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CYCLE XXXVI

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# Greening the downstream of biopharmaceuticals through process intensification in the research on innovative eco-compatible solvent

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# List of papers

This thesis is based on the results of the following papers obtained during the Ph.D. program, these are submitted or published papers. Reprints are appended at the end of the thesis.

**(I)** **Process Intensification for the Purification of Peptidomimetics: The Case of Icatibant through Multicolumn Countercurrent Solvent Gradient Purification**
C. De Luca, S.Felletti, D. Bozza, et al., MCSGP Industrial & Engineering Chemistry Research Vol 60, p. 6826-6834, ISSN: 0888-5885, doi: 10.1021/acs.iecr.1c00520

**(II)** **Downstream Processing of Therapeutic Peptides by Means of Preparative Liquid Chromatography**
C. De Luca, G. Lievore, D. Bozza, et al., Molecules, 2021, 26, p. 4688-1-4688-20, ISSN: 1420-3049, doi: 10.3390/molecules26154688

**(III)** **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, et al., SEPARATIONS, 2022, vol. 9, p. 125-1-125-9, ISSN: 2297-8739, doi: 10.3390/separations9050125

**(IV)** **Dimethyl carbonate as a green alternative to acetonitrile in reversed-phase liquid chromatography. Part I: separation of small molecules,**
S. Felletti, M. Spedicato , D. Bozza , C. De Luca , F. Presini , P. P. Giovannini , M. Carraro , M. Macis , A. Cavazzini , M. Catani , A. Ricci , W. Cabri , Journal of Chromatography A, 2023, doi: https://doi.org/10.1016/j.chroma.2023.464477

**(V)** **Dimethyl carbonate as a green alternative to acetonitrile in reversed-phase liquid chromatography. Part II: purification of a therapeutic peptide.**
D. Bozza , C. De Luca, S. Felletti, M. Spedicato, C. Nosengo, F. Presini , P. P. Giovannini , M. Carraro , M. Macis , A. Cavazzini , M. Catani , A. Ricci , W. Cabri , Journal of Chromatography A, 2023, https://doi.org/10.1016/j.chroma.2023.464530

# Published papers not included in this thesis:

**(I)** **Potency testing of cannabinoids by liquid and supercritical fluid chromatography: where we are, what we need.**

S. Felletti, C. De Luca, A. Buratti, D. Bozza, A. Cerrato, A. L. Capriotti, A. Laganà, A. Cavazzini, M. Catani, Journal of Chromatography A, 2021, 1651, p. 462304-1-462304-11. ISSN: 0021-9673 doi: 10.1016/j.chroma.2021.462304.

**(II)** **The Role of Adsorption and pH of the Mobile Phase on the Chromatographic Behaviour of a Therapeutic Peptide**

S. Felletti, C.De Luca, G. Lievore, A. Buratti, D. Bozza, M. Macis, A. Ricci, W. Cabri, A.Cavazzini, M. Catani, LCGC, 2021, Vol. 39, 14-20**.**

**(III)** **Recent developments in the high-throughput separation of biologically active chiral compounds via high-performance liquid chromatography,**

C. De Luca, S. Felletti, F.A. Franchina, D. Bozza, G. Compagnin, C.Nosengo, L. Pasti, A. Cavazzini, M. Catani, Journal of Pharmaceutical and Biomedical Analysis, 2023, 115794, ISSN 0731-7085, https://doi.org/10.1016/j.jpba.2023.115794.

# CONTENTS

# CHAPTER 1

# 1 INTRODUCTION

In recent years, climate change and environmental issues have increasingly sensitized society. Attention to these problems is supported by governments and international organizations that promote sustainable development programs, encouraging the transition to green industrial processes[1]. Research and industry are becoming more attentive to sustainable methods, materials, procedures, and technologies that can contribute to the application of the 12 principles of Green Analytical Chemistry (GAC) *Fig 1.1* [2], [3], [4], [5]. Solvents are an important part of the 12 principles. They are mentioned several times, especially in the fifth, "Safer solvents and auxiliaries," which calls for minimizing the use of solvents and limiting their use to harmless compounds. Furthermore, solvents are implicitly mentioned in the first, fourth, tenth, and eleventh principles, which consider waste production prevention, designing safer chemical substances by minimizing toxicity, and preventing the development of harmful substances.


*Figure 1.1 The twelve Principles of GAC*

These principles are based on reducing waste production, energy consumption, and volatile organic solvents. Large volumes of toxic, flammable, and/or hazardous solvents are used during chemical processes, and these properties are inherent in their application. The first step in replacing these harmful solvents has focused on synthesis processes, which have a greater environmental impact.

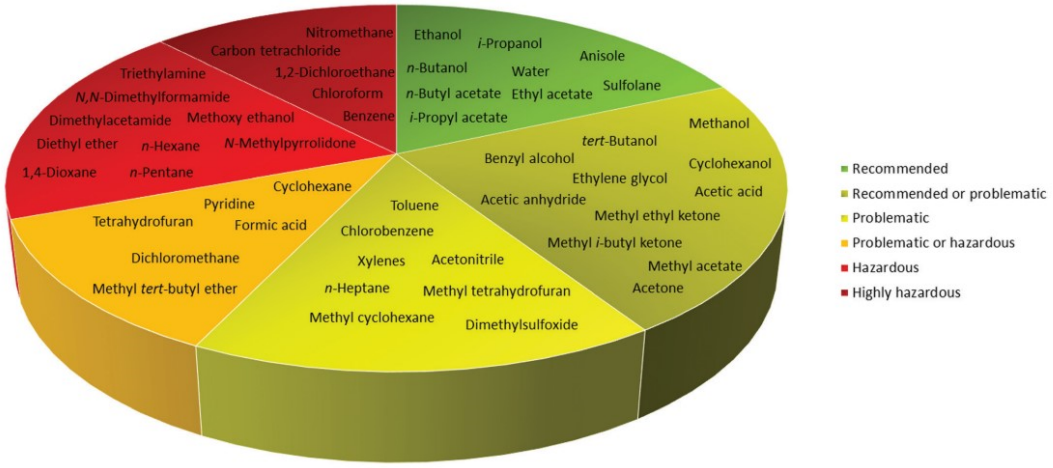


*Fig 1. 2 Overview of the most common solvent selection guides*

However, the interest and criticality of these solvents have involved all sectors of chemistry, including analytical chemistry. The interest of green analytical scientists is to reduce the use of organic solvents to avoid the accumulation of toxic chemical waste, such as the byproduct of chromatographic analysis. The pharmaceutical industry has focused on establishing true "rankings" of green solvents, a need that stems from the realization that the solvent is the primary component in a typical reaction during the production of an active pharmaceutical ingredient (API) [6], [7] (*Fig 1.2)*. This initiative is motivated by the realization that solvents play a key role in typical reactions in the production of active pharmaceutical ingredients. Criteria considered include safety, occupational health, environmental impact, quality, industrial constraints (such as boiling point, freezing temperature, density, recyclability) and cost. Several pharmaceutical companies have compiled solvent selection guides complete with data (*Fig 1.3).*

Pfizer has produced a concise two-page document that classifies classic solvents into three groups: preferred, usable, and undesirable. For undesirable solvents, the guide offers recommendations for substitution.

AstraZeneca's guide presents a table of 46 solvents rated on ten criteria, including two for safety, one for health and seven for the environment, incorporating life cycle analysis. Each criterion receives a rating from 1 to 10, accompanied by a colour coding system (green, yellow, and red) to facilitate analysis.

GlaxoSmithKline's guide follows a similar structure, including two safety criteria, one health criterion, three environmental criteria, and additional red flags, such as standards-regulated solvents. GCI-PR's solvent guide mirrors the formats of the AZ and GSK guides, with a table assessing one safety criterion, one health criterion, and three environmental criteria [6], [7], [8], [9], [10].

| Solvent | Astra Zeneca[a,b] | GCI-PR[a,c] | GlaxoSmithKline[a,d] | Sanofi[e] | Pfizer[f] |
|---|---|---|---|---|---|
| Methanol (MeOH) | 19 | 14 | 14 | | |
| Ethanol (EtOH) | 16 | 13 | 17 | | |
| i-Propanol (iPrOH) | 16 | 16 | 17 | | |
| *n*-Butanol (*n*BuOH) | 17 | 13 | 18 | | |
| *t*-Butanol (*t*BuOH) | 20 | 15 | 15 | | |
| Benzyl alcohol (BnOH) | — | 11 | 20 | | — |
| Ethylene glycol (MEG) | — | 13 | 21 | | |
| Acetone | 21 | 15 | 15 | | |
| Methyl ethyl ketone (MEK) | 21 | 16 | 15 | | |
| Methyl i-butyl ketone (MIBK) | 22 | 17 | 15 | | — |
| Cyclohexanone (CYC) | — | 14 | 20 | | — |
| Methyl acetate (MeOAc) | | 14 | 14 | | — |
| Ethyl acetate (EtOAc) | 18 | 15 | 16 | | |
| i-Propylacetate (iPrOAc, IPA) | 18 | 13 | 18 | | |
| *n*-Butylacetate (*n*BuOAc) | 13 | 14 | 21 | | — |
| Diethyl ether (DEE) | 27 | 21 | 3 | | |
| Methyl *tert*-butyl ether (MTBE) | 24 | 21 | 4 | | |
| Tetrahydrofuran (THF) | 23 | 16 | 4 | | |
| 2-Methyl tetrahydrofuran (2-Me-THF) | 24 | 15 | 11 | | |
| 1,4-Dioxane | 28 | 21 | 11 | | |
| Anisole (An) | 18 | 13 | 18 | | — |
| Dimethyl ether (DME) | 21 | 23 | 2 | | |
| Diisopropyl ether (DIPE) | — | — | 4 | | |
| *n*-Pentane (NPEN) | — | — | 7 | | |
| *n*-Hexane (NHEX) | 26 | 21 | 1 | | |
| *n*-Heptane (NHEP) | 21 | 17 | 14 | | |
| Cyclohexane (Cy) | 25 | 18 | 14 | | |
| Methyl cyclohexane (MeCy) | — | 17 | 16 | | |
| Benzene (Bn) | — | 21 | 1 | | |
| Toluene (Tol) | 22 | 18 | 11 | | |
| Xylenes (Xy) | 19 | 15 | 13 | | |
| Dichloromethane (DCM) | 20 | 18 | 5 | | |
| Chloroform ($CHCl_3$) | — | 18 | 4 | | |
| Chlorobenzene (ClBn) | 25 | 16 | 18 | | — |
| Carbon tetrachloride ($CCl_4$) | — | 19 | 3 | | |
| Dicyclohexyl ether (DCE) | — | 19 | 4 | | |
| Acetonitrile (ACN) | 24 | 14 | 14 | | |
| Dimethylformamide (DMF) | 20 | 17 | 7 | | |
| Dimethyl acetate (DMAc) | 20 | 16 | 4 | | |
| *N*-Methyl pyrrolidone (NMP) | 18 | 16 | 7 | | |
| Dimethyl sulfoxide (DMSO) | 8 | 15 | 14 | | |
| Sulfolane (Sul) | 9 | 13 | 21 | | |
| 1,3-Dimetil-3,4,5,6-tetraidro-2(1*H*)-pirimidinone (DMPU) | — | — | 14 | | — |
| Nitromethane | — | — | 1 | | — |
| Methoxy-ethanol | 21 | 20 | 3 | | — |
| Formic acid (HCOH) | 20 | 15 | — | | — |
| Acetic acid (AcOH) | 17 | 15 | 17 | | |
| Acetic anhydride ($Ac_2O$) | — | 16 | 15 | | — |
| Pyridine (Pyr) | 26 | 16 | 5 | | |
| Triethylamine (TEA) | 23 | 18 | 3 | | — |
| Water | — | — | 24 | | |

*Fig 1.3 The Sanofi and Pfizer guides use colour codes for the assessment of environmental friendliness. green = recommended; yellow = substitution advisable; red = disfavoured; dark red = forbidden.*

As it can be seen from the *Fig 1.3*, Acetonitrile for Astra Zeneca and GlaxoSmithKline is considered toxic and to be replaced but for Sanofi and GCI-PR is recommended, while ethanol for all the guides is considered safe. In the case of MeOH Astra Zeneca and GlaxoSmithKline guides agree that it would be more sustainable to replace it.

Liquid chromatography (LC) is used daily and is estimated to produce about 1.5 liters of waste per day, considering a common LC column (length 15-25 cm, diameter 4.6 mm, with a mobile phase flow of 1 mL/min). Approximately 26-50 million liters of chemical waste are produced each year [3], [4], [11]. Interest in green analytical methods is also growing in the field of biopharmaceutical production, especially in process intensification, i.e., the increase in the production of these compounds. The interest in this class of biomolecules is

due to their high specificity and the wide range of bioactivities they can trigger in the human body. Pharmaceutical peptides are increasingly used for treating symptoms associated with many diseases, including cancer, severe necrotizing soft tissue infections, hereditary angioedema, secondary hyperparathyroidism in people with chronic kidney diseases, etc. [7], [12], [13], [14], [15].

The separation and the downstream processes take place in Reversed-Phase LC (RPLC) conditions, which is the most common mode of quantitative and preparative applications. In RPLC is employed a hydrophobic stationary phase (usually made of silica particles functionalized with C18 or C8 chains) and the mobile phase is a more polar mixture of water and organic modifier. Acetonitrile (ACN) is the most employed for its excellent properties such as excellent strength elution, good miscibility in water, and UV transparency. However, from a green point of view, it is very unsafe for the environment and the human body due to the metabolic release of cyanide. For these reasons, the replacement of ACN with greener alternatives is becoming an urgent priority [9], [16]. The Environmental, Health, and Safety (EHS) describes the environmental indicators, health parameters, and safety specifications and it is used for the greenness of an organic solvent. Life cycle assessment (LCA) takes into consideration the effects of a product on the environment during the entire period, disposal, and potential recycling. LCA, unlike its counterpart, examines a product's impact on the environment throughout its entire lifespan—spanning production, usage, disposal, and potential recycling. To aid chemists in opting for eco-friendly alternatives, numerous pharmaceutical companies have crafted guides for selecting solvents, categorizing them based on their environmental impact [17], [18], [19], [20]. Noteworthy choices in these guides include ethanol (EtOH), isopropanol (IPA), acetone, ethyl lactate, and propylene carbonate, all recognized as green solvents and tested as eluents in RPLC [16], [21], [22], [23].

Recently, scientific attention has turned towards less mainstream alternatives like organic carbonates, known for their minimal eco-toxicity and complete biodegradability [24]. Dimethyl carbonate (DMC) is one such organic compound gaining prominence. As a nonpolar, flammable, transparent liquid with a scent reminiscent of methanol, it lacks irritating or mutagenic effects, ensuring safe handling. Extensively researched for applications across various product categories, DMC is particularly studied in the synthesis of pharmaceuticals, including taladafil [25], as well as in coatings and lithium-ion battery electrolytes [16], [19], [20], [21], [22], [23], [24], [25], [26], [27], [28].

In conclusion, the first approach to increase the greenness of the downstream processes is the replacement of toxic solvents with more eco-friendly ones. As mentioned, the bioactive molecules are purified under RPLC conditions using an ion-pair reagent added in the mobile phase. The ion pair plays a dual role in the separation process; it prevents ion exchanges by suppressing the ionization of unreacted silanols and charges the protein's basic side chain, forming ion pairs with the acid anion. Consequently, the retention of biopharmaceuticals is influenced by the addition of the ion-pairing agent and its concentration in the mobile phase. In liquid chromatography, Trifluoroacetic acid (TFA) has been a popular mobile phase additive for over two decades, serving not only for purification but also in peptide synthesis cleavage. However, its environmental impact is a concern, as it can result from the breakdown of numerous chemicals. TFA salts are stable and can accumulate in water bodies, posing potential hazards. Formic and acetic acids are considered more sustainable alternatives. Formic acid, especially, is advantageous for drugs and peptide-based therapeutics, as they often exist as acetate salts, eliminating the need for additional counterion exchange steps. Quaternary ammonium salts with phosphate counterions are emerging as alternatives in mobile phases. They offer UV transparency, enabling high resolution, loading, and recovery yields.

In batch liquid chromatography, maintaining a balance between purity, yield, and productivity poses a challenge. Overloading the column can decrease the resolution between the target product and similar impurities, impacting purity. This often results in a trade-off between purity and yield, as some impurities closely resemble the target, causing overlapping peaks. To address this, one can decrease sample volume or use less steep gradients, but this sacrifices productivity and increases solvent consumption. This purity-yield trade-off is a bottleneck in biopharmaceutical manufacturing. Continuous chromatographic techniques, involving counter-current movement of the stationary phase, offer a solution by reducing solvent usage. Additionally, exploring Supercritical Fluid Chromatography (SFC) shows promise, as it replaces organic solvents with eco-friendly options like carbon dioxide [7], [29].

### 1.1.1.Biopharmaceuticals

The terms "Biologics," defined by the Food and Drug Administration (FDA), and "Biologicals," adopted by the European Medicines Agency (EMA), refer to drugs derived from living cells or produced through biological processes. This categorization encompasses a diverse array of complex molecules, including proteins, carbohydrates, nucleic acids, genes, cells, blood products, monoclonal antibodies, cellular therapies, cytokines, and more.

In the 1980s, the term 'biopharmaceuticals' was coined to describe pharmaceutical substances that are inherently biological and produced using biotechnological methods. [30]. Widely used in both industry and the scientific community, this informal term encompasses various classes of compounds, including peptides and oligonucleotides. Despite their structural similarity or close relationship to physiological or natural compounds, these counterparts of biologicals are preferably synthesized through artificial rather than biotechnological processes. Despite some inconsistencies in nomenclature and internal differences, the entire category of biopharmaceuticals differs significantly from small organic molecules in terms of size, physicochemical properties, and production processes. Small molecules are typically between 0.1 and 1 kDa, while biologicals generally have a molecular weight above 1 kDa [30], [31], [32]. Small organic molecules represent the reliability of the pharmaceutical industry. Their structures are characterized by stability, discretion, and independence from variations in raw materials and manufacturing processes. In contrast, biologics present themselves as extremely intricate entities, characterized by heterogeneous structures and marked sensitivity to the specific manufacturing processes and raw materials used in their production. Regulatory agencies, when considering an alternative to the reference product, favor high similarity through extensive characterization of both the reference product and the proposed biosimilar, rather than a perfect physicochemical match. Because of their size and complexity, biopharmaceuticals undergo variation from batch to batch, and generic drugs must replicate the originally approved active pharmaceutical ingredient (API). Biopharmaceutical drugs are generally more sensitive to thermal, light, and chemical degradation, requiring considerable attention in the formulation of intermediate and final products. In this regard, biologics are usually molecules extremely sensitive to transit in the gastrointestinal apparatus and have strong limitations on cell penetrability due to their size, charge, and hydrophilicity. Consequently, they are typically administered parenterally through injection or other forms of administration, including ophthalmic, transdermal, and pulmonary, which are constantly emerging, at the expense of oral administration [33], [34]. Biopharmaceuticals have raised concerns about their long-term use compared with small molecules. Indeed, the safety and efficacy of biopharmaceuticals appear to be severely compromised by patients' ability to tolerate the drug. Prolonged treatment is associated with serious adverse events, including leukopenia, thrombocytopenia, and neuropsychiatric effects, which may necessitate dose reduction or even discontinuation of treatment in some patients. They offer significant advantages in revolutionizing the treatment of diseases. Despite concerns about the long-term use of biopharmaceuticals versus small molecules, their specificity allows them to target specific

molecules and receptors, minimizing side effects. Their potency allows for dose reduction and has commonly paved the way for better pharmacokinetic and pharmacodynamic properties than traditional synthetic drugs. Biopharmaceuticals play a leading role in the emergence of personalized medicine, offering tailored therapies with more precise responses and higher safety. 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 specializing in such development and production 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. A recent analysis revealed that the average production cost per piece of small molecules is about $5 per gram, whereas this value rises to about $60 for biologics per gram [35]. Small molecules are usually synthesized through strongly controlled and reproducible chemical reactions, followed by the conversion of the active ingredient and selected excipients into pharmaceutical forms suitable for the intended administration route. All the mentioned and additional downsides are, however, counterbalanced by the numerous advantages resulting from the use of biopharmaceuticals. With the advent and rapid growth of this new therapeutic class, multiple mechanisms to diagnose, prevent, treat, and cure diseases and medical conditions have been enabled. Biopharmaceutical drugs have revolutionized the treatment of a broad spectrum of diseases in almost all branches of medicine. Among their strengths, their specificity is recognized, targeting almost exclusively the aimed molecules and receptors, thus strongly decreasing the side effects associated with small-molecule drugs [13]. Their potency allows for reduced doses and has 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 the most accurate responses and highest safety margins [36]. Unlike synthetic drugs, biomolecules exhibit more articulated mechanisms 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 considering physiological mechanisms (e.g., insulins) to provide replacement of a patient's defective biomolecule and fulfill its absence due to genetic defects [33], [37], 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 for the ongoing Covid-19 pandemic [38]. The administration of the first-developed mRNA vaccines [36] was a turning point in

the evolution of the infection's spread and mortality. Indeed, recent data suggests that more than half a million human deaths have been averted through the vaccination campaign in the European Economic Area [38]. Recent trends and projections have been showing how the pharmaceutical R&D and manufacturing sector is shifting from small molecules toward biological products. A brief excursus on the advantages of adopting such macromolecules was presented, as well as on the rising economic pressure those intrinsically carry with them. The goal of this doctoral path and thesis was the evaluation of technologies capable of increasing the throughput of current manufacturing technologies and decreasing costs in biopharmaceutical production using a more environmentally friendly approach.

## 1.1.2 Biomanufacturing Process and Upstream Step

In the realm of biopharmaceutical production, particularly focusing on recombinant proteins, the biomanufacturing process follows a distinct sequence of steps, as illustrated in Fig. 1.4. Following the expression or synthesis of the protein, a recovery phase ensues, aimed at eliminating cells and cellular debris. This step is crucial for obtaining a clarified and filtered cell culture fluid, known as harvested cell culture fluid (HCCF) [40]. Subsequently, the developmental drug substance, obligated to meet all critical quality attributes (CQAs), undergoes the purification process. In the context of large-scale upstream processing, the emphasis shifts to cultivating cell lines in bioreactors under carefully controlled conditions. The precise regulation of parameters such as feeding, temperature, pH, and oxygen supply is essential to orchestrate the production of substantial quantities of recombinant biomolecules [39].

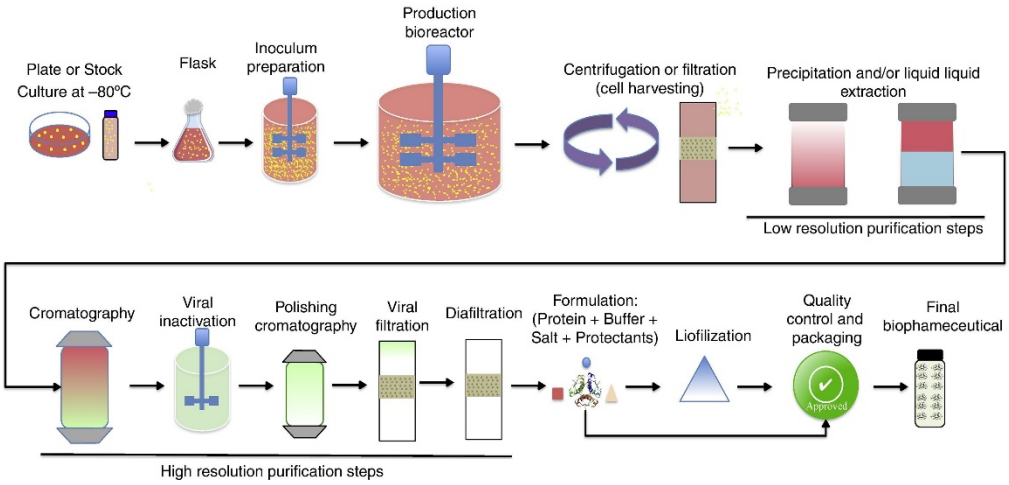


*Fig 1. 4 Biopharmaceuticals manufacturing*

The production processes between a biological product and large synthetic biopolymer molecules such as peptides and oligonucleotides are different. Various methodologies are employed for the synthesis of peptides, encompassing solid-phase peptide synthesis (SPPS), liquid-phase peptide synthesis (LPPS), semi-continuous/continuous peptide synthesis, chemoenzymatic peptide synthesis (CEPS), mechanochemical peptide synthesis, and recombinant production through fusion proteins. Notably, the latter technique is predominantly applicable for the synthesis of extended peptides containing natural amino acids, finding utility in the production of native, pharmacologically active peptides or in the semi-synthesis of analogs, particularly when the native sequence is sufficiently lengthy, as exemplified by liraglutide or semaglutide [7] .Regulatory authorities mandate stringent requirements for the manufacturing process, necessitating the delivery of a compound of exceptional purity, a criterion evaluated through a combination of analytical methods [41]. Solid-phase peptide synthesis (SPPS) stands as a venerable method, employing N,N-dimethylformamide (DMF) as a solvent for over fifty years. This methodology facilitates the efficient synthesis of extensive peptide sequences, ensuring high purity and yield across a spectrum of amino acids. SPPS involves the incremental expansion of a peptide sequence on a polymer-based, insoluble, solid support. Commencing with the anchoring of the initial amino acid, protected at the amino group, onto the resin via the acidic terminal end, the sequential development of the peptide sequence occurs through iterative reactions, including deprotection, washing, coupling, and further washing steps. Within the SPPS cycle, the widely adopted Fmoc/tBu orthogonal strategy entails the temporary protection of the growing α-amino terminus with an Fmoc group, subsequently removed during each deprotection step under basic conditions. Simultaneously, the amino acid side chains are shielded with acid-sensitive groups, removable as necessary during the peptide cleavage from the solid support. Throughout the SPPS cycle, the potential presence of impurities may be attributed to either process-related or product-related factors. Biocatalysis, especially chemo-enzymatic peptide synthesis (CEPS), plays a pivotal role in sustainable chemistry, finding widespread applications in diverse industrial sectors, including drug manufacturing. CEPS involves the application of various enzyme classes for the synthesis of pharmaceutical intermediates. Noteworthy advancements in technology have enabled the customization of enzymes for specific purposes, rendering enzymatic catalysis a potential sustainable alternative to traditional chemical ligation. Four enzyme classes, namely butelase, sortase, trypsiligase, and subtiligase variants, have undergone extensive scrutiny for their efficacy in facilitating specific peptide bond formation in water. While the promise of enzymatic steps in CEPS is evident, seamless integration with other technologies such as GSPPS and PA-

LPPS remains imperative. CEPS demonstrates its value in the synthesis of a diverse array of linear and cyclic peptides and protein conjugates, including highly complex substrates. Process-related impurities arise as a consequence of the inherent nature of the manufacturing process, particularly during cell line cultivation. This involves the recovery of expressed recombinant proteins alongside collateral harvesting of cell media components, substrates, salts, host cell proteins (HCPs), DNA, chromatographic media, solvents, and buffer components used in purification. Affinity chromatography emerges as a common strategy to eliminate process-related impurities [42]. Product-related impurities manifest as compounds structurally akin to the target product, including high and low molecular weight species (HMW, LMW) such as aggregates and fragments relevant to monoclonal antibodies. The catch&release method presents an innovative solution for the pre-purification of crude peptides, streamlining the RP-LC process. Unlike traditional liquid-liquid extraction methods that entail substantial volumes of volatile organic solvents, this method embraces sustainability by utilizing minimal amounts of water as the solvent. This approach introduces a capping step at each stage of the SPPS process. Capped fragments, unresponsive at the N-terminal with base-labile cleavable linkers, are identified through oxime-based and hydrazone-based ligation chemistry. During Trifluoroacetic Acid (TFA) cleavage, the peptide is liberated from the resin, concurrently removing the BOC-protecting group on the oxime. The liberated peptide undergoes treatment with aldehyde-modified agarose beads at pH 4.5 to generate the oxime. Following thorough washing to eliminate capped peptide fragments, the final peptide is released through treatment with ethanolamine. This pre-purification process has demonstrated successful application across diverse peptide structures.

