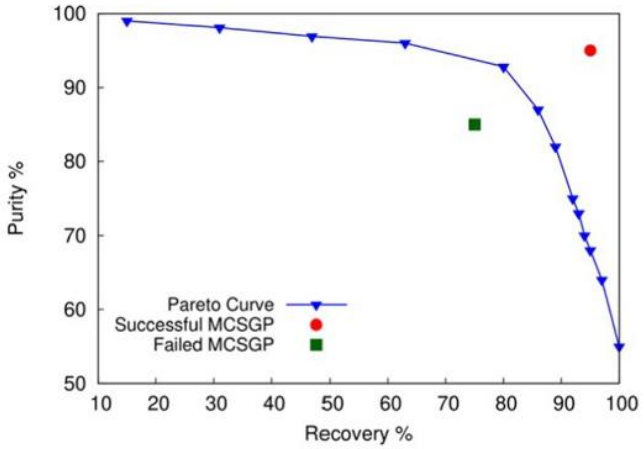

Fig. 5. Blue triangles: Pareto curve of a hypothetical design batch chromatogram. Red and green points: performance of two hypothetical MCSGP processes (red: successful; green: unsuccessful).

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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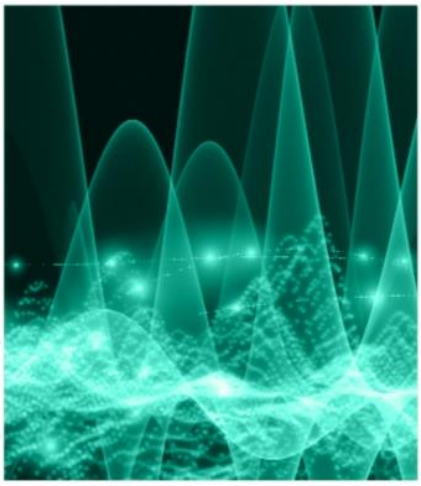
Paper V

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.

Chiara De Luca, Simona Felletti, Giulio Lievore, Alessandro Buratti, Mattia Sponchioni, Alberto Cavazzini, Martina Catani, Marco Macis, Antonio Ricci, and Walter Cabri

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
$\frac{A}{A+B+D} \times 100$	$\frac{A+B}{A+B+D} \times 100$

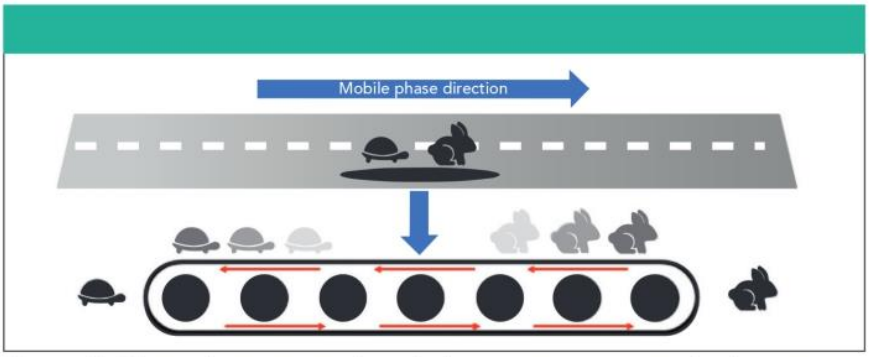

Figure 1: Schematic representation of the countercurrent mechanism; see text for details. Shadowed images of the turtle and the rabbit serve to simulate their movements. Modified with permission from reference (7).

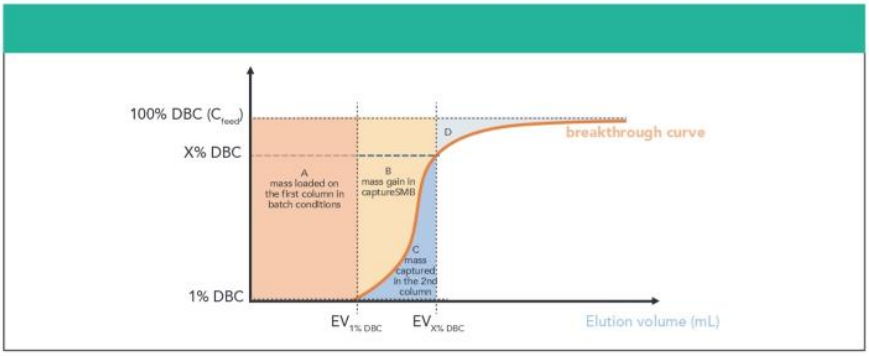

Figure 2: Schematic representation of a breakthrough curve. Area *A* represents the mass that can be loaded in the first column in batch conditions to reach 1% DBC. In twin-column captureSMB, the mass loaded on the first column is given by *A* + *B* while mass *C* is captured on the second column. The 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

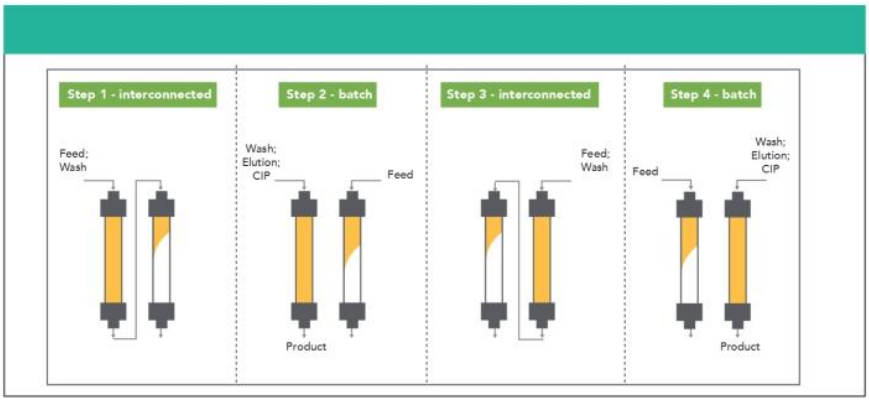

Figure 3: Schematic representation of twin-column captureSMB process. CIP stands for cleaning-in-place.

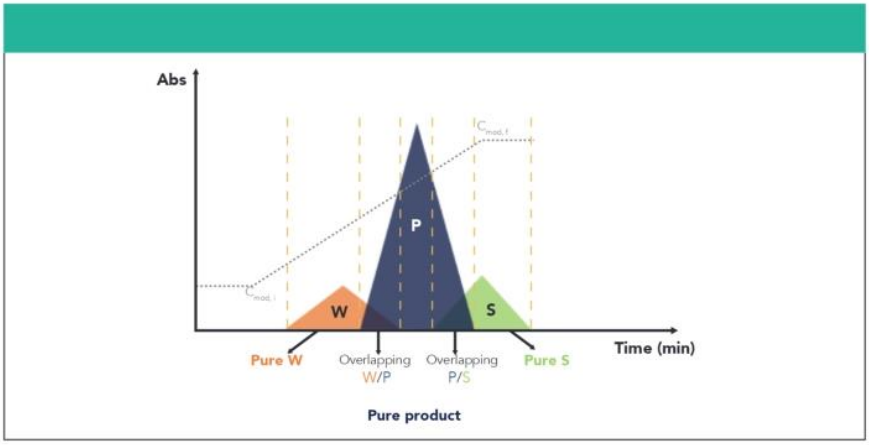

Figure 4: Schematic illustration of a ternary separation where the chromatographic peak of the target product (P) partially overlaps with those of two product related impurities. Here W refers to weakly adsorbing impurities and S to strongly adsorbed ones. Dotted grey line represents a hypothetical gradient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

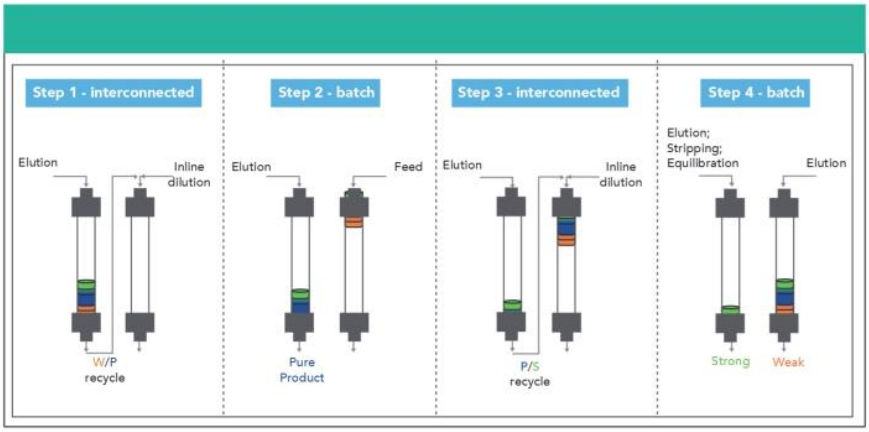

Figure 5: Schematic illustration of a twin-column MCSGP process.

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

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 VI

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Article

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

Chiara De Luca, Simona Felletti, Desiree Bozza, Giulio Lievore, Massimo Morbidelli, Mattia Sponchioni, Alberto Cavazzini,* Martina Catani,* Walter Cabri, Marco Macis, and Antonio Ricci

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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) often leading 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 tradeoff, 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, which is a peptidomimetic antagonist of bradykinin B2-receptor that 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.

1. INTRODUCTION

Protein-based drugs have become an important class of therapeutics since the 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.[3−6] They are constituted by a relatively short sequence of amino acids and therefore can be conveniently synthesized by solid-phase synthesis, which is 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, because of 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.[9−12] These impurities are usually referred to as "product-related impurities",[13,14] and they are very similar to the target product, differing, for example, with regard to just 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.[15−18] However, because of 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

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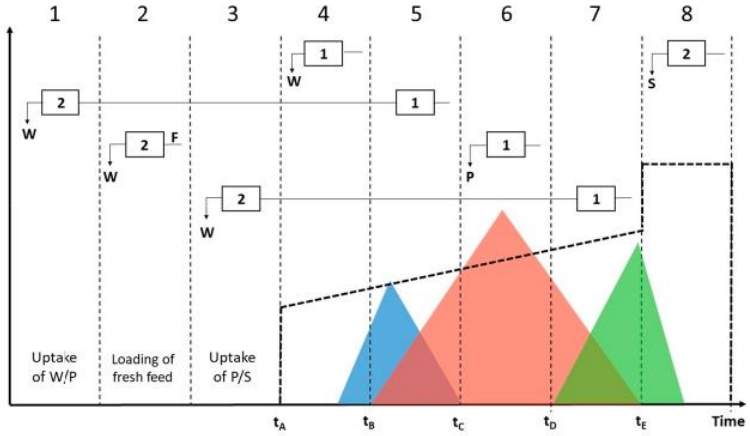

Figure 1. Schematic representation of a batch chromatogram and its different characteristic regions. The blue peak denotes weakly adsorbed impurities (W), the red one the target product peak (P) and the green one represents the strongly adsorbed impurities (S). [Reproduced with permission from ref 3. Copyright 2021, Elsevier.]

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 tradeoff", which dominates the performance of batch purification processes.[20,21] These constitute the core of the downstream portion of the protein manufacturing process, and generally provide the bottleneck of the commercial units in the entire biopharmaceutical industry.[22]

This tradeoff 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. In contrast, 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 time waste 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 ease of operation and reliability. In particular, it has been shown that MCSGP allows alleviating the purity−yield tradeoff typical of the batch processes, for example, providing much higher yields for the same purity values.[26−30]

The MCSGP process was developed ∼15 years ago.[31] In its more complex setup, 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, and oligonucleotides.[20,23,33−−43] The great advantage of MCSGP is that it 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.[2,42,43]

In this work, the MCSGP process has been applied to the purification of a crude mixture of icatibant, which is a small peptidomimetics of 10 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.[44−46] In addition, recent studies have revealed the potential of icatibant toward the improvement of oxygenation in patients affected by COVID-19 at an early stage.[47,48] The quantitative improvement of recovery, productivity, and solvent consumption is discussed, in comparison with the corresponding batch process.

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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. In contrast, 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 4−8, 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 the "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 the "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 that 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 that 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.[3,4,49]

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 binding conditions again. The overlapping portions of the peak, in fact, elute 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

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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:

$$\text{purity}\,(\%) = \frac{A_{\text{product}}}{A_{\text{total}}} \times 100$$

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:

$$\text{recovery}\,(\%) = \frac{m_{\text{prod collected}}}{m_{\text{prod injected}}} \times 100$$

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:

$$\text{productivity}\,(\text{mg/mL/h}) = \frac{m_{\text{prod collected}}}{V_{\text{col}} \times \text{time}}$$

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:

$$\text{solvent consumption}\,(\text{L/g}) = \frac{V_{\text{buffer}}}{m_{\text{prod}}}$$

The purity−yield tradeoff 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 high-performance liquid chromatography (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 %, accounting for, in addition to the impurities, salts, solvents and adsorbed water. 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 ∼6.8 with NH_4OH. After 1 h of agitation, the feed is filtered with 0.45 μm filters. The chromatographic purity of icatibant in the crude mixture was assessed to be ∼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 very high purity, a single-step purification procedure is sufficient to reach the purity specification of 99%. The separation was performed on a stationary phase that 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 mm × 4.6 mm (length (L) × inner diameter (ID)), whereas the dimensions of the two columns used in the MCSGP process were 150 mm × 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. The feed then is loaded to obtain a concentration of 10 $\text{mg/mL}_{\text{column}}$, 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),

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to improve the separation. In the batch run, fractions are collected during the gradient every 60 s, 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 must 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, CA, 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 mm × 4.6 mm Kromasil 5-100-C18, with a particle size of 5 μm and a pore size of 100 Å. 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 g/L 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 min along the gradient; next, it was increased to 84% in 3 min, and, after 4 min 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 ~88%) has been estimated through the percentage area of the target peak in the analytical HPLC chromatogram, reported in Figure 2A. Icatibant elutes at 10.9 min, while all the other minor peaks are impurities that must 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

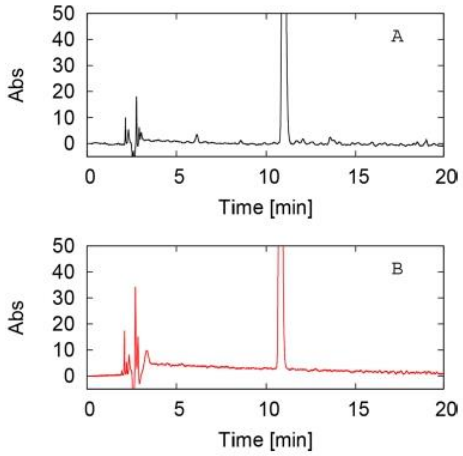

Figure 2. (A) Analytical HPLC chromatogram of the icatibant feed; (B) analytical HPLC chromatogram of a fraction with purity of >99%.

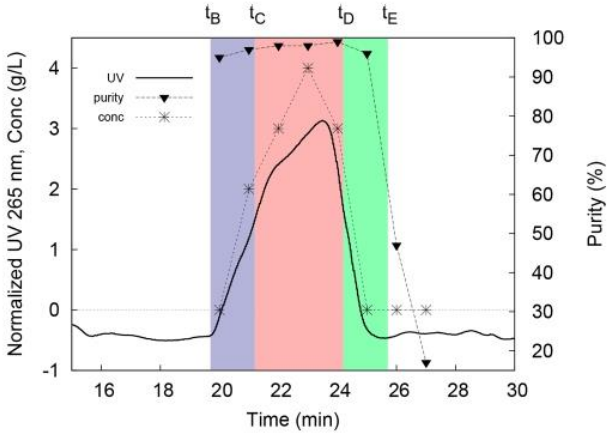

Figure 3. UV (at 265 nm), purity and concentration values in the column outlet stream, as a function of time for the design batch chromatogram.

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 >90%. The chromatogram under analytic conditions of a fraction with a purity of >99% is shown in Figure 2B.

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 tradeoff 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

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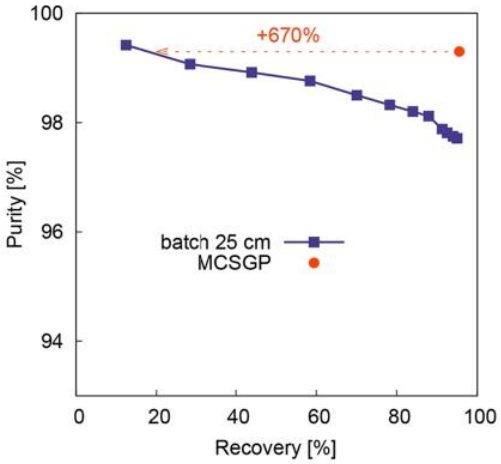

Figure 4. Pareto curve obtained for the batch run with the 25 cm column and the point representing the MCSGP performance at steady state.

