DOWNSTREAM PROCESSES FOR PEPTIDE MANUFACTURING: OPTIMIZATION STRATEGY AND LATEST TECHNICAL TRENDS

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ABSTRACT

This article highlights the key steps in the development and implementation of a proper purification strategy for the manufacture of peptide APIs. Our ambition is to recommend a structured methodology, including a good understanding of the separation mechanism to develop the process and to apply the latest technologies for the optimization of the purification step to reach the best performance of the manufacturing process.

KEYWORDS: Downstream, peptide chemistry, optimization strategy, process design, peptides.

INTRODUCTION

The recent publication of the 2020 FDA drug approvals shows again the growing interest of the pharmaceutical industries for oligonucleo- and pep-TIDES, representing around 10% of the new drug authorized this year (1), with two new oligonucleotides and three peptides. Over the period 2015-2019, peptides and peptides-containing molecules accounted for 7% of the total approved drugs (2).

Over 80% of the peptide currently developed are produced synthetically. The optimization of the synthesis process is strongly linked to the development of the right purification strategy, based on the identified critical impurities. As stated in a recent article (3), the development of new methodologies should minimize the number of iterations to select the optimal manufacturing strategy. The use of in-silico development tools is an emerging evolution in the practices to answer to the challenge to quickly get access to an optimized process with a Right First Time strategy.

Most of the publications related to peptide chemistry focus on the peptide synthesis, while the downstream part of the process is far less considered. However, when we consider the manufacturing costs of a synthetic peptide, the downstream part often represents at least half of the operational costs. This article aims to focus on the downstream steps, in particular the purification, and to show the available leverages to boost the overall process performance.

The first section will comment the methodology for the development of a purification process, and the second part will present the latest trends in the purification process used for peptide manufacturing.

METHODOLOGY FOR DSP OPTIMIZATION

The most significant changes for the industry probably occurred in the mid-80s with the move from low pressure silica resins to high performance chromatography on an industrial scale using spherical, 10-20 um, RP-silica. Lilly, Schering, and Ferring were pioneers in this field. The introduction of chromatographic media with smaller particle size and improved particle morphology has led to higher yield, throughput, purity, and increased resin lifetime.

Eka Chemicals and Osaka Soda (Daiso) were innovators in this field. Improved surface chemistry, high mechanical stability, enhanced physical stability at a wide range of pH (pH 2 -11) and improved re-usability are other recent advances. Novasep (formerly Prochrom) introduced high pressure axial compressed column (DAC) in the mid-80's, enabling the use of high-performance and small particle silica.

There are only a limited number of suppliers of reversed-phase silica being able to produce and supply spherical silica in multi-ton quantities.

The introduction of the high-performance concept in industrial scale chromatography has been fundamental in the peptide therapeutic area, since the complexity of peptides has increased over the last ten years. This is reflected in the increasing use of unusual amino acids, branched and dimeric peptides, polycyclic, stapled peptides, and conjugates.

The applied methodology to set up a purification process is typically divided in two main steps:

1. Development step: screening and selection of the phase system to establish the process
2. Optimization step: master the process performance and reliability

During the development step, many factors, potentially affecting the method are screened to determine the most important aspects and to reduce discrete variables, which are then further optimized. At this stage, so-called screening designs are applied. These designs allow evaluating the effects of a relatively high number of factors in a relatively small number of experiments.

Main targets of the Development step

- Reducing discrete variables.
- Relevant and representative crude should be used (spiked or actual).
- Identification of critical impurities (which have a negative impact upon yield, purity and throughput) and subsequent iteration process with synthesis and purification development.

- The transition steps from synthesis needs to be carefully considered: treatment of the cleaved deprotected crude peptide before injection (precipitation, neutralization, evaporation), assessment of bed contamination by strongly retained impurities, filtration…
- Selection of the chromatographic mode: e.g., gradients / isocratic, standard elution mode, stacked injections, steady state recycling, multicolumn chromatography…
- Selection of the number of the purification step (orthogonal approach)

Ideally, the process should be already locked at this stage, to avoid a major change of the purification process at a later stage, which could potentially impact the impurity profile of the purified API. This is the most challenging part of a good development strategy and methodology.

For the next optimisation steps, it is of considerable importance to acquire a detailed, enhanced understanding and experience about the product and the process variability in order to minimize risk failure, but maximize the probability of success and to generate the best process economy.

Response surface designs can be applied to examine the most essential factors from the screening step. Usually, two or three important factors are then optimized further, e.g., column loading, slope of gradient etc. For medium or high-risk unit operations, DoE is recommended both to identify important operational parameters with high impact on the performance parameters and to define safe operating ranges with respect to the quality attributes of the process.