### 1.1.3 The Polishing Step

Downstream processing, encompassing chemical and physical methodologies, is a critical phase for separating and purifying target biomolecules. Unfortunately, downstream processing has become a bottleneck in biopharmaceutical manufacturing due to its capacity lagging behind upstream production developments [43]. Purification steps contribute to around 50% of the total costs in the overall biomanufacturing stream [44]. Various techniques, including filtration, centrifugation, precipitation, flocculation, and gravity settling, have been evaluated. However, the poor scalability, inadequate resolving capacities, and lack of reproducibility of these methods have led to the prominence of preparative liquid

chromatography as the industrial gold standard for downstream processing [45]. Liquid chromatography offers robustness, selectivity, and high resolution, making it a preferred choice. In upstream processes, the expression of pharmaceutical biomolecules results in the co-production of various impurities. The second step, known as polishing, aims to enhance the purity of the captured material. Differentiation in polishing processes depends on the chemical structure of the crude product. Preparative liquid chromatography, once again, is utilized for the removal of such impurities. However, the specific challenges associated with each biopharmaceutical necessitate the exploitation of multiple or orthogonal chromatographic interaction mechanisms. For instance, ion exchange chromatography (IEC) is often employed to polish proteins, removing aggregates, fragments, HCPs, and leached protein A from the capture step. This technique takes advantage of the charged status of proteins, allowing adsorption either on cation exchange (CEX) materials if positively charged or on anion exchange (AEX) resins if negatively charged. Hydrophobic Interaction Chromatography (HIC) is gaining popularity for protein polishing due to its operation under non-denaturing conditions, avoiding product folding or denaturation. In HIC, proteins interact with hydrophobic ligands on the stationary phase, and retention is promoted using kosmotropic salts. Peptides, being more hydrophobic, are typically purified using Reversed Phase (RP) chromatography, a reliable and widely employable technique. RP chromatography employs stationary particles functionalized with hydrophobic structures, such as aliphatic chains or aromatic rings, and uses organic modifiers like acetonitrile, ethanol, methanol, or isopropanol. To address limitations like low loadability, high cost, and limited selectivity, Ion-Pair Reversed Phase chromatography was developed. This technique utilizes ion-pair reagents to improve chromatographic retention and selectivity for charged analytes. While these purification techniques offer efficiency, challenges such as low loadability and high costs persist. The biomanufacturing industry is increasingly focused on selecting greener and easier-to-dispose solvents to align with industry goals. Despite these challenges, ongoing efforts aim to enhance purification techniques and meet the evolving needs of biopharmaceutical manufacturing.

## 1.1.4 Intensification Process

A batch chromatographic process involves separating components through adsorption in a single-column setup. A mixture with both the desired compound and impurities is introduced into the stationary phase. Elution using mobile phases follows, resulting in product recovery. The column is then purged and re-equilibrated before initiating a new purification cycle. Currently, batch processes are widely utilized in biopharmaceuticals, pharmaceuticals, fine chemicals, and food processing. Despite their dominance, these processes have historical drawbacks and limitations. For example, in capture processes, the adsorbent cannot be fully loaded to its static binding capacity to prevent material breakthroughs, leading to reduced throughput and productivity [42]. The operation is discontinuous, and the overall process is slowed by equilibration, strip, and cleaning-in-place (CIP) procedures, which must be repeated for each purification cycle. Additionally, continuous operator intervention is necessary for software operation, fraction collection, and system refilling with new collecting material. Analytical characterization of fractions for each batch further burdens QC and QA departments. Work under batch conditions is influenced by the trade-off between yield and purity. When impurities show structural similarities with the target product, their chromatographic peaks often coelute, appearing in the front or rear region of the target peak. This challenge is particularly pronounced in the synthesis of biopolymers, such as peptides and oligonucleotides, where ternary separations are common.

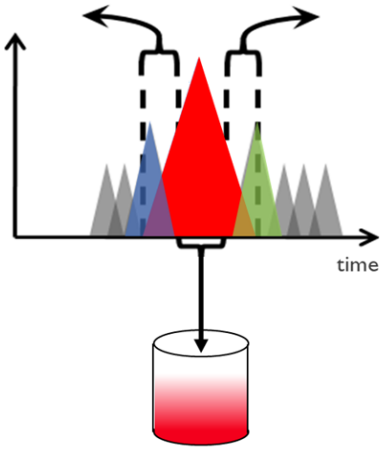


*Fig 1.5 Ternary separations affected by yeld-purity trade off*

*Fig 1.5* graphically illustrates these phenomena. To solve this problem, efforts have been directed towards the development of more efficient purification solutions. One approach involves improving the performance of stationary resins, with the aim of outperforming

existing ones and improving separation capabilities, thereby reducing material overlap and expanding regions of pure material. In analytical applications, advances such as the reduction of particle sizes in columns (down to a range of less than 2 μm) by ultra-high performance liquid chromatography (UPLC) have led to increased efficiency and separation capabilities, withstanding pressures of up to 1200 bar. However, this strategy is not feasible in preparative chromatography. Preparative processes face significantly lower back pressure limits, typically up to 100 bar for laboratory equipment and up to 50 bar for production facilities. In addition, larger particles are essential in preparative chromatography to maximize the binding of the feed material and increase productivity [46]. In Chapter 3, a digression on the origin and latest innovations in continuous chromatography will be presented, while the Results and Discussion section will describe and discuss the research work carried out in the field of continuous countercurrent chromatographic processes for the purification of biopharmaceuticals and greener applications.

# CHAPTER 2

## 2.1 MODELS OF CHROMATOGRAPHY

The modelling of chromatographic processes can be explained through equilibrium or kinetic theories. Equilibrium theories are used to describe ideal chromatography and are employed when the mobile phase and the stationary phase reach instantaneous equilibrium [47]. This occurs when mass transfer rate is considered infinite, and the efficiency of the column also occurs in mass transfer and adsorption. There is no band-broadening effect due to kinetic theories, but the shape of the resulting peak is only given by a thermodynamic factor. However, in non-ideal chromatography, equilibrium is not reached because it takes time for the solute in the mobile phase to encounter the adsorbent material in the stationary phase. Kinetics theories better describe the real chromatographic system, which is also referred to as the dispersive model. Mass transfer is slow, so equilibrium effects or adsorption processes take place but there is a diffusive term that takes into account all the dispersive effects that influence band broadening. Moreover, chromatography can be divided also into *linear* and *nonlinear* in ideal chromatography. In *linear conditions* of infinitive dilution, the concentration of solute adsorbate is low, the molecules do not compete for the adsorption in the active site in the stationary phase. The retention time of the analyte is independent of its composition and concentration. Band profiles correspond to a Gaussian peak, excluding the extra phenomena column. The isotherm that describes the equilibrium between the stationary phase and the mobile phase, presents a linear behavior. In *nonlinear chromatography,* the concentration of solute adsorbate is high, and this creates competition for the adsorption in the stationary phase, in fact, preparative chromatography determines the tailing profile. "In a chromatographic system, the equilibrium isotherm, which describes

the distribution of components between the mobile and stationary phases, is not necessarily linear. It can exhibit either an upward concavity (referred to as anti-Langmuir) or a downward concavity (referred to as Langmuir). This behaviour is observed while assuming that the mass of each injected component remains constant. This assumption is valid under the condition that there are no chemical reactions occurring between the components within the column. Essentially, it means that the components are not undergoing any chemical changes as they move through the chromatographic system. As a result, it becomes feasible to develop a mass balance equation for each component and establish a set of initial boundary conditions that describe both ideal and non-ideal models [48].

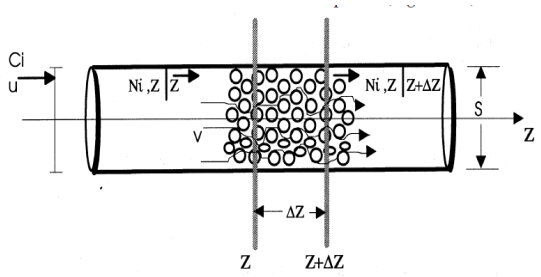


*Fig 2. 1 Differential mass balance in a column slice*

## 2.1.1The Ideal Model

The ideal model assumes that the efficiency of the column and the rate of the mass transfer is infinite and there is no kinetic origin for the band broadening. The equilibrium is reached immediately between the mobile phase and the stationary phase [47], [48], [49], [50]. The differential mass balance equations are a set of partial differential equations and solving this one is possible to obtain the chromatographic band profiles.

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = 0 \qquad (2.\ 1)$$

In this differential equation the first term, $C$, is the concentration of the analyte in the mobile phase; the second term, $q$, is respectively the stationary phase. Instead, the phase ratio is $F = \left(\frac{(1-\epsilon_t)}{\epsilon_t}\right)$ and the total porosity of the column is by $\epsilon_t = \frac{V_0}{V_{col}}$ , $V_0$ is the hold-up volume and the $V_{col}$ is the column volume.

It's a possible rewrite of Equation *(2.1)* and can be used for observing important information about the thermodynamic system from the shape of the band.

$$\frac{\partial C}{\partial t} + \frac{u_z}{1+F\frac{dq}{dC}}\frac{\partial C}{\partial z} = 0$$

*(2. 2)*

This equation shows that the migration velocity of a given concentration zone, $u_z$ is associated to the mobile phase concentration, and to the local curvature of the isotherm. The partition coefficient $D_a$ governs the rate, and it is tangents to isotherm, instead the from the slope of the isotherm the study of the migration and the change of a band profile in a chromatographic experiment.

To solve the system of mass balance equations, proper initial and boundary conditions must be defined [51], [52], [53]. The initial conditions are the following:

$$C_i(z,t) = \ 0 \qquad i = 1{,}2$$

*(2. 3)*

And the boundary:

$$C_i(z,t)\begin{cases} C_{i,0} & 0 \le t \le t_{inj} \\ 0 & t > t_{inj} \end{cases}$$

*(2. 4)*

## 2.1.2 The Dispersive Model

However, these equations describe the ideal model, for the non-ideal where the kinetics transfers are not infinitive rapid due to the diffusion term and all the factors that consider the band broadening such as external mass-transfer, inner diffusivity into pores, the adsorption-desorption kinetics, and the homogeneity of the flow rate[48], [52]. The contribution of the axial dispersion and finite mass transfer kinetic is given by coefficient Da, which is the apparent axial coefficient dispersion term.

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

*(2. 5)*

*L* is the length of the column, and *N* is the number of theoretical plates.

The mass transfer resistance is low to be considered as an additional contribution to $D_a$. Every single-component system be explained using only one partial differential equation, namely, the equation for the mass balance.

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

*(2. 6)*

Where the first term on the right represents the axial diffusion coefficient, this equation is based on the dispersive equilibrium, the kinetics of mass transfer, and the efficiency of the column is high but not infinitive. In addition, the system is described by another equation:

$$q = f(C)$$

*(2. 7)*

Where $q$, is the isotherm equation.

To solve the system of mass balance equations, proper initial and boundary conditions must be defined [51], [52], [53]. The initial conditions are the following:

$$C_i(z,t) = \ 0 \qquad i = 1{,}2$$

*(2. 8)*

And the boundary:

$$C_i(z,t)\begin{cases} C_{i,0} & 0 \le t \le t_{inj} \\ 0 & t > t_{inj} \end{cases}$$

*(2. 9)*

## 2.1.3 The General Rate Model

The general rate model takes into consideration all contributions from transport and kinetic phenomena that lead to band broadening, precisely for these reasons this model is used to characterize a chromatographic process. It encompasses two distinct equations for mass conservation of the analyte: one pertaining to the mobile phase that moves through the interstitial spaces among packed particles, and another concerning the immobile phase residing within the pores. Additionally, diffusion is presumed to occur between the flowing mobile phase stream and the stagnant mobile phase, while adsorption-desorption kinetics take place between the motionless mobile phase and the surface of the adsorbent [47], [54]. The mass balance equation for the bulk mobile phase within the interstitial volume of particles is formulated as follows:

$$\frac{\partial C}{\partial t} + u_h \frac{\partial C}{\partial z} + \frac{3}{r_\rho}\frac{F_e N_0}{\partial z} = D_L \frac{\partial^2 C}{\partial z^2}$$

*(2. 10)*

$u_h$ is the interstitial velocity of the mobile phase, $r_\rho$ is the diameter of the particles $N_0$ is the mass flux of the analyte from the mobile phase to the external surface of the stationary phase and $F_e$ is a parameter correlated to the interstitial porosity. $D_L$ is the axial dispersion coefficient expressed as the sum of the molecular and eddy diffusion coefficients. The equation that described the diffusion of the solute within the pores of the stationary phase particles is:

$$\epsilon_p \frac{\partial C_p}{\partial t} + (1 + \epsilon_p) \frac{\partial C_s}{\partial t} = D_p \left( \frac{\partial^2 C_p}{\partial r^2} + \frac{2}{r} \frac{\partial C_p}{\partial r} \right)$$

*(2. 11)*

where $\epsilon_p$ is the particle porosity, $C_p$ and $C_s$ are respectively the concentration of the analyte inside pores and adsorbed pores on the stationary phase, $D_p$ is the pore diffusivity coefficient.

To solve the system of mass balance equations, proper initial and boundary conditions must be defined [51], [52], [53]. The initial conditions are the following:

$$C_i(z, t) = 0 \qquad i = 1,2$$

*(2. 12)*

And the boundary:

$$C_i(z, t) \begin{cases} C_{i,0} & 0 \leq t \leq t_{inj} \\ 0 & t > t_{inj} \end{cases}$$

*(2. 13)*

# 2.2 ADSORPTION EQUILIBRIA

The interactions between solutes and both the mobile and stationary phases play a significant role in shaping adsorption isotherms. These isotherms offer valuable insights into the thermodynamic properties governing the formation of band profiles and the occurrence of chromatographic separations. This is particularly pertinent in conditions that are non-linear. Within the literature, one can find several adsorption models [48]. Each of these models is used to fit experimental data accurately. These models consider the heterogeneous nature of the adsorbent surface as well as the diverse potential interactions that can transpire between pairs of adsorbates and adsorbents, as well as among different adsorbate molecules. To simplify, the earlier discussion explores how the concentration of an analyte influences its interaction with the particles of the stationary phase during adsorption.

At low concentrations, this relationship follows a linear behavior. However, as concentrations increase, the bonding becomes more intricate and culminates in saturation, when no more substances can be adsorbed [55]. To investigate these interactions, various models have been employed to clarify how analytes and particles interact in both the mobile and stationary phases. The choice of a suitable model depends on factors such as the nature of the substance to be adsorbed, the properties of the particles (homogenous or heterogeneous) and the composition of the material to be processed. In some cases, different substances may compete for available adsorption sites. Understanding these models is crucial when designing processes involving adsorption, especially when the processes are

non-linear or involve the separation of substances. Some models commonly used to elucidate these interactions are briefly illustrated.

## 2.2.1 Linear Isotherm

The linear isotherm proves valuable in analytical conditions that frequently involve low concentrations and small quantities of analyte under study. It establishes a direct proportionality between the concentrations in the mobile phase (C) and the stationary phase (q).

$$q = aC \tag{2. 14}$$

The parameter $a$ ,which is the slope of the isotherm, is equal the product of the retention factor $k$ of the analyte and the phase ratio $F$ as

$$a = \frac{k}{F} \tag{2. 15}$$

However, its limitation lies in its inability to effectively describe situations of nonlinear or preparative chromatography, where the interactions among adsorbed molecules play a significant role that cannot be ignored.

## 2.2.2 Langmuir Isotherm

The Langmuir isotherm is a basic and widely used model in describing how molecules get adsorbed in liquid chromatography. This model assumes that the adsorption process occurs in a single layer, without interactions between the adsorbed molecules. It also assumes that there's only one type of adsorption site on the surface, making the adsorption consistent throughout.

Mathematically, the Langmuir isotherm is defined as an equation involving the saturation capacity $q_s$ and the adsorption constant $b$.

$$q = \frac{q_s bC}{1 + bC} \tag{2. 16}$$

Interestingly, when the concentration $C$ is very low ($C \to 0$), this equation simplifies to the linear isotherm equation $q = q_s b$, with $q_s = a$ where $a$ is the Henry's adsorption constant.

Among the various available models, the Langmuir model is widely used to explain, adapt to data, and simulate how molecules get adsorbed in liquid chromatography. This model has been successfully applied in numerous cases involving compounds such as peptides in biopharmaceuticals [55].

## 2.2.3 Bilangmuir Isotherm

This class of isotherms assumes localised adsorption that occurs on a heterogeneous surface without significant interactions between the adsorbed molecules. The bilangmuir adsorption model extends this concept by considering two distinct types of homogeneous sites located on the adsorbent surface, each covered by different chemical groups. Consequently, the isotherm equation emerges from the sum of two distinct contributions, corresponding to the independent behaviours of these two distinct adsorption sites, labelled sites 1 and 2. This model assumes non-uniform adsorption surfaces, such as mixed-mode resins or from unintentional features such as unmodified silyl groups. The Bilangmuir model considers the possibility of similar or dissimilar interaction mechanisms between the analyte and the two available adsorption sites [47], [48], [49], [50], [56], [57], [58].

$$q = \frac{q_{s,1} b_1 C}{1 + b_1 C} + \frac{q_{s,2} b_2 C}{1 + b_2 C} \tag{2. 17}$$

Here, $q_{s,1}$ and $q_{s,2}$ indicate the saturation capacities of site 1 and site 2, respectively, while $b_1$ and $b_2$ indicate the equilibrium constants associated with these two sites. This model has mainly been used to elucidate enantiomer separations on chiral stationary phases (CSPs). However, it has also demonstrated its applicability in describing the behaviour of biopharmaceutical entities [55].

## 2.2.4 Bet Isotherm

These types of isotherms consider surfaces where adsorbed molecules interact uniformly, allowing for the possibility of multiple layers of adsorption. The extended liquid-solid BET isotherm proposes that adsorption can occur on the surface of bare silica or on an existing layer of adsorbed analyte, characterized by equilibrium constants $b_s$ and $b_l$. The equation is as follows:

$$q = \frac{q_s b_s C}{(1 - b_l C)(1 - b_l C + b_s C)} \tag{2. 18}$$

If $b_l$ is less than 1, the above equation becomes identical to *Eq (2.16)*, which represents the traditional Langmuir isotherm with a curved upward shape. However, if the interaction

energy between two adsorbate molecules increases, the behavior of the system is depicted by a curved downward isotherm [48], [59], [60].

## 2.2.5 Excess Isotherm

The excess isotherm describes the adsorption behavior of components in the mobile phase (typically binary solvent mixtures) onto the surface of the adsorbent. For this reason, understanding it is crucial for a deep comprehension of chromatographic and separation processes. The components of the mobile phase, due to their characteristics, can be selectively adsorbed or adsorbed in varying amounts on the surface of adsorbent particles, significantly influencing the retention behavior of analytes. Excess isotherms can provide insights into the differing adsorption of solvents, which can describe packing properties such as polar properties (quantity of free silanols), hydrophobic properties (coverage density), and the uniformity of bound ligands [61], [62]. Determining excess isotherms is important because it enables the assessment of the influence of adsorbed mobile phase components in the stationary phase on the chromatographic retention of analytes, describing the equilibrium distribution between the mobile and stationary phases. Excess isotherms have been calculated using the so-called Perturbation on the Plateau (PP) method [48]. In the PP method, a distinction is made between the rate of concentration, which describes the propagation of solute concentration along the column, and the rate at which molecules move within the column. This distinction is not usually made in chromatography because it is always performed under conditions of infinite dilution, and thus the two rates are equivalent. For the calculation of the excess isotherm, the method known as the perturbation method (also known as the "peak on a plateau" method) has been used. This involves introducing a small perturbation, achieved by injecting an excess of one of the components of the mobile phase compared to the other, once equilibrium between the mobile and stationary phases has been reached [63]. This perturbation can generate peaks that move through the column at a linear velocity defined as:

$$u_{\phi A} = \frac{F_v L}{V_0 + S\left(\frac{d\Gamma_A}{d\phi_A}\right)} \qquad (2.\ 19)$$

Where $\phi A$ is the volume fraction of component A in the bulk of the mixture, $Fv$ is the volumetric flow rate, $V_0$ is the thermodynamic void volume, $S$ is the total surface area of the

adsorbent in the column, and $\Gamma A$ is the excess surface of component A. The retention volume of the perturbation peak can be written as:

$$V_{R,A} = \frac{F_v L}{u_{\phi A}} = V_0 + S\left(\frac{d\Gamma_A}{d\phi_A}\right) \quad (2.\ 20)$$

Through the integration of *Equation (2.20)* it is possible to calculate the excesses of component A

$$\Gamma_A = \frac{\int_0^1 \left(V_{R,A}(\phi_A) - V_0\right) d\phi_A}{S} \quad (2.\ 21)$$

$V_0$ is calculated as follows:

$$V_0 = \int_0^1 V_{R,A}(\phi)\, d\phi \quad (2.\ 22)$$

To obtain the complete dependence of the excess quantity (or the retention times of the perturbation) of one of the two components across the entire concentration range, it is necessary to repeat the experiment with various mobile phase compositions, covering the range from one pure component to the other. It is possible to rewrite the excess surface using volumes and volume fractions of concentrations:

$$\Gamma_A = V_A^a - V^a \phi_A \quad (2.\ 23)$$

$V_A^a$ represents the volume of component A adsorbed, and $V^a$represents the volume of the adsorbed phase. By extrapolating the slope of the excess isotherm in the decreasing branch within its linear region, it is possible to estimate the total quantity of component A adsorbed onto the adsorbent phase. The equation of the tangent is given by:

$$V_A^a \equiv \Gamma_A + V^a \phi_A \equiv b_1 + a_1 x_1 \quad (2.\ 24)$$

with a and b the slope of the inflection tangent and the y-intercept.

## 2.3 MASS TRANSFER KINETICS IN LC

In this section are reported the foundational theoretical principles of mass transfer kinetics in liquid chromatography. The comprehensive examination of various sources contributing to kinetic band broadening is feasible primarily under linear conditions. In these conditions, the impact of thermodynamic equilibria on band profiles becomes negligible [48].

The van Deemter equation establishes a correlation between the height equivalent to a theoretical plate, H (or its dimensionless form $h = H/d_p$ with $d_p$ being the particle diameter), and the mobile phase velocity. This equation provides a way to connect theoretical plate characteristics with interstitial velocity, offering insights into column efficiency [53] as:

$$h = a\,(v) + \frac{b}{v} + \; c_s v + c_{ads} v + h_{heat} \tag{2. 25}$$

where $a\,(v)$ is the eddy dispersion, $b$ the longitudinal diffusion, $c_s$ represents the solid-liquid mass transfer resistance and $c_{ads}$ is the term for slow adsorption-desorption kinetics usually in RPLC is missing because the adsorption-desorption process is fast but not for large molecules, such as biopharmaceuticals [64]. $h_{heat}$ is due to the friction between the eluent at high flowrate and particles. The interstitial velocity is defined as:

$$v = \frac{F_v d_p}{\pi r^2 \epsilon_e D_m} \tag{2. 26}$$

$r$ is the column radius, $\epsilon_e$ the external porosity and $D_m$ the bulk molecular diffusion coefficient.

The examination of individual factors influencing band broadening becomes feasible by integrating appropriate models of diffusion in porous media with experimental measurements.

### 2.3.1 Eddy Dispersion

The Eddy dispersion term $a$, is caused from the unpredictable flow patterns within the through-pores of packed beds. It encompasses trans-channel eddy dispersion, short-range inter-channel eddy dispersion, and trans-column eddy dispersion. In *Eq. (2.18)*, the eddy dispersion term persists as a manifestation of irregularities in the stream path within through-pores of packed beds. However, a comprehensive mathematical expression capturing the

accurate structure of packed beds remains elusive. Consequently, $a$ can be experimentally estimated by subtracting values of $b$ and $c$ from $h$ [65]. In achiral system the term $c_{ads}$ is equal 0, so the equation for the eddy dispersion is given by:

$$a\,(v) = h - \frac{b}{v} - c_s v \qquad (2.\ 27)$$

In chiral system, the term $c_{ads}$ cannot be neglected so the following equation is derived:

$$a\,(v) + \ c_{ads} v \ = h - \frac{b}{v} - c_s v \qquad (2.\ 28)$$

## 2.3.2 Longitudinal Diffusion

The longitudinal diffusion term $b$ is estimated by peak parking (PP) and describes the band broadening due to the relation of axial gradient concentration through the porous particles and the interstitial volume, in absence of flow. The PP consist in an injection of the analyte at a constant linear velocity, when the middle of the column is reached, suddenly stop the flow rate. In this phase the band is free to diffuse inside the porous media during a certain parking time $t_p$, after that the flow rate is resume at the same previous liner velocity. The effective diffusion coefficient $D_{eff}$ can be calculated from PP, when the slope of $\sigma_x^2$ is plotted vs $t_p$.

$$D_{eff} = \frac{1}{2}\frac{\Delta\sigma_x^2}{\Delta t_p} \qquad (2.\ 29)$$

The longitudinal term can be calculated through the $D_{eff}$ as follow:

$$b = 2\,(1 + k_1)\frac{D_{eff}}{D_m} \qquad (2.\ 30)$$

Where $D_m$ is the molecular diffusion coefficient of the analyte in the mobile phase mixture.

## 2.3.3 Models of Diffusion in porous media

The coefficient $D_{eff}$ considers all the contributions to diffusion of the analyte in the composite material made of the porous zone impregnated by the eluent $D_p$ and the bulk mobile phase $D_m$. There are many models available to describe the diffusion processes in

porous media but only the more realistic Effective Medium Theory (EMT) of Maxwell will be reported here [66], [67], [68], [69].

The time-averaged or parallel model was elaborated by Knox, describes that the mass fluxes inside and outside the particles are additives:

$$D_{eff} = \frac{\gamma_e D_m + \frac{1-\epsilon_e}{\epsilon_e}\,(1-\rho^3) D_p}{1+k_1} \tag{2. 31}$$

Where the $\gamma_e$ is the obstructive geometrical factor. This is the simple description of distribution of the samples in different phases, provides acceptable values for $D_{eff}$.

The Effective Medium Theory (EMT) provides a much more accurate description of $D_{eff}$ because describe microstructure of particles. The Maxwel Model is the basic and simple variant of all the EMT.

$$b = 2(1+k_1)\gamma_e = \frac{2}{\epsilon_e}\,\frac{1+2\beta(1-\epsilon_e)}{1-\beta\,(1-\epsilon_e)} \tag{2. 32}$$

Where $\beta$ the polarizability constant is described as:

$$\beta = \frac{\alpha_{part}-1}{\alpha_{part}+2} \tag{2. 33}$$

Where $\alpha_{part}$ is the relative particles permeability that is the ratio between the permeability of the particle zone $D_{part}$ (including diffusion in the stagnant mobile phase and surface diffusion), $D_m$, over that of the interstitial zone:

$$\alpha_{part} = \frac{\epsilon_e k_1}{1-\epsilon_e} \cdot \frac{D_{part}}{D_m} \tag{2. 34}$$

### 2.3.4 Solid-liquid mass transfer Resistance

The solid-liquid mass transfer resistance is described by the terms $c_s$ in the *Eq. (2.25)*. It is a consequence of the diffusion across the porous particle. Is possible to calculate by the Laplace transform of the general rate model of chromatography [65], [70], [71].

$$c_s = \frac{1}{30} \cdot \frac{\epsilon_e}{1-\epsilon_e} \cdot \left(\frac{k_1}{1+k_1}\right)^2 \left[\frac{1+2\rho+3\rho^2-\rho^3-5\rho^4}{(1+\rho+\rho^2)^2}\right] \cdot \frac{D_m}{D_p} \tag{2. 35}$$

### 2.3.5 Adsorption- Desorption Kinetics

The term $c_{ads}$ in *Eq. (2.25)* takes into consideration the finite rate at which analyte molecules adsorb to and desorb from the adsorbed layer and the pores filled with eluent. For small molecular weight compounds, the impact on h is minimal, given their very high adsorption rate constant, $k_a$ [65], [70]. Conversely, for proteins and the most retained enantiomer in chiral analytes, the adsorption-desorption process can significantly affect the separation efficiency. The derivation of the $c_{ads}$ term follows the Laplace transform of the general rate model of chromatography [48], [72], [73], [74].

$$c_{ads} = 2 \cdot \frac{\epsilon_e}{1-\epsilon_e} \cdot \frac{1}{1-\epsilon_p} \cdot \frac{1}{1-\rho^3} \left(\frac{k_1}{1+k_1}\right)^2 \left(\frac{k_p}{1+k_p}\right)^2 \frac{D_m}{k_{ads} d_p^2} \tag{2. 36}$$

With $k_p = \frac{(1-\epsilon_p)}{\epsilon_p} K_a$ and $K_a$ is the adsorption equilibrium constant and $k_{ads}$ the kinetic adsorption constant.