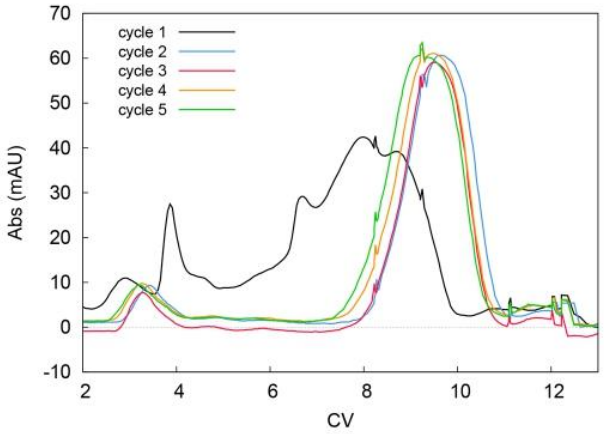

Figure 5. Ultraviolet (UV) spectrum in the stream leaving the upstream column during the first switch of five MCSGP cycles.

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 us imagine transferring this chromatogram from batch to continuous. The overlapping regions, which would be kept within the unit during the interconnected phase, contain an overall percentage of 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, in contrast, 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.

Table 1. Comparison of the Performance Parameters of the Batch and MCSGP Processes and Summary of Performance Improvements by Using MCSGP

	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%

In order to complete the comparison between batch and continuous operation, we must also consider 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 min. 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 min. Finally, also the solvent consumption plays a relevant role on the final production cost and must 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. In contrast, 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.

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Note 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, generally, that 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 tradeoff 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, because of 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 <5% with the MCSGP unit. This is also seen in terms of process intensification, where the productivity increase from 1.13 g/L/h to 7.6 g/L/h implies a reduction of ∼7 times in the unit volume or duration of operation for producing the same amount of purified target product.

Note that the performance parameter 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 under 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.

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Notes

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Paper VII

molecules

MDPI

Review

Downstream Processing of Therapeutic Peptides by Means of Preparative Liquid Chromatography

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Abstract: The market of biomolecules with therapeutic scopes, including peptides, is continuously expanding. The interest towards this class of pharmaceuticals is stimulated by the broad range of bioactivities that peptides can trigger in the human body. The main production methods to obtain peptides are enzymatic hydrolysis, microbial fermentation, recombinant approach and, especially, chemical synthesis. None of these methods, however, produce exclusively the target product. Other species represent impurities that, for safety and pharmaceutical quality reasons, must be removed. The remarkable production volumes of peptide mixtures have generated a strong interest towards the purification procedures, particularly due to their relevant impact on the manufacturing costs. The purification method of choice is mainly preparative liquid chromatography, because of its flexibility, which allows one to choose case-by-case the experimental conditions that most suitably fit that particular purification problem. Different modes of chromatography that can cover almost every separation case are reviewed in this article. Additionally, an outlook to a very recent continuous chromatographic process (namely Multicolumn Countercurrent Solvent Gradient Purification, MCSGP) and future perspectives regarding purification strategies will be considered at the end of this review.

Keywords: peptide; biomolecules; biopharmaceuticals; solid phase peptide synthesis; purification; preparative chromatography; continuous chromatography; MCSGP

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1. Introduction

Peptides are organic polymers composed of 2–50 amino acids linked to each other by means of covalent amide (=peptide) bonds. The composition, length and sequence of the amino acid chain have a dramatic influence on the activity of the peptide itself, for example in the human body. Peptides are called bioactive if they have a beneficial impact on body functions, on biological processes and, as a consequence, on health [1–3]. The literature about different kinds of bioactivity that peptides can exert on the human body is broad. They can interact with different organs and systems, such as digestive, cardiovascular, nervous and immune systems. This is the reason why they have been extensively studied for their potential applications in cosmetics, food and pharmaceutical fields. Some peptides derived from milk proteins, for instance, are considered promising alternatives to classical treatments for cancer therapy, targeting cancer cells specifically, without affecting healthy ones [1]. Other peptides, as well as some proteins, are considered to be "biomarkers", which means species that can give an indication about the effectiveness of a treatment [4]. Recently, some peptides have also been tested to treat some symptoms related to COVID-19 disease. For instance, icatibant (a peptidomimetic constituted of 10 amino acids, antagonist

of bradykinin B2 receptors and effective against symptoms of hereditary angioedema) has recently shown its potentiality to improve oxygenation in patients affected by COVID-19 at early stage [5,6]. Moreover, some peptidic vaccines have also been designed to provide immunity to SARS-CoV-2 [7].

This class of molecules represents an effective alternative to small molecule drugs. Peptides, especially if endogenous, exhibit remarkable advantages such as high bioactivity, specificity to the target tissues, broad range of therapeutic effects, no toxicity nor tendency to accumulate in the human body [8–13]. For instance, classical drugs with anticancer or anti-carcinogenic activity usually cannot recognize cancer cells from normal ones, and this causes an intrinsic toxicity of "traditional" therapies. Peptide-based treatments not showing this negative effect are being considered more and more appealing [1].

In 2014, about 60 peptide drugs had already been approved and launched on the market. At that time, the peptide market represented about 1.5% of all pharmaceuticals sales [14]. The increasing number of peptides entering clinical trials year by year indicates that there is a growing interest from the side of pharmaceutical companies in the use of this class of molecules as drugs [14–16]. To date, the number of therapeutic peptides approved is 70 [17].

Peptides can be classified as endogenous, if they are synthesized inside the human body, or exogenous if they are introduced into the organism from an external source. For instance, it is well-known that proteins acquired through food are an essential source of amino acids and it has been demonstrated that some specific portions of dietary proteins exert biological functionalities. Very important sources of bioactive peptides include dairy food, fish, eggs, soybean, rice, corn, peanuts etc. In most cases, the amino acid sequence of the bioactive peptide is contained within a parent protein, where it is inactive, and it can be released by enzymes during digestion [18–20]. In other cases, the peptide of interest can be synthesized. Recently, peptidic analogues possessing remarkable pharmaceutical properties have been developed and obtained by means of chemical synthesis [21].

The most suitable technology for the production of a given peptide strongly depends on the molecular size of the target molecule [22]. Even though often highly selective, all production methods lead to mixtures where the target peptide is present together with a series of other molecules (impurities). The target Active Pharmaceutical Ingredient (API) must be separated from all impurities produced during the manufacturing process, because strict regulations are imposed to all kinds of pharmaceuticals, for quality and safety reasons. Particularly, impurities can be divided into two groups [23,24]:

- Process-related impurities, deriving from the production method employed (salts, pieces of cells or of DNA, …)
- Product-related impurities, that are species chemically similar to the target product.

The first type of impurities can be easily separated from the peptide of interest, e.g., through affinity chromatography, during the so-called capture step [25]. In this chromatographic technique, the ligand specifically binds to the target molecule, whereas the impurities are flushed through the column. The peptide or protein is then eluted with a suitable buffer.

Elimination of product-related impurities (which is technically defined as the polishing step of the purification process) is much more challenging because of their similarity to the target molecule, that makes the use of affinity chromatography impracticable [24]. In the vast majority of cases, even this step is performed by means of liquid chromatography.

It is worth mentioning that the capture step is only performed for purification of peptides manufactured with recombinant methodologies, therefore capture will not be discussed any further; on the other hand, the different solutions proposed to perform the polishing of complex mixtures will be subject of this review. The paper will conclude with an overview on the future perspectives in the field of chromatography for the purification of peptides and other biomolecules, including the emerging continuous chromatographic processes.

2. Methods of Production (Upstream Processing)

Production of the peptide occurs during the upstream part of the manufacturing. There are several ways to obtain the target peptide. Some of them imply to extract it from the parent protein, where the peptide is contained but inactive. The release is performed through the action of enzymes or by microbial fermentation. Other processes involve to synthesize the peptide-chain starting from single amino acids, adding one amino acid at a time [18].

2.1. Enzimatic Hydrolysis and Microbial Fermentation

Food is a valuable source of amino acids and peptides. For example, proteins contained in food can release peptides with bioactive functions during their fermentation or when exposed to enzymes with hydrolytic activity. The outcome of the hydrolysis, namely the type of peptides produced starting from a single parent protein, depends on many experimental factors (type of enzyme or microorganism used, combination of vitro enzymatic hydrolysis with microbial fermentation, etc.). Therefore, the number of bioactive peptides obtainable from food proteins is essentially unlimited [3]. Dairy products, for instance, are an important source of proteins in the human diet and, from them, a number of bioactive peptides can be obtained. Peptides with antihypertensive, antibacterial and immunomodulatory activity have been released by casein and by whey proteins using pepsin, trypsin and chymotripsin as enzymes [26–29]. Additionally, the enzyme thermolysin has been employed to obtain hypotensive peptides from other kind of foods, such as corn and porcine skeletal muscle [30–33]. The traditional mode to perform hydrolysis of proteins is to operate in batch, which means through a discontinuous process inside a reactor. This method, anyway, has resulted to be less efficient than continuous methods employing enzymatic membrane reactors, where protein hydrolysis, product collection and catalyst recovery happen in the same unit [34,35].

Dairy products can release bioactive peptides also when subjected to the action of particular bacteria that trigger the fermentation of this kind of foods. For example, it has been demonstrated that *Lactobacillus helveticus*, *Enterococcus faecalis*, yoghurt and cheese bacteria and Lactic Acid bacteria can hydrolyze milk proteins to produce peptides with ACE-inhibitory activity (antihypertensive) [28,36–41]. Similarly, peptide with this kind of bioactivity were produced from chicken meat, using *Aspergillus* protease [42].

Another kind of bioactivity that was observed in protein hydrolysates deriving from beef meat is the antioxidant one, especially against lipid oxidation. The employment of bioactive peptides as antioxidant additives in food could be pivotal in the substitution of artificial antioxidants, whose potential health risks are already recognized [43,44].

2.2. Chemical Synthesis

Despite the success of other production methods, the technique of choice to produce small to medium peptides, especially for pharmaceutical applications, is still chemical synthesis [45]. The main reasons are two: firstly, a synthesis method is developed starting from standard and well-established procedures, so its development is less complex and less time-consuming. Secondly, in the synthetic approach, differently than recombinant approach (see Section 2.3), modified amino acids can also be incorporated in the peptide chain. These characteristics make chemical synthesis the preferred technique for peptide production.

Chemical synthesis can be performed either in liquid- or in solid-phase. Both strategies are based on similar reaction mechanisms, where amino acids and/or fragments of the desired peptide are added successively to the mixture, to react with the growing chain. In Solid Phase Peptide Synthesis (SPPS), firstly developed by Merrifield [46], one N-protected amino acid reacts with the peptide chain, which is anchored to a solid support (a resin) and, after that, the terminal amino acid is deprotected. Then, the following amino acid undergoes the same procedure. Functionalities on the aminoacid side chain need to be protected as well, in order to avoid side reactions. After the procedure is terminated and

all the amino acids have reacted, the peptide is released from the resin during the cleavage step. A scheme representing the Solid Phase Peptide Synthesis is depicted in Figure 1.

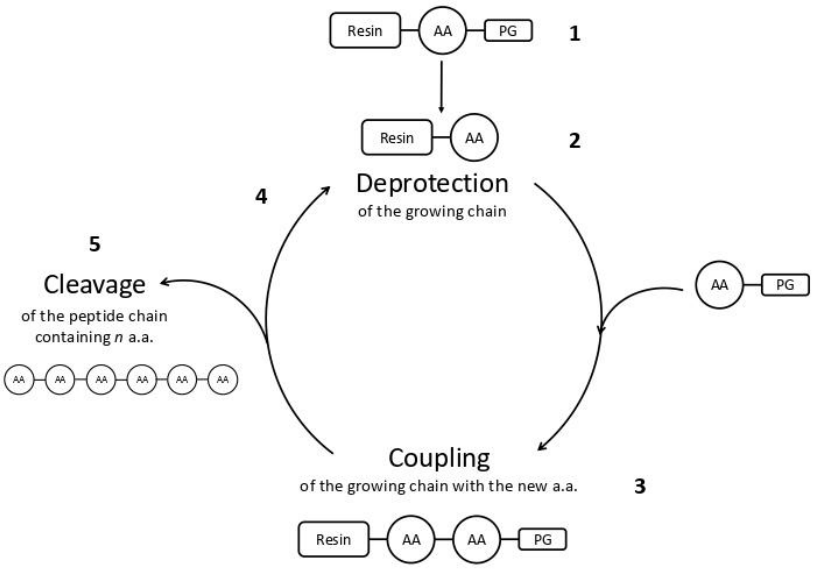

Figure 1. Scheme representing Solid Phase Peptide Synthesis. (1) An amino acid (AA) is protected on the functional group that must not react, and it is bonded to an insoluble resin. Then, its protecting group (=PG) is removed (2), so that the following amino acid, which in turn is protected, can bond to the growing chain (3). Successively, also the second amino acid is deprotected, to add the third one (4). After all the amino acids have been added, the peptide chain is recovered from the synthesis mixture with the step called cleavage (5).

The presence of the solid support allows one to recover the product simply by filtration: in this lies the reason of the success of synthesis in solid phase. Moreover,the process can be automated [14,22,47–49]. Currently, huge efforts are being made in order to make the synthetic processes as green as possible, by introducing the use of protecting groups and alternative solvents more sustainable than the traditional ones [50–52].

Liquid phase synthesis plays an important role in the manufacturing of short peptides (up to 10 amino acids). Recently, this approach gained importance for the manufacturing of longer peptides through the coupling of its previously synthesized fragments [53].

2.3. Recombinant Approach

This technique is the preferred one to produce peptides containing only natural amino acids on large scale. Compared to isolation from proteins and chemical synthesis, recombinant approach represents the most cost-effective and green way for large-scale peptide manufacturing. Particularly, *Escherichia coli* is the most widely used host. With this genetic engineering process, nevertheless, only peptides containing natural amino acids can be produced. Moreover, the biotechnological process requires great research efforts to develop a suitable procedure, and it is also time-consuming. Generally, the steps followed are: selection of an appropriate expression system, construction of expression vectors, development of the bioprocess. The operating conditions can be tailored for the specific product considered [14,47,54].

3. Purification Techniques (Downstream Processing)

None of the aforementioned upstream methods to obtain the peptide of interest leads to a single product. Actually, a series of impurities are produced together with the target. They must be removed during the downstream step of manufacturing. The reason is that

every impurity could potentially exert adverse biological activity on the human body. Therefore, very strict purity requirements are applied to pharmaceuticals.

Different purification methods have been developed, with their own advantages and disadvantages. The purification strategy must be evaluated for every single case, in light of imposed requirements that, in turn, vary depending on the particular application [55].

If a peptide is produced through hydrolysis, for instance, it can be separated by means of ultrafiltration from the enzyme employed during the process and from other protein residues with higher molecular mass. Generally, in this case, the membranes of choice have a low molecular mass cut-off and the size of their pores depends on the molecular weight of the desired peptide. Anyway, the main disadvantage of this technique is the poor selectivity of the membrane [1,56].

A technique particularly useful in case of separation of charged peptides and proteins is IsoElectroFocusing (IEF), which is based on the same separation principles as electrophoresis. The sample is injected in a chamber where an electric field is applied, in presence of a pH gradient. Acidic species move towards the anode and basic ones towards the cathode. When a species reaches a zone with pH identical to its isoelectric point, it stops migrating. Then it can be moved to a detection windows to be identified. Therefore, IEF separates analytes depending on their isoelectric point [57]. Several modes of IEF have been developed, some of which can be used on analytical while others on preparative scale, which is the case of IEF in solution [58] or in a cellulose-based separation medium [59]. Anyway, IEF lies outside the main topic of this review and therefore it will not be treated any further.

A common purification issue is the separation of a complex mixture of peptides deriving from solid-phase synthesis. The separation can only be done after cleavage and it is frequently challenging because impurities may differ from the target peptide by a single amino acid or a single functional group, resulting in very similar chemical properties [60]. Typical by-products due to solid phase synthesis include peptides with one amino acid that did not react or reacted in the wrong position, peptides with side-chain modification (oxidation, deamidation, epimerization, alkylation, ring closure or opening, incomplete deprotection) and truncated peptides [61,62].