## 2.4 LINEAR SOLVENT STRENGTH MODEL

Chromatographic techniques used for biopharmaceutical separation, both analytical and preparative, predominantly rely on gradient elution. In gradient elution, the organic modifier fraction $\phi$, which affects the eluotropic strength of the mobile phase, is progressively increased during chromatographic runs. This method is particularly useful for separating complex mixtures like proteins and peptides, where analyte retention factors strongly correlate with the modifier percentage in the mobile phase. Unlike isocratic separations, the

equilibrium isotherm in this scenario isn't constant, introducing time and location dependencies on velocity according to the ideal model. To accommodate these changes, it's assumed that isotherm parameters are related to ϕ, independently of the gradient. The linear solvent strength (LSS) model is employed in reversed-phase LC. This model describes the retention factor (k) variation with ϕ. Adsorption isotherm analysis must consider this, as the isotherm changes with the evolving organic modifier concentration in gradient elution. Consequently, under these conditions, variations in the retention factor can be accurately described.

$$\ln k\,(\phi) = \ln k_0 - S\phi$$

*(2. 37)*

When the modifier concentration $\phi$ is at its initial value $\phi = 0$ , the retention factor k is denoted as $k_0$. This can be thought of as an extrapolated point on the retention factor scale. S is a characteristic coefficient relates to the composition of the solute and the mobile phase composition. Consider the Langmuir isotherm parameters, $a$ and $b$, and the organic modifier is easily got by recognizing that the retention factor $k$ is equivalent $k = aF = q_s bF$. This relationship can be expressed as follows:

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

*(2. 38)*

Where $a_0$ (which is the same as $k_0$) as a special value when there's no modifier present $\phi = 0$. If the $q_s$ doesn't change at different values of the modifier $\phi$ the two values $b$ and $\phi$ are connected using the same *Eq (2.31)*

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

*(2. 39)*

$b_0$ is the adsorption constant at $\phi = 0$. Combining the latter equation and the Langmuir equation, is possible derive how the adsorption is influenced under a linear gradient.

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

*(2. 40)*

# CHAPTER 3

## 3.1 PREPARATIVE CHROMATOGRAPHY

The improvement in biopharmaceutical manufacturing primarily relies on liquid chromatography, a separation technique by the Russian botanist Tswett in 1903. This process is based on the differences in migration velocity between the various components of a mixture when they are transported by a moving fluid through a column composed of solid particles. [75]. Within this system, an equilibrium is established for all mixture components, influenced by factors such as temperature, and the nature of stationary and mobile phases. A deep understanding of the interactions occurring within the separation media enables a detailed characterization of the process. This, in turn, facilitates model-based predictions and optimizations. Consequently, there has been a growing interest in modelling chromatographic processes, driven by the desire to minimize process development costs and challenges. Typically, the development of industrial chromatographic purification processes involves labour-intensive, time-consuming lab-scale experiments using columns like those in the manufacturing plant but with smaller dimensions. This experimental approach demands significant amounts of expensive feed material, which is often limited in quantity. As a solution to these challenges, there is a solid momentum in adopting modelling alternatives. These alternatives aim to circumvent the drawbacks associated with extensive experimentation, identify critical process parameters, and optimize the overall process design. The limitations and drawbacks of batch chromatography, coupled with the imperative to cut downstream costs and enhance efficiency, have spurred significant efforts in chemical engineering. The goal is to develop novel, sustainable manufacturing solutions. In single column processes, partially pure side fractions often undergo re-chromatography, where the same or a similar chromatographic operation is performed using these side fractions as load material. While this process can recover a purer fraction of the product, the separation is more challenging due to higher impurity content in the load material compared

to the regular feed material. Re-chromatography comes with operational disadvantages, including regulatory limitations, challenges inside fraction storage and handling, and issues with side fraction stability and quality control. Continuous chromatography processes offer a solution by automatically recycling impure portions. Moreover, they use the same resin and solvents as batch chromatography without requiring additional separation optimization. The transition to continuous processes leads to significant improvements in resin and buffer consumption, recovery of partially pure regions, and results in key process parameters such as throughput, yield, purity, and process mass intensity (PMI) [76]. The beginning of continuous processes dates back to the early 1950s, and shortly thereafter the crosscurrent fashion of the mobile and stationary phase principle was applied to chromatographic separations [77]. One of the pioneering innovations in this field is continuous annular chromatography (CAC), characterized by a rotating annular bed of stationary phase subjected to a continuous countercurrent flow of the mobile phase. By continuously introducing the feed at a fixed point, separation in both bed length and angular coordinates is achieved [78]. However, it has become apparent that incorporating countercurrent motion between the mobile phase and chromatographic media optimizes mass transfer efficiency, leading to higher adsorbent material utilization [79]. Application of this phenomenon to continuous processes would further improve separation efficiency. In the early stages, a system that facilitated true countercurrent flow of the stationary phase material and liquid was termed the True Moving Bed (TMB) process. However, scalability problems emerged due to the friction and pressures caused by the actual movement of the microspheres. To overcome this problem, the concept of simulated moving bed (SMB) was introduced, in which the stationary phase is packed into smaller columns connected to different parts of the process. Instead of physically moving the solid microspheres, the inlet and outlet lines are moved relative to the stationary chromatographic microspheres, resulting in simulated motion [80]. SMB found its roots in hydrocarbon separation in 1961 [81] and has since been widely applied in various manufacturing sectors, including petrochemical, pharmaceutical and chemical [82]. However, the integration of SMB technology into biopharmaceuticals has encountered an inherent limitation: it does not support gradient elution. Most chromatographic purifications of biopharmaceuticals involve ternary separations that require linear gradients for sufficient resolution between early eluting impurities, the target product, and late eluting impurities. Early attempts to introduce gradients into SMB processes involved supercritical eluents and altered pressure levels and mobile phase densities [83]. While these solutions were continuous, they did not consider the countercurrent movement of the two phases, which is critical for optimizing mass transfer efficiency. A significant

development emerged in 2007 with the invention of the multicolumn countercurrent solvent gradient purification process (MCSGP). This innovative approach enabled continuous processes and countercurrent movement of the two phases while simultaneously applying a linear solvent gradient [84]. This marked a significant advance in chromatographic technology. By finding less solvent usage [85], [86].

### 3.1.1 Process Parameters

Quality by design is an approach geared towards ensuring the quality of pharmaceuticals through the application of statistical, analytical, and risk-management methodologies in their design, development, and manufacturing. A key objective within quality by design is the comprehensive identification, explanation, and management of all sources of variability that impact a given process, utilizing appropriate measures. This concept, introduced by the FDA and EMA, seeks to enhance the understanding of the relationship between critical quality attributes (CQAs) and the clinical properties of the product. In the context of manufacturing processes, including preparative chromatography methods, a focus on robustness, reproducibility, and sensitivity is crucial. This applies to both laboratory-scale equipment and plant infrastructure. The eluate obtained from preparative chromatography is typically collected in fractions, each of which must undergo offline analytical characterization. Depending on the biopharmaceutical nature of the product, various analytical techniques may be employed. The goals of a characterization campaign include defining the structure of the major product and related impurities, establishing the relationship between structure and function, and quantifying the ratio of target to impurities derived from the process. Comprehensive characterization not only contributes to fundamental product knowledge but also facilitates the future implementation of meaningful comparability protocols supporting manufacturing changes. Depending on the attributes being investigated and the nature of the analyte, various analytical assays may be applied. High Pressure Liquid Chromatography (HPLC) is one of the most widely used technologies for obtaining multiple types of information from a sample. The data obtained from analytical chromatography plays a critical role in estimating parameters that quantify and qualify the performance of a purification process by four parameters: chromatographic purity, recovery, productivity, and solvent consumption. The purity is expressed in percentage from the ratio between the target peak area and the total area (includes impurities):

$$Purity\ (\%) = \frac{A_{target}}{A_{total}} \times 100 \qquad (3.\ 1)$$

Recovery is described as the proportion of peptide mass within a specific fraction or a set of fractions in relation to the overall peptide mass introduced into the system.

$$Recovery\ (\%) = \frac{m_{target\ collected}}{m_{target\ injected}} \times 100 \tag{3. 2}$$

Relating the quantity of the retrieved target compound to both the overall duration of the preparative method and the complete column volume of the stationary phase defines productivity. This parameter serves to articulate the amount of the target compound purified within a specific timeframe per column volume (CV).

$$Productivity\ (mg/L/h) = \frac{m_{pool\ collected}}{CV\ \times\ time} \tag{3. 3}$$

The factor for the solvent consumption indicates the volume of solvent necessary to deliver a mass of target within purity specifications. This parameter is important to take into account the consumption to achieve the lowest value possible.

$$Solvent\ consumption\ (mL/mg) = \frac{Total\ Volume}{m_{target\ collected}} \tag{3. 4}$$

## 3.1.2 Approaches to improvement of the downstream

In a batch chromatographic process, a single-column mode is employed for an adsorption-based separation process. A feed mixture containing both the pure compound and impurities is injected into the stationary phase. Elution via mobile phases is performed, the product is recovered, and the column is purged and re-equilibrated before starting a new purification process. While batch processes are widely used in various industries, they have drawbacks, such as decreased throughput during capture processes to avoid material breakthrough, leading to lower productivity. The operation is discontinuous, and equilibration, strip, and cleaning procedures need to be repeated for each purification, requiring constant operator intervention. Additionally, there's a yield-purity trade-off, especially when impurities are structurally similar to the target product. There are three potential approaches to address this problem. The first involves using highly efficient stationary phases, such as those with high mass transfer rates, to improve elution peak sharpness [87]. This can be achieved by using smaller resin particles, but this involves increasing the pressure drop [30]. As a result, low flow velocities are required, which often results in reduced throughput. Alternatively, lengthening the column length can yield similar results [78]. A second option is to modify

the operating conditions of the process, such as reducing the amount of loading or the gradient slope, to minimize regions of overlap [32]. However, this modification tends to decrease productivity and increase solvent consumption, resulting in higher processing costs [88], [89]. The third possibility involves replacing the single-column batch operation with a multi-column process. In this approach, the overlapping region of the chromatogram is recycled within the unit, while only the fraction of the target product that meets the purity specifications is extracted. This allows simultaneous improvements in purity and yield, mitigating the trade-offs mentioned earlier [90]. It is important to note that these processes pose greater challenges in terms of design and operation than single-column processes. Efforts have been made to enhance purification solutions, such as continuous methods like Multicolumn Countercurrent Solvent Gradient purification (MCSGP).

It is necessary replace a single column step for the downstream processes to a continuous method, increasing the greenness of the procedure.

Moreover, new stationary resins and the use of mixed-mode resins with orthogonal separation mechanisms in a single column. Doped Reversed materials, using separate ligands for specific interactions, show promise in achieving higher loadings and improving chromatographic selectivity. The introduction of doping ion-exchange ligands influences the retention strengths on the stationary resin, leading to better resolution of chromatographic bands and potentially eliminating the purity-yield trade-off in batch chromatography. One approach involves the adoption of mixed-mode resins, combining orthogonal separation mechanisms within a single column [91]. Crude materials often contain varying impurities with different chemical structures, such as hydrophobicity, carried charge, and dimension. Mixed-mode chromatography (MMC) resins utilize ligands capable of multiple interaction modes with analytes, enhancing chromatographic selectivity through diverse interaction combinations. MMC has demonstrated significantly higher loadings in certain applications. A recent innovation in this field is Doped Reversed materials (DRP), which use two separate ligands, each exhibiting its own type of interaction. This distinction allows for an extreme specific concentration of each ligand, unlike most mixed-mode materials where different ligands are generally equally distributed [91], [92].

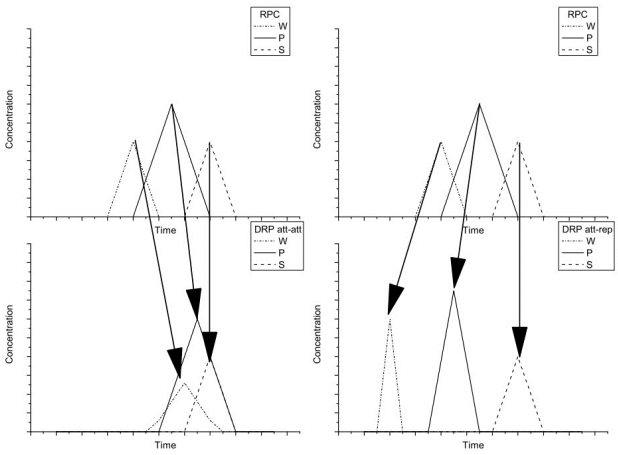


*Fig. 3. 1 Representation of chromatograms in RPC (top) and DRP (bottom). The arrows show the effect of the doping IEX groups. Attractive–repulsive (right) Attractive–attractive (left)* [92]

The basic RP structure of DRP resins retains hydrophobic analytes. However, biopharmaceutical crudes often have analytes and impurities with varying hydrophobic, hydrophilic, and charged regions. Introducing doping ion-exchange ligands influences final retention strengths on the stationary resin. In RP separation, more hydrophobic components are strongly bound and elute later. In biopolymer polishing applications, introducing a repulsive ion-exchanger in a RP resin has a positive impact. For a three-element separation (product, weak impurity, and strong impurity), the weak impurity is shielded from adsorption by repulsion, eluting earlier. The product is slightly affected, and the strongly hydrophobic impurity is minimally affected. This leads to better-resolved chromatographic bands, eliminating overlapping peak regions and improving purity-yield trade-off. A pure region of the product can be pooled without sacrificing yield. However, using a doping ion-exchanger of opposite charge to analytes may have a detrimental impact on separation outcome, boosting the retention of weak impurities.

## 3.2 THE MULTICOLUMN COUNTERCURRENT SOLVENT GRADIENT PURIFICATION PROCESS

The advent of MCSGP technology has been a crucial development in enabling the application of countercurrent continuous separation to the biopharmaceutical manufacturing domain. This technology relies on the countercurrent movement of two phases (or more) and the application of a linear gradient, facilitating the collection of pure fractions and the internal recycling of contaminated product fractions, such as bands of product overlapping with impurities. With the utilization of MCSGP technology, the longstanding trade-off between purity and yield can be alleviated, even in complex ternary separations [93]. A key

feature of this technology is the ability to deliver the target compound with high yield and purity simultaneously through internal recycling, thus saving substantial amounts of expensive materials and eliminating the need for reprocessing operations. In its initial implementation, MCSGP operated with six columns. However, recognizing the complexity of the hardware, the process was simplified, initially reducing the columns to four and eventually to three units [91]. In its latest iteration, the technology is facilitated by twin columns, significantly reducing the need for tubes and valves. This reduction in components reduces overall system costs, complexity, and footprint, making the process more efficient and economical[94]. Continuous improvement involves refining the process using consistent buffers, resins and chromatographic conditions, reflecting benchmarks.

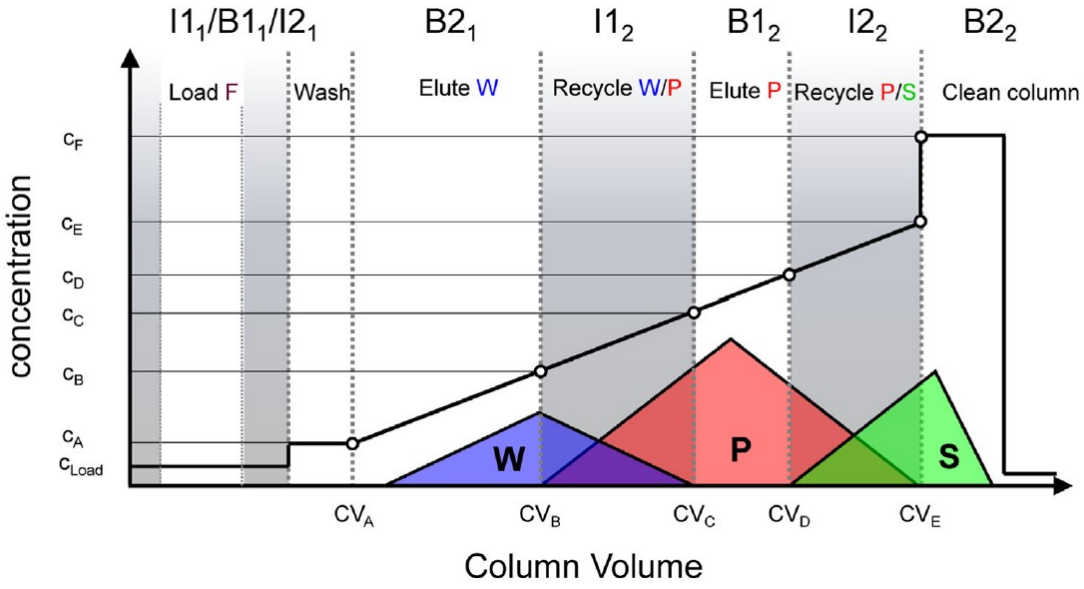


*Fig. 3. 2 Design chromatogram used to setup a MCSGP process.* [95]

To initiate the design of a MCSGP process, it's essential to choose a design batch chromatogram. The selection process is usually based on identifying a specific fraction of the chromatogram in which the purity of the product meets the specified criteria, although the yield is typically between 30% and 80%. Consequently, the approach involves creating a design chromatogram in which a section of the chromatogram obtained by gradient elution meets the product purity requirements while accepting a slightly lower yield. This chromatogram serves as the foundation for determining crucial parameters such as flow rates, resin loading, and gradient slope. In addition, recycling sections are delineated using offline batch analysis, accurately delineating retention times in overlapping regions. To understand the principles and immense potential of MCSGP, it is necessary observe the batch chromatogram illustrated in *Fig. 3.2*. This chromatogram depicts a centre-cut or ternary separation conducted under gradient elution conditions. The general scheme involves

the elution of a main compound located between weak and strong impurities (blue and green triangles), resulting in partial overlapping of the peaks. [13]. The chromatogram is divided into distinct zones.

Zone $I1_1/B1_1/I2_1$: The column, pre-equilibrated with the eluent, is loaded with a fresh feed and also with the impure side fractions recycled from the upstream column. Once the analyte is adsorbed onto the stationary phase, the modifier gradient begins.

Zone $B2_1$: Weakly adsorbing impurities (referred to as W) with lower retention than the target product begins eluting from the column.

Zone $I1_2$: The product (P) starts eluting, but the weakly adsorbing impurities are still in the elution phase. Due to poor resolution between W and P, their peaks overlap. The product in this zone doesn't meet purity requirements due to contamination by species W. However, it cannot be discarded and must be recovered to achieve optimal process yield.

Zone $B1_2$: The target compound elutes, meeting purity requirements for pharmaceutical purposes.

Zone $I2_2$: Another overlapping region where the target compound coelutes with strongly adsorbing impurities (S).

Zone $B2_2$: The column undergoes stripping with a high percentage of organic modifier to eliminate S impurities. It is then equilibrated again with the initial eluent composition at the beginning of the gradient.

Fractions of the eluate are systematically collected during the gradient and subsequently analysed via HPLC to generate a purity profile encompassing zones $B2_1$ to $I2_2$ [13].

To fully delineate the operational conditions of the process, it is necessary to define the operational parameters for each of the steps. These parameters include the flow rates (Q) of the two pumps involved in each phase, the switching times (t) and, in the case of the gradient pump (P1), the modifier concentration. (c). Moreover, while keeping the feed composition, buffer system, resin material and column unchanged. The operating parameters for the continuous process are obtained from the design chromatogram shown schematically in *Fig. 3.2*, where the elution profiles of the components to be separated are shown as a function of column volume (CV)[95].

$$CV = \frac{\int_0^t Q\ (t')dt'}{V_{col}} \qquad (3.\ 5)$$

In the $CV_A$ to $CV_E$ range, a linear modifier concentration gradient is applied, starting with $c_A$ and ending with $c_E$. The MCSGP process adopts a similar gradient elution shown in *Fig 3.2*,

recycling overlapping non-pure fractions ($CV_B$– $CV_C$ and $CV_D$–$CV_E$) within the unit during interconnected states. The initial design step involves defining these regions on the chromatogram by selecting values for boundaries $CV_B$, $CV_C$, $CV_D$, and $CV_E$. The starting point of the modifier gradient in the design chromatogram is set as $CV_A$, determining the gradient slope ($m$) accordingly.

$$m = \frac{c_E - c_A}{CV_E - CV_A} \tag{3. 6}$$

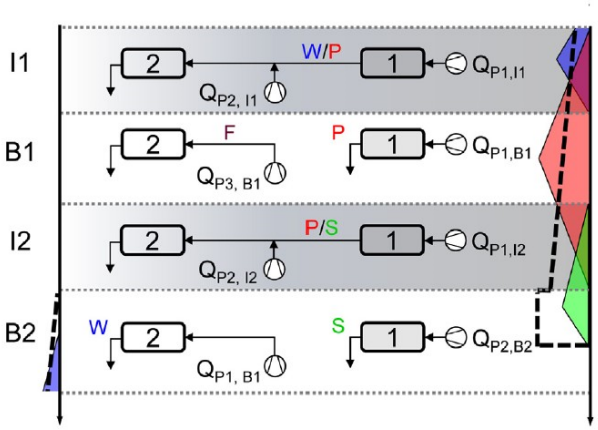


*Fig. 3.3 Flow scheme, half a cycle of a twin-column MCSGP process is illustrated with time progressing from top to bottom* [95]

*Fig 3.3* outlines the schematic chromatograms from columns 1 and 2 on the left and right respectively. In the initial step (I1), performed at time $t_{I1}$, the gradient pump P1 is connected to column 1. The inlet flow has a flow rate of $Q_{P1,I1}$ (representing the flow rate of pump P1 during step (I1) with the modifier concentration increasing from $c_B$ to $c_C$, reflecting the values of the design chromatogram in *Fig 3.2*. Consequently, the overlap region of pump P1 was changed. Consequently, the overlapping region of the chromatogram containing W and P is fed into column 2. In this column, resorption is carried out according to the modifier concentration. In this column, readsorption of the target product is performed by mixing the outflow from column 1 with a compensating flow $Q_{P2,I1}$, characterised by an appropriate lower modifier concentration of $c_{P2}$. In this first interconnected step the flowrates of the gradient pump $Q_{P1,I1}$ of the compensation pump P2 $Q_{P2,I1}$, as well the duration $t_{I1}$, is defined as:

$$Q_{P1,I1} \leq Q_{max} \frac{c_{crit} c_{P2}}{c_c c_{P2}} \tag{3. 7}$$

$$Q_{P2,I1} = Q_{max} - Q_{P1,I1}$$

*(3. 8)*

$$t_{I1} = \frac{(CV_C - CV_B)V_{col}}{Q_{P1,I1}}$$

*(3. 9)*

The equations are derived from the constraint that, during phase I1, the total flow entering column 2 and the buffer concentration within this column must not exceed the specified limits $Q_{max}$ and $c_{crit}$.

In the next step (B1), conducted at time $t_{B1}$, both columns operate in batch: On column 1, product elution is achieved by extending the linear gradient from $c_C$ to $c_D$ at flow $Q_{P1,B1}$. Simultaneously, fresh feed is introduced to column 2 via pump P3 at a flow rate of $Q_{P3,B1}$.

$$Q_{P1,B1} = \frac{(CV_D - CV_C)V_{col}}{t_{B1}}$$

*(3. 10)*

And the flow rate for loading fresh feed into the 2 column is given by:

$$Q_{P3,B1} = \frac{V_{feed,MCSGP}}{t_{B1}}$$

*(3. 11)*

Where the $V_{feed,MCSGP} = (1 - y)V_{load,batch}$

*(3. 12)*

$y$ is defined as the fraction of feed target product to be recycled in the MCSGP, it's possible to calculated from the design chromatogram. It is given by:

$$y = \frac{\int_{CV_B}^{CV_C} c_P(CV)dCV + \int_{CV_D}^{CV_E} c_P(CV)dCV}{\int_{CV_B}^{CV_E} c_P(CV)dCV}$$

*(3. 13)*

$t_{B1}$, is determined by the longer of the two phases, both of which must be run at $Q_{max}$. It is important to note that in the initial transition of the inaugural cycle, when the unit starts up, there is obviously no product available for recycling. Therefore, it is practical to load the same amount of the designed batch chromatogram Eq (3.12) with y = 0.

Following this, in step (I2), the two columns are once again operated in an interconnected mode for a time $t_{I2}$. The overlapping region of the chromatogram P/S is expelled from column 1 by increasing the modifier concentration from $c_D$ to $c_E$ at a flow rate of $Q_{P1,I2}$.

Similar to the previous interconnected step, product re-adsorption on column 2 is accomplished by appropriately reducing the modifier concentration with the compensation stream $Q_{P1,I2}$.

$$Q_{P1,I2} \leq Q_{max} \frac{c_{crit} c_{P2}}{c_E c_{P2}} \tag{3. 14}$$

$$Q_{P2,I1} = Q_{max} - Q_{P1,I2} \tag{3. 15}$$

$$t_{I2} = \frac{(CV_E - CV_D) V_{col}}{Q_{P1,I2}} \tag{3. 16}$$

In the subsequent step (B2), the columns operate batch-wise with a distinct pump connection. As all the product has been eluted from column 1, the regeneration process commences with pump P2 at a flow rate $Q_{P2,B2}$ of over a duration of $t_{B2}$. Meanwhile, the gradient pump P1 is used to facilitate the elution of weakly adsorbed impurities from column 2. This is achieved by raising the modifier concentration from $c_A$ to $c_B$ , accompanied by a flow rate of $Q_{P1,B1}$.

$$Q_{P1,B2} = \frac{(CV_A - CV_B) V_{col}}{t_{B2}} \tag{3. 17}$$

The extended phase, often associated with rebalancing $t_{B2}$ plays a key role in this design approach. It should be noted that the flow rate in the MCSGP unit during the elution phase (phases I1 and I2 in column 1 of *Fig. 3.2*) is kept lower than in the initial batch. This deliberate adjustment helps to improve performance by minimising the impact of mass transfer resistances. Alternatively, the process can be operated with a constant $Q_{P1} < Q_{max}$. To summarize the design setup, we start with a design chromatogram, which specifies the loading amount and gradient. Next, the user defines the recycling windows, and the method presented derives all the operational parameters for the MCSGP process. The expected performance of the process, measured in terms of yield, and purity that can be estimated based on the placement of these windows within the design chromatogram[95].

# CHAPTER 4

## 4.1 RESULTS AND DISCUSSION

In this chapter the results of published papers or already submitted at the time I am writing this thesis. This is organized in different sections, each summarizing the results obtained in a specific research topic. In the discussion, some sensitive data have been intentionally omitted. For further details, the reader is referred to reprints of the full papers appended at the end of the thesis.

## 4.2 DOWNSTREAM PROCESSING THROUGH MULTICOLUMN CONTINUOS PREPARATIVE LIQUID CHROMATOGRAPHY (Paper I-II)

The scope of this thesis is to show how it is possible to intensify the downstream processing as well as to increase its greenness. In this section the two identical columns continuous countercurrent chromatographic process is applied for the purification of biopharmaceutical compounds. In chapter 3 the Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is described.

In **Papers I** and **II**, the scenario of continuous chromatography was explored, and we introduced the rationale, mechanisms, results, and possible applications of the MCSGP process. From an experimental point of view, successful applications of the above technology to a biopharmaceutical (Icatibant) were described in Paper II.

## 4.2.2 From batch to continuous chromatography in the polishing of peptides

The chromatographic processes in the downstream steps often involve a batch operating methodology, which employs a single column. Typically, these purification processes encounter a ternary separation, in which the target peptide elutes between two sets of impurities, one with lower retention and another with higher retention on the stationary phase. The challenge arises from the similarity between the target product and related impurities, which causes their peaks to overlap on both the front and tail of the product peak. This overlap intensifies at higher loads. As a result, two limiting scenarios emerge: narrow the collection window to achieve higher purity at the expense of recovery or widen it to achieve higher yields, sacrificing purity as some of the impurity peaks are also collected. In batch chromatography, achieving high purity and high yield simultaneously is virtually impossible, resulting in a trade-off between yield and purity. A schematic representation of this trade-off is shown in *Fig. 4.1*.

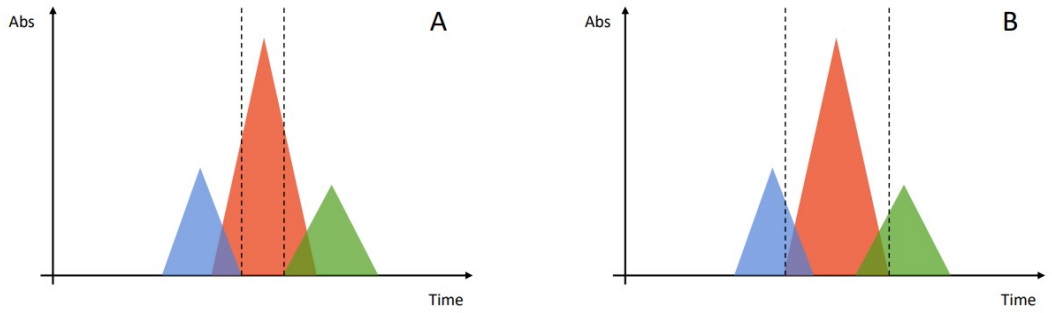


*Fig 4.1 Yield-Purity trade-off. Weak and strong impurities are respectively the blue and green triangles. The target product in red.*

Multicolumn chromatographic processes offer a solution to the challenges often encountered in the purification of complex mixtures of biopharmaceuticals. These techniques involve the use of at least two columns with identical dimensions and stationary phases. The key principle behind multicolumn processes is the countercurrent movement of the mobile phase in relation to the stationary phase, achieved through a series of switching valves that control the mobile phase's path. Countercurrent movement improves recovery, and the continuous nature of multicolumn processes automates the entire procedure, reducing the time of manual operations and improving reproducibility. A specific technique, Multicolumn Countercurrent Solvent Gradient Purification (MCSGP), developed about fifteen years ago, has found wide application in the purification of various biomolecules, including

monoclonal antibodies, pegylated proteins, oligonucleotides, cannabidiols, and peptides. MCSGP is distinguished by its ability to use a solvent gradient for elution, unlike other continuous chromatographic techniques limited to isocratic conditions. This flexibility is critical because the retention of biomolecules depends on the composition of the mobile phase. In addition, MCSGP, unlike typical SMB processes, can effectively manage ternary mixtures through a single unit operation. Process improvements arise from the overlapping regions of internal recycling, in which product and impurities partially elute on the head and tail of the main peak, respectively. The switching of inlet and outlet column valves regulates internal recycling, altering the path of the mobile phase. The combination of continuous and countercurrent concepts, along with internal recycling and the use of a solvent gradient program, enables the separation of ternary mixtures. This achieves high product purities and elevated yields, exceeding traditional single-column processes in terms of productivity.

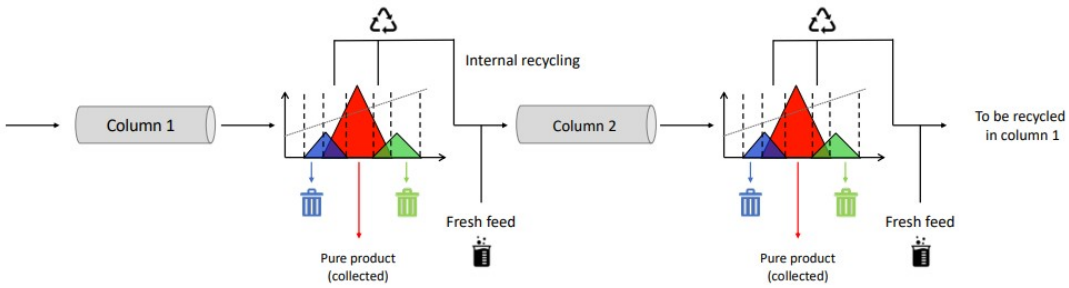


*Fig 4. 2 The MCSGP technique*

For instance, a six-column MCSGP process significantly improved the purification of a 32 amino acids synthetic peptide, calcitonin, increasing the yield from 66% in batch purification to nearly 100% in continuous processing, with a purity of 93%. Similar success was observed in the separation of an industrial crude of glucagon, with the recovery increasing from 71% to almost 88% and purity reaching 89%.

In Paper II, the discussion delves into one case that elucidate the transition from single-column batch chromatography to continuous chromatography for polypeptide downstream, exemplified by dowstream process of Icatibant, a therapeutic peptide composed of 10 amino acids used in treatment of symptoms of hereditary angioedema because it is an antagonist of bradykinin B2 receptors. Moreover, it is potential used toward the improvement of oxygenation in patients affected by COVID-19 in early stage. The initial step involved the evaluation of the Icatibant case, where a crude solution of the peptide, synthesized through Solid Phase Synthesis by Fresenius Kabi iPsum (Villadose, Italy), showed a chromatographic purity of 88% *Fig. (4.3)*.

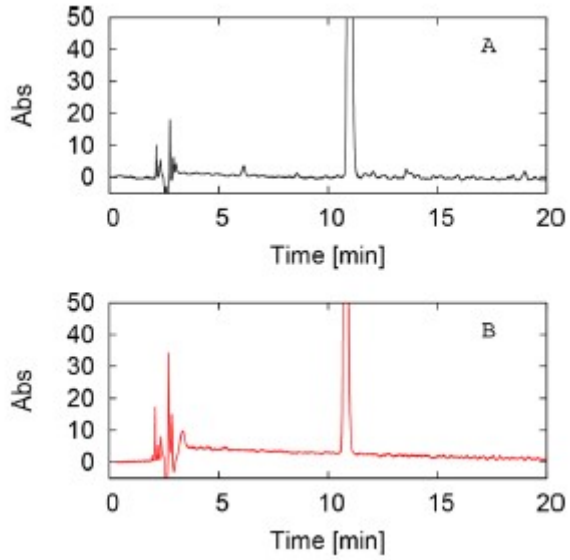


*Fig 4.3 The black analytical chromatogram for the feed of Icatibant, the redone the purest fraction of Icatibant*

The chromatographic purity in the data suggests that a one-step purification method is adequate to reach the 99% purity requirement. The separation process utilized a common stationary phase, Daisogel-SP-120-10-ODS-BIO, with a pore size of 120 Å and particle size of 10µm, consistent across both batch and MCSGP processes. The batch column, with dimensions 250 mm × 4.6 mm, and the two MCSGP columns, each 150 mm × 4.6 mm, shared identical stationary phase characteristics. For the batch run, the column volume (CV) was 4.2 mL, while each MCSGP column had a CV of 2.5 mL. The mobile phases employed in the gradient elution were 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). MP-A also played a role in the in-line dilution of the overlapping fraction during the interconnected phase of MCSGP operation. Following column equilibration for 2 CVs with 12% MP-B, feeding was initiated, succeeded by a gradient elution over 18 CVs from 12% to 37% MP-B. To conclude, a regeneration and re-equilibration protocol was executed. The obtained batch chromatogram was used to setup the MCSGP.

The utilization of diverse mobile phases is essential to ensure the smooth operation of chromatographic system pumps and minimize the impact of slight inconsistencies in buffer preparation. The conditions selected for purifying a 25cm column were later applied to a 15cm column of the same resin, establishing the baseline chromatogram for the MCSGP process. Shifting to 15cm columns serves two purposes: firstly, to facilitate a fair comparison of productivity values between single column and continuous chromatography, ensuring comparable overall resin volumes. Ideally, MCSGP column lengths should be half of those in batch chromatography. Secondly, for the twin-column continuous experiment, maintaining consistent geometric dimensions is crucial. Our experimental setup necessitated

a compromise due to the unavailability of two 12.5cm packed hardware. *Fig. 4.4* displays the design batch chromatogram outcome, along with purity values and the target product concentration profile, derived from fraction analysis.

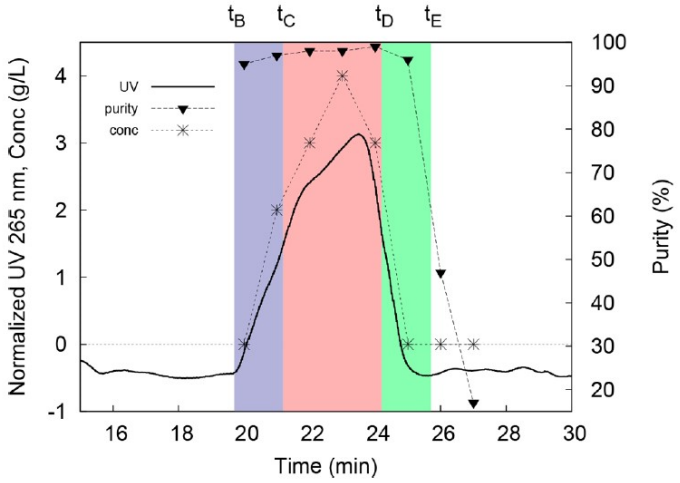


*Fig 4.4 Design batch chromatogram, through offline analysis obtained target concentration as a function of time for the design batch chromatogram.*

This information enabled a precise selection of both the recycling and product collection timeframes. The collection window in red signifies the eluate portion meeting purity specifications without the need for further processing. In contrast, the recycled portion contains material only partially purified, typically discarded in traditional batch chromatography but here designated for continuous reprocessing or separate storage. Using the ChromIQ software operating the preparative system CUBE, specific valve-switching times were determined. As illustrated graphically, a blue zone contains the target product and weakly adsorbing impurities (W/P), while a green zone comprises the product and strongly adsorbing impurities (P/S). A played a role as an in-line dilution agent at different stages within the MCSGP process. The UV signals obtained at the first column's outlet during the initial switch in each of the five cycles are depicted in *Fig. 4.5*. The first switching signal differs significantly from subsequent ones. This variation can be attributed to a combination of factors rather than a single cause. First, the composition ratio of components in the column undergoes continuous changes during cycles, eventually reaching a relatively stable proportion after a few switches. In addition, the recycling of specific component regions in the downstream column requires extra flow rates from in-line dilution streams. This is due also to the stat-up procedure where the first switch the feed duration is higher compared to the subsequent switches of the process. These flows, influenced by the chosen starting point of the gradient, can slightly change the composition of the mobile phase along the gradient. In addition, more complex factors, such as displacement effects or stagnation of organic solvents in the pores, could contribute to the initial mismatch.

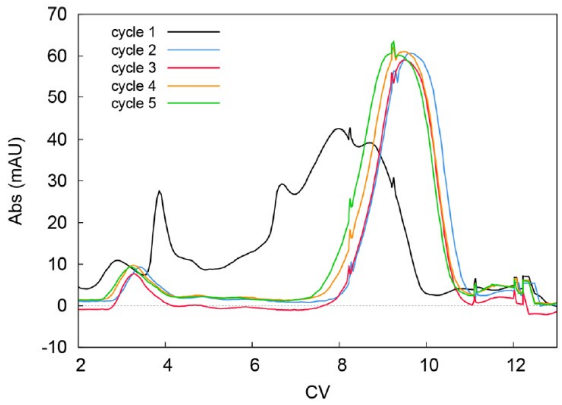


*Fig 4.5 UV profile of Icatibant in the flowrate leaving the upstream column during the five MCSGP cycles.*

However, assessment of the establishment of cyclic steady state is based on UV signals from the second cycle onward. A more pronounced consistency is evident in subsequent cycles, corroborated by chromatographic analysis of the collected samples. The quality and concentration of the eluate remained consistent and comparable, affirming the sustained separation ability of the resins in multiple stages. The strength and effectiveness of the multicolumn experiment were validated through process comparison. In the table are shown process values to characterize the two separate 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 4.1 Performance parameters obtained of the batch and MCSGP.*

The recovered injected material exhibited a purity exceeding 99%, with a notable 95.5% obtained through pooling via MCSGP. This significant difference is evident in the pareto curve depicted in *Fig 4.6*. It is important to note that the slight disparity in productivity increase compared to mass recovery is attributed to the higher consumption of stationary resins in MCSGP. Ultimately, the solvent consumption per gram of material produced at a certain purity is approximately five times less in continuous runs. As the pharmaceutical industry moves towards more sustainable and green processes, the reduction of mobile phases becomes increasingly significant. Additionally, the conservation of consumables during production positively impacts manufacturing economics. Notably absent from the previous analysis in *Table 4.1* are the advantages conferred by process automation, including reduced human intervention, decreased chances of errors and dead times, and enhanced consistency in product quality across manufacturing batches.

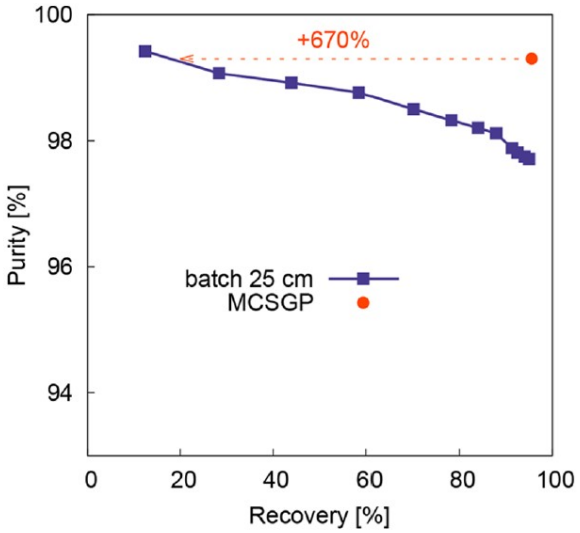


*Fig 4.6 Pareto curve obtained for the batch and MCSGP processes.*

MCSGP demonstrated outstanding results in the purification of icatibant, achieving a purity greater than 99% for both batch and continuous methods. Continuous processes significantly improved recovery from around 12% to over 95%, with a process gain of +670%. Productivity increased by more than 5 times, and solvent consumption reduced by 80%.

## 4.2.2 Closing Remark

There is an urgent need to improve bioprocessing because the biopharmaceutical industry faces several challenges, with a focus on reducing the gap between upstream and downstream processes to improve efficiency and resource utilization. Although continuous countercurrent techniques are recognized as viable substitutes for batch purifications, they face some reluctance because of their complexity. The multicolumn approach explored in this thesis offers a promising solution to mitigate the typical trade-offs associated with single-column chromatography. In our experimental work, we have demonstrated that MCSGP is able to consistently to provide purified material with high quality specifications and avoid disposal of material (yield reduction), thereby increasing the productivity of the process in of material (yield reduction), thereby increasing the productivity of the process in purifying peptides therapeutic peptides. This technology has demonstrated full automation, robustness and cost-effectiveness, cornerstones of the upcoming biopharmaceutical industry. In addition, as demonstrated in Paper II using MCSGP there is a gain in solvent consumption, resulting in less production of organic waste solvent. It therefore turns out that such an approach is necessary to achieve a lower environmental impact.

# 4.3 REPLACING ACETONITRILE AS ORGANIC MODIFIER IN RP-LC FOR THE SEPARATION OF BIOACTIVE PEPTIDES (Paper V-VI)

The purpose of this section is to show how the downstream processing is rather reducing the environmental footprint of the downstream processing. In order to do this, we have tested the chromatographic properties of dimethyl carbonate as green organic modifier in RPLC. **Paper IV** reports a fundamental study where these innovative solvents were used for the separation of probe molecules. **Paper V** reports the application of these environmentally sustainable solvents in a downstream process of a pharmaceutical peptide (Icatibant).

## 4.3.2 Separation of probe molecules using eco-alternative to ACN

**Paper IV** reports the first studies that were initially conducted on the retention behavior and kinetic performance of two different probe molecules, namely paracetamol and caffeine under RPLC conditions with UV-Vis detection, using different organic modifiers ethanol (EtOH) , isopropyl alcohol (IPA) in aqueous solution, including dimethyl carbonate (DMC). In this study, the performance of DMC was evaluated in comparison to traditional RPLC solvents (EtOH, IPA and ACN). An in-depth investigation was conducted to analyze all factors contributing to band broadening and retention. The goal was to unravel the mass transfer phenomena and diffusion properties of analytes in DMC/H2O. This is the first paper to report a fundamental study of retention mechanisms in RPLC using DMC as an organic modifier and it represents the basis for further understanding of the possibility of also using this solvent for industrially relevant chromatographic applications.

In Chapter 2.3 the theory for the evaluation of the kinetic performance of a chromatographic column is described. Since DMC has been barely applied in RPLC before, at the beginning we have considered its physicochemical properties. It was found that DMC has a cutoff wavelength of 220 nm, the highest among the four solvents examined. Although this may be a limitation for samples requiring wavelengths shorter than 220 nm in complex analyses, for most LC applications dealing with aromatic rings or absorption maxima at longer wavelengths, this limitation is not significant. In addition, the maximum amount of DMC allowed in an aqueous mobile phase is about 10% percent, thus minimizing the baseline noise caused by DMC absorption at wavelengths shorter than 220 nm.

In comparison to the other solvents, DMC has a higher boiling point and density and an intermediate viscosity between ACN and EtOH, but no harmful effects. These aspects must

be carefully considered when evaluating the overall sustainability and feasibility of a chromatographic method. In this context, reducing the amount of organic solvent used in a chromatographic run, as in the case of DCM (max 10% v/v), contributes to reduce the environmental impact resulting from its production. Next, the retention of different organic modifiers was investigated. From the comparison, it is evident that DMC exhibits the highest elution strength in all cases, since it shows lower retention factors ($k$) than the other solvents (*Fig 4.7*).

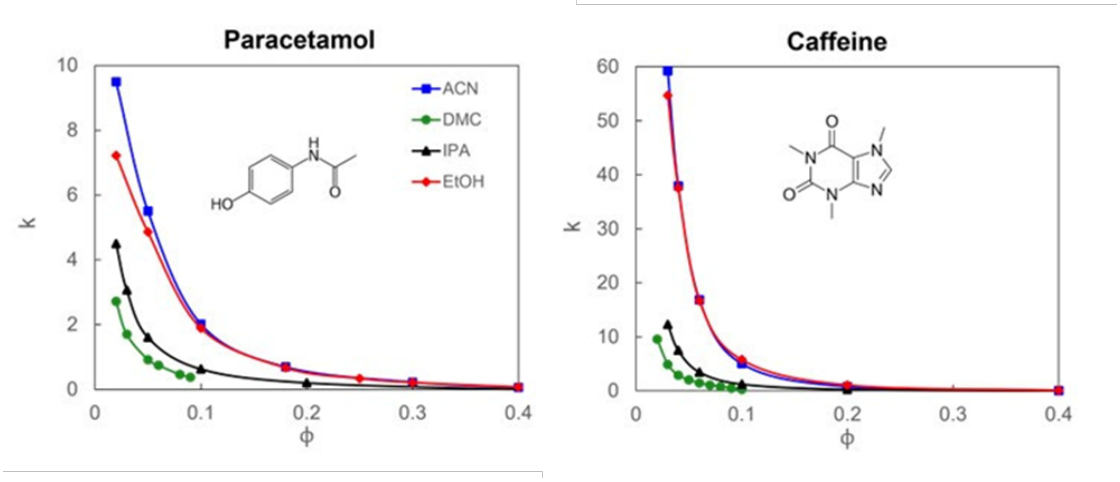


*Fig 4.7 Retention curves obtaneid for Paracetamol (left) and Caffeien (right)*

This characteristic is a significant advantage, especially from an industrial and large-scale perspective, as it helps to reduce solvent consumption and solvent disposal costs. In fact, a minimal amount of DMC is sufficient for efficient elution. This behavior is elucidated by considering its polarity, indicated by the log $Kow$ values. EtOH and ACN emerge as the most polar solvents, with very low and similar log $Kow$ values (about -0.3). In contrast, IPA and DMC have higher log $Kow$ values (0.05 and 0.35, respectively). As a result, the polarity of DMC is closer to that of the C18 stationary phase compared with other solvents. This result is translated in a decrease in the partition coefficient and, consequently, a decrease in retention, as the analytes will be characterized by a higher affinity toward the DMC.

The impact of organic modifiers on efficiency was analysed by constructing the van Deemter curves for caffeine and paracetamol (*Fig. 4.8*) and through peak parking experiments.

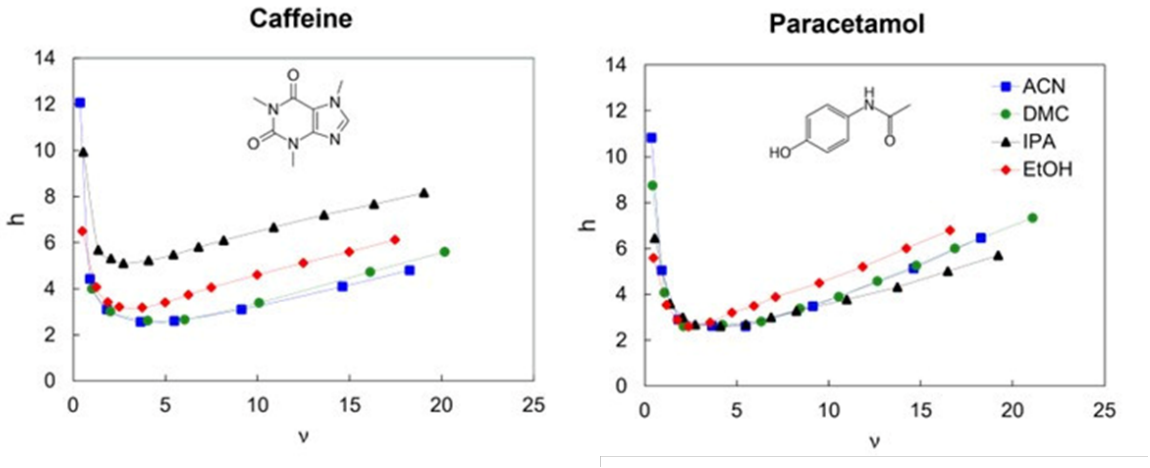


*Fig 4.8 van Deemter curves for Caffeine (left) and Paracetamol (right).*

The data obtained revealed that the diffusion coefficients depend on the nature of the organic modifier, remaining consistent for both sample molecules. The $b$ term, which constitutes about 40-45% of $h_{\min}$, showed slightly higher values for caffeine due to higher retention, with a more pronounced impact in less viscous solvents such as ACN and DMC than in EtOH and PAH. Alcohols hindered the diffusion process, following the trend: ACN > DMC > EtOH > IPA for $Deff$. The $c_s$ term, just 2-3% of $h_{\min}$, , followed the trend: IPA > EtOH > DMC > ACN. Finally, $a(v)$ was determined by subtracting $b$ and $c_s$ terms from $h$, revealing comparable eddy dispersion contributions for ACN and DMC, with smaller values for caffeine.

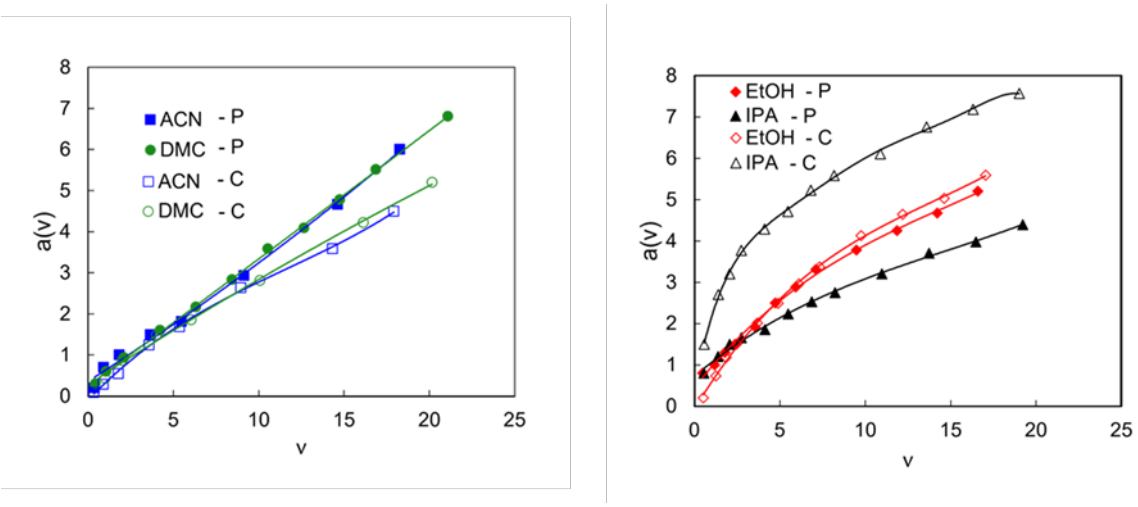


*Fig 4.9 eddy dispersion curves for paracetamol (full points) and caffeine (empty points) measured with ACN (blue squares), DMC (green points), IPA (black triangles) and EtOH (red diamonds)*

IPA stands out as the solvent with the highest eddy dispersion for caffeine across the entire $v$ range. To delve deeper into this behavior, additional aspects were considered. Initially, a detailed examination of molecular diffusion on the porous particle surface was conducted, revealing that solvents like ACN or DMC, with low viscosity, accelerate molecule diffusion along the hydrophobic surface. In contrast, alcohols (EtOH and IPA) restrict the surface

mobility of analytes. The observed larger $D_{part}$ /$D_s$ ratio for caffeine compared to paracetamol suggests a stronger interaction between caffeine and the layer of organic modifier on the particle surface, known as excess adsorption. This effect is more pronounced with alcohols, potentially due to their ability to form hydrogen bonds. As a result, caffeine spends more time on the stationary phase, coupled with a smaller contribution of surface diffusion, enhancing eddy dispersion.

### 4.3.3 Purification of a therapeutic peptide using greener alternatives to ACN

In **Paper V** a proof-of-concept study is reported where DMC has been employed for the very first time for semi-preparative purposes, with the scope of verifying whether it can be effectively used as organic solvent in place of ACN for the purification of a biopharmaceutical (Icatibant). Initially, the retention behavior of the Icatibant was studied using ACN, DMC, EtOH and IPA and then these solvents were used for the downstream processing of the target peptide by comparing the purification performance in terms of process purity, recovery and productivity as a function of the solvent employed. The study initiated by exploring the retention behavior of Icatibant by varying the composition of the mobile phase (MP). The MP, a TEAP buffer (pH = 8), was modified with varying percentages of four organic modifiers: ACN, DMC, EtOH, and IPA. *Fig. 4.10* illustrates the logk vs. Φ plots of Icatibant, where k is the retention factor. The curves exhibit a typical reversed-phase behavior, with a near-linear decrease in the logarithm of retention as the organic solvent percentage increases. Notably, DMC enhances the elution strength significantly, requiring about 9%(v/v) DMC for k=1, compared to higher percentages for other solvents (22% IPA, 30% ACN, and 35% EtOH). The slopes of the retention curves for ACN, IPA, and EtOH are similar, while the slope of DMC curve is slightly steeper, indicating a more pronounced effect of changes in mobile phase on retention.