Chromatography is the most suitable technique for the purification of valuable products, which is the case of pharmaceuticals, where high resolution and selectivity are required [63,64]. This technique allows one to obtain very high efficiency in the separation of complex mixtures, where the components have similar chemical properties. It is flexible and adaptable since a wide selection of stationary and mobile phases to choose from is available. Additionally, a number of well established chromatographic methods have already been developed at industrial level and are available for practical biopharmaceutical applications [55]. A disadvantage of chromatographic methods is that it is difficult to handle viscous mixtures, which cause increase in the backpressure; moreover, organic solvents are used almost always as mobile phase, and this poses environmental concerns regarding their toxicity and disposal [65]. Anyway, chromatography remains the technique of choice for the purification of biomolecules at laboratory, preparative and industrial level. Currently, industrial processes for biopharmaceuticals employ almost exclusively chromatography both for capture and for polishing steps, whose difference has already been explained in Section 1.

Due to the complexity of the peptide mixtures, generally a combination of chromatographic techniques based on different separation principles is required to improve the resolution power [66]. Therefore, at least two different chromatographic modes are applied consecutively, either online or offline, resulting in a multidimensional separation (e.g., two-dimensional liquid chromatography, 2D-LC). A separation performed on the basis of different types of interactions is often referred to with the term "orthogonality" [67], meaning that the two dimensions of the separation can remove different impurities. For example, ion-exchange, HILIC and reversed-phase chromatography separate the analytes depending on different features (charge, hydrophilicity and hydrophobicity) and therefore they can be

used as dimensions orthogonal to each other [68]. Otherwise, also reversed-phase in acidic conditions and in basic conditions can be considered to be orthogonal separation methods and have shown very good results in terms of peak capacity when applied to purifications of peptide mixtures [69]. In the case where the two chromatographic modes are coupled offline, the product eluting from the first column is collected and then re-analyzed in the second dimension. This approach is quite labor-intensive and time-consuming. On the other side, in online multidimensional chromatography the product eluting from the first column is immediately injected into the second column, and this allows one to speed up a lot the analysis time, but requires compatibility between the solvents used in the two dimensions. For preparative scale purification, the heart-cutting mode is generally the preferred choice, meaning that only the peak of interest is further separated in the second dimension [70]. On the contrary, for analytical scale analysis, meaning to identify components in the mixture, comprehensive multidimensional separations are usually performed, where the eluent from the first dimension is injected into the second column over the entire first separation time [71]. Generally, mass spectrometry is coupled to multidimensional chromatography, especially at the outlet of the second column [72].

Multidimensional chromatography is based on the same principles as one-dimensional chromatography and therefore will not be further discussed. In the next paragraphs, different modes of chromatography will be considered in detail. Additionally, different purification techniques described in this Section and in the next ones are summarized in Table 1.

Table 1. In this table, the main techniques employed for peptide purification and their interaction mechanisms are summarized.

Purification or Identification Method	Mechanism
Ultrafiltration	Target peptide is separated from other species depending on their size
IsoElectroFocusing (IEF)	Peptides are separated on the basis of their isoelectric point through an electric field and a pH gradient
Single-column chromatography	Different modes of chromatography have been developed, depending on the chemical features of the analytes: • RP-LC: hydrophobic character • Ion-Exchange: charge • HILIC: hydrophilic character • Mixed-Mode: combination of two ligands on the same stationary phase with orthogonal interection mechanisms
Multicolumn chromatography	Combination of two or more orthogonal chromatographic modes applied consecutively
MCSGP	Same separation principles as single-column chromatography but with the use of two or more identical columns. The performance parameters increase due to internal recycling of impure fractions into the system.

3.1. Reversed-Phase Liquid Chromatography (RP-LC)

The mode of chromatography most frequently encountered when it comes to the purification of peptide and protein mixtures is reversed-phase liquid chromatography [73]. This technique separates the analytes depending on their hydrophobic properties [1]. Generally, C18 ligands are the most employed stationary phases in RP-LC, but occasionally C8 or even C4 ligands have shown better retentive characteristics, especially in case of very hydrophobic peptides. In other cases, also monolithic, poly(styrene-divinylbenzene)-based columns have been used [74]. All these stationary phases are able to distinguish diastereomers (peptide epimers for instance) but not enantiomers.

In RP-LC, the retention of macromolecules, such as peptides, decreases drastically with the content of organic modifier [48,75,76]. Therefore, it is recommended for macromolecules to use gradient elution, which also contributes to improve the separation of the target peptide from its product-related impurities, since species with similar structure can show very different adsorption behaviour at a given mobile phase composition [49]. Several peptide mixtures have been separated by means of gradient elution in RP-LC, such as insulin from its main degradation product (A21-desamido insulin) [77] and octreotide from impurities [49]. Using a very shallow gradient (0.1% ACN per min), Harris and coworkers [78] managed to purify mixtures of different synthetic polypeptides, with length ranging from 23 to 51 amino acids, containing closely eluting impurities. Sample amounts varying from 145 to 900 mg of peptide mixture could be purified with this one-step chromatographic method, including peptides modified with non-proteinogenic substituents (e.g., biotin, carboxyfluorescein); the purities reached were almost always above 95%.

Besides ACN and other more eco-friendly solvents have been tested and compared to it. Ethanol, for instance, has shown elution strength and separation characteristics similar to ACN during the separation of three peptides (bradykinin,angiotensin II, angiotensin I), thus resulting to be a promising candidate to substitute ACN in some cases [79].

Acidic ion-pairing agents (trifluoroacetic acid or formic acid) are often added to the mobile phase to pair with basic amino acids, that are positively charged at acidic pH, improving peak shape [4,80]. A study demonstrated that TFA concentration affects the recovery of the peptide or protein: at low TFA concentrations, below 0.05%, strong ion-exchange interactions can establish between analytes and hydrolyzed silanols, thus causing peak broadening [81].

Mazzoccanti et al. [82] developed a d-ERRP (dynamic Electrostatic-Repulsion Reversed-Phase) method (a variation of the classical static ERRP) based on the repulsion between the basic peptide and a hydrophobic ion-pairing agent adsorbed on the alkyl chains of the stationary phase, both positively charged at acidic pH. The hydrophobic agent used in that research was tetrabutylammonium, dissolved in the mobile phase together with TFA. This innovative chromatographic mode was successfully applied to the purification of glucagon (containing 29 amino acids) from its epimer [D-His]^1-Glu and other four critical synthetic impurities deriving from deamidation or racemization of some amino acids. This technique is called "dynamic" because the repulsion is generated by the flow of the mobile phase, in opposition to static ERRP that will be discussed later.

A remarkable advantage of RP-LC over other chromatographic modes is that, thanks to the solvents employed, it can be easily coupled with Mass Spectrometry, a detection technique very popular for the characterization of macromolecules such as peptides and proteins [83]. This technique has allowed one, for instance, to separate bioactive peptides and tryptic digests of different proteins in RP-LC under both acidic and alkaline conditions, using trifluoroacetic acid or a buffer made of triethylamine and acetic acid respectively, and applying a gradient of ACN [74]. The detectability of peptides in conditions of full-scan negative-ion electrospray ionization mass spectrometry after the separation at high pH was two to three times lower with respect to their detectability in conditions of full-scan positive-ion electrospray ionization mass spectrometry after the separation at low pH.

3.2. Ion-Exchange Chromatography (IEX)

In ion-exchange chromatography, the separation mechanism is based on the electrostatic interactions between the opposite charges of analytes and stationary phase. Separation is modulated by the amount of competitive ions present in the mobile phase [1]. This technique is particularly suitable when dealing with peptide purification, because most peptides have a net charge that can be varied depending on the pH of the mobile phase. At acidic pH, carboxylate groups and basic residues (Arg, Hys, Lys) are mainly protonated. Therefore, IEX is often used to characterize charge variants of peptides and proteins, even

though lately it has been more and more replaced by RP-LC because of its incompatibility with MS detection [84].

To identify peptide modifications such as deamidation or acetylation, that are detected with difficulty through RP-LC, IEX reveals to be a good choice. Moreover, this technique is able to distinguish between analytes with similar hydrophobicity. For example, using a strong cation-exchange column (Luna SCX containing a phenyl sulfonic acid exchanger) with salt gradient (namely, potassium chloride) it was possible to separate bradykinin variants differing only slightly in hydrophobicity or only by one charge [85].

Crimmins [86] verified that, during the IEX separation of a complex mixture of synthetic peptides positively charged from +1 to +7 at pH 3, the order of elution followed the order of charge monotonically; the most retained compound was the peptide with charge +7 (see Figure 2). In that case, the stationary phase used was a sulfoethyl aspartamide (a hydrophilic strong-cation exchange adsorbent), whereas mobile phases were MP_A = 5 mM sodium phosphate pH 3, 25% acetonitrile (ACN), and MP_B = 5 mM sodium phosphate, 500 mM NaCl pH 3, 25% acetonitrile. At low levels of ACN, retention and selectivity are mainly governed by the presence of basic amino acids and positive charges contained in the peptide. The same stationary phase has been tested by different research groups to analyze several peptides (up to 50 in a single study, ranging from 5 to 20 amino acid residues) [87–89]. However, the elution order does not always follow the order of charge. By analysing with the same method described above the peptide fragments produced by digestion of myoglobin, it was noted an inversion of the elution order with respect to the charge order [90]. This behaviour was attributed to the overall hydrophobicity of the peptide and to the fact that, for steric reasons, the charged residues do not interact simultaneously with the ion-exchange resin. Probably, there is a limit related to peptide molecular weight above which the monotonic relation between the elution order and the global charge of the analyte is no longer valid.

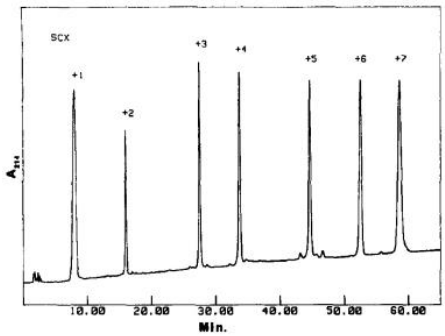

Figure 2. Separation of seven synthetic peptides bearing different charges by means of Ion-Exchange chromatography. Reproduced with permission from [86].

Ion-exchange chromatography has been employed also for pre-purification of the peptide of interest. In a previous study, lactoferrin was hydrolyzed with porcin pepsin A. The hydrolysate was then loaded in a SP-Sepharose Fast Flow column (where SP is sulphopropyl, a strong cation exchanger) and eluted with a gradient of ammonia solution. During the gradient elution, only impurities were eluted. At the end of the gradient, a wash was performed with NaCl 2 M, in order to recover the target peptide (LFcin-B) [91]. Otherwise, it is possible to modulate the experimental conditions in order to trap the impurities on the resin whereas the peptide passes through the column with no retention. For example, this procedure was applied for the purification of C-peptide. This molecule contains 31 amino acid residues, with only one being basic, therefore it has a very low isoelectric point, around 3. By using a strong cation exchange and applying a mobile phase with pH slightly above its isolectric point, this strategy resulted to be successful, as it was

confirmed by a comparison of LC-MS chromatograms of the sample before and after the purification [92].

Last, IEX is often employed for peptide mapping, to demonstrate protein identity, as in the case of cytochrome C tryptic digest and hemoglobin [84].

3.3. Hydrophilic Interaction Chromatography (HILIC)

Hydrophilic Interaction Chromatography (HILIC) and can be considered as a variant of normal-phase chromatography. As it is shown in Figure 3, it is likely that HILIC involves a partitioning mechanism between the more hydrophobic mobile phase and a layer of solvent rich in water adsorbed on the stationary phase, but the phenomena responsible for such a behaviour are still not well understood [93]. Alpert [87] demonstrated that, if at low percentage of ACN the separation mechanism is mainly based on ion-exchange, on the other hand retention increases dramatically at high levels of ACN (e.g., greater than 50%). This is due to hydrophilic interactions. At the same time, electrostatic effects diminish in importance. The order of elution is the opposite than in reversed-phase chromatography, meaning that retention increases from least to most hydrophilic component [94].

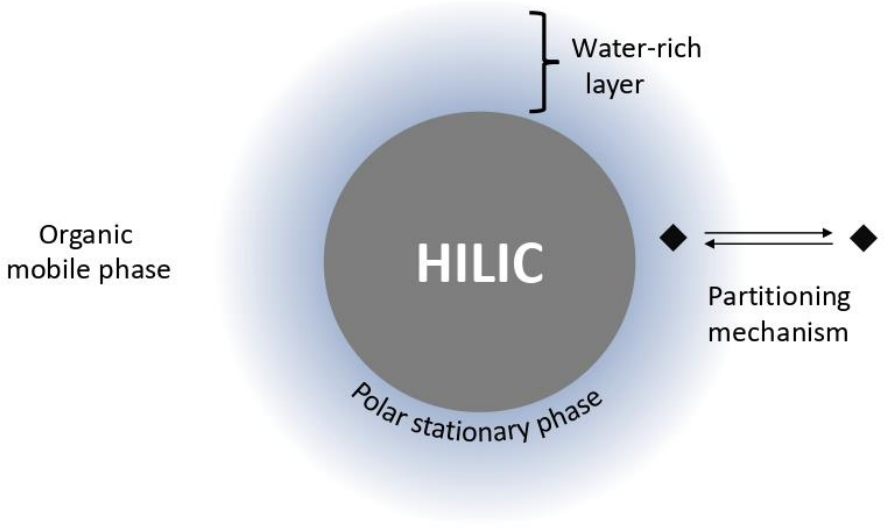

Figure 3. Scheme of the mechanism regulating elution in HILIC. The polar stationary phase is surrounded by a layer rich in water, whereas the mobile phase is an organic solvent. Probably, the analytes are retained because of partitioning mechanism. It results that more hydrophilic components are more retained.

HILIC has been employed for different applications. Nine peptides present in an illicit drug mixture, including oxytocin, leuprorelin, sermorelin, epitalon and melanotan II, have been separated within 35 min using a gradient elution method employing LC-DAD-MS. The mobile phases used for the gradient were MP-A: acetonitrile, deionized water and 100 mM ammonium formate pH 3.0 (80:10:10, $v/v/v$), and MP-B: acetonitrile/deionized water/100 mM ammonium formate pH 3.0 (40:50:10, $v/v/v$). The column was a ZIC HILIC column, made of bare silica modified with sulfobetaine. In addition, other stationary phases were tested, including CORTECS (made of bare silica) and BEH HILIC column (composed of unbound porous particles hybridized via ethylene crosslinking) [95]. A comparative study between three HILIC columns (XBridge amide from Waters, bare silica Kinetex HILIC from Phenomenex and silica with quaternary ammonium and sulphonic acid Nucleodur HILIC from Macherey Nagel) was accomplished in the context of quantification of proteotypic tryptic peptides, where chromatography was coupled with ESI-MS. It resulted that a higher sensitivity was obtained using the amide column without salt buffer

in the mobile phases [96]. HILIC technique proved to be a good solution also for the separation of immunoglobulin deriving peptides containing modified residues (deamidated asparagine and oxidized methionine) from their native forms, a procedure necessary for the identification and quantification of critical impurities. The method involved the use of a Penta-HILIC column and a gradient elution program where the percentage of strong solvent (water containing 0.1% formic acid and 50 mM ammonium formate) increased from 5 to 70% within 90 min, whereas the content of ACN decreased [97].

A variation of the classical HILIC technique, developed by Mant et al. envisaged to perform the peptide separation using a salt gradient (sodium perchlorate) in the presence of isocratic high content of organic solvent, specifically ACN around 80–90%. The column employed was a Halo Penta-HILIC column. This technique was applied to different mixtures of synthetic peptides differing in structure, number of amino acids (from 10 to 26) or charge, and in those circumstances it resulted to be more suitable for peptide separations than the traditional version of HILIC in terms of resolving capabilities [98]. For instance, Figure 4 shows the differences in the separation of a mixture of three synthetic α-helical peptides by using reversed-phase chromatography (panels A and B), HILIC (panel C), isocratic HILIC (panel D) and HILIC/SALT (panel E). The peptides considered have the same structure (Ac-ELEKLL**X**ELEKLLKELEK-amide) except for the amino acid **X** in position 7, which in the three cases is either Ser (LS7), Thr (LT7) or Val (LV7). Ser and Thr are much more polar than Val, which is non-polar, and this explains the order of elution.

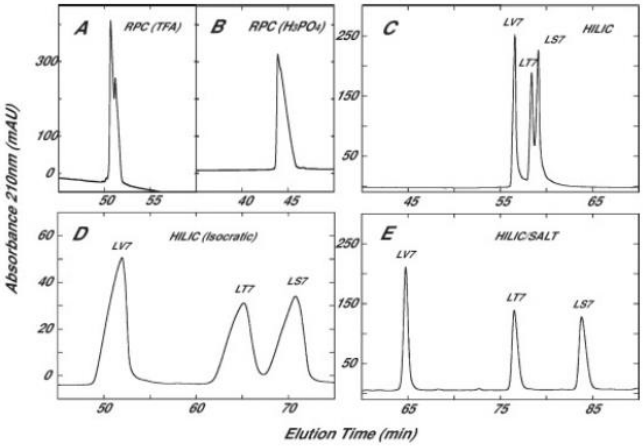

Figure 4. Separation of a mixture of LV7, LT7 and LS7 peptides by RP-LC with TFA (panel **A**) or phosphoric acid (panel **B**) as the ion-pairing reagent, HILIC (decreasing concentration of acetonitrile, panel **C**), isocratic HILIC (isocratic concentration of acetonitrile, panel **D**) and HILIC/SALT (isocratic concentration of acetonitrile and salt gradient, panel **E**). Reproduced with permission from [98].

3.4. Mixed-Mode Chromatography

Peptide mixtures contain a huge number of impurities with different chemical properties. Therefore, a single chromatographic technique is often insufficient to obtain a good separation. Both hydrophobicity/hydrophilicity and charge play a key role in the purification of peptide samples. Recently, innovative stationary phases have been developed, that combine two separation mechanisms (reversed-phase or HILIC and ion-exchange) [99,100]. The presence of two different ligands enables two separation mechanisms: a first one based on the hydrophobic or hydrophilic character of the compound of interest and the second one based on its ionic properties, namely its net charge at the pH of the mobile phase [60,101]. An example of stationary phase containing both hydrophobic chains and positively charged ionic groups is shown in Figure 5.

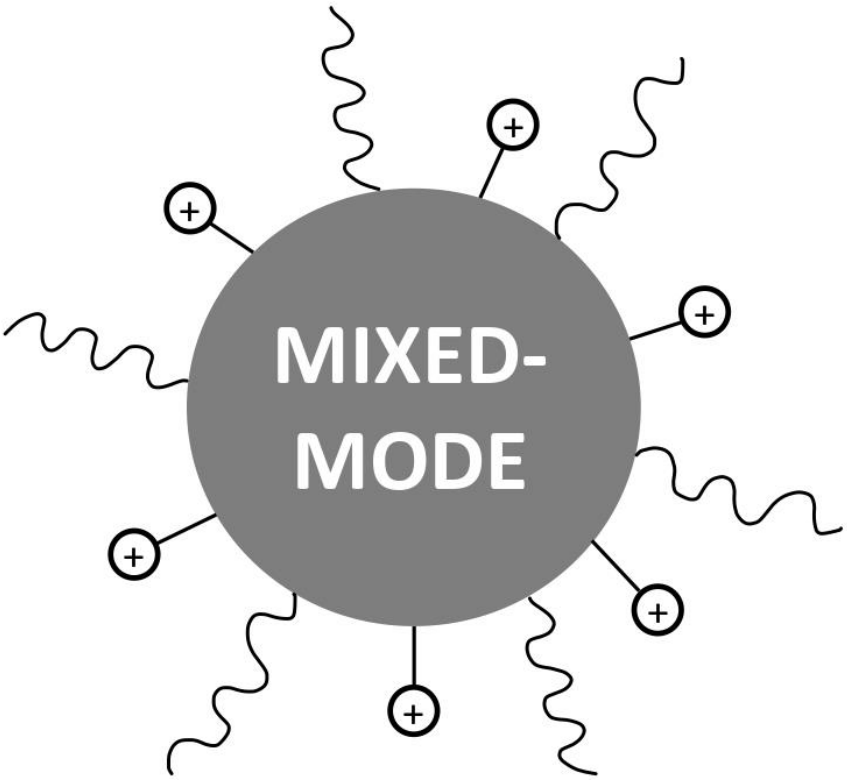

Figure 5. Scheme of a typical stationary phase used for Mixed-Mode Chromatography. The particle is functionalized with alkylic chains (e.g., C8) and charged groups (e.g., quaternary amines).

Moreover, depending on the ion-exchanger nature, on the pH of the mobile phase and on the pI of the peptide of interest, the ion-exchange mechanism can work either in attractive or in repulsive way. In 2014, Gritti and Guiochon [102] applied the static ERRP concept by using BEH-C18 columns modified with different loadings of quaternary amino groups. Since the positive charges are chemically bonded to the silica surface, this ERRP can be defined "static" in opposition to dynamic ERRP, described in Section 3.1. They applied this technique to several peptides and proteins, with molecular mass ranging from 0.9 to 80 kDa and pIs between 4.7 and 11.3. Water and ACN with TFA were used as mobile phases (pH = 3); being the pH of the eluent lower than all the PIs of the analytes, it resulted that all the peptides and proteins as well as the amines were positively charged, and therefore the ion-exchange mechanism worked in repulsive way. As expected, the retention times of all compounds decreased with increasing surface density of the amino groups on the stationary phase, because of the greater electrostatic repulsion between the positively charged analytes and the ionic groups bonded on the BEH-C18 stationary phase.

Khalaf and coworkers [100] used Zeochem columns functionalized with C8 chains and with quaternary amines. The mobile phases tested were aqueous buffers containing different salts (sodium acetate, ammonium acetate) and a percentage of ACN between 3 and 50% v/v. When pH is lower than pI both the ion-exchangers and the peptide are positively charged; therefore, their interaction is repulsive, and, as a consequence, the global effect is to decrease the retention times. In that research, Khalaf demonstrated that significant improvements in separation performance were obtained, both in analytical and in preparative scale: selectivity and productivity were increased up to twice as high, whereas yield was improved by around 20%.

Kadlecová et al. [103] compared two columns with positively charged ionic ligands (XSelect CSH C18, containing pyridyl group, and Atlantis PREMIER BEH C18 AX, contain-

ing a quaternary alkylamine) for the separation of 14 peptides, especially ten dipeptides, three pentapeptides and one octapeptide. The analytes have been separated in gradient conditions using ammonium formate 5 mM (pH 3) and ACN. All the peptides were baseline separated within four minutes using PREMIER BEH C18 AX column and a quite steep gradient. To obtain a comparable degree of separation, a more shallow gradient had to be used on XSelect CSH C18, achieving a complete separation in 10 min.

On the other hand, for the purification of a goserelin mixture, Bernardi et al. [99] used Zeochem columns functionalized with C8 chains and sulfonic groups (strong cation-exchange ligands). The mobile phases were aqueous buffers containing sodium acetate in different percentages and a known quantity of ACN (from 6 to 50% v/v). pH was around 4, while pI of goserelin was 11.5: therefore, the electrostatic mechanism was attractive, because the analytes and the ionic ligands on the stationary phase bore different charges. They demonstrated that the adsorption strength increases with increasing the percentage of sulfonic groups on the surface of particles: higher Henry coefficient values were found for higher ion-exchanger concentrations.

Beside hydrophobic compounds, also hydrophilic analytes can be separated in mixed-mode. Litowski et al. [60] used a polysulfoethyl A strong cation-exchange column, that presents a hydrophilic character, to separate in gradient conditions a mixture of a 21-residues synthetic amphipathic α-helical peptide from its impurities modified on the side-chain, particularly acetylated on three different serine residues (positions 3, 10 and 17). Eluent A was 10 mM aqueous TEAP, pH 6.5, containing 65% ACN and eluent B was identical to eluent A but contained also 350 mM $NaClO_4$. The ability of HILIC/CEX of separating these four species is outstanding, since the analytes exhibit the same charge and extremely similar hydrophilicity, features that require both separation mechanisms to isolate the target compound.

4. Current Challenges and Future Perspectives

4.1. The Need to Investigate the Theoretical Basis of Adsorption

The goal of purification procedures is to achieve high purity products with elevated recovery, possibly by means of high throughput and economically viable processes. Combining all these requirements is challenging. Moreover, during the development of purification processes, often a "trial-and-error" strategy is followed, an approach which is time-consuming and costly. Indeed, it is estimated that around 50 to 70% of the whole manufacturing cost is attributable to the downstream processing [49,65,104]. In this regard, it appears necessary to model the chromatographic behavior of the species of interest through theoretical and computational instruments, in order to define a priori proper experimental conditions for the purification of the target [105,106]. Particularly, knowing the thermodynamic properties (that is, the adsorption isotherms) involved in the adsorption of the target molecule is the basis to predict its behaviour in a wider range of working conditions, which makes trial-and-error optimization superfluous. Additionally, mechanistic models can be a useful tool to predict the impact of process parameters and experimental conditions on product quality [107]. Computational methods, such as Inverse Method, allow one to obtain thermodynamic data starting from very little amount of compound, which is particularly advantageous when the material of interest is expensive or present in limited amount, and this could promote the need for model building among pharmaceutical companies during process development phases. Some of the authors of this review have published a study regarding the modeling of the chromatographic behaviour of a peptide in non-linear conditions [49].

The investigation of thermodynamic adsorption equilibria regulating the separation in batch of the compounds contained in the peptide mixture could also be used to scale the process to continuous chromatography, a field where the knowledge of non-linear chromatography has not been deeply investigated yet.

4.2. Continuous Chromatographic Techniques

The chromatographic processes performed during the polishing steps usually employ a single column; this operative methodology is called "batch". The classic situation encountered in these purification processes is a ternary separation, meaning that the peptide of interest elutes as intermediate between two groups of impurities: a first group with lower retention (more weakly adsorbed on the stationary phase) and a second group with higher retention (more strongly adsorbed). The similarity between the target product and its related impurities leads unavoidably to an overlap between their peaks, on both the front and the tail of the product peak; the situation becomes even worse for higher loadings. As a consequence, two borderline cases are possible: the collection window can be narrowed, in order to obtain a higher purity, to the detriment of recovery, or it can be widened, and this leads to higher yields but lower purity, since also a part of the impurities peaks is collected. The qualitative example depicted in Figure 6, shows that in batch chromatography it is practically impossible to reach both a high purity and high yield at the same time (yield-purity trade-off) [108,109].

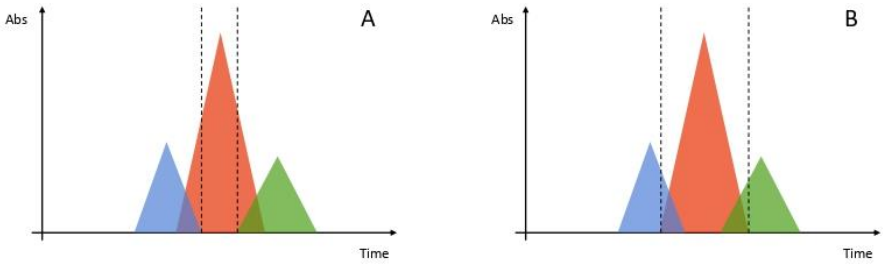

Figure 6. The red peak represents the target product, eluting between a group of impurities more weakly adsorbed (blue peak) and a group of impurities more strongly adsorbed (green peak). When deciding which portion of the main peak to collect, two cases are possible: (**A**) narrow collection window leads to higher purity but lower recovery; (**B**) broad collection window leads to high recovery but low purity, since also a portion of impurities peaks is collected. This is the so-called "purity-yield trade-off".

Multicolumn chromatographic processes can help to overcome this limitation, very frequent for complex mixtures of biopharmaceuticals. This group of techniques includes processes where at least two columns, identical in dimensions and in stationary phase, are used. The concept on which multicolumn processes are based is the countercurrent movement of the mobile phase with respect to the stationary phase, simulated through a series of switching valves, that can influence the path accessible to the mobile phase. In most cases, the countercurrent movement of the two phases allows to improve the recovery. The second fundamental aspect of multicolumn processes is that they work continuously, making the whole procedure cyclic and automatic. This permits to reduce times related to operations that the operator would accomplish manually and, as a natural consequence, it allows to improve reproducibility.

Particularly, to deal with polishing of complex biomolecules mixtures, a process called Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) has been developed around fifteen years ago by Aumann and Morbidelli [110]. During the years, this appealing technology has found a wide application range, since it has been used for several cases of biomolecules purification where intensification of the downstream steps was needed, such as the purification of monoclonal antibodies [111–113], PEGylated proteins [114], oligonucleotides [115], cannabidiols [116], and of course of several peptides [48,110,117–119].

The most important feature of the MCSGP process is the possibility to use a solvent gradient for the elution, differently than other continuous chromatographic techniques that only work in isocratic conditions, for example Simulated Moving Bed [25,120]. This requirement is mandatory because the retention of biomolecules strictly depends on the

mobile phase composition (percentage of organic modifier, salt concentration, etc.), as already stated above [49,105,118,121–124].

The improvements gained with MCSGP are due to the fact that the overlapping regions (the regions where product partially elutes with weak and strong impurities, on the head and on the tail of the main peak respectively) are recycled internally into the unit in order to be reprocessed. The mechanism regulating the internal recycling lies in the movement (switching) of the inlet and outlet column valves, which can connect and disconnect the columns and, as a consequence, change the path executed by the mobile phase. In Figure 7, a scheme illustrating the working principle of MCSGP is depicted.

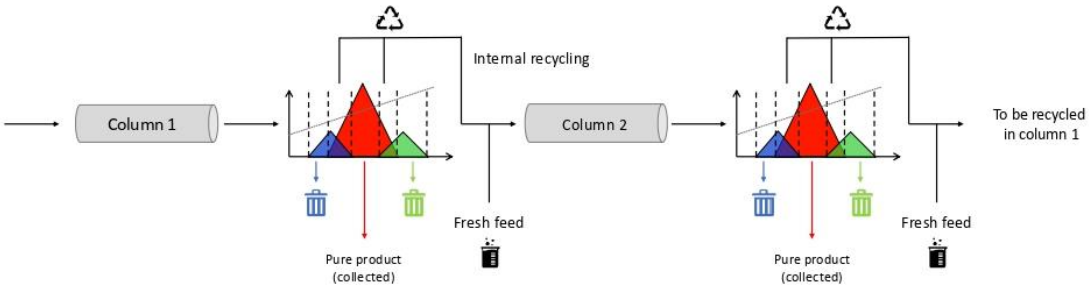

Figure 7. A scheme illustrating the MCSGP technique is shown. The gradient elution is performed in the first column. The main peak (red) overlaps in the front and in the tail with other groups of impurities (blue and green). The overlapping regions are therefore internally recycled into the second columns in order not to waste product, whereas the central part of the red peak (pure target product) is collected. On the contrary, windows where only impurities elute with no product are discarded. The same procedure is repeated at the outlet of the second column, where the overlapping regions are recycled into the first one. During the recycling, also some fresh feed is loaded into the same column, in order to keep the total amount of product constant into the system.

The combination of continuous and countercurrent concepts coupled with the internal recycling and the possibility to use a solvent gradient program enables separation of ternary mixtures and allows to reach high product purities and elevated yields at the same time, outperforming the traditional single column processes, in many cases also in terms of productivity [125,126]. One of the first applications of the six-column MCSGP process concerned the purification of a 32 amino acids synthetic peptide, namely calcitonin, whose initial purity was 46%. It was found that, using a reversed-phase method, the yield increased from 66% for the batch purification to almost 100% for the continuous process, with a purity of 93% [110]. Another case of a complex peptide mixture that has been successfully separated through MCSGP is an industrial crude of glucagon, a synthetic peptide containing 29 residues. The study published [48] shows how to transfer a batch method to an MCSGP process; Figure 8 illustrates the batch chromatogram starting from which the MCSGP method has been setted. The results obtained indicate that the recovery can be increased from 71% to almost 88% with twin-column MCSGP, reaching a purity value equal to 89%. Last, a very recent research [119] reports an application where MCSGP has led to outstanding results in all the performance parameters. In the case of purification of icatibant in reversed-phase conditions, indeed, the purity obtained for the target product was greater than 99% for both the batch and the MCSGP method, but the continuous processes allowed to improve the recovery from around 12% to more than 95%, with a process gain of +670% in terms of recovery. Additionally, the productivity was improved by more than 5 times whereas the solvent consumption was reduced by 80%. For an in-depth description of the MCSGP technique, further examples of application and how to develop an MCSGP method starting from a design batch chromatogram, the interested reader is referred to a review specifically focused on this topic, written by some of the authors [24].