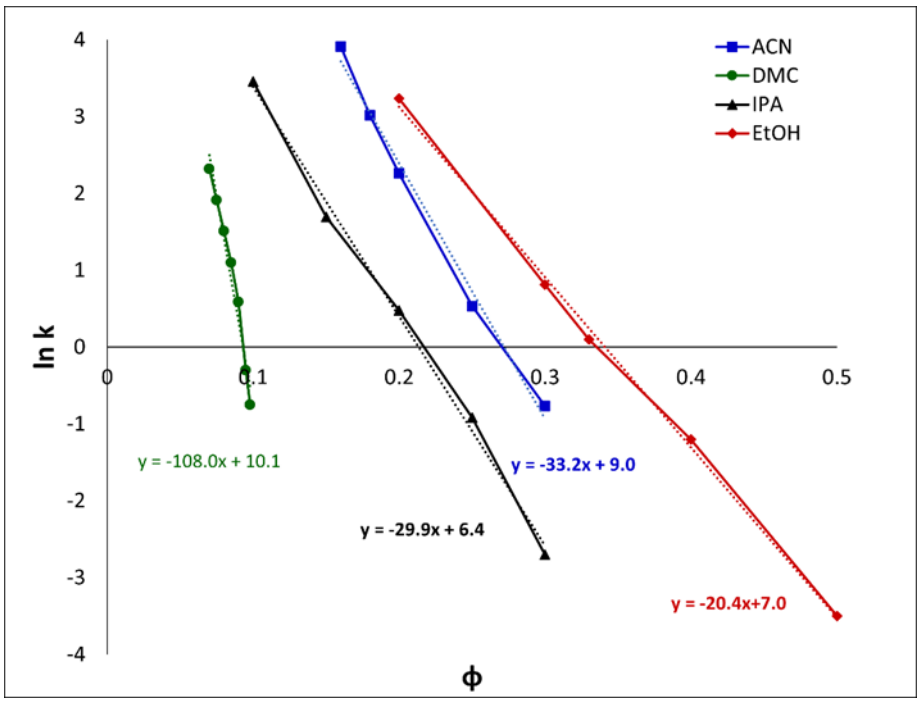


*Fig 4.10 retention curves of Icatibant in ACN, DMC, EtOH and IPA*

Despite this, under the experimental conditions used in this work, no substantial deviations in retention times were observed. To enhance reproducibility, premixed mobile phases with varying proportions of aqueous buffer and organic modifier were employed.

The study of retention led to the development of semi-preparative LC methods for the purification of Icatibant by varying the organic modifier within suitable ranges (*Table 4.2*, *Fig. 4.11*)

| Modifier | Duration of the gradient (CV) | Flow rate (mL/min) | Variation of organic modifier along the gradient (%) |
|---|---|---|---|
| DMC | 10.3 | 1.0 | 7.3-10.0 |
| ACN | 18.0 | 1.5 | 14.8-24.8 |
| IPA | 10.3 | 1.0 | 14.0-22.0 |
| EtOH | 10.3 | 1.0 | 20.0-28.0 |

*Table 4.2 Experimental conditions for the purification of the feed of Icatibant in DMC, ACN, IPA and EtOH.*

The limited variation of DMC, due to its higher elution strength, results in reduced consumption and waste of organic solvents, with consequent environmental benefits. Despite the similar shape of the target peak for all solvents, ACN and DMC generate narrower peak shapes than alcohols. This is crucial for peptides, as larger peaks in alcohols can lead to significant overlap between the target and impurities, affecting the trade-off between product, purity and recovery.

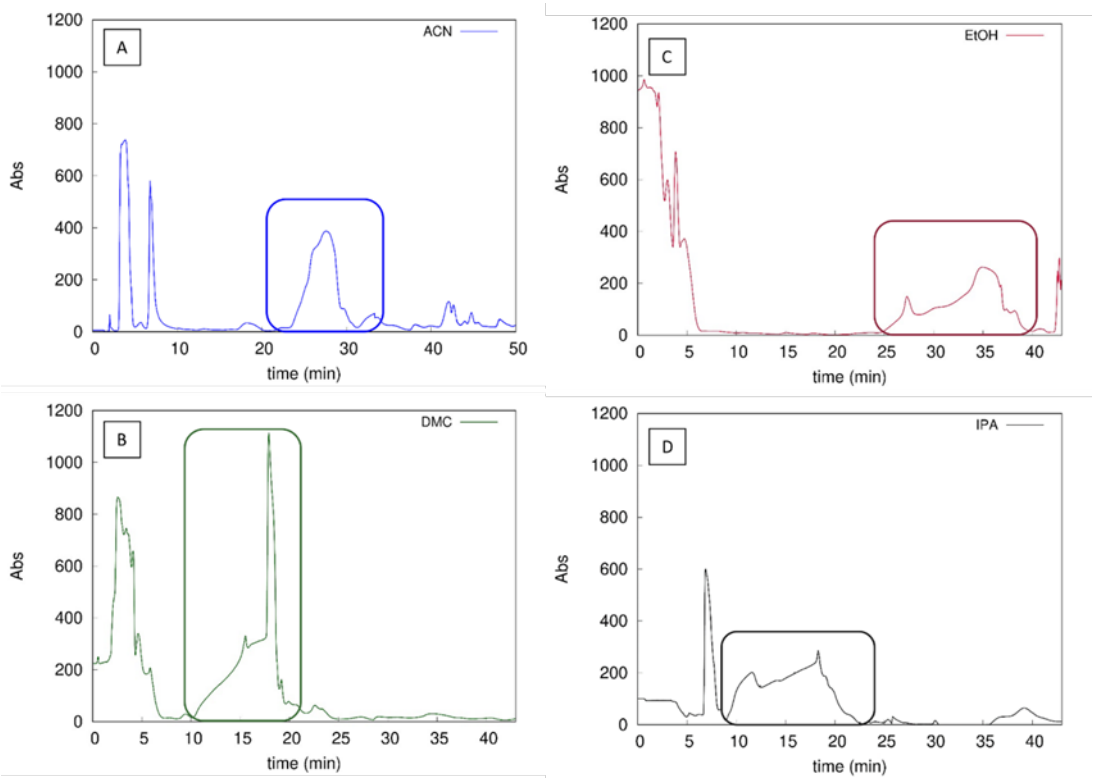


*Fig 4.11 Preparative chromatograms obtained in ACN (blue), EtOH (red), DMC (green) and IPA (black)*

The collected fractions from the target peaks were analyzed through offline UHPLC, and the data were used to create Pareto curves, showcasing the trade-off between purity and recovery In general, this relationship varies inversely: the purest fraction collected contains a marginal amount of peptides, whereas when the collection window is enlarged, higher recoveries are obtained at the cost of purity losses. (*Figure 4.12*).

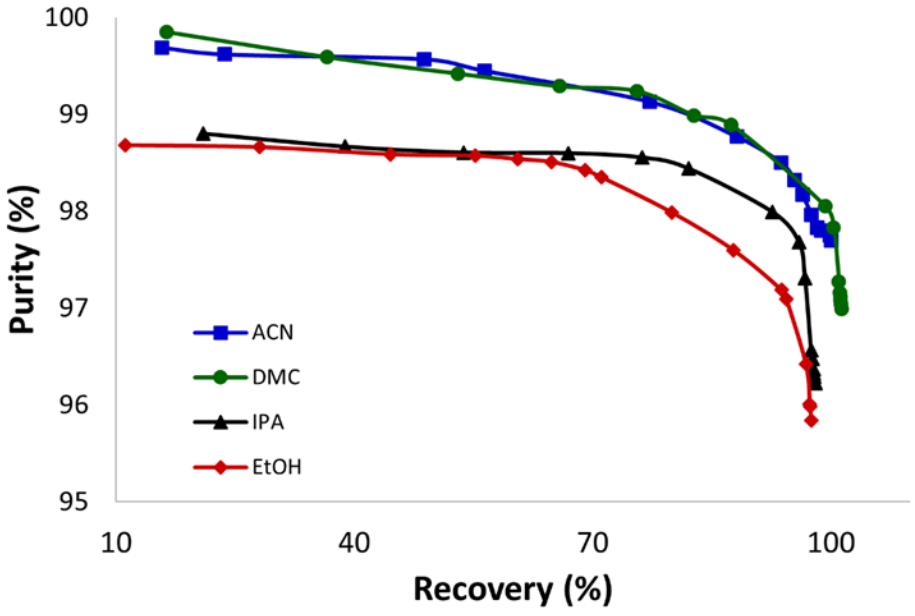


*Fig 4.12 Pareto Curve obtained in ACN, DMC, IPA and EtOH*

These curves, constructed for each of the four solvents, offer crucial insights into the

performance of preparative-scale purification methods. DMC demonstrates purification performance comparable to ACN, with nearly superimposed curves. The purest fraction, represented by the leftmost point, shows a purity of approximately 100% for both ACN and DMC, while EtOH and IPA yield slightly lower purities (around 99%). Detailed data on recovery, purity, and productivity are presented in *Table 4.3* for clarity.

| ORGANIC MODIFIER | RECOVERY (%) | PURITY (%) | PRODUCTIVITY (mg/L/h) |
|---|---|---|---|
| ACN | 15.8 | 99.7 | 2.8 |
| | 99.9 | 97.7 | 11.9 |
| | 94.0 | 98.5 | 11.2 |
| DMC | 16.4 | 100.0 | 2.4 |
| | 99.9 | 96.9 | 14.1 |
| | 93.5 | 98.5 | 13.9 |
| EtOH | 11.2 | 98.7 | 1.6 |
| | 97.4 | 96.0 | 13.9 |
| | 64.8 | 98.5 | 9.4 |
| IPA | 21.0 | 98.8 | 2.3 |
| | 98.1 | 95.8 | 14.2 |
| | 79.0 | 98.5 | 11.2 |

*Table 4. 3 Performance parameters obtained for the downstream processes in ACN, DMC, EtOH and IPA.*

Considering the maximum yield scenario, where the entire product collection is pooled, all solvents achieve roughly 100% recovery. ACN yields the highest purity (98%), followed by DMC (97%) and alcohols (96%), with a consistent purity gain of about 30% from the initial feed purity of 76%. Notably, productivity at 100% recovery is comparable for all solvents, except for ACN, which is lower due to its longer gradient duration. While Pareto curve endpoints assess the yield-purity trade-off, real-case scenarios often target a specific purity level for pharmaceutical compliance. Setting a target peptide purity of 98.5%, both ACN and DMC lead to the highest recovery (around 94%) compared to EtOH (65%) and IPA (80%). This suggests that ACN and DMC offer highly pure products with minimal mass loss (around 6%). DMC also exhibits the highest productivity, surpassing ACN even with its longer gradient.

In the context of Icatibant purification, potential alternative solvents, especially DMC, demonstrate results comparable to ACN in terms of recovery and purity. Additionally, depending on the target purity, EtOH or IPA can serve as effective and environmentally friendly alternatives to ACN.

### 4.3.4 Closing Remark

This study marks the first comprehensive evaluation and comparison of the kinetic properties and mass transfer characteristics of DMC in comparison to more traditional solvents used RPLC. The results are promising, showing that DMC allows to achieve comparable efficiency to ACN while using a significantly smaller volume of solvent. Notably, DMC exhibits excellent efficiency for analytes like paracetamol and caffeine, outperforming the commonly considered green alternatives, such as EtOH and IPA, whose efficiency is influenced by analyte chemistry. DMC emerges as an optimal candidate for replacing ACN in RPLC due to its similar kinetic performance without causing detrimental effects on column backpressure, thanks to its comparable viscosity. Despite the challenge posed by DMC limited solubility in water, this drawback is mitigated by its high elution strength. The study lays the groundwork for future investigations, particularly in the separation of biomolecules, addressing the growing need for greener solvents in the biopharmaceutical industry.

In the purification of the therapeutic peptide Icatibant, DMC, EtOH, and IPA have shown promising results, achieving roughly 100% recovery and >95% purity. However, for higher purity targets (>98.5%), both ACN and DMC outperform alcohols in terms of recovery, with DMC demonstrating the highest productivity. This positions DMC as a promising candidate for preparative conditions in the isolation of biopharmaceuticals, offering potential advantages in shorter runs, reduced instrumentation usage, and decreased organic solvent waste.

## 4.4 DOPED REVERSED PHASE MATERIALS (Paper III)

In this work we have used an innovative stationary phase, named Doped Reversed Phase, that is applied to the difficult polishing process of a large bio peptide (Liraglutide). In this work, it is demonstrated that using this stationary phase it is possible to reduce the time of the analysis, saving solvents. The results are presented in **Paper III.**

### 4.4.2 The applications of the Doped Reversed Phase columns

Liraglutide is a 31 amino acids polypeptide, similar to the physiologically secreted hormone GLP-1, used in the therapy of type-2 diabetes and obesity. This was kindly given by Fresenius Kabi iPsum (Villadose, Italy). In the initial phase of upstream treatment, the aim

is to overcome pharmacokinetic challenges and improve pharmacodynamic characteristics. In the process of enhancing the original structure with the addition of a fatty acid side chain and a glutamic spacer, complications arise during typical reverse phase peptide polishing steps due to increased hydrophobicity. Thus, the incorporation of an aliphatic fatty chain significantly intensifies hydrophobic interactions with the resin, leading to irreversible adsorption and a decrease in chromatographic selectivity between the product and impurities under RPLC conditions. Consequently, the initial polishing protocol required two separate methods to achieve optimal purity, employing orthogonal techniques with different types of interactions. To address these challenges, we devised a purification protocol using Doped Reversed Phase (DRP) material as a stationary phase. DRP materials represent a peculiar class of mixed-mode resins that address two problems simultaneously. Considering the experimental conditions and analyte loadings, the ion exchangers can function attractively or repulsively. Given the robust hydrophobic interaction existing between the analytes and the RPC resin in our context, the strategic choice has been to select ion exchangers that induce analyte repulsion, consequently leading to a final reduction in overall retention. Liraglutide, with an isoelectric point (pI) 4.9, is extremely soluble at pHs above 8.5, but tends to precipitate under more acidic conditions. To solve this problem, the crude mixture was dissolved in preparative MP-A: 25mM triethylamine phosphate buffer, pH 8.5 (adjusted with 85% orthophosphoric acid). The negative charge led to the selection of sulphonated groups (CIEX ligands) in a 10% ratio to induce repulsion between the elements. The experimental objective was to compare the downstream process using a standard RP stationary phase and the DRP phase (90% C8 - 10% sulphonate groups). The mobile phases used for the downstream process were: MP-A: triethylamine phosphate buffer 25 mM, with a pH corrected to 8.5 and MP-B: ACN.

In *Fig 4.13* are reported the chromatograms obtained using the C8 e Mixed-mode resins.

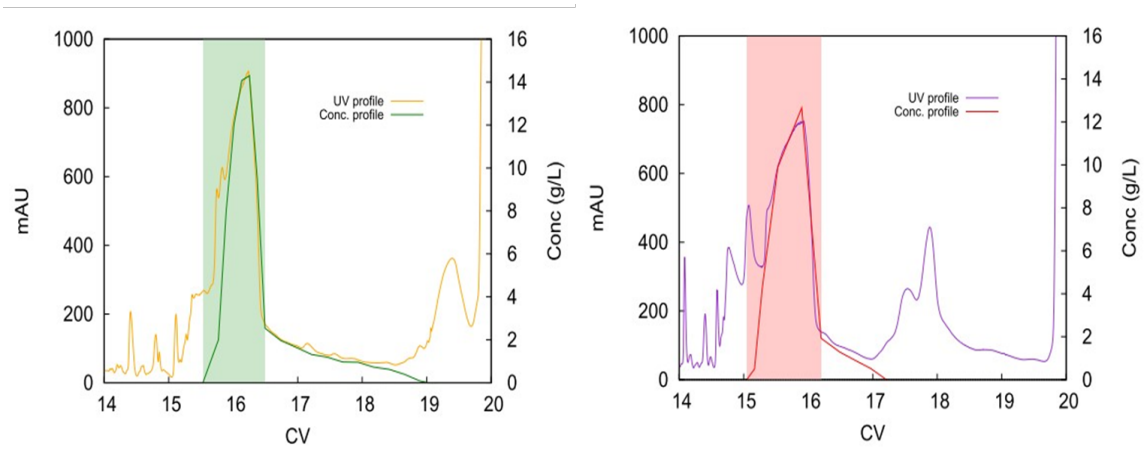


*Fig 4.13 UV profile and concentration profile of Liraglutide with the C8 column (left) and the DRP column (right). The colored regions represent the pooled windows*

From the UV profiles, it can be seen that the entire peak, with the doped column (purple), elutes in about 2 CV, whereas with the C8 column (yellow), about 3.5 CV are required to completely elute the product peak. It can also be seen that this is due to the repulsive effects between the charged ionic groups on the stationary phase and Liraglutide, both of which are negatively charged by considering that the pH of the mobile phase (8.5) was more basic than the pI (4.9) of the peptide. In addition to being narrower, the product peak elutes sooner when treated with the mixed-mode column, confirming the general impact of repulsive ionic charges on retention. It can also be seen that the concentration profile is less affected by tailing using a DRP column. A Pareto curve, a diagram relating purity and recovery, was plotted for both separations *Fig. 4.14.* A clear increase in purity values is obtained by separating the crude with the mixed-mode column.

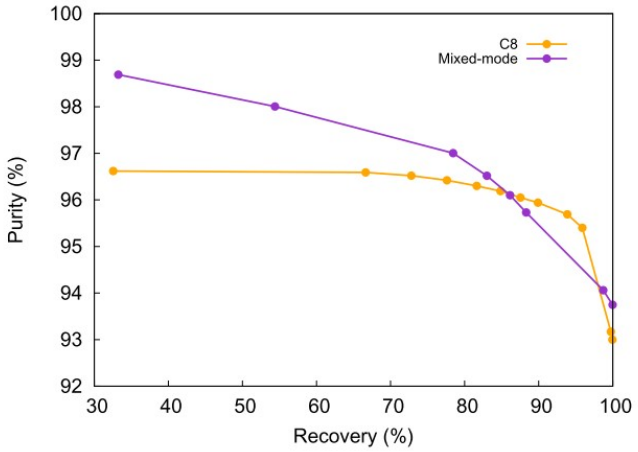


*Fig 4.14 Pareto curve obtained for the downstream process using the C8 and DPR colum.*

After setting the purity threshold for the collected pool, the side regions of the main peak, which overlap with the narrow eluting impurities, must be subjected to reprocessing to avoid wasting significant amounts of the target peptide. Although this additional purification step is time-consuming and expensive, an interesting option is to collect the main peak excluding the tail, as shown in the chromatograms in *Figure 4.13*. The performance comparison of the two columns, focusing on this chromatographic portion, is presented in *Table 4.4*.

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

*Table 4.4 Comparison of Recovery, Purity for the C8 and Doped column*

The results show that the use of the mixed-mode column is more efficient than traditional C8 column. The results show that the use of the mixed-mode column slightly improves purity (+2.5%) and increases recovery by +15%. Consequently, a smaller percentage of the target product must be reprocessed in a subsequent purification step (about half that of the C8 column), aligning with the liraglutide peak recovered from the discarded tail. Moreover, the productivity of the processes using the mixed mode increased from 4.7 mg/mL/h, as obtained using the C8 resin, to 5.4 mg/mL/h.

### 4.4.3 Closing Remark

A novel approach on a stationary phase based on hydrophobicity and charge interactions was investigated in this work. At pHs above the pI of the peptide, a repulsive effect is established between the target and charged cation exchange ligands doping the stationary phase, both of which are negatively charged. Narrower peaks are obtained and their retention decreases, thus allowing for a less solvent consumption for their elution.

# CHAPTER 5

## 5.1 CONCLUSION

The primary objective of this research is to show the feasibility of implementing a more environmentally friendly downstream processing method for biopharmaceuticals. The biopharmaceutical industry is currently facing several challenges, particularly in bridging the gap between upstream and downstream processes to enhance efficiency and resource utilization. Although continuous countercurrent techniques are acknowledged as potential alternatives for batch purifications, there is reluctance due to perceived complexity.

In this study, is explore the multicolumn approach as a promising solution to address the usual trade-offs associated with single-column chromatography. Through experimental endeavors, we demonstrate that Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) consistently delivers purified material with high-quality specifications, minimizing material disposal and thereby increasing process productivity for purifying therapeutic peptides. This technology, characterized by complete automation, robustness, and cost-effectiveness, represents a significant advancement for the evolving biopharmaceutical industry.

Moreover, as presented in Paper II, reveal that MCSGP contributes to a reduction in solvent consumption, leading to a decrease in the production of organic waste solvents. This approach proves essential for achieving a lower environmental impact in bioprocessing.

The study also marks a pioneering effort in comprehensively evaluating and comparing the kinetic properties and mass transfer characteristics of Dimethyl Carbonate (DMC) in comparison to traditional solvents like Acetonitrile (ACN) used in Reversed-Phase Liquid Chromatography (RPLC). Encouraging results indicate that DMC can achieve comparable efficiency to ACN while using significantly less solvent volume. Notably, DMC exhibits exceptional efficiency for analytes such as paracetamol and caffeine, outperforming

commonly considered green alternatives like EtOH and IPA, whose efficiency is influenced by analyte chemistry. DMC emerges as a promising substitute for ACN in RPLC, offering similar kinetic performance without adverse effects on column backpressure due to its comparable viscosity.

In the purification of the therapeutic peptide Icatibant, reveal promising results with DMC, EtOH, and IPA, achieving approximately 100% recovery and >95% purity. However, for higher purity targets (>98.5%), both ACN and DMC surpass alcohols in terms of recovery, with DMC demonstrating the highest productivity. This positions DMC as a potential candidate for preparative conditions in the isolation of biopharmaceuticals, offering potential advantages such as shorter runs, reduced instrumentation usage, and decreased organic solvent waste.

Additionally, a novel approach based on hydrophobicity and charge interactions on a stationary phase was explored in this work. At pH levels above the isoelectric point of the peptide, a repulsive effect is established between the target and charged cation exchange ligands doping the stationary phase, both of which carry negative charges. This results in narrower peaks, decreased retention, and consequently, reduced solvent consumption for elution. These innovative findings pave the way for future investigations, especially in the separation of biomolecules, addressing the increasing demand for environmentally friendly solvents in the biopharmaceutical industry.

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# *Paper I*

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

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**ABSTRACT:** Biopharmaceuticals are subjected to very strict purity requirements to be marketed. At the same time, peptides and other biomolecules are industrially synthesized through techniques (e.g., solid-phase synthesis) 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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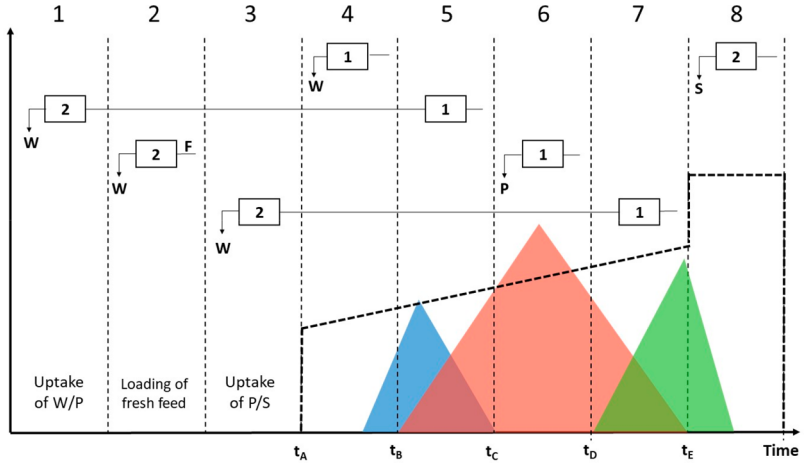


**Figure 1.** Schematic representation of a batch chromatogram and its different characteristic regions. The blue peak denotes weakly adsorbed impurities (W), the red one the target product peak (P) and the green one represents the strongly adsorbed impurities (S). [Reproduced with permission from ref 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 $\mu$m 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 $\mu$m. 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 $\mu$m and a pore size of 100 Å. The wavelength chosen was 226 nm, while the injected volume was 2 $\mu$L. 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

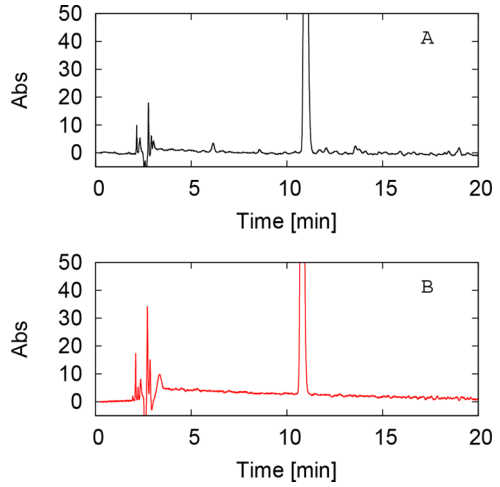


**Figure 2.** (A) Analytical HPLC chromatogram of the icatibant feed; (B) analytical HPLC chromatogram of a fraction with purity of >99%.

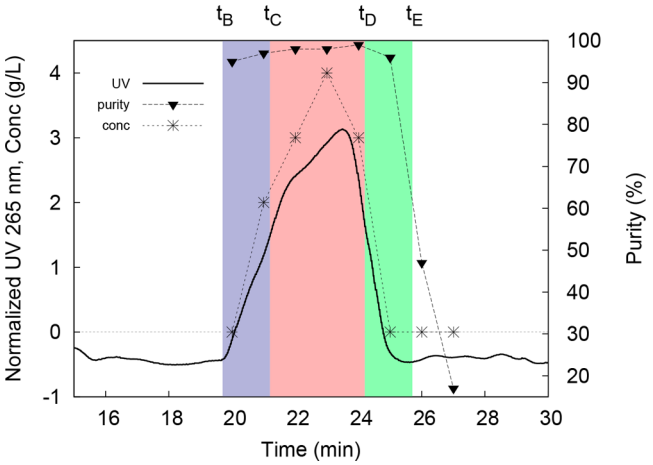


**Figure 3.** UV (at 265 nm), purity and concentration values in the column outlet stream, as a function of time for the design batch chromatogram.

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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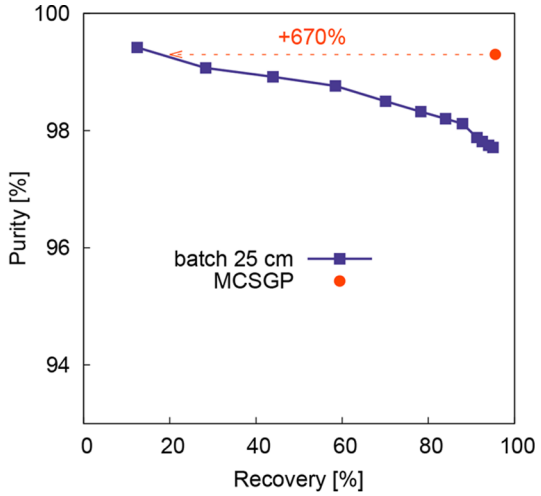


**Figure 4.** Pareto curve obtained for the batch run with the 25 cm column and the point representing the MCSGP performance at steady state.

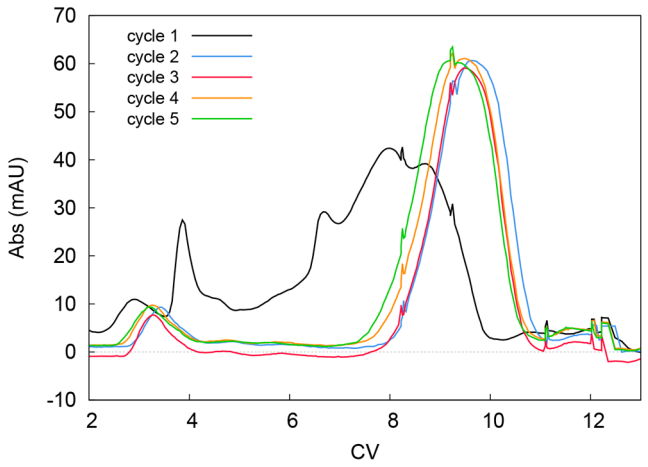


**Figure 5.** Ultraviolet (UV) spectrum in the stream leaving the upstream column during the first switch of five MCSGP cycles.

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

The authors declare no competing financial interest.

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*Paper II*

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.

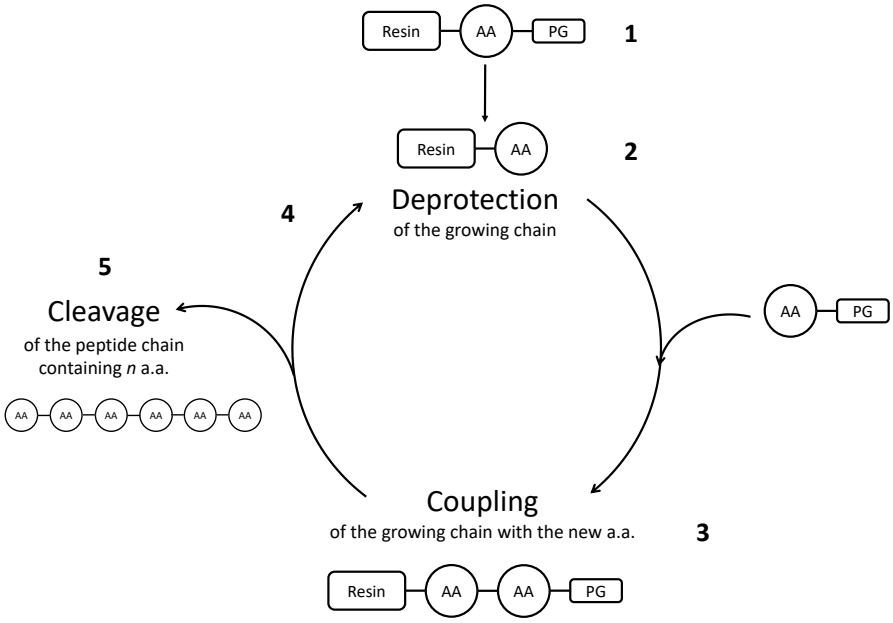


**Figure 1.** Scheme representing Solid Phase Peptide Synthesis. (1) An amino acid (AA) is protected on the functional group that must not react, and it is bonded to an insoluble resin. Then, its protecting group (=PG) is removed (2), so that the following amino acid, which in turn is protected, can 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:<br>• RP-LC: hydrophobic character<br>• Ion-Exchange: charge<br>• HILIC: hydrophilic character<br>• 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.

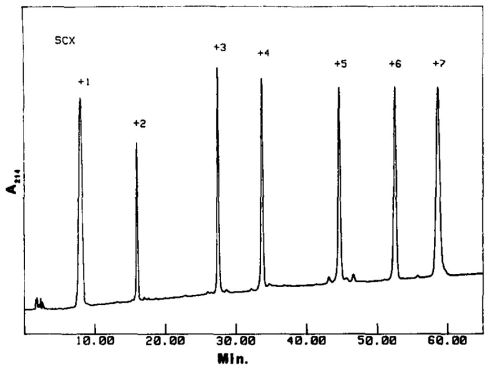


**Figure 2.** Separation of seven synthetic peptides bearing different charges by means of Ion-Exchange chromatography. Reproduced with permission from [86].

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].

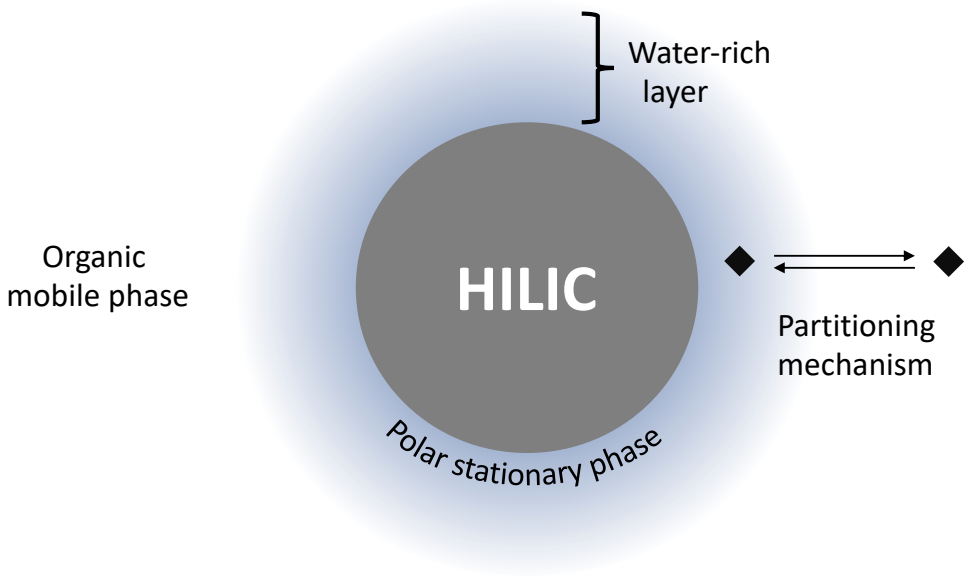


**Figure 3.** Scheme of the mechanism regulating elution in HILIC. The polar stationary phase is surrounded by a layer rich in water, whereas the mobile phase is an organic solvent. Probably, the analytes are retained because of partitioning mechanism. It results that more hydrophilic components are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

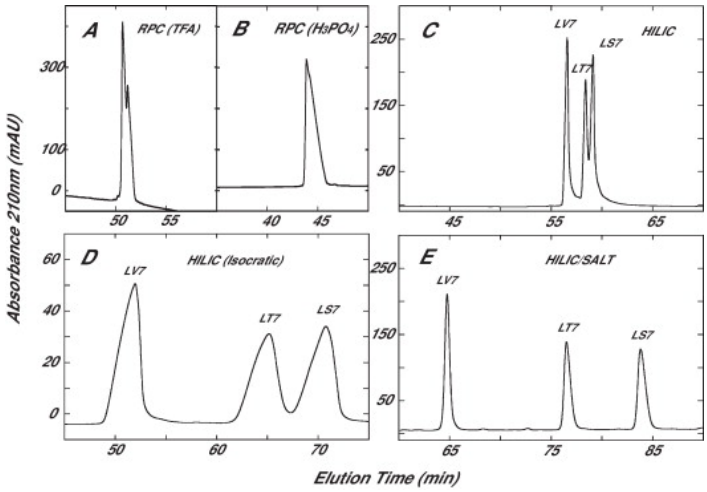


**Figure 4.** Separation of a mixture of LV7, LT7 and LS7 peptides by RP-LC with TFA (panel **A**) or phosphoric acid (panel **B**) as the ion-pairing reagent, HILIC (decreasing concentration of acetonitrile, panel **C**), isocratic HILIC (isocratic concentration of acetonitrile, panel **D**) and HILIC/SALT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

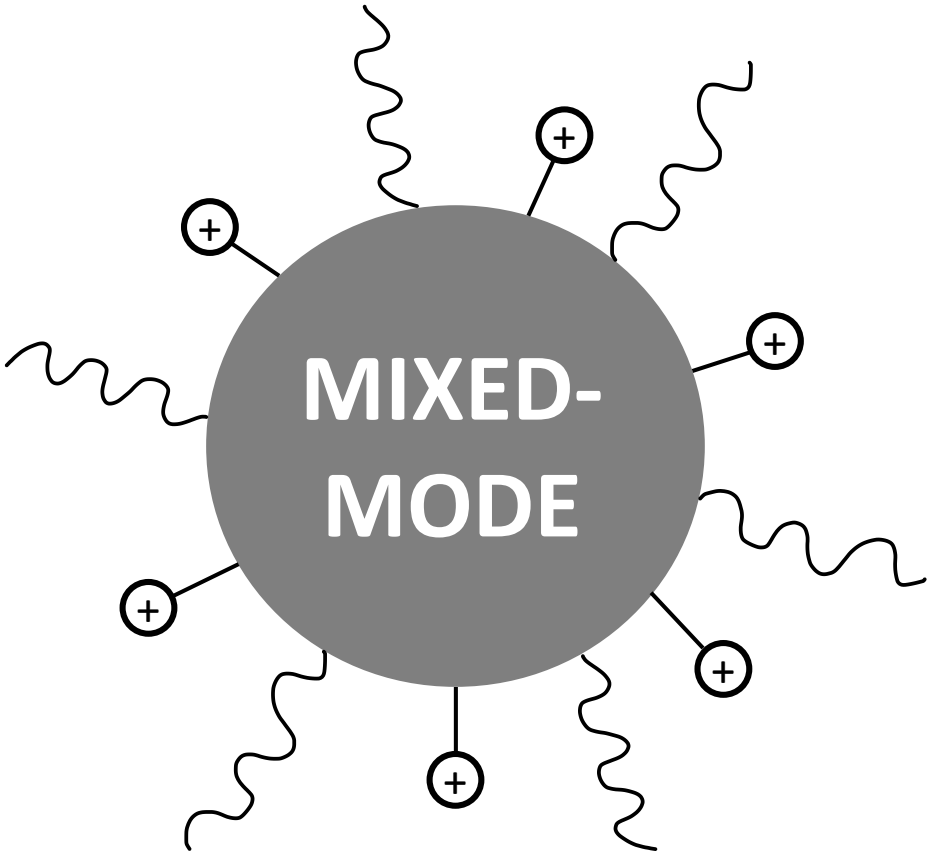


**Figure 5.** Scheme of a typical stationary phase used for Mixed-Mode Chromatography. The particle is functionalized with alkylic chains (e.g., C8) and charged groups (e.g., quaternary amines).

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 $\alpha$-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].

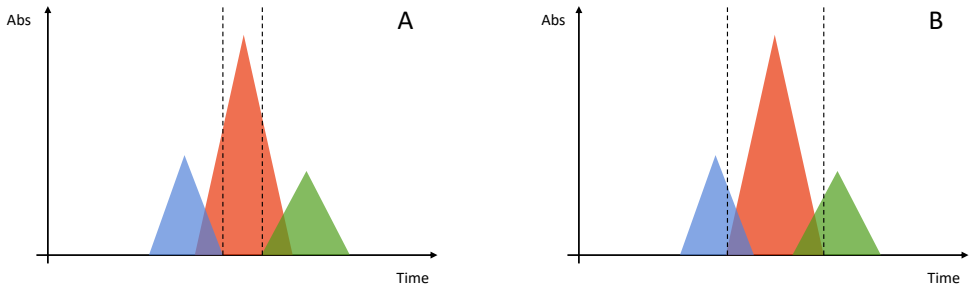


**Figure 6.** The red peak represents the target product, eluting between a group of impurities more weakly adsorbed (blue peak) and a group of impurities more strongly adsorbed (green peak). When deciding which portion of the main peak to collect, two cases are possible: (**A**) narrow collection window 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.

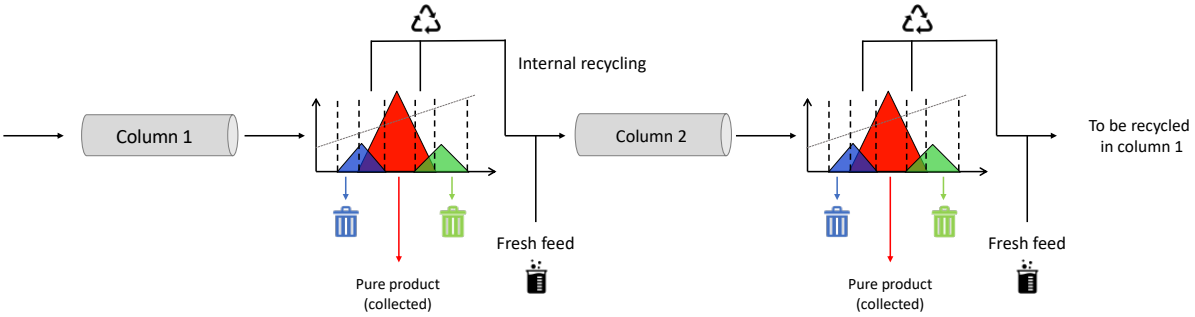


**Figure 7.** A scheme illustrating the MCSGP technique is shown. The gradient elution is performed in the first column. The main peak (red) overlaps in the front and in the tail with other groups of impurities (blue and green). The overlapping regions are therefore internally recycled into the second 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].

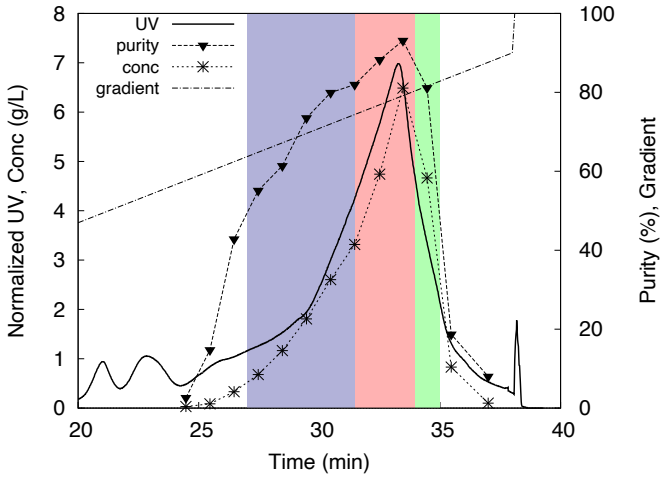


**Figure 8.** Batch chromatogram obtained for the purification of a glucagon crude mixture. The blue and green windows represent the impure portions of the main peak that need to be recycled into the second column. On the other side, the red window represents the target product fulfilling the purity 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 III*

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:

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

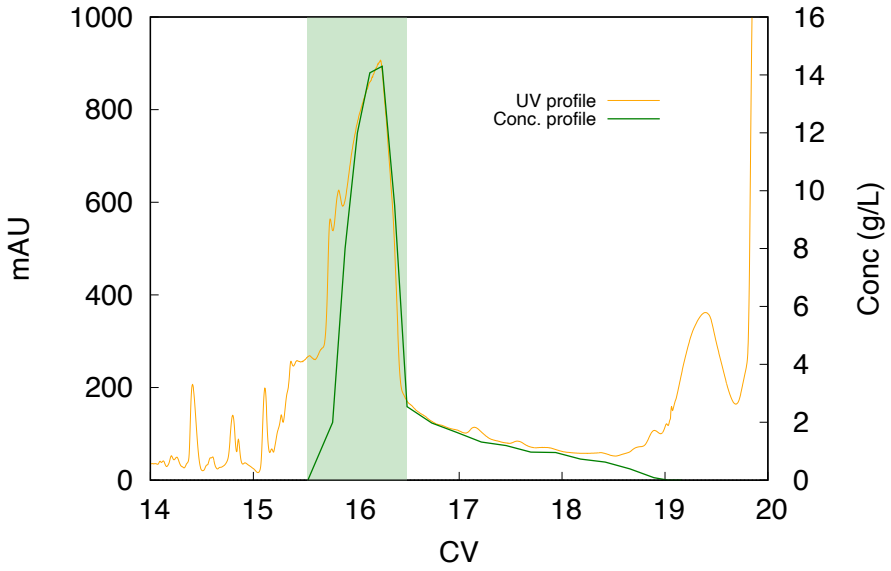
Besides purity, recovery can also be evaluated for each fraction. It is defined as

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

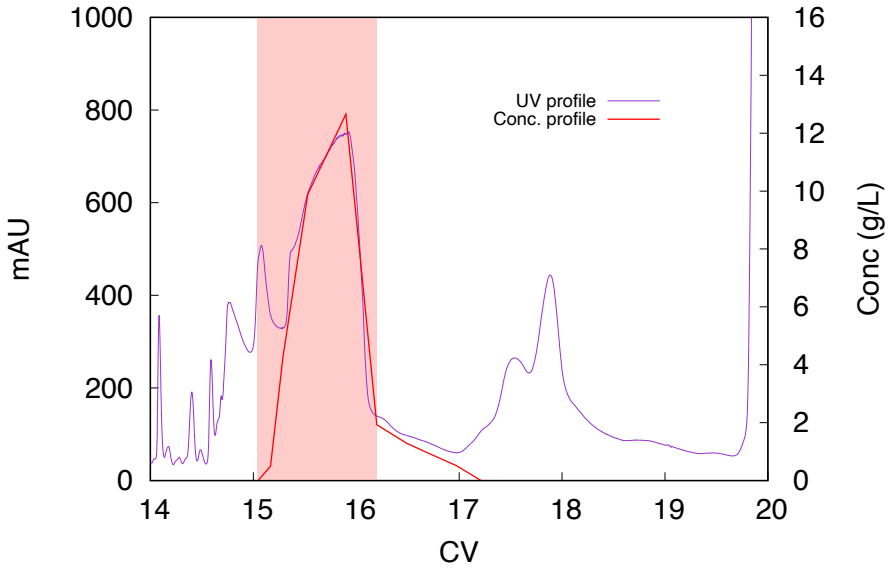
which is the mass of the peptide contained in a fraction divided by the total peptide mass injected.

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.

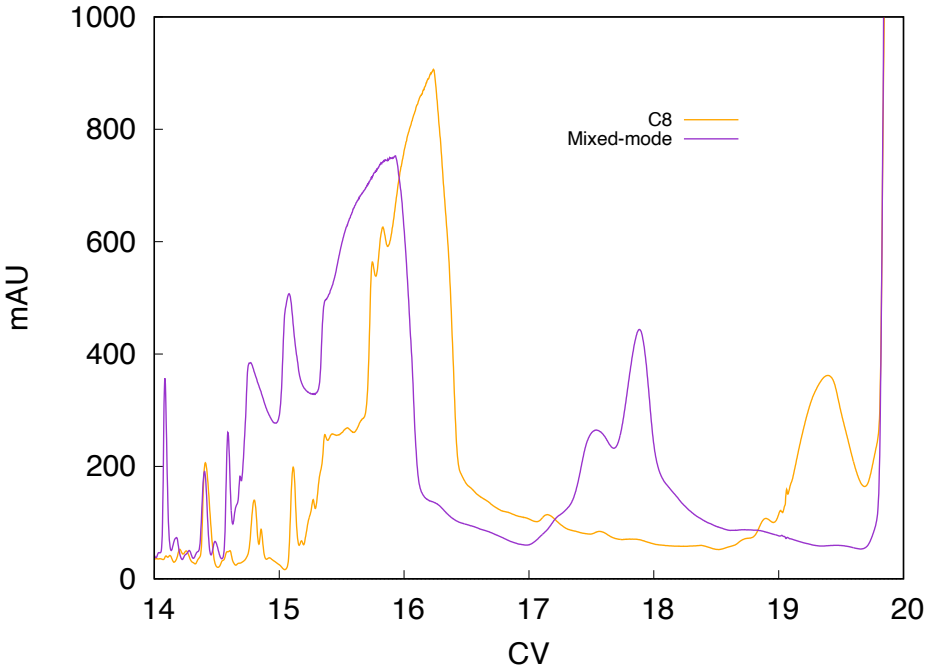



**Figure 2.** Comparison between chromatographic profiles obtained with C8 (orange) and mixed-mode (violet) columns. The main peak and other species elute earlier because of the repulsive effects established between doping ligands and the feed components, both negatively charged.

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.

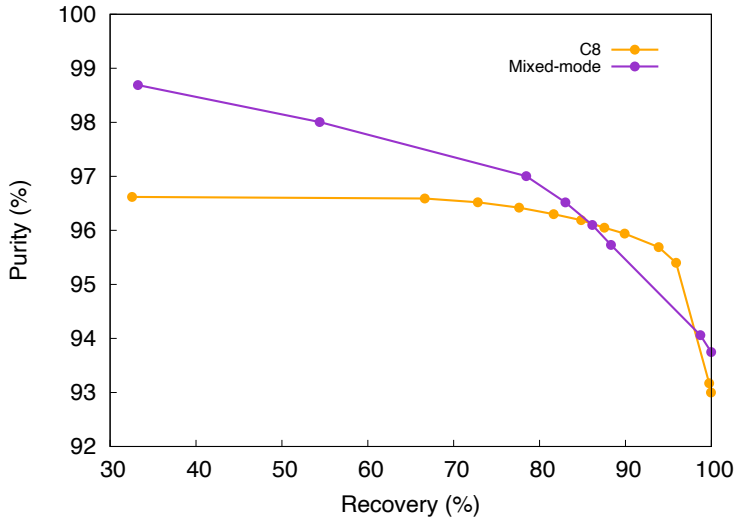



**Figure 3.** Comparison between the Pareto curves obtained with the two columns in the same chromatographic conditions (C8: orange; MM: violet).

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 IV*

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# Dimethyl carbonate as a green alternative to acetonitrile in reversed-phase liquid chromatography. Part I: Separation of small molecules

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ABSTRACT

Nowadays, environmental problems are drawing the attention of governments and international organisations, which are therefore encouraging the transition to green industrial processes and approaches. In this context, chemists can help indicate a suitable direction. Beside the efforts focused on greening synthetic approaches, currently also analytical techniques and separations are under observation, especially those employing large volumes of organic solvents, such as reversed-phase liquid chromatography (RPLC). Acetonitrile has always been considered the best performing organic modifier for RPLC applications, due to its chemical features (complete miscibility in water, UV transparency, low viscosity etc); nevertheless, it suffers of severe shortcomings, and most importantly, it does not fully comply with Environmental, Health and Safety (EHS) requirements. For these reasons, alternative greener solvents are being investigated, especially easily available alcohols.

In this work, chromatographic performance of the most common solvents used in reversed-phase chromatography, i.e., acetonitrile, ethanol and isopropanol, have been compared to a scarcely used solvent, dimethyl carbonate (DMC). The analytes of interest were two small molecules, caffeine and paracetamol, whose kinetics and retention behaviour obtained with the four solvents have been compared, and all contributions to band broadening have been assessed. Results about kinetic performance are very promising, indicating that a small amount (7 % v/v) of DMC is able to produce the same efficiency as a 2.5-times larger ACN volume (18 % v/v), and larger efficiency than alcohols.

This paper reports, for the first time, fundamental studies concerning the mass transfer phenomena when DMC is used as an organic solvent in RPLC, and, together with the companion paper, represents the results of a research whose final aim was to discover whether DMC is suitable for chromatographic applications both in linear and preparative conditions.

## 1. Introduction

In recent years, the awareness of modern society on global threats such as climate change and environmental issues has constantly increased. Governments and authorities have promoted specific programmes to enable sustainable development, such as the European Green Deal which aims to reach climate neutrality by 2050 and to boost the economy through green technologies [1].

In this context, the twelve principles of Green Chemistry represent the basis to develop sustainable processes for the production of materials and goods [2–5]. In particular, the replacement of harmful solvents with greener ones (which is directly mentioned in the 5$^{th}$ principle and indirectly referred to in almost every other) has become one of the main driving factors in laboratories and industries to enable a sustainable growth. This special attention is attributable to the fact that large volumes of toxic, flammable, or hazardous solvents are handled daily in

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chemical processes. These characteristics are often related to specific requirements needed for the application. For instance, volatile solvents are often employed for extraction or purification purposes since they can be easily evaporated, but they can generate unwanted air emissions which increase the risk of workers exposure.

Early efforts to substitute toxic solvents have been especially focused on production/synthetic processes which could have the greatest environmental impact. However, in the last few years, there has been a growing interest in applying Green Chemistry principles to almost every sector of chemistry. One of these areas is analytical chemistry, by considering, especially, analytical laboratories where liquid chromatography (LC) is routinary employed. Indeed, it is estimated that a liquid analytical chromatography instrument, using a conventional LC column (15-25 cm long, 4.6 mm diameter, packed with 5 μm particles) at a mobile phase flow rate of 1 mL/min, produces about 1.5 L of waste per day, which means about 500 L of effluent per year. Therefore about 26-50 million litres of chemical waste are generated every year worldwide [6,7].

For these reasons, Green Analytical Chemistry (GAC) is increasingly considered a new and important sub-area of Green Chemistry, which has started emerging in the 2000s [8]. The goal of GAC is to adapt the concepts of Green Chemistry (which have been specifically formulated for synthetic processes) to the field of analytical chemistry to reduce its environmental impact without compromising outcome and performance [2]. Obviously, the ideal solution would be the development of innovative solventless analytical procedures, possibly based on the only use of aqueous solutions, or the recycling of the solvent in order to produce a minimal amount of waste and to reduce the environmental impact of the process. However, many analytical processes require the use of organic solvents, and, in those cases, GAC suggests replacing toxic, harmful chemicals with greener alternatives [2,4,6].

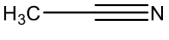
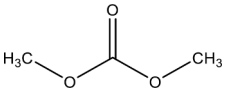
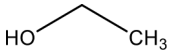
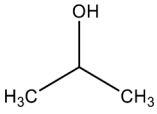
Among these processes it is worth mentioning separations in reversed-phase LC (RPLC) conditions, which represent the most popular mode of chromatography for quantitative and preparative applications, being estimated to be used in almost 80 % of the cases [9,10]. RPLC is based on the use of a hydrophobic stationary phase (typically made of silica particles functionalized with $C_{18}$ or $C_8$ chains) and a more polar mobile phase, which is usually a mixture of water and an organic modifier. Acetonitrile (ACN) is by far the most employed organic solvent for RPLC due to its intrinsic properties such as low viscosity, excellent elution strength, UV-transparency (cut-off wavelength at 190 nm, see Table 1), and complete miscibility with water. Despite these characteristics, which are very convenient from a chromatographic point of view, acetonitrile also possesses other properties that make it unsuitable from a GAC perspective. Indeed, it shows dramatic shortcomings associated with the production of acrylonitrile, since ACN is produced as a by-product of its production [9,10]. Furthermore, ACN is associated to toxic effects on the human body due to the metabolic release of cyanide. For these reasons, the replacement of ACN with greener alternatives is becoming an urgent priority.

The greenness of an organic solvent can be defined on the basis of Environmental, Health and Safety (EHS) requirements and Life-Cycle Assessment (LCA). The first criterion comprehends environmental indicators (such as persistence and water/air hazards), health parameters (e.g., chronic toxicity) and safety specifications (including flammability, explosion risk and stability). LCA, on the other hand, evaluates the effects of a product on the environment over the entire period of its life, thus including production, uses, disposal and potential recycling. In order to help chemists in the choice of a green option, many pharmaceutical companies have drawn up solvent selection guides, which rank solvents according to their “greenness” [11,12]. Among these, ethanol (EtOH), isopropanol (IPA), acetone, but also ethyl lactate and propylene carbonate are popular green solvents which have already been tested as eluents in RPLC [2,13–15].