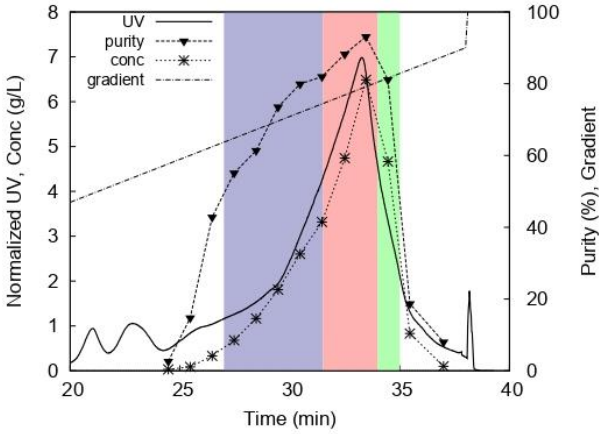

Figure 8. Batch chromatogram obtained for the purification of a glucagon crude mixture. The blue and green windows represent the impure portions of the main peak that need to be recycled into the second column. On the other side, the red window represents the target product fulfilling the purity requirements. Beside the UV profile, also the purity and concentration profiles and the gradient are shown. For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article. Reproduced with permission from [48].

5. Conclusions

Bioactive peptides show a broad range of activities, that vary from antimicrobial, antihypertensive, immunomodulatory, antioxidant, etc; therefore, they can be employed as food additives or pharmaceutical ingredients for the therapy of some diseases. They can be produced by means of hydrolysis of the parent protein or they can be chemically synthesized, especially through Solid Phase Peptide Synthesis. Their application as biopharmaceuticals requires the peptide to fulfill very strict purity and quality specifications; as a consequence, choosing proper purification processes is one of the most important parts of the manufacturing process design. Liquid chromatography is usually the best technique employed for purification, especially when used in reversed-phase mode. Additionally, ion-exchange chromatography and hydrophilic interaction chromatography are frequently used, depending on the hydrophobic/hydrophilic properties of the peptide and on its charge. Innovative mixed-mode stationary phases, on the other hand, combine reversed-phase and ion-exchange features. So, depending on the chemical characteristics of the peptide, for a large class of compounds a suitable chromatographic technique can be easily found.

Thanks to important technological advancements, continuous chromatographic techniques based on the concept of countercurrent chromatography are becoming established. MCSGP represents a remarkable case of process which is sparking interest, because of improvements gained in product quality, economic advantages related to higher productivity and, nonetheless, a simplification of the process due to the automation of operations.

Although huge progress has been done in the purification processing, theoretical studies focusing on the modeling of the process should be implemented, to favour a quick optimization of the procedures. Additionally, some obstacles and barriers must still be faced from the point of view of the regulation, in order to assure to meet Good Manufacturing Practise (GMP).

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Paper VIII

separations

MDPI

Communication

Benefits of a Mixed-Mode Stationary Phase to Address the Challenging Purification of an Industrially Relevant Peptide: A Proof-of-Concept Study

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Abstract: Peptides are a class of biomolecules with a great potential from the therapeutic point of view, because of their unique biological properties. Industrially, the production stategies adopted produce both the target peptide and a series of impurities that must be removed. Preparative chromatography is the technique of choice for the large-scale purification of biomolecules, generally performed in reversed-phase mode, using hydrophobic adsorbents (e.g., C8 stationary phases). A promising and innovative alternative is represented by mixed-mode columns, which bear two different ligands on the particle surface, exploiting two different retention mechanisms to improve the separation. This work represents a proof-of-concept study focused on the comparison of a hydrophobic adsorbent and a mixed-mode one (bearing both hydrophobic groups and charged ones) for the purification of a crude peptide mixture. Thanks to more-favourable thermodynamics, it was found that, when collecting the whole peak excluding fractions of the peak tail, the mixed-mode column led to an increase in the recovery of roughly +15%, together with a slight improvement in purity at the same time, with respect to the traditional hydrophobic column. In addition, if the whole peak, including the tail, is collected, the performance of the two columns are similar in terms of purity and recovery, but the pepetide elutes as a narrower peak with the mixed mode. This leads to a collection pool showing a much-higher peptide concentration and to lower solvent volumes needed, which is a beneficial achievement when targeting more sustainable processes. These results are very advantageous from the industrial viewpoint, because they also involve a decrease in the peptide amount contained in the peak tail, which must be reprocessed again to satisfy purity requirements.

Keywords: stationary phases; mixed-mode chromatography; reversed-phase chromatography; peptide purification; preparative chromatography

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1. Introduction

In the last decades, the market of biopharmaceuticals, including protein-, peptide- and oligonucleotide-based therapeutics, has experienced an impressive increase due to the biological properties of this new class of molecules being potentially promising for medical applications [1–4]. Recent advancements in the production strategies, such as in genomics, proteomics recombinant strategies and peptide synthesis, have pushed even further the development of these new drugs [5–8]. Compared to traditional small molecules, biopharmaceuticals exhibit higher specificity and potency, which derive from their complex

three-dimensional structures [9,10]; in addition, their action results are effective even at very-low concentrations [11]. Due to their structural complexity, their synthesis processes, which make use of technologies such as solid-phase peptide synthesis (SPPS) or chemo-enzymatic peptide synthesis (CEPS) [12–15], often deliver the principal product together with several unwanted impurities, lowering the overall upstream yield. Accordingly, purification steps are required to achieve the purity constraints set by regulatory agencies, such as FDA, for active pharmaceutical ingredients (APIs) [16].

The increasing interest towards peptides as a new class of pharmaceuticals has contributed to intensify the demand for innovative and better-performing purification methods. The main technique employed for peptide purification is preparative chromatography, especially carried out in revesed-phase conditions (RPC) due to the hydrophobicity of peptides [17]. Generally, however, a single purification step is not sufficient to separate the target molecule from all other species [18]; therefore, different chromatographic modes are often used consecutively to get rid of several types of impurities. This approach, which is of utmost importance at an industrial level, is referred to as *orthogonality*: two techniques are orthogonal if they separate compounds depending on two different types of interactions. The combination of orthogonal methods helps to address separation issues that could not be solved using a single technique [19]. Three chromatographic modes with orthogonal selectivities, for instance, are reversed-phase chromatography, size-exclusion chromatography and ion-exchange chromatography, which separate analytes mainly based on their hydrophobicity, dimensions and charge, respectively.

In addition to applying two different techniques consecutively, orthogonal separations can be achieved within the same column when the stationary-phase particles are functionalised with two different ligands, to combine two separation mechanisms in a single chromatographic step [20]. The technique where the stationary phase bears ligands with different reactivities is referred to as *mixed-mode chromatography* [21]. Traditional mixed-mode materials contain two functional groups with different chemistries on the same ligand, while more recent mixed-mode stationary phases exhibit two different ligands, one with a higher superficial concentration and a second one having a much lower surface density. For example, some reversed-phase stationary phases can be doped with small amounts of ion-exchange ligands, forming a so-called doped reversed phase (DRP). Since, in these cases, the ligands are two distinct functional groups, the surface concentration of each on their solid-phase particles can be accurately chosen. It has been demonstrated by Khalaf et al. that the surface density of the doping groups is linearly dependent on their concentration in the mixture used to derivatize the stationary phase [18,22,23].

The doping ion-exchange ligands can work in "attractive" or "repulsive" ways, depending on the experimental conditions used [20,22,24]: they exhibit repulsion towards the analytes having their same charge and attraction towards the ones with an opposite charge sign. On the other hand, the reversed-phase ligands act in an attractive way towards hydrophobic molecules, and, therefore, the whole stationary phase can work globally in "attractive–attractive" or "attractive–repulsive" ways. In traditional reversed-phase chromatography, more hydrophobic species are more retained, whereas less-hydrophobic (including charged) components elute first. When working in attractive–attractive mode, compounds charged with the opposite charge of the dopants experience an increase in retention. This leads to a decrease in resolution because those that would be the first eluting peaks in traditional RP chromatography, in this case move towards more retained ones, whose position is not influenced by the dopants. On the contrary, in attractive–repulsive mode, the repulsion performed by the dopants on the analytes with the same charge sign push them to elute earlier. Again, the hydrophobic species are not affected by the presence of doping ligands. This leads to an increase in the resolution between hydrophilic charged species and more retained hydrophobic peaks. From this purely theoretical explanation, it seems clear that DRP materials are expected to perform better in attractive–repulsive mode. When dealing with peptides, their isoelectric point (pI) defines the sign of their charge depending on the pH of the mobile phase. Particularly, when the mobile-phase

pH is below the pI, the peptide is positively charged, whereas at a pH higher than the pI, the peptide is negatively charged. Therefore, to work in attractive–repulsive mode, for mobile phases where pH < pI, anion-exchange materials (AIEX) must be chosen; otherwise, for phases with pH > pI, cation-exchange resins (CIEX) must be used [23].

This study is intended to be a proof of concept that, since the combination of attractive and repulsive interactions causes an increase in the resolution of peaks of analytes with different chemistry, the use of these doped stationary phases can be potentially beneficial for preparative applications. The compound that has been purified in this study is liraglutide, an analog of human GLP-1 (glucagon-like-peptide 1), a potent blood-glucose-lowering hormone physiologically secreted in the duodenum in response to food intake. It is a 31 amino acids polypeptide, acylated with a group -Glu-palmitoyl on Lys(1) (sequence: H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(1)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-(OH)-palmitoyl-Glu(1)-OH) with a pI of 4.9 and a molecular mass of 3751 g/mol [25]. Derivatization with a fatty acid side chain and a glutamyl spacer is needed to improve pharmacokinetic and pharmacodynamic properties, to prolong its plasma half-life time [26,27]. In this study, reversed-phase and doped reversed-phase resins were tested and compared for the purification of a mixture of this peptide industrially obtained by means of SPPS. Particularly, the mixed-mode stationary phase was a reversed-phase resin loaded with a defined percentage of cation-exchange groups. By operating at a pH above the pI of liraglutide, the electrostatic interactions between the negatively charged peptide and the cation-exchange groups on the resin become repulsive, while the hydrophobic C8 chain positively interacts with the hydrophobic peptide, globally resulting in the already-mentioned "attractive–repulsive" effect.

2. Materials and Methods

2.1. Peptide

The crude mixture of liraglutide was synthesized by means of solid-phase synthesis by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy). The peptide constitutes 34% of the crude mixture based on weight. All crude peptide samples (from now on called feed) were prepared in order to obtain a concentration of 1 g/L; in addition, the feed was filtered before injection using a Millipore apparatus with 0.45 µm membrane filters produced by Carlo Erba Reagents (Milan, Italy). Liraglutide in the feed has a chromatographic purity of 49%, measured with the analytical method reported in Section 2.4.

For the HPLC calibration curve (area vs. concentration), standard solutions of liraglutide were prepared by dissolving pure liraglutide, provided by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy), as described for the feeds, in a concentration range from 0.1 to 2 g/L.

2.2. Columns and Buffers

Two different reversed-phase resins with the same column dimensions (250 × 4.6 mm) were employed in this study: a Daisogel-SP-10 µm-C8-Bio column with pore size 120 Å, by Dr. Maisch Daisogel (Ammerbuch, Germany), functionalised with C8 chains, and a doped-reversed phase ZEOsphere-DRP-10 µm-C8-C10 column with pore size 120 Å, produced by Zeochem AG (Uetikon am See, Switzerland), functionalized with both C8 chains and 10% of sulfonates group (CIEX ligands). All reagents for buffers preparation were purchased from Sigma-Aldrich (St. Louis, MI, USA), including acetonitrile, triethylamine, phosphoric acid 85%, trifluoroacetic acid. Particularly, for preparative applications, the mobile phases used were MP-A: triethylamine phosphate buffer 25 mM, with pH corrected to 8.5 using orthophosphoric acid 85%, and MP-B: ACN. The mobile phases used for analytical experiments were MP-A: 0.05% TFA in water, and MP-B: methanol:water:acetonitrile:TFA 80:15:5:0.05.

2.3. Preparative Chromatographic Equipment and Methods

Preparative chromatography experiments were performed at room temperature using an ÅKTA pure 25L instrument (GE Healthcare, Uppsala, Sweden), equipped with a fraction collector and operated through the Unicorn software. The detector wavelength was set at 300 nm.

The purification method used included an equilibration step at 15% MP-B 1 CV long, where CV is the geometrical column volume, corresponding to 4.2 mL for a 250 × 4.6 mm column. Then the sample was loaded into the column in order to obtain a concentration of 10 mg of peptide per mL of stationary phase, which corresponds to a loaded volume of 42 mL of feed with a concentration of 1 g/L. After the loading, the column was washed for 2 CVs with 15% MP-B. A first gradient was used to increase rapidly the MP-B percentage (from 15 to 34.5% in 1 CV). A second, much-shallower gradient was used for the main purification step, where MP-B percentage increased from 34.5 to 39.5% in 6 CVs at a very low flow rate (0.5 mL/min). The washing step lasted for 10 CVs, at a constant percentage of MP-B (75%). In all the steps but the second gradient, the flow rate was 2 mL/min.

During the elution, fractions were periodically collected (1 fraction every mL eluted), diluted with water, if necessary, and analysed offline (see Section 2.4).

2.4. Analytical Chromatographic Equipment and Methods

Analytical chromatographic analyses were performed on an Agilent 1100 Series Capillary LC (Agilent, Santa Clara, CA, USA), equipped with a binary pump system, an autosampler and a diode array detector set at 220 nm. The column thermostat was set at 25 °C and the injection volume was 8 µL. The column employed for analytical experiments was a Cortecs C18+, 150 × 4.6 mm, 2.7 µm (Waters, Milford, MA, USA).

The elution was performed in gradient conditions, with MP-B varing from 80 to 95% in 30 min, followed by a second gradient from 95 to 100% B in 5 min, a washing step with 100% B and then a re-equilibration step, at the initial conditions.

3. Results and Discussion

The purification method described in Section 2.3 was used to purify the liraglutide crude mixture in order to increase the peptide purity from 49 to at least 90% after collecting and pooling the fractions eluted along the gradient. All the fractions were analysed offline in order to obtain, for each, the peptide concentration, purity and recovery. By identifying the concentration and the volume of every fraction (see Section 2.3), the mass of the peptide could be determined.

For a single fraction, purity, which is the most-important parameter to consider when evaluating the outcome of a purification, is the area of the target peak ($A_{pept\ frac}$) divided by the total area of all the peaks integrated ($A_{tot\ frac}$) in that fraction, which also includes impurities:

$$Purity\,(\%) = \frac{A_{pept\,frac}}{A_{tot\,frac}} \tag{1}$$

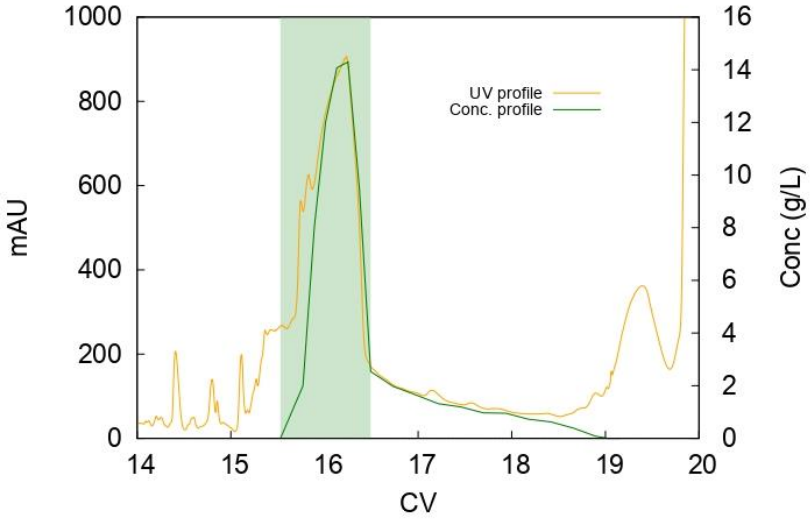
Besides purity, recovery can also be evaluated for each fraction. It is defined as

$$Recovery\,(\%) = \frac{m_{pept\,frac}}{m_{pept\,inj}} \tag{2}$$

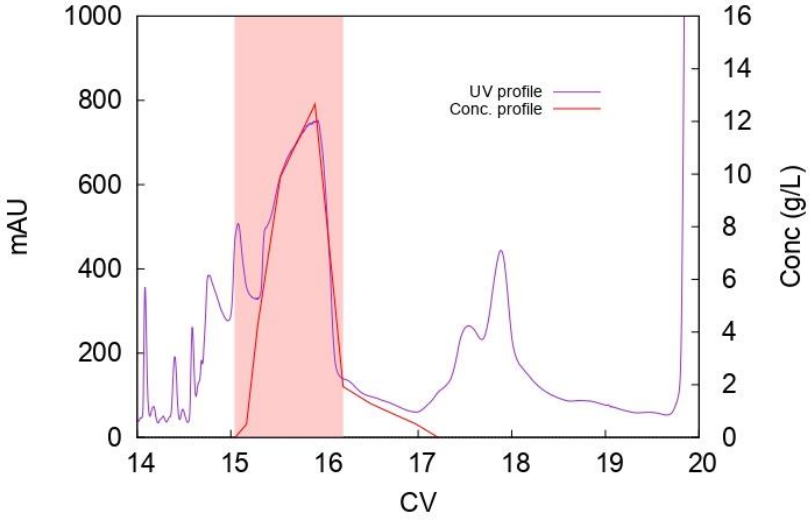
which is the mass of the peptide contained in a fraction divided by the total peptide mass injected.

Both recovery and purity were calculated as percentage.

Figure 1a shows the preparative chromatogram, zoomed between 14 and 20 CV elution, obtained by using the traditional reversed-phase column (C8), in orange, and the concentration profile of liraglutide along the gradient in green. Right before CV = 20, the UV signal increases sharply because the gradient has ended and the stripping procedure is being performed. As can be seen, the peptide concentration profile is very broad and tailed. On the other side, the main peak is much less tailed when using a mixed-mode column (C8+ 10% of cation-exchange groups), as shown in Figure 1b, where the UV profile is in violet and the concentration profile is shown in red. By comparing the two concentration profiles, it can be noted that, with the doped column, the whole peak elutes in about 2 CVs; whereas with the C8 column it elutes with in 3.5 CVs, results that are much broader.



(**a**)



(**b**)

Figure 1. UV profile and concentration profile of the target peptide using (**a**) a C8 column and (**b**) a mixed-mode column. The coloured regions represent the pooled windows (see the text for further details).

Beside being narrower, the peak also elutes earlier when using the mixed-mode column, as can be seen by superimposing the two chromatograms (see Figure 2). This is due to the repulsive effects established between the charged ionic groups on the stationary phase and the peptides, both negatively charged. Indeed, the pH of the mobile phase, which was 8.5, was higher than the isoelectric point of liraglutide (4.9), so the peptide exhibits a negative charge in these chromatographic conditions. Therefore, in these experiments, the column was employed in attractive–repulsive mode. Beside the main peak, the decrease in retention using the doped column is also quite evident when considering a late-eluting

group of impurities. These species elute just before the stripping phase, around 19 CVs, with the C8 column, but they elute earlier, between 17 and 18 CVs, with the mixed-mode column.

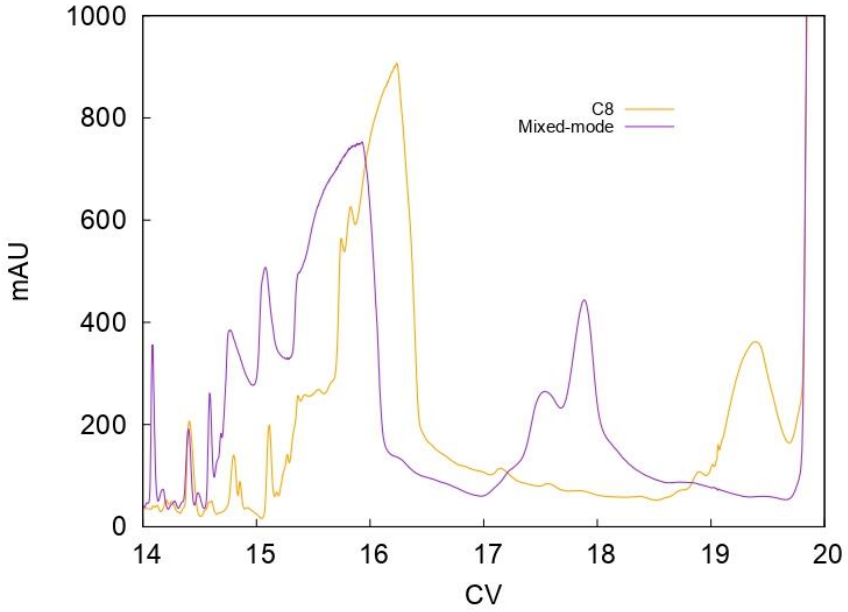


Figure 2. Comparison between chromatographic profiles obtained with C8 (orange) and mixed-mode (violet) columns. The main peak and other species elute earlier because of the repulsive effects established between doping ligands and the feed components, both negatively charged.

For each column, a so-called Pareto curve was also obtained, starting from the offline analysis of the fractions collected along the gradient. This curve reports the variation in purity with recovery [2,16,28]. These two parameters vary inversely: the purest fraction of the peak only contains a small percentage of the peptide mass injected. On the other hand, when the collection window is broadened, the recovery increases, but some side impurities are collected together with the target compound. This results in a decrease in the purity. The Pareto curve is obtained by considering first the purest fraction, which also exhibits the lowest recovery, and then pooling it with the purest adjacent fraction. This pool is then, again, pooled with the purest adjacent fraction in order to increase the recovery, and so on and so forth until the whole target peak has been collected. This corresponds to the case where the purity is as low as possible.

The Pareto curves obtained using the two columns with the same preparative method as described in Section 2.3 are shown in Figure 3. It is worth noting that, at very high values of purity, the Pareto curve obtained with the mixed-mode column lies above the curve obtained with the C8 column. This means that, in this particular region of the Pareto curve, the doped column allows for higher purities than the C8, with the recovery being similar. A similar behavior is also present at very-high recoveries, even if to a lesser extent. In the central region, however, the purity results are higher for the C8 column. The reason why, when using these particular conditions, the doped column Pareto curve does not lie above the other curve for the whole range of recovery could be that the charged peaks result is much narrower but they also elute closer to each other because of the attractive–repulsive effect. For instance, the late-eluting peaks on the right of the target peak possibly overlap with the tail of liraglutide in the case of the mixed-mode column.

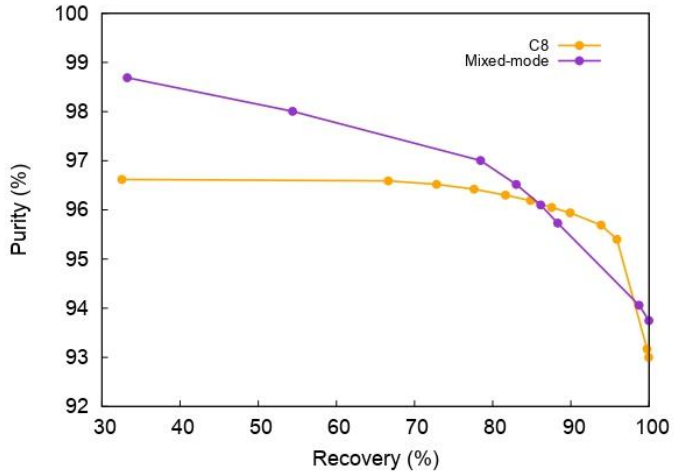


Figure 3. Comparison between the Pareto curves obtained with the two columns in the same chromatographic conditions (C8: orange; MM: violet).

One of the major advantages of using the mixed-mode column is the peak shape obtained, which is much-narrower and less-tailed. From the industrial point of view, after deciding the purity limitation that the pool collected must fulfill, the side regions of the main peak, which overlap with close-eluting impurities, must be reprocessed, in order to not waste large amounts of the target peptide. It is clear that this further purification step is not beneficial in terms of time, cost and waste. Therefore, an interesting option that shows the benefits provided by the doped column would be to collect the main peak excluding the tail, as highlighted in the chromatograms in Figure 1a,b. The performance obtained for the two columns when pooling this portion of the chromatogram are reported in Table 1. It results that, with the mixed-mode column, the purity can be slightly improved (+2.5%) while the recovery increases by +15%. A direct consequence is that a smaller percentage of the target product must be reprocessed in a following purification step when using the mixed-mode column (about half, with respect to the C8 column), which corresponds to the peptide recovery of the the liraglutide peak tail discarded.

Table 1. Performance parameters obtained with the two columns when pooling the target peaks with the exception of their tails, and amount of peptide that must be reprocessed in both cases because of liraglutide under the discarded tails.

	Recovery (%)	Purity (%)	Amount to be Reprocessed (%)
C8 column	77.6	92.8	22.4
Doped column	88.9	95.0	11.1

Alternernatively, if the whole peak satisfies the purity requirements, it can be entirely collected. In this case, it elutes completely in 2 CVs with the mixed-mode column and 3.5 CVs with the C8 column. This results in a pool with a higher concentration, in the first case. In addition, the duration of the method could be reduced and the gradient could be stopped after the main peak elution, with a considerable saving of time and solvents.

4. Conclusions

Mixed-mode resins are alternative, promising stationary phases that exploit two retention mechanisms generally based on hydrophobicity and charge interactions. Their use can be particularly beneficial when dealing with complex mixtures such as peptide crude samples, for which more traditional stationary phases do not allow to obtain enough chemoselectivity. At pHs above the peptide isoelectric point, a repulsive effect is estab-

lished between the target compound and the charged cation-exchange ligands doping the stationary phase, which are both negatively charged. As a result, the peaks become narrower and their retention decreases; in addition, the main peak is much less-tailed.

In this study, an industrial crude liraglutide mixture was purified with both a C8 column and a C8 stationary phase doped with 10% cation-exchange groups. It was shown that, if collecting the whole peak or if collecting the purest fractions, the mixed-mode column shows slightly better performance with respect to the C8. The most relevant aspect is that, when the target peak is collected by discarding the tailed part, the mixed-mode column allows to increase the recovery by +15%, at a slightly higher purity. From an industrial viewpoint, it is convenient for the target peak to be as little tailed as possible, in order to minimize the side portions of the peak, which must be recycled in a subsequent purification step. This leads to an increase in productivity and to an improvement in the overall process performance, also from the point of view of the greenness of the process, since smaller solvent amounts would be required. This research is to be intended as a proof-of-concept study for the particular purification of liraglutide, but similar outcomes are expected also for different crude-peptide mixtures when using these resins.

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Paper IX

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Enrichment and recovery of oligonucleotide impurities by N-Rich twin-column continuous chromatography

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ABSTRACT

N-Rich is a twin-column continuous chromatography technology well suited for small-scale isolation and the enrichment of product related impurities. For the first time, N-Rich was used for impurity isolation from a double-stranded RNA (dsRNA) therapeutic synthetic oligonucleotide (ON), produced by solid-phase synthesis. By employing the N-Rich process, where the desired impurities are recycled and selectively enriched, and interfering substances are depleted, it was possible to obtain substantial amounts of high purity marginal impurities with a reproducible, automatized, and productive method. The productivity-purity tradeoff inherent to traditional impurity isolation methods, i.e., analytical chromatography, was effectively alleviated. Using N-Rich, satisfactory purity values and mass recoveries of several low-concentrated impurities could be obtained simultaneously.

A performance comparison demonstrated an up to 15-fold increase for purity values and up to 20-fold mass impurity isolation and concentration with the N-Rich technology in comparison to conventional isolation procedures, drastically reducing processing times, manual handling, and waste production.

1. Introduction

Synthetic oligonucleotides (ONs) are short nucleic acid chains able to control the expression of target genes and accordingly modify expression of disease-related proteins that are normally inaccessible by small molecules and proteins. After overcoming some major hurdles (e.g., poor pharmacokinetics, ineffective target delivery, insufficient biological activity, off-target toxicity) therapeutic ONs are now going through a tremendous growth in the biopharmaceutical sector [1,2]. An increasing number of synthetic ONs are being developed for a wide range of research, diagnostic and therapeutic applications. To date, 13 ON-based therapeutics have been approved by the FDA and, as reported on ClinicalTrials.gov, more than 180 ON-based candidates have been approved for clinical trials for various applications [3,4].

Synthetic oligonucleotides comprise several classes of chemical entities differing by their chemistry and structure–activity relationship (SAR): small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO) and aptamers, to name a few [5]. These are typically generated using solid-phase synthesis, a cyclic approach, where the progressive addition of nucleotide monomers eventually results in a polymeric chain of predefined structure and length, *n* [6]. Despite a high coupling yield, solid-phase synthesis generates many structurally closely related impurities, among which are shortmers (e.g., *n-1*, *n-2*), resulting from incomplete coupling reactions, longmers (e.g., $n + 1$, $n + 2$), and other by-products coming from deamination, depurination, oxidation and other side reactions [7]. Moreover, diastereomers are frequently formed due to the chirality of sulfur atoms presents on phosphorothioate oligonucleotides, the widest adopted backbone modification [3].

A mandatory aspect of the pre-clinical drug development of biopharmaceuticals is the isolation and characterization of impurities for further structural, biochemical, and toxicological elucidation, and cell or animal-based safety assays [8]. Currently regulatory agencies have yet to provide definitive reporting, qualification, and identification threshold values for ON impurities. However, a series of white papers from Capaldi et al. regarding the regulatory environment of oligonucleotides suggested the values of 0.2 % as reporting, 1 % as identification and 1.5 % as qualification limits [9,10,11]. However, it is anticipated that these limits will become more stringent as higher performing processing technologies are standardized.

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Prior to characterization, isolation of product related impurities is currently carried out using inefficient and time-consuming methods. A standard approach is the application of linear scale reversed phase (RP) or ion exchange (IEX) chromatographic methods, either using analytical U(H)PLC or preparative systems linked to a fraction collector [12,13]. The operator faces a tradeoff between productivity and purity, typical of single column chromatography [14–17]. These methods operate in the linear range of the adsorption isotherm, thereby achieving a high resolution and minimizing overlap between components, and thus obtaining high purity values. However, this comes at the cost of restricted injection amount per run (micrograms), very low final target concentration, and prolonged processing times due to the need to pool target material from multiple chromatographic runs.

Given the limitation of standard methods, employing the N-Rich process, a twin-column continuous chromatography technique, we can potentially alleviate bottlenecks with the result of providing larger amounts of high purity product-related impurities in a short operation time [18–20]. This technology was already successfully applied for the enrichment of antibody isoforms and peptide impurities [12,14]. This work describes the design and operation of the twin-column N-Rich process, applied for the first time to a therapeutic oligonucleotide, a 24-mer double-stranded RNA. Operating under reversed phase conditions, the process performance and product quality obtained with N-Rich and semi-preparative batch processes were evaluated and compared.

1.1. The N-Rich rationale

Continuous chromatography is increasingly used by industrial separation specialists as a scalable manufacturing technology capable of reducing bottlenecks in downstream processing of biopharmaceuticals in capture and polishing applications [21–24]. In contrast, application of the technology in product development for reducing bottlenecks in the generation of impurity standards or other difficult-to-purify or low abundant compounds, is relatively new. N-Rich is an automated process using two identical columns to enrich and purify a desired compound from a complex mixture. Conventional resin material can be used for separations with N-Rich, such as ion exchange (IEX), hydrophobic interaction (HIC) or reversed phase (e.g., C18). N-Rich can be set up to target a single compound, or a region of the chromatogram containing several compounds. The N-Rich process is composed of four steps (Fig. 1). The first method (Startup) begins by loading feed material onto the first column and performing a linear gradient elution. During the second method (Enrichment), a region of the chromatogram containing target impurities is transferred from the first column, with in-line dilution, and re-adsorbed to the second column. All fractions not intended for recycling, including main product and other side-impurities, can be either discarded in waste or, if valuable, separately collected for other purposes. In the meantime, fresh feed is loaded onto the second column in addition to the recycled target. This step leads to an enrichment of the target molecules relative to other compounds in the mixture. The process step is repeated in a cyclic fashion between the two columns, progressively increasing the concentration of the target impurities. Phase three (Depletion) is a single switch without addition of new feed. This step depletes non-target compounds while internally recycling the accumulated target impurities before the final elution step. The depletion step greatly improves the final purity obtained for impurities that are closely eluting with the product peak. Finally, in the fourth phase (Final Fractionation), the enriched target material is eluted with a shallow gradient over two columns in-series and the target material is collected performing a fine fractionation. This strategy maximizes the resolution of the enriched compounds, and the pure target material is recovered at a higher concentration than with batch methodology.

The advantages of N-Rich over analytical and batch-wise elution include reduced processing times and consumables, in addition to increased final resolution, recovered mass, and purities. Furthermore, the automatization of the recycling and elution processes requires fewer operator activities, e.g., pooling, analyzing, merging, and reprocessing fractions, and consequently reduced operational footprint and qualified personnel time consuming [16,17].