Very recently, the attention of scientists has also focused on other less popular options such as organic carbonates, which show a very low eco-toxicity and are completely biodegradable [16]. An example of these organic compounds is dimethyl carbonate (DMC). This is a nonpolar flammable transparent liquid, with a similar smell to that of methanol. It does not have irritating or mutagenic effects; therefore, it can be safely handled. It is extensively studied for applications in several product groups: synthesis of pharmaceuticals (such as taladafil [17]), coatings, and lithium-ion battery electrolytes [18]. In addition, it is commonly used as an environmentally friendly substitute of dimethyl sulphate and methyl halides for acid-catalysed carboxymethylation reactions [19]. According to EHS criteria, DMC is considered among the "recommended” solvents, therefore it belongs to the same class of water and alcohols [11]. However, the application of DMC in chromatography is still very limited. The first report is dated 2021, where this solvent has been used as an organic modifier in RPLC for the separation of different probe molecules of pharmaceutical interest by using inductively coupled mass spectrometry (ICP-MS) as detection method. It was found that, thanks to its higher hydrophobicity, a lower percentage of DMC was necessary to elute analytes in comparison to MeOH or ACN, allowing for a better stability of the plasma [20]. The other recent paper about DMC reports on its use as an organic solvent for normal phase LC (NPLC) and hydrophilic interaction chromatography (HILIC). The authors reported that DMC is a weaker solvent both for NPLC and HILIC applications, at least under the experimental conditions used in that case, but it allowed to obtain slightly better efficiencies [21].

In this work, fundamental studies concerning retention behaviour and kinetic performance of two different probe molecules, namely

**Table 1**
Chemo-physical characteristics of ACN, DMC, EtOH and IPA [42].

| Properties | Acetonitrile (ACN) | Dimethyl carbonate (DMC) | Ethanol (EtOH) | Isopropanol (IPA) |
|---|---|---|---|---|
| Structure |  |  |  |  |
| Pictograms |  |  |  |  |
| Molecular weight (g/mol) | 41.05 | 90.08 | 46.07 | 60.10 |
| log $K_{ow}$ | -0.34 | 0.35 | -0.31 | 0.05 |
| Boiling point (°C) | 81.6 | 90.4 | 78.2 | 82.3 |
| Melting point (°C) | -43.8 | 4.7 | -114.1 | -89.5 |
| Density at 25°C (g/cm$^3$) | 0.786 | 1.069 | 0.785 | 0.781 |
| Viscosity at 25°C (mPa•s) | 0.334 | 0.589 | 1.074 | 2.038 |
| Cut-off (nm) | 190 | 220 | 210 | 210 |

paracetamol and caffeine, were investigated under RPLC conditions with UV-Vis detection by using different organic modifiers in aqueous solutions, including the innovative DMC. Particularly, DMC performance was compared to traditional RPLC solvents, such as ACN, EtOH and IPA. A detailed investigation of all the contributions to band broadening has been performed in order to unravel mass transfer phenomena and diffusion properties of the analytes in DMC/$H_2O$ as well as in all the other mobile phase compositions.

To the best of our knowledge, this is the first work reporting on a fundamental study of retention mechanisms in RPLC by using DMC as an organic modifier and it represents the basis to further understand the possibility to also use this solvent for chromatographic applications also of industrial relevance. These further aspects will be the object of the second part of this work.

## 2. Theory

### 2.1. Efficiency

The evaluation of kinetic performance of a chromatographic column is commonly done through the well-known van Deemter equation which correlates the efficiency, in terms of plate height ($H$), to the mobile phase linear velocity. The reduced version of this equation involves the use of adimensional coordinates which allow the evaluation of kinetic performance independently from particle diameter ($d_p$) and type of analyte:

$$h = a(\nu) + \frac{b}{\nu} + c_s\nu \tag{1}$$

being $h$ ($=H/d_p$) the reduced plate height and $\nu$ the reduced interstitial velocity, expressed as:

$$\nu = \frac{F_v d_p}{\pi r^2 \varepsilon_e D_m} \tag{2}$$

where $F_v$ is the flow rate, $r$ the column radius, $D_m$ the molecular coefficient of the analyte in the mobile phase and $\varepsilon_e$ the interstitial porosity of the packed bed. The latter is defined as the ratio between the interstitial volume, $V_e$ (which can be calculated through Inverse Size Exclusion Chromatography [22]), and the geometrical volume of the column, $V_{col}$.

The three terms appearing in Eq. (1) refer to the main sources of band broadening in a chromatographic column and they can be independently evaluated. The $b$-term is the longitudinal diffusion which is defined as:

$$b = 2(1+k_1)\frac{D_{eff}}{D_m} \tag{3}$$

where $D_{eff}$ is the effective diffusion coefficient in the porous zone (see later on) and $k_1$ is the zone retention factor, which is referred only to the interstitial volume. It is correlated to the retention factor $k$ with the following relationship:

$$k_1 = \frac{(1+k)\varepsilon_t}{\varepsilon_e} - 1 \tag{4}$$

being $\varepsilon_t$ the total porosity, which is the ratio between the void volume, $V_0$, and $V_{col}$.

The interpretation of $D_{eff}$ in light of a proper model of diffusion in porous media [23–28] allows to evaluate the different contributions to diffusion in the adsorbent, including the intraparticle diffusivity, $D_{part}$, which accounts for diffusion in the intraparticle volume. In this work, the more advanced Effective Medium

Theory (EMT) [23–25,27] has been employed. According to the Maxwell's model, $D_{eff}$ can be written as:

$$D_{eff} = \frac{1}{\varepsilon_e(1+k_1)}\left[\frac{1+2(1-\varepsilon_e)\beta}{1-(1-\varepsilon_e)\beta}\right]D_m \tag{5}$$

where $\beta$ is the polarizability constant, defined as:

$$\beta = \frac{\alpha_{part}-1}{\alpha_{part}+2} \tag{6}$$

and $\alpha_{part}$ is the relative permeability:

$$\alpha_{part} = \frac{D_{part}K_{part}}{D_m} = \frac{D_{part}k_1\varepsilon_e}{D_m(1-\varepsilon_e)} \tag{7}$$

with $D_{part}$ the diffusion coefficient through the porous particles and $K_{part}$ the whole particle-based equilibrium distribution constant.

The $c_s$-term reported in Eq. (1) accounts for the solid-liquid mass transfer resistance, which represents the main source of band broadening at high flow rates. Its expression is the following:

$$c_s = \frac{1}{30}\,\frac{k_1}{(1+k_1)^2}\,\frac{D_m}{D_{part}} \tag{8}$$

$a(\nu)$ can be calculated by subtraction of $b$- and $c_s$-terms from accurately measured $h$-values:

$$a(\nu) = h - \frac{b}{\nu} - c_s\nu \tag{9}$$

## 3. Materials and methods

### 3.2. Columns and materials

Caffeine and paracetamol were purchased from Merck Sigma-Aldrich (Darmstadt, Germany). All solvents and reagents were purchased from Carlo Erba Reagents (Milan, Italy), except for DMC which was from Thermo Fisher Scientific (Waltham, Massachusetts, USA), with a purity $\geq$ 99 %. A Kromasil C18 column (250×4.6 mm i.d.) packed with 5 µm fully porous particles was purchased from Merck Sigma-Aldrich (Darmstadt, Germany). A 33 × 4.6 mm Micra column (Eprogen, Inc., Downers Grove, IL, USA) packed with 1.5 µm non-porous silica particles was purchased from DBA Italia s.r.l. (Milan, Italy) and employed for the estimation of bulk molecular diffusion coefficients.

### 3.2. Equipment

All measurements were carried out on an Agilent 1290 Infinity LC System, equipped with a binary solvent pump (max pressure: 1200 bar), a column thermostat, an autosampler and a photodiode array detector. Detection wavelength was 254 nm. Temperature was set at 25°C.

### 3.3. Retention studies

The dependence of the retention factor, $k$, on the fraction of organic modifier, $\Phi$, was evaluated at different mobile phase compositions for two small molecules (paracetamol, caffeine). ACN, DMC, IPA and EtOH were investigated as organic modifiers. It is worth noting that $\Phi_{DMC}$ ranged from 0.01 to 0.1 due to its miscibility limit in water. The flow rate was 1 mL/min. Injection volume was 0.5 µL. Retention factors, $k$, were corrected for the extra-column residence time. The hold-up time, $t_0$, was calculated with inverse size exclusion chromatography, ISEC (for further details the reader is referred to Ref. [29]).

### 3.4. Peak parking experiments

Peak parking measurements were employed for the purpose of estimating diffusion coefficients, $D_{eff}$ and $D_m$, of caffeine and paracetamol with all the organic solvents tested in this study (ACN, DMC, IPA, EtOH)

[30,31]. $D_{eff}$ was calculated by considering that the spatial peak variance $\Delta\sigma_x^2$ is directly proportional to the parking time, $t_{park}$, through the following relationship:

$$\Delta\sigma_x^2 = 2D_{eff}t_{park} \quad (10)$$

Experimentally, spatial peak variance was calculated as $\Delta\sigma_x^2 = L^2/N$, where $L$ is the column length and $N$ the number of theoretical plates given by the software (determined with the method of moments). All the data obtained were corrected for the extra-column peak variance. Parking times employed were 0, 2, 5, 7, 10, 15, 20, 25, and 30 min and the flow rate was 0.2 ml/min.

Molecular diffusion coefficients, $D_m$, of probe molecules in all the solvents mentioned above were estimated by performing peak parking experiments in a column packed with non-porous particles (Micra column) at a flow rate of 0.2 ml/min.

In this case:

$$D_m = \frac{D_{eff}}{\gamma_e} \quad (11)$$

where $\gamma_e$ is the external obstruction factor, a geometrical parameter which describes the constriction and the tortuosity of inter-particle channels [32]. In order to determine the value of $\gamma_e$ an experiment of peak parking was carried out using a molecule whose $D_m$ is known from literature. Thiourea dissolved in pure water was chosen for this purpose ($D_m = 1.33 \times 10^{-5}$ cm$^2$/s) [33]. The value of $\gamma_e$ was found to be 0.65.

### 3.5. van Deemter curves measurements

van Deemter curves were measured for each organic modifier by choosing an appropriate mobile phase composition to keep the retention factors of paracetamol and caffeine constant at around 0.65 and 1.20, respectively. The mobile phase compositions were the following: (i) ACN/$H_2O$ 18:82 % (v/v); (ii) DMC/$H_2O$ 7:93 % (v/v); (iii) EtOH/$H_2O$ 18:82 % (v/v) and (iv) IPA/$H_2O$ 10:90 % (v/v).

All measurements were performed through stepwise increments of flow rate, starting from 0.02 mL/min, up to 1 ml/min with ACN and DMC or up to 0.7 ml/min with EtOH and IPA, due to higher back pressures. Retention time and column efficiency (given as number of theoretical plates) of eluted peaks were automatically processed by the software (calculated through the method of moments) and corrected for the extra-column contribution.

## 4. Results and discussion

Firstly, physico-chemical properties of DMC were evaluated in order to understand possible advantages and limitations related to its applicability in LC. By looking at Table 1, it can be evinced that DMC is characterised by a cut-off wavelength of 220 nm, which is the highest among the four solvents that were considered in this study. This could represent a limitation when dealing with complex samples that would require wavelength below this value but for most applications in LC (involving molecules with aromatic rings or with absorption maxima at higher wavelengths) it does not represent an issue. Also, it must be kept in mind that the maximum amount of DMC in an aqueous mobile phase is around 10 %, therefore the baseline noise caused by DMC absorption at wavelengths below 220 nm should be very limited.

DMC shows a higher boiling point and density if compared to the other three solvents, an intermediate viscosity between ACN and EtOH, but no harmful effects. These points should be carefully evaluated when considering the overall greenness, environmental impact, and feasibility of a chromatographic method. Furthermore, the energy and material required for the production of the organic solvent has to be taken into account. In this contest, lowering the amount of organic solvent used into the chromatographic run, such in the case of DCM (max 10 % v/v is used), will lower the environmental impact coming from its production. On the basis of these points, DMC can be considered a comparable, even if not better in some feature, alternative solvents from a greenness point of view respect to the other ones investigated in our study.

### 4.1. Effect of mobile phase composition on retention

The dependence of the retention factor on the amount of organic modifier (Φ) was evaluated for caffeine and paracetamol (Fig. 1) by using the four different organic modifiers (ACN, DMC, IPA, EtOH).

In reversed phase chromatography, retention decreases by increasing the percentage of organic modifier in mobile phase due to the increase of solubility and affinity of analytes with the organic-rich phase, or in other words, to the reduction of the partition coefficient [34,35]. By comparing the figures, it can be evinced that DMC shows the highest elution strength in all cases, indeed, for the same Φ, DMC shows smaller $k$ if compared to other solvents. This represents a clear advantage, especially from the industrial and large-scale viewpoint, in terms of environmental impact, solvent consumption and solvent disposal costs, since a limited amount of this solvent would be sufficient for the elution.

This behaviour can be explained by taking into account the polarity, in terms of log $K_{ow}$ (Table 1), of the organic modifiers used. From these data it is clear that EtOH and ACN are the most polar solvents, having very low (and similar) values of log $K_{ow}$ (around -0.3), followed by IPA (0.05) and DMC (0.35). Therefore, DMC polarity is the closest to the $C_{18}$

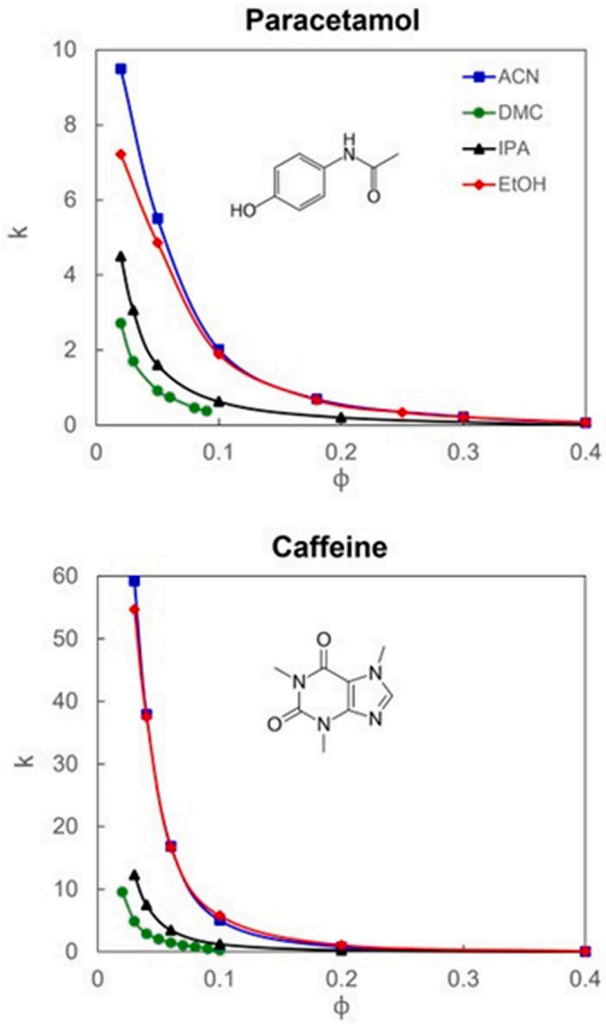



**Fig. 1.** Dependence of the retention factor ($k$) on the amount of organic modifier (Φ) for paracetamol (top) and caffeine (bottom). ACN (blue squares), DMC (green points), IPA (black triangles) and EtOH (red diamonds).

stationary phase, with respect to the other solvents. This leads to a decrease of the partition coefficient, hence of retention, since analytes show high affinity towards DMC.

### 4.2. Efficiency

The effect of organic modifiers on efficiency was evaluated for the two small molecules (caffeine and paracetamol) by calculating their van Deemter curves (Fig. 2). Firstly, from these plots it can be evinced that, for paracetamol ($k = 0.65$), relatively good kinetic performance (with $h$ values as low as 2.6) was achieved with all solvents. It is worth noting that, at high $\nu$, IPA leads to the best efficiency, followed by ACN and DMC, while EtOH leads to the worst performance. Conversely, van Deemter curves of caffeine ($k = 1.20$) are not overlapping. In this case, a different trend is observed, indeed IPA leads to the worst kinetic performance. As an example, at $\nu = 20$, $h$ values of 8.5, 7.0, 5.3 and 5.6 were measured for IPA, EtOH, ACN and DMC, respectively. Moreover, the $c$-branch of van Deemter curves obtained with alcohols for the two molecules are parallel, with almost the same performance obtained with EtOH. Surprisingly, IPA leads to very high efficiency for paracetamol but, when the analyte is caffeine, a huge loss of efficiency is observed.

It is noteworthy that van Deemter curves of ACN and DMC are perfectly superimposable, independently from the probe molecule under study, indicating that DMC is able to produce the same efficiency as ACN. In this case, the $c$-branch is steeper for paracetamol.

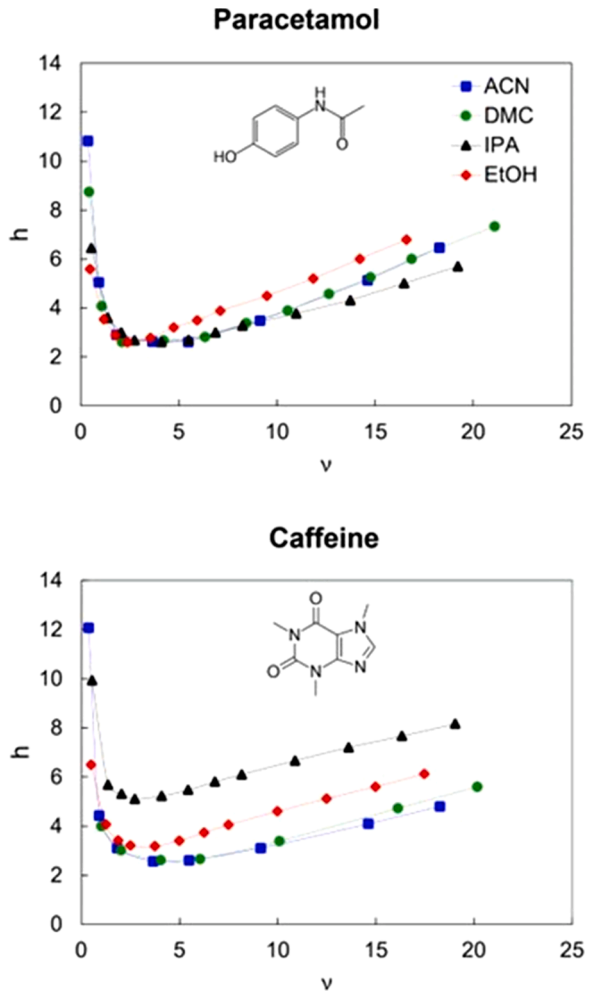



**Fig. 2.** van Deemter curves of paracetamol (top) and caffeine (bottom) using four different organic modifiers: ACN (blue squares), DMC (green points), IPA (black triangles) and EtOH (red diamonds).

In the following, the individual terms of the van Deemter equation (Eq. (1)) will be independently evaluated by combining stop-flow experiments (peak parking) and the Maxwell's model [23]. Once effective and molecular diffusion coefficients, $D_{eff}$ and $D_m$, have been calculated through the peak parking method, longitudinal diffusion terms of the two probe molecules were then evaluated through Eq. (3). The application of the Maxwell's model permits to calculate the diffusion in the porous zone ($D_{part}$), allowing to access the $c_s$ term (Eq. (8)). All these data are reported in Table 2. As it can be easily noticed, diffusion coefficients depend only on the nature of the organic modifier used, being practically the same for the two sample molecules. As expected, $b$-terms are slightly larger for caffeine than for paracetamol, due to the higher retention. Overall, the $b$-term represents approximately 40-45 % of $h_{\min}$, a value in line with what is reported in literature [36]. The contribution of $b$ on $h_{\min}$ is more pronounced for less viscous solvents, like ACN and DMC, with respect to EtOH and IPA (see Table 1). Alcohols, on the contrary tend to disfavour diffusion process due to their large viscosity, hence $D_{eff}$ follows the trend: ACN > DMC > EtOH > IPA. The $c_s$-term represents only 2-3 % of $h$(min). From Eq. (8) one can notice that it depends on retention and on the ratio between $D_m$ and $D_{part}$. The ratio $D_m/D_{part}$, and as a consequence $c_s$, follow the trend: IPA > EtOH > DMC > ACN.

Finally, $a(\nu)$ was calculated by subtracting $b$ and $c_s$ terms from $h$ (Eq. (9)). The results are plotted in Fig. 3 for ACN and DMC (top) and alcohols (bottom). By this mean of representation, it can be easily evinced that, once again, ACN and DMC show the same eddy dispersion contributions for the two probes, with smaller values obtained for caffeine, result which is consistent with literature, where the higher the retention the smaller the eddy dispersion [37,38]. Nevertheless, when using alcohols, especially IPA, the opposite behaviour is observed. Indeed, the eddy dispersion contribution measured for caffeine with IPA is consistently higher than that of paracetamol. IPA turns out to be the solvent with the highest eddy dispersion for caffeine over the whole range of $\nu$ investigated, followed by EtOH. As an example, at $\nu$= 19 the use of IPA leads to a 60 % loss of efficiency if compared to ACN or DMC.

In order to explain this behaviour, further aspects have been evaluated. Firstly, a more detailed description of molecular diffusion occurring on the surface of the porous particle has been investigated. To this end, the contributions of pore and surface diffusions to intraparticle diffusion have been separately calculated by assuming the parallel diffusion model [30,39]:

$$D_{part} = \varepsilon_p \gamma_p F(\lambda_m) D_m + (1 - \varepsilon_p) K_A D_s \tag{12}$$

with $\varepsilon_p$ being the particle porous zone porosity, $\gamma_p$ the internal obstruction factor, $F(\lambda_m)$ the hindrance diffusion factor, $K_A$ the Henry's constant of adsorption and $D_s$ the surface diffusion coefficient. Calculated $D_s$ coefficients are shown in Table 2, where it can be noticed that: (i) $D_s$ follows the trend already observed for other diffusion coefficients ($D_{part}$ and $D_{eff}$), i.e. ACN > DMC > EtOH > IPA, (ii) caffeine shows $D_s$ values which are more than 30 % smaller than paracetamol, (iii) $D_s$ coefficients measured with ACN are more than 6-fold higher than with IPA for both molecules. These results indicate that solvents like ACN or DMC, thanks to their small viscosity, are able to speed up the molecule diffusion along the hydrophobic surface of the particle, while alcohols (EtOH and IPA) limit the surface mobility of the analytes. It is interesting to notice that the ratio $D_{part}/D_s$ is systematically larger for caffeine with respect to paracetamol (Table 2). This may be linked to a stronger interaction between caffeine and the layer of organic modifier adsorbed on the surface of the porous particles, the so-called excess adsorption [40]. This effect is more pronounced with alcohols, probably due to the possibility to form hydrogen bonds. Caffeine, as a consequence, will spend more time on the stationary phase, with respect to paracetamol. This, in combination with a smaller contribution of surface diffusion that does not favour the reduction of concentration gradients originating from velocity variations occurring inside the packed bed, will cause an

**Table 2**
Effective ($D_{eff}$), molecular ($D_m$), particle ($D_p$) and surface ($D_s$) diffusion coefficients, longitudinal diffusion (b) and solid-liquid mass transfer resistance term ($c_s$) for caffeine and paracetamol measured for the four organic modifiers.

| Compound | Paracetamol | | | | Caffeine | | | |
|---|---|---|---|---|---|---|---|---|
| Organic modifier | ACN | DMC | EtOH | IPA | ACN | DMC | EtOH | IPA |
| $D_{eff}$ ($cm^2/s$) | $5.2\times10^{-6}$ | $4.1\times10^{-6}$ | $2.8\times10^{-6}$ | $2.4\times10^{-6}$ | $5.1\times10^{-6}$ | $4.1\times10^{-6}$ | $2.8\times10^{-6}$ | $2.3\times10^{-6}$ |
| $D_m$ ($cm^2/s$) | $7.4\times10^{-6}$ | $6.5\times10^{-6}$ | $5.4\times10^{-6}$ | $4.9\times10^{-6}$ | $7.5\times10^{-6}$ | $6.6\times10^{-6}$ | $5.5\times10^{-6}$ | $4.9\times10^{-6}$ |
| $D_{part}$ ($cm^2/s$) | $4.2\times10^{-6}$ | $2.9\times10^{-6}$ | $1.7\times10^{-6}$ | $1.3\times10^{-6}$ | $4.1\times10^{-6}$ | $3.1\times10^{-6}$ | $1.9\times10^{-6}$ | $1.5\times10^{-6}$ |
| $D_s$ ($cm^2/s$) | $4.1\times10^{-6}$ | $2.8\times10^{-6}$ | $1.0\times10^{-6}$ | $0.6\times10^{-6}$ | $2.8\times10^{-6}$ | $1.7\times10^{-6}$ | $0.6\times10^{-6}$ | $0.4\times10^{-6}$ |
| $D_m$ /$D_{part}$ | 1.8 | 2.2 | 3.2 | 3.9 | 1.8 | 2.1 | 3.0 | 3.4 |
| $D_{part}$ /$D_s$ | 1.0 | 1.1 | 1.6 | 2.1 | 1.5 | 1.8 | 3.0 | 3.3 |
| $b$ | 4.0 | 3.4 | 2.9 | 2.6 | 4.5 | 4.3 | 4.0 | 3.5 |
| $c_s$ | 0.014 | 0.017 | 0.025 | 0.030 | 0.013 | 0.015 | 0.018 | 0.022 |

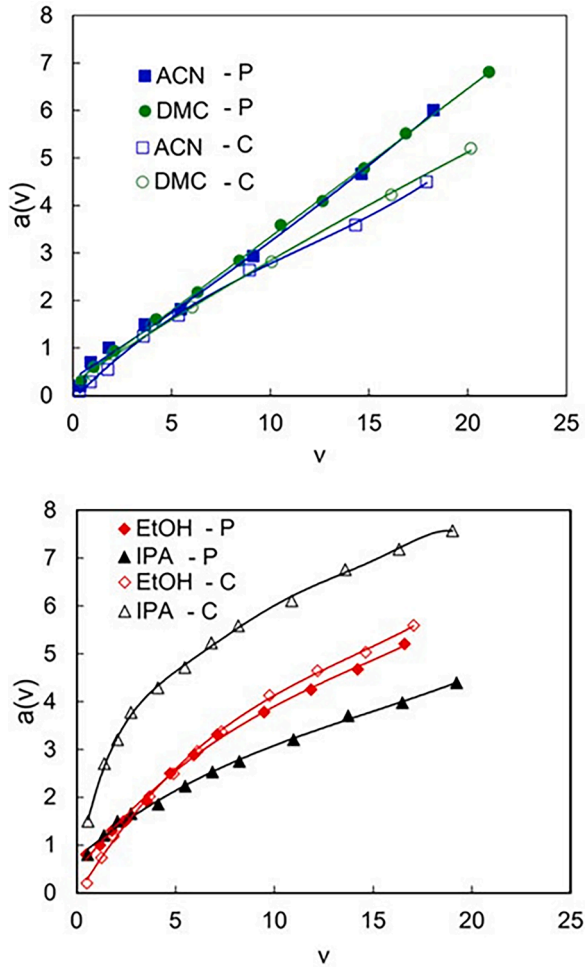



**Fig. 3.** Eddy dispersion curves ($a(\nu)$) of paracetamol (full points) and caffeine (empty points) measured with ACN (blue squares), DMC (green points), IPA (black triangles) and EtOH (red diamonds).

enhancement of eddy dispersion. This effect has been recently observed also in chiral chromatography [41].

## 5. Conclusions

In this work, for the first time, the evaluation and the comparison of kinetic properties and mass transfer characteristics of DMC with more traditional solvents commonly employed as organic modifiers in RPLC have been performed with two probe molecules. Results are very promising, indicating that DMC is able to produce comparable efficiency to ACN, obtaining the same retention with much smaller volume of solvent. On the other hand, efficiency measured with EtOH and IPA, which are considered common green alternatives to ACN, is deeply influenced by the analyte chemistry.