2. Materials and methods

2.1. Chemicals

HPLC-grade acetonitrile (ACN) and methanol (MeOH) from Merck Millipore (Darmstadt, Germany) in addition to deionized water from a Milli-Q Advantage A10 were employed as mobile phases. An ion-pairing system TEA/HFIP was added, prepared with triethylamine (≥99.5 %) and Hexafluor-2-propanol (≥99.5 %), both purchased from Sigma-Aldrich (St. Louis, MO, USA). The feed material was a 24-mer double-stranded RNA oligonucleotide, synthetized by means of solid-phase synthesis, solubilized in 30 % ammonium hydroxide at the concentration of 44.8 g/L with a chromatographic purity of 75.6 %. The feed was diluted to 0.5 g/L with preparative chromatography buffer A.

2.2. Preparative chromatography conditions

Batch-wise and N-Rich chromatographic experiments were performed on a Contichrom® CUBE 30 continuous chromatography system (ChromaCon AG, A YMC Company, Zurich, Switzerland). The system was equipped with a fraction collector. Mobile phases were kept at a constant temperature of 60 °C using an AZURA® CT 2.1 column thermostat from Knauer (Berlin, Germany). The solvents used for preparative chromatography were as follows: 0.2 % ACN, 0.2 % TEA, 1 % HFIP (Buffer A); 1.66 % MeOH, 15 % ACN (Buffer B); a stripping buffer of 5 % MeOH, 45 % ACN (Buffer C). Two Triart Prep C18-S, 12 nm-10 µm 4.6 mm i.D. × 150 mm columns from YMC (Kyoto, Japan) were used as stationary phases. The UV signals were monitored at 280 nm.

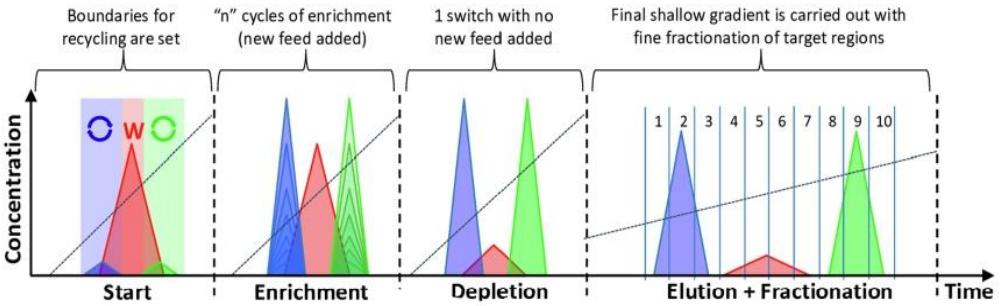

Fig. 1. The N-Rich process automatically enriches and isolates compounds in fractions that are initially highly dilute. This is done in four methods: 1) Start; 2) Enrichment of target compounds (blue, green); 3) depletion of interfering compounds (red); 4) Final elution with fractionation.

2.3. Offline analytics

Feed analytical characterization and pooled fraction evaluations were conducted by injecting 2 µL sample volume on a 1290 Infinity II LC System (Agilent, Santa Clara, CA, USA) equipped with an Acquity UPLC Oligonucleotide BEH C18, 1.7 µm, 2.1 mm i.D. × 50 mm column (Waters Corporation, Milford, MA, USA). Mobile phase A () was constituted of 0.2 % TEA, 1 % HFIP in water whilst mobile phase B () was a solution of 10 % MeOH and 90 % ACN. The mobile phase flow was set at 0.3 mL/min, the signal recorded at UV 260 nm wavelength and the column thermostat set to 62 °C. The analytical protocol began with a one-minute initial equilibration at 1 %B, followed by a four-minute gradient from 1 %B to 6.5 %B and a ten-minute gradient from 6.5 %B to 8.5 %B. Finally, after three minutes of stripping the stationary phase with 30 %B, the percentage of was decreased to the initial value.

2.4. Design batch chromatogram

The first step to design a continuous enrichment process is to select a single column batch "design chromatogram". The batch protocol, in this case serving both the purpose of representing the single column benchmark and the reference method for the continuous chromatography setup, included a 3 column volumes (CVs) equilibration with Buffer A, a 0.2 CV feed load (0.25 g per liters of resin load) and a 2 CVs wash after load with Buffer A performed at 300 cm/h. Thereafter, a 200 cm/h flow rate was used to perform a linear gradient elution from 15 % to 70 % of Buffer B in 12 CVs. Eventually, a resin regeneration step with strip Buffer C and a re-equilibration procedure with Buffer A were applied for 2 and 3 CVs, respectively, at the increased flow rate of 600 cm/h. During the elution stage, a fine fractionation was performed to both characterize the impurities in order to establish suitable recycling windows for the N-Rich process.

2.5. N-Rich operating parameters

The N-Rich process uses the same column, solvents, and elution protocols as the single column preparative batch process. By means of the N-Rich design software (N-Rich wizard) included with the ChromIQ® operating software of the Contichrom® CUBE system, the previously obtained batch "design chromatogram" is imported and used for process design. At this stage, based on the findings from the analytical evaluations of the batch experiment, the recycling windows for the impurities to be accumulated are selected. Therefore, the chromatogram is divided into 4 zones (see example in Fig. 2) using five section borders (-). They correspond to the switching times that compose the N-Rich elution protocol. In particular, corresponds to the modifier gradient starting time and it is employed by the wizard to set dilution parameters, is the trigger point where the elution of the weakly adsorbing impurities begins that are internally recycled to the second column. The window defined by and corresponds to a region of nearly pure main compound, which undergoes depletion at each switch. However, it is worth noting that the pure product region could have been also collected instead of discarded and used for other purposes. Eventually, in the to interval, strongly adsorbing impurities are eluted and inline diluted before entering the second column. Inline dilution ensures re-adsorption of the target compound in the downstream column by lowering the modifier concentration. Simultaneously, corresponds to the end of the elution stage and the begin of the stripping procedure. In specific, the operational switching times were as follows: t_1=14.8 min, t_2=31.3 min, t_3=52.8 min, t_4=54.3 min and t_5=64.3 min.

Once section borders for impurity recycling and product removal are configured, the wizard automatically determines the load per switch, the in-line dilution factors for the recycling phases, and the gradient start and end concentrations of each section to be operated by the CUBE system pumps. For each switch, the elution sections enclosed between and (weak recycling region) and and (strong recycling region) were in-line diluted and transferred to the relative downstream column.

The wizard computes the inline dilution flow rates for this operation. However, taking into consideration bed height and pressure constraints, the operator can autonomously optimize this feature to reduce processing times and thus increase productivity. Selected values were respectively 1.17 mL/min and 1.22 mL/min for W and S compounds.

Moreover, with the aid of the Dynamic Process Control, i.e., a UV-based software control part of the ChromIQ® operating system, it is

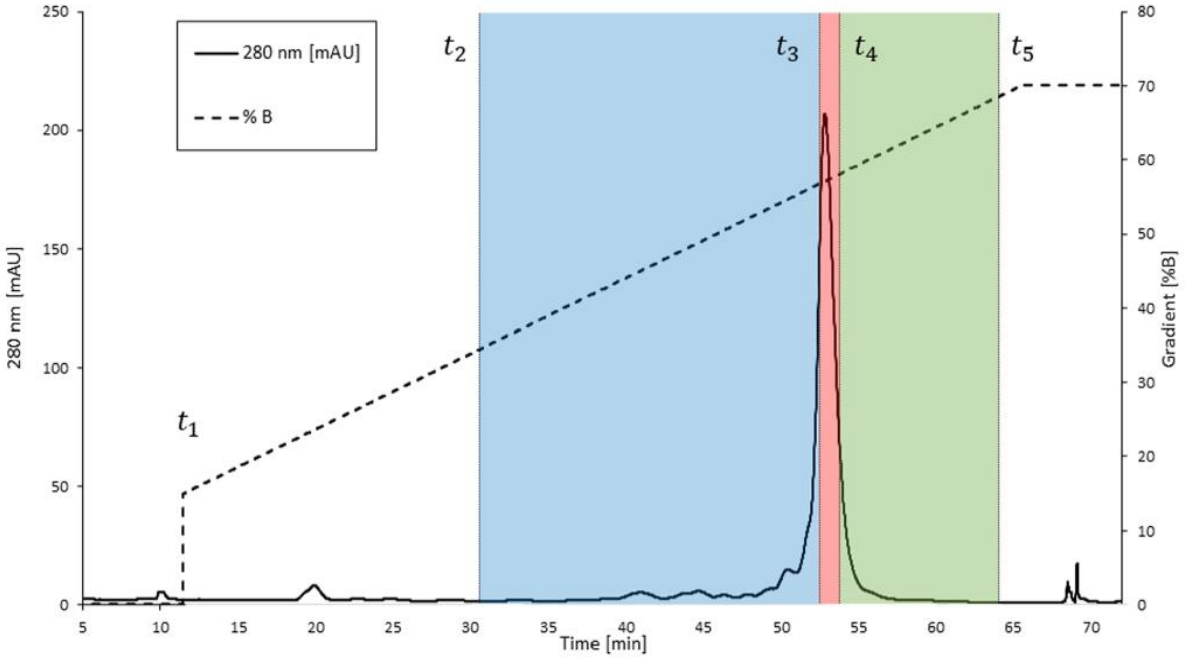

Fig. 2. Batch chromatogram. The colored regions are determined with the selection of section borders (t_1-t_5): in blue the weakly adsorbing impurities region, in red the region containing the pure product and in green the strongly adsorbing impurities area.

possible to establish a threshold intensity value which triggers product removal by UV threshold. This feature is introduced so that any shift in product retention time due to column aging, buffer variabilities, and other possible inconsistencies over multiple cycles, would be compensated for and have no impact on process performance. In this way, consistent product quality and yields are ensured. To achieve maximal reproducibility, product removal was triggered with UV-based dynamic process control, at an intensity value of 200 mAU. The accumulation step was performed for a total of 10 cycles. Upon finalization of the design parameters, the N-Rich wizard automatically creates the operating methods described in the N-Rich rationale section (Startup, Accumulation, Depletion & Elution). [11].

3. Results

3.1. Batch chromatography

In the investigated case, the starting material of a preparative chromatographic polishing process (AIEX) contained 22 % impurities which were reduced to 7 % by the polishing step. Despite the well optimized process, several impurities exceeded reporting thresholds, and therefore qualify for further characterization according to regulatory guidance. In this current example, N-Rich was designed with the aim to accumulate not only a single compound, e.g., a closely eluting impurity, but rather a heterogeneous group of impurities above the threshold, for further characterization.

As a precursor to N-Rich design, the first step was to generate a single column batch chromatogram with sufficient resolution using the Contichrom CUBE (Fig. 2). In the current drug development process, impurities are pooled from multiple high-resolution batch runs for further characterization. For this reason, the preparative batch chromatogram, besides being a template for the N-Rich experimental design, was also used as performance benchmark. Batch chromatography conditions are described in section "2.2 Preparative Chromatography Conditions" and the batch chromatogram is shown in Fig. 2. Fractions generated during the experiment were evaluated with analytical HPLC in order to qualify the separation capabilities of single column chromatography and to highly resolve and identify the contained species, generally pure oligonucleotide chain and structurally similar species. Close-eluting compounds, including shortmers, longmers and other structurally related impurities, require deep characterization and understanding.

Therefore, based on analytical evaluations and on relative retention times and purity values, two different recycling zones of the design chromatogram, comprising large amounts of close-eluting species, were defined by means of the ChromIQ® software, as illustrated in Fig. 2. A weakly adsorbing impurity zone (W), highlighted in blue and enclosed between min 31.3 - 52.8 min (t_2-t_5), containing more weakly adsorbing compounds than the pure oligonucleotide chain. A strongly adsorbing impurity zone (S), highlighted in green and enclosed between 54.3 - 64.3 min (t_4-t_5), conversely populated by impurities more strongly adsorbed on the stationary phase. Finally, a zone (P) is shown in red, where the analytical evaluations delivered the highest purity values for the main oligonucleotide product. During the continuous chromatography run development, such recycling zones were selected across a wide temporal range for both weak and strong adsorbing impurities, with the final intent to collect in a single run as much as possible enriched byproducts species.

3.2. Continuous chromatography

The N-Rich process was developed starting from the design batch chromatogram and the relative offline analytics data. The triggering

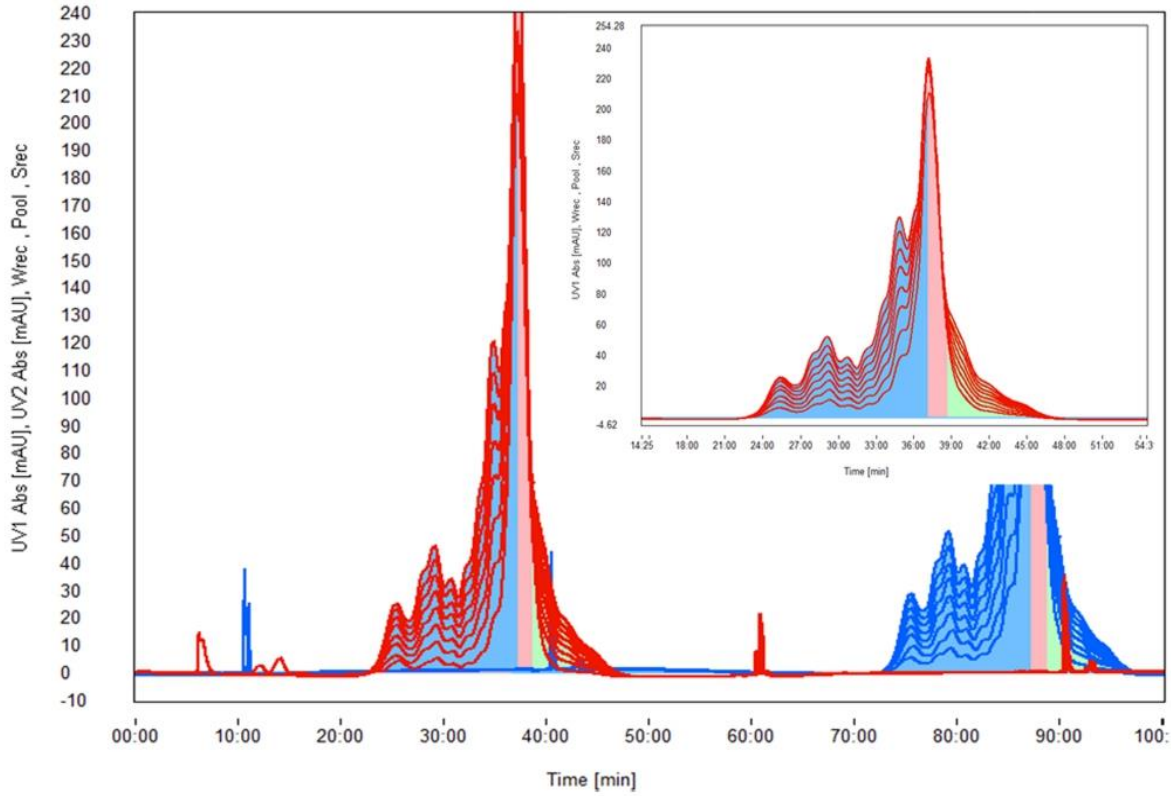

Fig. 3. Accumulation phase in N-Rich. The signals of column 1 are shown in red, with a zoomed perspective on the upper right corner, and the signals of column 2 are shown in blue. A progressive and gradual increase of the concentration of the target compounds from cycle to cycle is demonstrated. Interfering substances are instead constantly depleted and reduced in the overall percentage.

times for recycling and the rationale behind their selection are introduced in the previous section. Fig. 3 shows the outcomes of the accumulation phase: a nearly perfect match in elution profiles and progressive growth of all impurities chromatographic profile in the recycling portions was achieved. Meanwhile, the main compound region, devoid of critical impurities, was successfully removed at each switch and consequently resulted at the same intensity values of a single column batch run. In other words, cycle after cycle, the undesired substance (pure oligonucleotide chain) was depleted from the system, whereas targeted substances (impurities) were isolated and loaded, previous inline dilution, on the respective downstream column. The steady removal of the main compound prevents non-linear effects, such as displacement effects, which could negatively impact the isolation. Once the "Accumulation" cycles were completed, the "Depletion" method was applied. This method corresponds to a switch without loading new feed and thus leads to further depletion of any excess non-target compounds that would otherwise contaminate the desired target impurities. Consequently, a major increase of the target compounds purities and their relative enrichment was obtained.

As illustrated in Fig. 4, the conclusive "Final Fractionation" phase comprising final elution and fine fractionation was executed. The elution gradient was designed to boost separation capacities, exploiting the whole stationary resin. Indeed, the two columns employed in the continuous recycling of components were positioned in-series and operated with a mobile phase flowrate of 100 cm/h, performing a 20 % to 60 % of in 25 CVs shallow gradient. Fractions were collected and their analytical characterization allowed to identify those containing impurities of a sufficiently high quality and to establish a comparison between the batch and continuous experiment outcomes.

4. Discussion

To compare N-Rich vs batch processes we measured the purity and the productivity of each approach. Purity is a major consideration in impurity collection since optimal characterization requires a high degree of purity for each compound. The purity value was calculated from the analytical chromatographic profiles of all the pooled fractions obtained with the preparative system. The second parameter measured was the process productivity, i.e., the overall amount of time and chromatographic resources to achieve an adequate impurity mass recovery. In fact, not only a satisfactory degree of purity is required, but also enough material to fully characterize with multiple techniques.

A graphical evaluation of the N-Rich outcome is shown in Fig. 5 where the analytical chromatogram of the feed material is presented (A) followed by its overlay with the chromatograms obtained injecting equal amount of material evaluating several fractions obtained during the final elution phase containing the enriched impurities (B). In the latter graph, singular peaks of high intensity, each representing an isolated target impurity, can be identified both on the weakly and strongly adsorbing regions of the chromatogram. This graphical comparison clarifies the enrichment capabilities of the continuous chromatography approach. The throughput of linear isotherm range HPLC separation (Fig. 5A), with minor substances barely reaching detection limits, is outperformed by the N-Rich technology which progressively accumulated targeted substances and allowed their final separation and collection. In this regard, the reader should note as well how the regions outside the recycling windows were not subjected to peak intensity grow, demonstrating the technique selectivity and precision.

For the numerical comparison eight impurities isolated both batch-wise and towards continuous chromatography were chosen. Table 1 shows their retention time and relative values obtained from purity and mass recovery assessments. For each listed compound, identified by its retention time, the highest purity values obtained via HPLC fraction analysis were selected as representative of batch or N-Rich isolation capabilities. Once selected the fractions containing the highest value of purity of each of the eight compounds, we then proceeded estimating their concentration, i.e., the mass of material dissolved in the pooled eluate. To do so, the intensity of the peak obtained in the analytical evaluation was introduced in the calibration line previously built with a standard of the feed material. The linearity characteristics in the

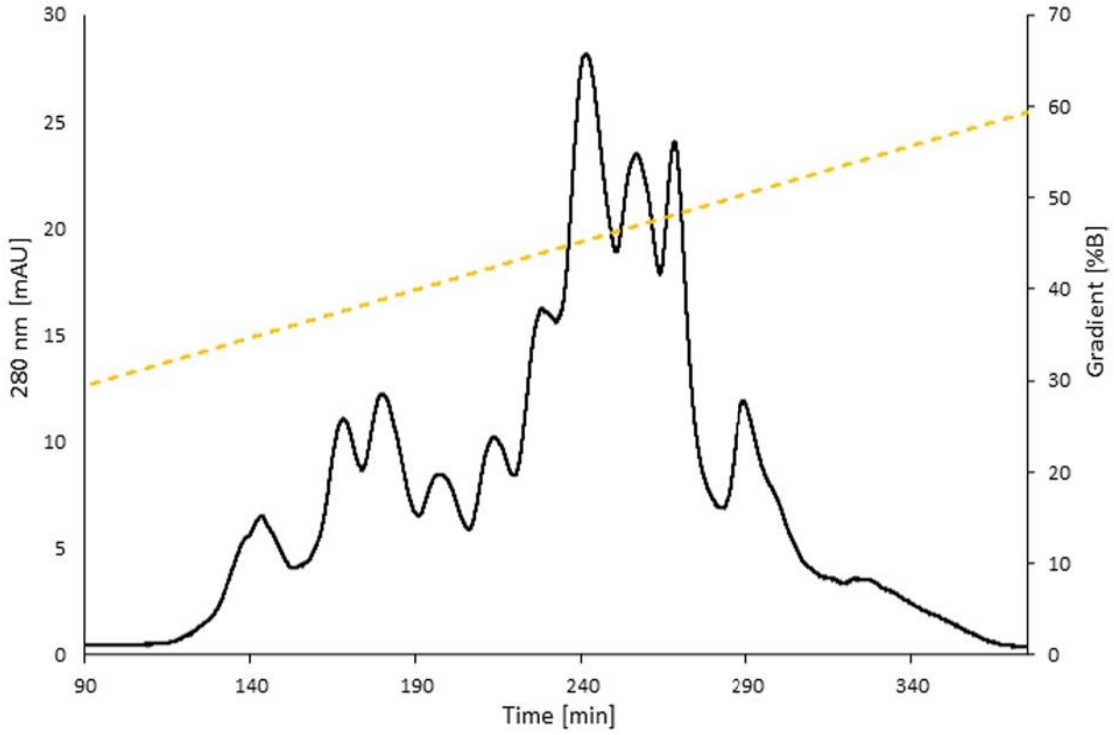

Fig. 4. Final Fractionation phase. The enriched regions are eluted using a shallow gradient over both the column in series applying a shallow gradient and a fine fractionation.

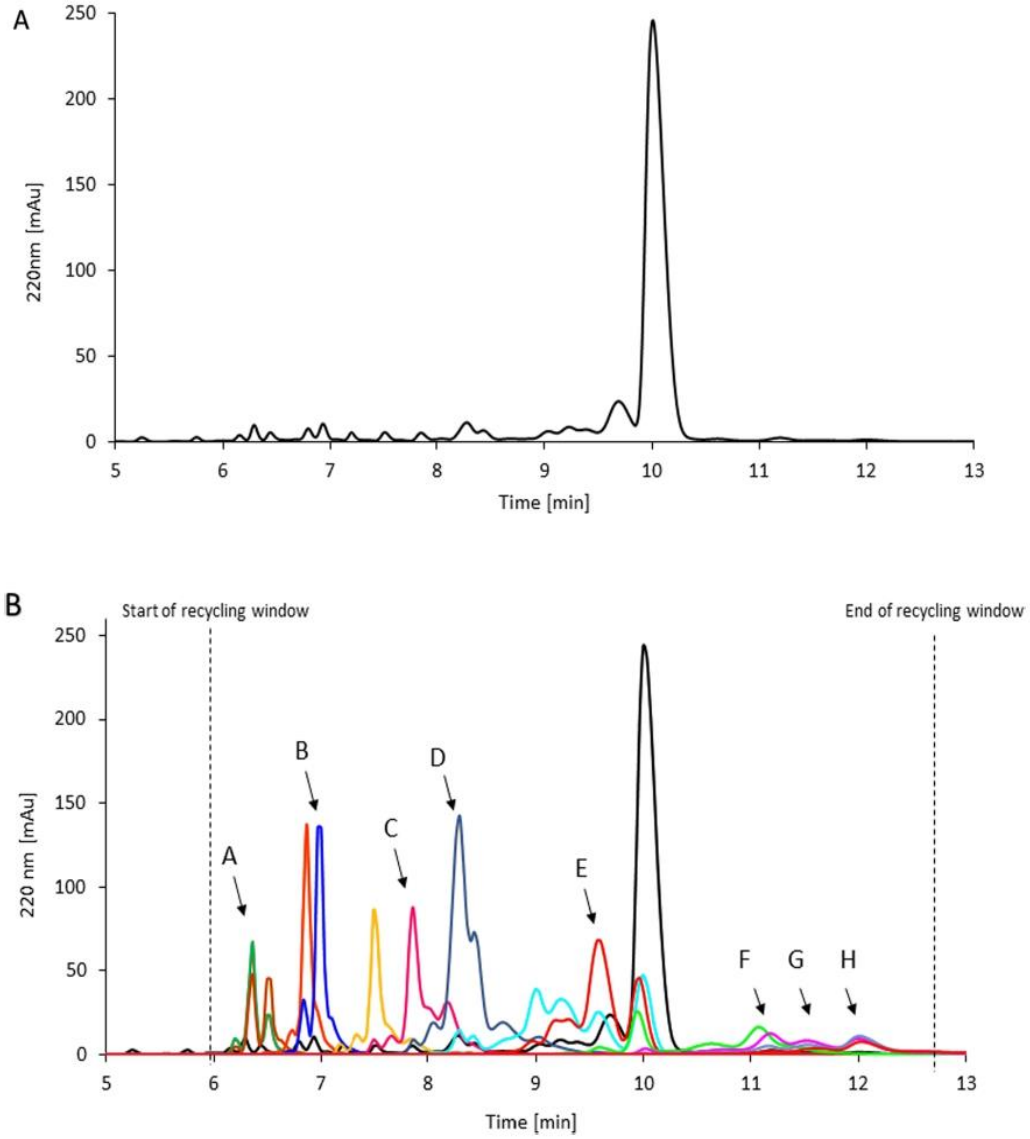

Fig. 5. A) Analytical chromatogram of the 24-mer oligonucleotide feed B) Overlay of the feed analytical chromatograms (black) and of the enriched impurities chromatograms.

Table 1
Purity and concentration performance of batch and N-Rich processes carried out on the CUBE system with the same stationary resin and mobile phase buffers.

Compound	Retention time (min)	Batch		N-Rich	
		Purity (%)	Concentration (μg/mL)	Purity (%)	Concentration (μg/mL)
A	6.3	25.2	1.85	60.8	6.58
B	7.2	11.9	0.46	59.9	6.81
C	7.7	20.9	1.81	59.8	17.02
D	8.2	12.9	1.11	53.7	21.57
E	9.5	21.1	15.61	49.3	19.38
F	11.3	3.6	1.27	49.5	5.74
G	11.6	1.5	0.15	22.8	2.48
H	12.2	4.6	0.46	38.3	3.69

analytical range of different byproducts were approximated to be constant. Fractions obtained by means of continuous chromatography were characterized by higher purity values. For instance, Compound A (6.3 min.) and Compound B (7.2 min), both weakly adsorbing impurities, were respectively pooled with purities 2.5-fold and 5-fold higher using continuous chromatography. Even superior outcomes were achieved with Compound G (11.6 min.), a minor strongly adsorbing impurity, which was obtained 15-fold purer in comparison with Compound G fractions from batch runs. Indeed, the molecule chromatographic profile completely overlaps with the main product and other strongly adsorbed impurities to the point that with normal batch processing the highest purity achieved was only 1.5 %. This value was increased to 22.8 % by using N-Rich. Although it was not carried out in this study, it is conceivable that a more targeted N-Rich experimental set-up on this specific molecule would result in higher purity outcomes. Using a restricted recycling zone would result in a progressive enrichment of this singular compound with the simultaneous depletion of the other closely eluting impurities. The absence of these interfering substances would eventually boost the purity values in the "Final Fractionation" method for the specific impurity.

The comparison of impurities obtained between single column batch and N-Rich also demonstrates fraction concentrations up to 19-fold higher in N-Rich (Table 1). In this respect, the purest fraction containing Compound C was obtained batchwise at a concentration of 1.81 μg/

mL, while the corresponding fraction delivered by N-Rich contained 17.02 µg/mL. The available amount for molecule characterization was almost tenfold increased. A productivity scenario for the collection of 1 mg of an impurity is presented in Fig. 6. The times (days) provided were calculated considering the experimentally obtained throughput (mg) and the required duration to run each individual method. These factors were then normalized per column volume of stationary resin (CV). Since continuous chromatography is a scalable process, therefore feasible to be operated with larger stationary resin volumes consequently diminishing the required processing time per mg of compound without losing chromatographic resolution, it was possible to consider a scale-up scenario from 0.46 cm (CV = 2.5 mL) to 1.0 cm i.D. (CV = 11.8 mL) columns. With this normalization it was computed the number of runs, and consequently of time, required in order to separate and concentrate 1 mg of compound D (8.2 min). The N-Rich process would require 8 days of processing time. On the other hand, obtaining the same amount of material using semi-preparative batch chromatography and analytical chromatography would result in an operational time of 10 and 87 days, respectively. Additionally, for the same compound, the purity values obtained batch-wise were 4 times lower than those obtained with N-Rich, thanks to the progressive depletion of interfering substances at each cycle and to the final two column in series elution method. In theory, similar purity values could be achieved batch-wise with a single column or two columns in series process applying very shallow gradient or reducing the injected mass. However, this scenario would be counterbalanced by an exponential increase of the processing times and solvents requirement that would result in a drastically reduced the productivity. Purities reported in the graph are shown according to experimental data. For UHPLC, this value was omitted as it was not possible to collect the compound in question (or any other) with a sufficient concentration for re-analysis.

Another advantage of N-Rich that should be noted is the ability to control for process variability (e.g., column deterioration, variation in buffer composition, etc.) by means of the Dynamic Process Control ("Autopeak") supported by the Contichrom CUBE® system. Shifts in retention time are compensated by means of UV-triggered valve switching so that the main, non-target compound is consistently removed at the correct time. In contrast, the process variabilities can have a significant impact on the retention time of single column batch chromatograms resulting in the need to carefully pool fractions from individual runs.

In this study, impurity isolation and their relative purity assessment were not investigated with U(H)PLC. Despite the potential for purity values that could be in the range of those obtained with N-Rich or higher, the enormous processing time that this methodology would require to achieve a reasonable amount of product casts shadows on the actual implementation of U(H)PLC for production of impurity standards. In addition, the solvents requirements for the production of 1 mg compound G with N-Rich and U(H)PLC were calculated, and for the continuous set-up, 57 L of mobile phases would be required, while the hypothetical projection to produce 1 mg with U(H)PLC was approximatively 178 L of solvents, corresponding to a more than 3-fold increase.

5. Conclusion

In this research paper the advantages of continuous chromatography over semi-preparative batch and analytical scale chromatography concerning biotherapeutic impurities separation and collection for analytical characterization were presented. The innovative impurities enrichment method N-Rich was applied to a 24-mer double-stranded RNA (dsRNA) therapeutic oligonucleotide (ONs) and its outcomes compared with traditional impurities collection methods. The process was designed as a proof of concept aiming to recycle, amplify and collect a broad range of minor side-compounds through a completely automatized procedure.

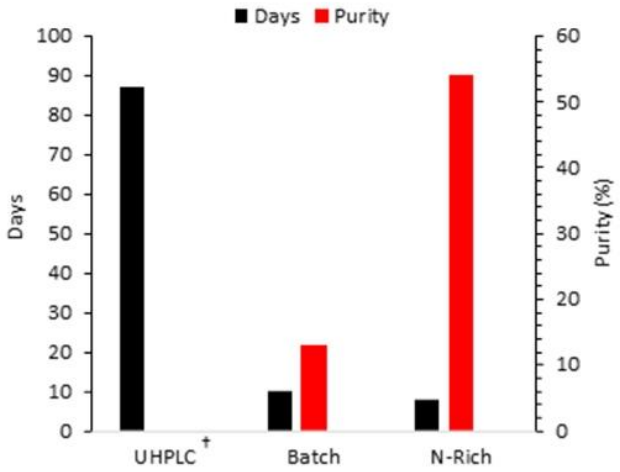

Fig. 6. Comparison of processing times (days), considering a scale-up scenario for batch and continuous experiments, from 0.46 cm i.D. to 1 cm i.D. columns, and achieved purity values (%) obtained through U(H)PLC, batch single column chromatography and continuous chromatography (N-Rich). The time was calculated for producing 1 mg of the impurity compound D. (see Table 1). † Purity values were not assessed for the U(H)PLC technique, in consideration of the extremely unfavorable processing times.

N-Rich was proven able to deliver analytical impurity standards with greater efficiency than traditional techniques optimizing crude material processing, and minimizing the number of analytical evaluations, expensive and long concentration steps and reducing waste production in terms of solvents and consumables (e.g., valuable feed material, collection materials). Furthermore, the chromatographic resins are preserved due to decreased processing times and the reduced number of harsh cleaning procedures. A remarkable reduction of time requirements in order to isolate the impurity standard compounds was demonstrated. Eventually, this technique leads to a positive effect on the constant need for qualified operators in carrying out time-consuming repetitive experiments. Moreover, it significantly reduces the need for manual handling, pooling, and sample storage and management. In the constant demand for a more efficient, effective, and greener biopharmaceutical production, continuous chromatography and the N-Rich process offer a powerful and robust tool.

CRediT authorship contribution statement

Giulio Lievore: Investigation, Data curation, Writing – original draft. **Richard Weldon:** Conceptualization, Resources, Writing – review & editing. **Martina Catani:** Data curation, Validation. **Alberto Cavazzini:** Formal analysis, Funding acquisition. **Thomas Müller-Späth:** Writing – review & editing, Supervision, Funding acquisition, Project administration.

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.

Data availability

Data will be made available on request.

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