All these data indicate that DMC can be considered as an optimal candidate for the replacement of ACN in RPLC, since it leads to similar kinetic performance without detrimental effects on the column back-pressure, due to a very similar viscosity. The main issue is related to the scarce solubility of DMC with water, but this aspect cannot represent a critical drawback by considering the high elution strength of DMC.

Results of this work aim to lay the foundations for further studies involving the use of DMC for more specific fields, such as the separation of biomolecules of industrial interest and their purification through preparative LC. These aspects are discussed in the companion paper.

## CRediT authorship contribution statement

**Simona Felletti:** Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Matteo Spedicato:** Investigation, Writing – original draft. **Desiree Bozza:** Investigation, Validation. **Chiara De Luca:** Methodology, Writing – review & editing. **Francesco Presini:** Validation, Data curation. **Pier Paolo Giovannini:** Validation, Data curation. **Marco Carraro:** Methodology, Data curation, Resources. **Marco Macis:** Methodology, Data curation, Resources. **Alberto Cavazzini:** Supervision, Funding acquisition, Writing – review & editing. **Martina Catani:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Antonio Ricci:** Conceptualization, Resources, Supervision, Project administration, Writing – review & editing. **Walter Cabri:** Project administration, Supervision.

## 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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# *Paper V*

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# Dimethyl carbonate as a green alternative to acetonitrile in reversed-phase liquid chromatography. Part II: Purification of a therapeutic peptide

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ABSTRACT

Preparative liquid chromatography in reversed phase conditions (RPLC) is the most common approach adopted in the downstream processing for the purification of therapeutic peptides at industrial level. Due to the strict requirements on the quality imposed by the Regulatory Agencies, routinary methods based on the use of aqueous buffers and acetonitrile (ACN) as organic modifier are commonly used, where ACN is practically the only available choice for the purification of peptide derivatives. However, ACN is known to suffers of many shortcomings, such as drastic shortage in the market, high costs and, most importantly, it shows unwanted toxicity for human health and environment, which led it among the less environmentally friendly ones. For this reason, the selection of a suitable alternative becomes crucial for the sustainable downstream processing of peptides and biopharmaceuticals in general.

In this paper, a promising green solvent, namely dimethyl carbonate (DMC) has been used for the separation of a peptide not only in linear conditions but also for its purification through non-linear overloaded chromatography. The performance of the process has been compared to that achievable with the common method where ACN is used as organic modifier and to that obtained with two additional solvents (namely ethanol and isopropanol), already used as greener alternatives to ACN.

This proof-of-concept study showed that, thanks to its higher elution strength, DMC can be considered a green alternative to ACN, since it allows to reduce method duration while reaching good purities and recoveries. Indeed, at a target purity fixed to 98.5 %, DMC led to the best productivity with respect to all the other solvents tested, confirming its suitability as a sustainable alternative to ACN for the purification of complex biopharmaceutical products.

## 1. Introduction

Peptides are organic polymers composed by a series of amino acids (usually from 2 to 50), with molecular weight between 500 and 5000 Da. Many of them are called bioactive because they can have a beneficial impact on biological functions and, thus, on human health [1,2]. The research into this class of biomolecules has started at the beginning of the 20th century, especially by focusing on natural human hormones, including insulin, which has been synthetized for the first time in 1921. Reduced half-lives and poor oral availability have been the main challenges in peptide drug development, but recent advancements in synthetic strategies have allowed to produce novel candidates with better pharmacokinetics and specificity which have revamped the interest around this class of biomolecules. Today, more than 80 peptide-based drugs have been approved and commercialized, but almost 600 more candidates are under preclinical studies [3].

Among the many methods that can be employed to synthetize peptides, solid phase peptide synthesis (SPPS), introduced by Merrifield in

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early 60′s [4], is the most popular and widely used for industrial purposes, also thanks to the fact that it can be easily automated. Other methods involve the use of specific enzymes able to generate a peptide bond in water (chemoenzymatic synthesis) or recombinant technologies through an appropriate expression system [5]. However, none of these techniques allows to produce the target peptide alone and a series of unwanted impurities is also synthetized which has an impact on the purity of the final product. Among the impurities, process related ones (e.g., host cell proteins, viruses, etc.) can be easily removed; on the opposite, product related impurities (molecular variants of the target peptides, including fragments, diastereomers and aggregates) are much more challenging because they share common features with the target product [6,7]. For this reason, one or more purification steps are required to meet quality constraints for (bio)pharmaceuticals imposed by Regulatory Agencies before product commercialization.

Liquid chromatography (LC) in preparative conditions is routinary employed in industry for this purpose. This technique makes use of columns of higher dimensions with respect to common analytical LC and thus it requires the use of larger flow rates (in the order of 10–50 mL/min in preparative conditions up to several L/min in production scale chromatography) [8].

Peptides are commonly purified in reversed-phase conditions, by using hydrophobic stationary phases (C18 or C8, depending on the hydrophobicity of the peptide) and a mixture of aqueous solutions and an organic modifier as mobile phase. Since the retention of peptides is highly influenced by changes in organic modifier concentration [9,10], they are usually purified in gradient conditions, which are also mandatory to improve the resolution of the target peptide from its product-related impurities [11].

However, among all the techniques available for the purification of (bio)products, preparative chromatography is the highest demanding one in terms of energy and waste generation, due to the high dilution required to perform the process and the energy used to control and operate the gradient required for an efficient purification as well as the energy required to evaporate the solvent from the pool containing the purified product.

Consequently, several liters of aqueous and organic mixtures are generated daily as waste which needs to be disposed, with relevant costs and environmental impact, making the downstream process a critical bottleneck from the point of view of sustainability in the entire production process of a target (bio)pharmaceutical [5].

Furthermore, the most widely used organic modifier by (bio)pharmaceutical industries is acetonitrile (ACN), due to its excellent characteristics from a chromatographic point of view, including UV transparency. In particular, as of today, ACN is the only possible choice for the purification of therapeutical peptides at industrial level. However, this solvent suffers of severe shortcomings, including shortage periods linked to the industrial production of acrylonitrile, known as SOHIO process, where ACN is obtained as by-product of its process [12], and very high prices. Moreover, ACN is known to release cyanide upon metabolism in the hepatic cells, with all the drawbacks associated to the toxicity of this product, both from the human and the environmental point of view [13]. For this reason, the International Conference of Harmonization (ICH) guidelines classified ACN as Class 2 solvent with a residual allowed limit of not more than (NMT) 410 ppm in the pharmaceutical drugs [14]. The importance of the environmental and safety impact of the organic solvents is further demonstrated by the Green Chemistry roundtable established American Chemical Society, whose aim is to introduce the concepts of Green Chemistry into pharmaceutical manufacturing [15]. Even if, at the beginning, the focus was only on synthetic drugs, lately the attention has been moved also to biopharmaceuticals, drafting a detailed solvent selection guides which rank them according to their “greenness” impact [5,16–19].

In this optic, the search for alternative solvent with a lower impact from an environmental/toxicological point of view and with a broader availability in the market will become pivotal for ensuring a robust and sustainable downstream process.

In the companion paper, we have investigated the possibility of replacing ACN with dimethyl carbonate (DMC), one of the most promising emerging green solvents used for many industrial applications but barely applied to chromatography [20–26]. In that case, two small molecules were used to understand its retention properties from a fundamental viewpoint. In the comparison, also ethanol (EtOH) and isopropanol (IPA) were considered, since they are the most common green alternatives to ACN used as organic modifiers in LC [27].

This work is intended as a proof-of-concept study where DMC has been employed for the very first time in overloaded non-linear condition with the scope of verifying whether DMC can be effectively used as organic solvent, in replacement of ACN, for the purification of a pharmaceutically relevant peptide. To this end, an industrial perspective was adopted, where the goal is the comparison of the purification outcome in terms of process purity, recovery and productivity as a function of the solvent employed [10,28,29]. After comparing the retention behavior of the peptide using ACN, DMC and also EtOH and IPA, these solvents have been used for the isolation of the target product.

## 2. Theory

The purpose of preparative chromatography is the purification, isolation or accumulation of the target product; therefore, in contrast to analytical chromatography, preparative applications require large amount of sample to be processed [8,30]. The eluate is collected at the outlet in different fractions that are subsequently offline analyzed. Hence, chromatographic performance is estimated from analytical (ultra) high performance liquid chromatography ((U)HPLC), by quantifying four parameters: chromatographic purity, recovery, productivity, and solvent consumption. (Bio)pharmaceuticals must respect severe purity requirements, hence the first parameter to take into consideration is the chromatographic purity. It is obtained from the ratio of the target peak area ($A_{target}$) to the total area ($A_{total}$ ) of all the peaks present in the analytical chromatogram, which also includes impurities, and it is expressed as a percentage:

$$Purity\ (\%) = \frac{A_{target}}{A_{total}} \times 100 \tag{1}$$

Recovery is defined as the ratio of the mass of peptide contained in a fraction or pool of fractions ($m_{target\ collected}$) to the total peptide mass injected ($m_{target\ injected}$) into the system:

$$Recovery\ (\%) = \frac{m_{target\ collected}}{m_{target\ injected}} \times 100 \tag{2}$$

The productivity represents the ratio of amount of target peptide recovered in a fraction or a pool per unit of time and volume of stationary phase (which could be approximated with the geometrical volume of the column ($CV$)), which is calculated as the geometrical volume of the column:

$$Productivity\ (mg/L/h) = \frac{m_{pool\ collected}}{CV\ \times\ time} \tag{3}$$

Finally, the last parameter to take into account when determining the goodness of a purification process is solvent consumption, expressed as the ratio of the total MP volume used during the whole run and the mass of peptide contained in a fraction or pool. This parameter is important from the industrial point of view; indeed, it is convenient to achieve the lowest solvent consumption as possible. This parameter is defined as:

$$Solvent\ consumption\ (mL/mg) = \frac{Total\ Volume}{m_{target\ collected}} \tag{4}$$

## 3. Materials and methods

### 3.1. Sample preparation

The crude mixture (feed) of Icatibant, a cyclic peptide composed by ten amino acids was kindly given by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy) and it was obtained by means of solid-phase synthesis. The product content in the crude mixture was 43 % based on weight. A defined amount of feed was dissolved in 50 mM ammonium acetate/ACN solution, 97/3% v/v [28]. The final concentration of Icatibant was 2.5 g/L. Then, the solution was left under agitation for one hour and filtered with 0.20 μm filters prior to injection. For the purposes of this work (see further on), the feed (same concentration) was prepared also by employing either IPA, EtOH or DMC in place of ACN.

### 3.2. Non-linear overloaded chromatographic conditions

ACN, EtOH and IPA from Carlo Erba Reagents (Rodano, Milano, Italy) and all other reagents for buffers were from Merck-Sigma Aldrich (St. Louis, MI, USA). DMC (purity > 99 %) was from Thermo Scientific (Waltham, Massachusetts, USA).

Purifications were performed on a ÄKTA pure 25 L instrument (Cytiva/GE Healthcare, Uppsala, Sweden), equipped with a fraction collector, a detector set at 265 nm and operated through the Unicorn software. A 250 × 4.6 mm Daisogel-SP-120-10-ODS-BIO column, with a pore size of 120 Å and a particle size of 10 μm, was used for preparative runs; its geometrical volume was 4.15 mL. The feed prepared in Section 3.1 with a concentration of 10 mg/mL$_{column}$ was injected into the column by using a dedicated pump working at 3 mL/min. 10 mg/mL$_{column}$ corresponds to 1 % loading (expressed as mg$_{target}$/μL$_{column}$), which allows to efficiently perform purification of the desired product in overloaded condition with satisfactory productivity. Since the maximum concentration of DMC is roughly 10 % (v/v) in pure water [22], the "organic" mobile phase (MP-B) used for the gradient elution was a mixture of 20 mM triethylamine phosphate (TEAP) buffer (pH=8 adjusted with $H_3PO_4$):DMC 90:10%(v/v). Then, the "aqueous" mobile phase (MP-A) was prepared by mixing 90 % of 20 mM TEAP and 10 % of MP-B. The mixed phases were prepared by adding first DMC and then the TEAP buffer. The solution was shaken and sonicated with ultrasound for 30 min until complete dissolution of the bubbles.

The mobile phases prepared for the purification method using EtOH and IPA were the following. MP-A was a mixture of 20 mM TEAP:EtOH (or IPA) 90:10 % (v/v) while MP-B was 20 mM TEAP:EtOH (or IPA) 50:50% (v/v). The details of the purification methods with DMC, EtOH, and IPA are shown in Table 1. During the elution step, the flow rate was 1 mL/min, and fractions were collected every 1 mL. The purification methods were compared with an existing method in ACN already applied for the purification of Icatibant in [28], where the mobile phases were 20 mM TEAP:ACN 90:10% (v/v) as MP-A and a mixture of 20 mM TEAP:ACN 50:50% (v/v) as MP-B. The flow rate during the elution with the latter method was 1.5 mL/min and fractions were collected every 1 mL. A lower flow rate was used when DMC, EtOH and IPA were used as organic modifiers due to the higher backpressure generated by these solvents with respect to ACN.

**Table 1**
Experimental details used for the purification of Icatibant with the four different solvents.

| Modifier | Duration of the gradient (CV) | Flow rate (mL/min) | Variation of organic modifier along the gradient (%) |
|---|---|---|---|
| DMC | 10.3 | 1.0 | 7.3–10.0 |
| ACN | 18.0 | 1.5 | 14.8–24.8 |
| IPA | 10.3 | 1.0 | 14.0–22.0 |
| EtOH | 10.3 | 1.0 | 20.0–28.0 |

### 3.3. Analytical method

For the offline analysis of all collected fractions, an Agilent 1290 Infinity UHPLC system (Agilent, Santa Clara, CA, USA), equipped with a binary solvent pump, a column thermostat set at 50 °C, a diode array detector (DAD) set at 226 nm and an autosampler, was employed. The column was a 250 × 4.6 mm Kromasil 5-100-C18, with particle size of 5 μm and a pore size of 100 Å. The analytical method has already been described in [28]. Diluted standard solutions of Icatibant, used for the calibration curve, were prepared by dissolving the pure peptide, provided by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy), as described for the crude mixture, in a concentration range from 0.1 to 3 g/L. The feed purity measured with HPLC was about 76 %.

## 4. Results and discussion

This study began with the investigation of the retention behavior of Icatibant by changing the composition of the MP. The MP was a solution of TEAP buffer (pH = 8) modified with variable percentages of four different organic modifiers, namely ACN, DMC, EtOH and IPA. The retention curves of Icatibant are presented as log$k$ vs. φ plots in Fig. 1, being $k$ the retention factor, defined as $k = (V_R - V_0)/V_0$, with $V_R$ the retention volume and $V_0$ the hold-up volume of the column.

The trend reflects the traditional reversed phase behavior, with a quasi-linear decrease in the logarithm of retention when increasing the organic solvent percentage in the mobile phase. These curves display quite different operative ranges depending on the type of organic modifier that is employed. The most important difference is that, as already observed for small molecules in the first part of this work, with DMC the MP elution power is strongly enhanced. Indeed, it is possible to observe that to achieve a retention factor of $k = 1$ for Icatibant, about 9 %(v/v) DMC in the MP is needed, while higher percentages of the other solvents are required, such as 22 %(v/v) of IPA, 30 %(v/v) of ACN and 35 %(v/v) of EtOH, which shows the lowest elution power. From Fig. 1, it can be also observed that the slopes of the retention curves obtained with ACN, IPA and EtOH are very similar whereas that of DMC is a bit steeper, which implies that a small change on the concentration of organic modifier may have a significant effect on retention, at least for this analyte and in these conditions. However, albeit this may affect reproducibility with respect to the other solvents, no considerable deviations in terms of retention times were observed during the experiments performed in this work. Furthermore, the potential variability associated to the retention curve slope can be avoided through the use pre-mixed mobile phases containing both the aqueous buffer and the organic modifier in different proportions.

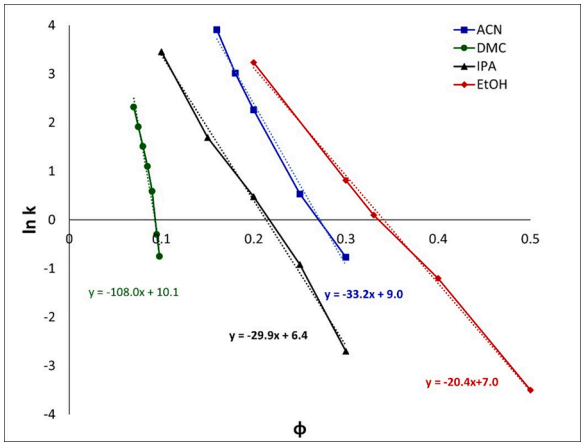


**Fig. 1.** Dependence of the retention factor, expressed as ln $k$, on the fraction of organic modifier $\phi$ in MP, using ACN (blue squares), DMC (green circles), EtOH (red diamonds) and IPA (black triangles).

From the data obtained in the retention study, several overloaded LC methods were developed for the purification of Icatibant by choosing suitable ranges of φ, depending on the nature of the organic modifier (see Table 1 and Fig. 2A–D). As it can be seen, the variation of organic modifier is limited for DMC, due to its higher elution strength. This translates into a smaller consumption (and waste) of organic solvent, with considerable advantages from the disposal and environmental viewpoints.

Focusing on the target peak, it can be noted that its shape is similar for the four solvents, with a diffused front and a shock in the rear part, more or less pronounced, suggesting an anti-Langmuirian adsorption mechanism. However, it can be also observed that the peaks obtained with ACN and DMC show "better" peak shapes if compared to alcohols. This aspect should be carefully taken into account when dealing with complex mixtures, as for peptides, since broader peaks may result in significant peak overlap between the target and impurities, with detrimental effects on the final trade-off between product purity and recovery.

The fractions subtended to the target peaks were collected and off-line analyzed through UHPLC. From the data obtained, it was possible to plot the so-called Pareto curve which shows how the purity varies with the recovery, as shown in Fig. 3. This curve is obtained by first considering the purest fraction and its recovery (upper point on the left). Then, this sample is pooled with the purest adjacent fraction to increase the recovery, and so on until the entire target peak is collected, as described in [28]. By enlarging the collection window, recovery will increase while purity will decrease. The result is a purity-yield trade-off, according to which it is very difficult to obtain high purity and high recovery at the same time.

These curves have been constructed for each of the four solvents used in this work and they provide fundamental information about the performance of a purification method on preparative scale. For the sake of clarity, data related to recovery, purity and productivity are reported in Table 2. From Fig. 3 and Table 2 it can be noted that DMC provides purification performance comparable to ACN, since their curves are almost superimposed. Concerning the purest fraction, i.e., the first point on the left, either ACN or DMC lead to a purity of about 100 %, while it is slightly lower with EtOH and IPA (roughly 99 %).

If the other limiting case is considered, corresponding to the maximum yield (achievable when the entire product collection window is pooled), it can be observed that with all solvents roughly 100 % recovery has been obtained. In this case, ACN provides the highest purity (98 %), followed by DMC (97 %) and alcohols (96 %). All solvents show a comparable gain in purity, roughly 30 %, with respect to the initial feed, whose purity was 76 %. It is worth noting that the productivity measured at 100 % recovery is the same for all solvents except for ACN, for which it is lower due to the longer gradient duration (see Eq. (3)).

As it was pointed out, the two end points of the Pareto curve are useful to check the goodness of different purification methods, in terms of yield-purity trade-off. Furthermore, the pharmaceutical contest demands high purity values for the isolated final product, usually >98 %. Accordingly, a peptide purity of 98.5% has been set as target for the experiments performed in our study. In Table 2, recovery and productivity values have been calculated in correspondence with this purity for all the solvents. As it can be noted, both ACN and DMC lead to the highest recovery (roughly 94 %) if compared to EtOH (65 %) and IPA (80 %). This result indicates that the former solvents are able to provide highly pure product with a minimal loss of mass (around 6 %). Concerning productivity, the maximum value has been obtained with DMC. Also in this case ACN shows a productivity value smaller than DMC, due to the longer gradient used.

All the potential alternative solvents to ACN have shown interesting outcomes for the purification of Icatibant. Nevertheless, it has been seen that, for this specific case, especially DMC can provide very similar results to ACN, in terms of recovery and purity. It is worth mentioning that depending on the imposed target purity also EtOH or IPA can be

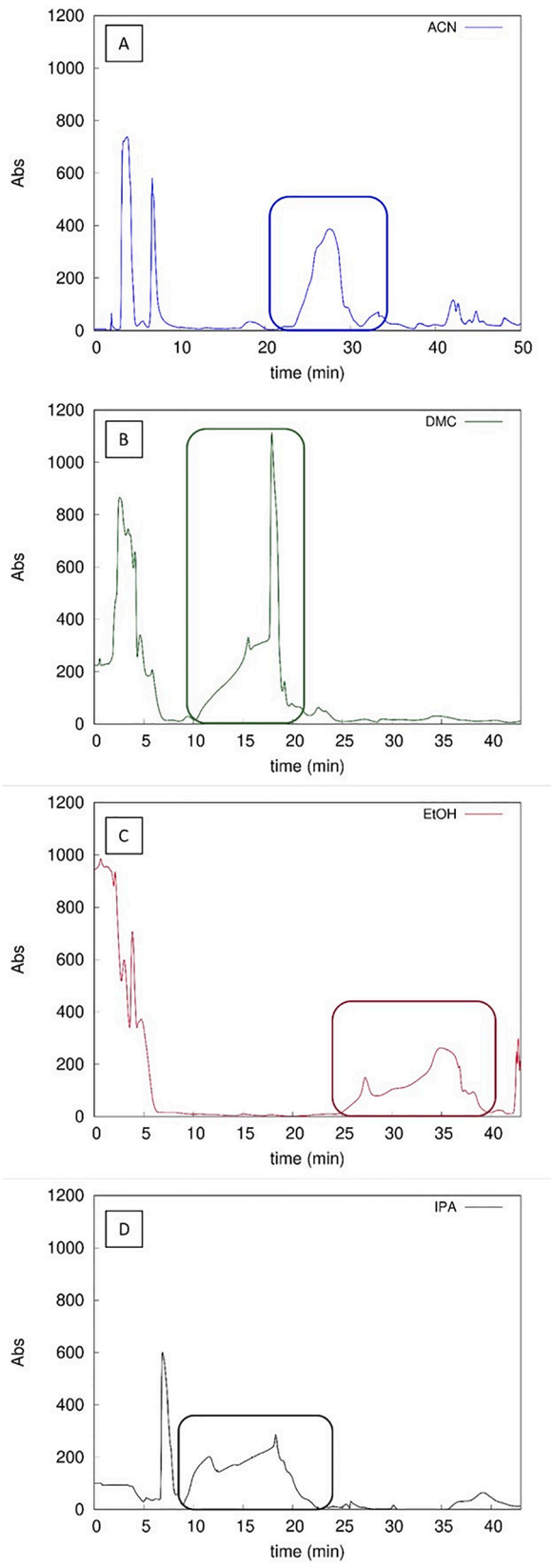


**Fig. 2.** UV profiles of Icatibant along the gradient step obtained using ACN (A), DMC (B), EtOH (C) and IPA (D). The target peak has been highlighted with a box. Time=0 identifies the beginning of the gradient.

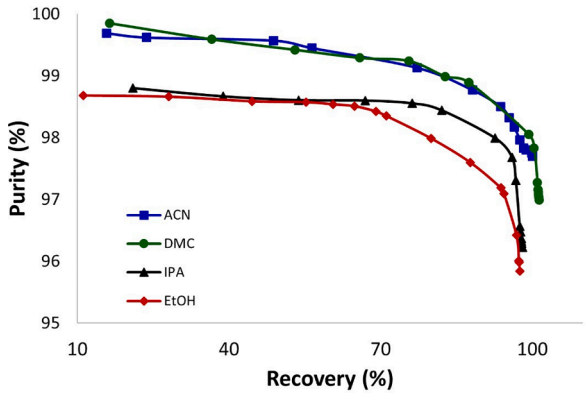


**Fig. 3.** Pareto curves for the purification of Icatibant by using ACN (blue squares), DMC (green circles), EtOH (red diamonds) and IPA (black triangles).

**Table 2**
Comparison of the performance parameters using different organic modifiers, at the two extremities of the Pareto curve (highest purity and highest yield) and at a target purity equal to 98.5%.

| ORGANIC MODIFIER | RECOVERY (%) | PURITY (%) | PRODUCTIVITY (mg/L/h) |
|---|---|---|---|
| ACN | **15.8** | **99.7** | **2.8** |
| | **99.9** | **97.7** | **11.9** |
| | **94.0** | **98.5** | **11.2** |
| DMC | **16.4** | **100.0** | **2.4** |
| | **99.9** | **96.9** | **14.1** |
| | **93.5** | **98.5** | **13.9** |
| EtOH | **11.2** | **98.7** | **1.6** |
| | **97.4** | **96.0** | **13.9** |
| | **64.8** | **98.5** | **9.4** |
| IPA | **21.0** | **98.8** | **2.3** |
| | **98.1** | **95.8** | **14.2** |
| | **79.0** | **98.5** | **11.2** |

effectively used as green alternatives to ACN.

## 5. Conclusions

The replacement of ACN with greener solvents is becoming an urgent need in the biopharmaceutical industry. In this study, several potential alternative solvents, namely DMC, EtOH and IPA, have been tested for the purification of a therapeutic peptide, Icatibant.

This study revealed that all the solvents selected are able to effectively purify Icatibant, with roughly 100 % recovery and > 95 % purity. Nevertheless, when a pharmaceutically acceptable purity is selected (e.g. > 98.5 %), ACN and DMC show much better results in terms of recovery compared to alcohols. Remarkably, the highest productivity is obtained when using DMC as organic solvent.

In this context, DMC can be considered as a promising candidate to be used also in preparative conditions for the isolation of biopharmaceuticals. In particular, as demonstrated also in the companion article for some small molecules, this solvent shows a higher elution strength with respect to ACN, as well as the other alcohols that have been tested (in particular EtOH, which is among the solvents available today in terms of greenness). These features will potentially translate into shorter runs, less instrumentation usage, and a considerable decrease of organic solvent waste, the disposal of which is generally an unnecessary cost and burden to the environment. All these aspects are very important from the point of view of the greenness of the process. The overall aim of these two companion articles is to propose DMC as a green alternative to the well-established ACN, IPA and EtOH, an alternative which had almost never been investigated before for chromatographic applications, especially in overloaded conditions. Its suitability for the purification of a peptide mixture of industrial interest was demonstrated at semi-preparative level, showing comparable performance result respect to ACN. Similar results have to be expected at industrial preparative level, ensuring to keep the same stationary/mobile phases and the% of product loaded respect to the dimension of the column selected.

The benefits obtained with DMC could even be amplified in the future, by employing it in highly productive manufacturing processes possibly based on continuous multicolumn chromatographic techniques. Multicolumn Countercurrent Solvent Gradient Purification (MCSGP), for instance, has led to impressive improvements in productivity and solvent consumption in the purification of Icatibant through ACN, with respect to traditional single-column chromatography [28]; therefore, even better results are expected by employing DMC with this technique, contributing to develop greener manufacturing processes of peptides [31].

## CRediT authorship contribution statement

**Desiree Bozza:** Investigation, Validation, Writing – original draft, Visualization. **Chiara De Luca:** Methodology, Formal analysis, Writing – original draft. **Simona Felletti:** Methodology, Formal analysis, Writing – review & editing. **Matteo Spedicato:** Investigation, Validation. **Francesco Presini:** Validation, Data curation. **Pier Paolo Giovannini:** Validation, Data curation. **Marco Carraro:** Methodology, Data curation, Resources. **Marco Macis:** Methodology, Data curation, Resources. **Alberto Cavazzini:** Supervision, Funding acquisition, Writing – review & editing. **Martina Catani:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Antonio Ricci:** Conceptualization, Resources, Supervision, Project administration, Writing – review & editing. **Walter Cabri:** Project administration, Supervision.

## 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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