Main target of the Optimization step(s):

- Structured and systematic experimental work with a reduced number of variables, i.e., enhanced cause effects understanding and understanding of parameter interactions.
- Robustness (Design space), Defining the Design and Control spaces of the process, in which critical process parameters and operating ranges are identified and controlled.
- Design space validation - Estimation of ranges, Robustness testing at target set points and quantification of risks.
- Stability studies / Storage conditions of the solutions in the purification process.

The development and optimization of the phase system are performed using several chromatographic resins, buffer/pH values, and solvent systems in an organized way and by using analytical columns packed with the same preparative material as will be used on a large scale in a later stage.

The use of these analytical columns will keep the peptide sample and solvent consumption low, and since modern preparative chromatography is probably one of the very few chemical processes that can be directly scaled up without any specific adjustments when the technology is well mastered: a process developed on an analytical column of a few mm internal diameter is representative of the results obtained on a large scale preparative column, and the obtained performance can easily be extrapolated.

There are many discrete variables to consider and optimize for a required purity in a downstream purification process:

Variable	*Early stage*	*Late stage*
Number of steps	(X)*	X
Type of stationary phases**	(X)	X
Type of mobile phases***	(X)	X
Product loading		X
Yield and throughput		X
Process economy		X
Type of mass overloaded chromatographic mode ****		X
Flow rate and slope of gradient		X
Fractionation/cut points		X
Bed length		X
Product hold time		X
Stationary phase lifetime		X
Solvent consumption		X
Size and type of supply system		X

**Partly optimized,*
*** Include supplier, surface chemistry (IEC, RPC, polymeric), pore size, particle size, ligand length.*
**** Include type of buffer, pH, ion strength, salt addition, type of organic solvent.*
**** *Isocratic, gradient, standard elution mode, pre purification steps, stacked, continuous etc.*

The above suggested methodology can be used as a baseline… but reality is often far more complex! The best practice is the agility to adapt the development and optimization protocol to the project constraints: pressure on product delivery at early stage (reducing the opportunity for an extended screening), optimization efforts (find the right development efforts vs gain on manufacturing costs)…. All this should however not compromise on the reliability and reproducibility of the process for commercial cGMP manufacturing.

Process robustness

To improve the process robustness and avoid contamination of a high-performance bed, it is a good idea to subject a crude synthetic peptide mixture to a pre-purification or a capturing step, in which most of the impurities are removed.

This can be achieved by e.g., implementing an upstream ion exchange step, which almost completely removes non peptide-related hydrophobic impurities as well as neutrals originating from the protecting groups and the scavengers.

Besides these impurities, the IEC step will also remove or minimize closely-related peptide impurities and frequently show an orthogonal selectivity relative to the reversed phase silica step.
Furthermore, these polymeric resins used in ion exchange chromatography allow us to use sodium hydroxide regeneration to elute any strongly retained impurities without any resin degradations.

The IEC step will generate a well-defined feed composition resulting in an extended lifetime of the silica-based resins downstream.

Figure 1 below illustrates a typical DSP flow scheme of a peptide process. Figure 2 illustrates the in-process control of a multistep purification process of a 40-mer peptide starting from 62 % purity and finally resulting in a purity of +99 %.

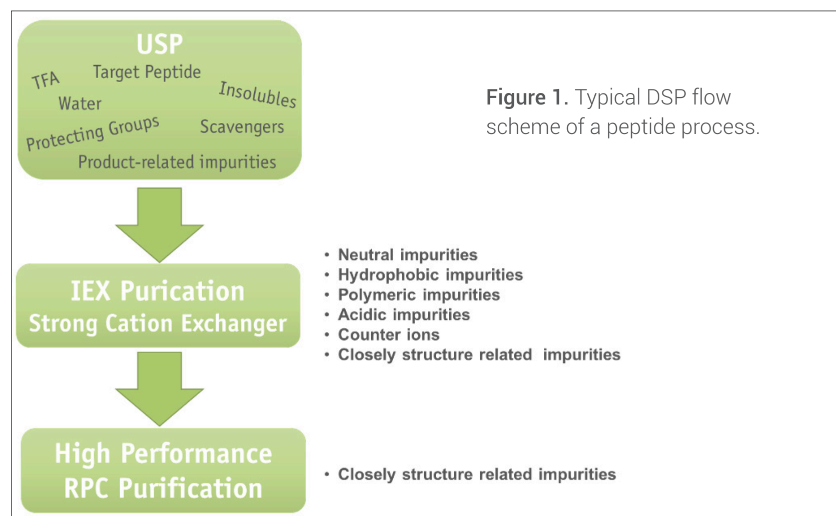


Figure 1. Typical DSP flow scheme of a peptide process.

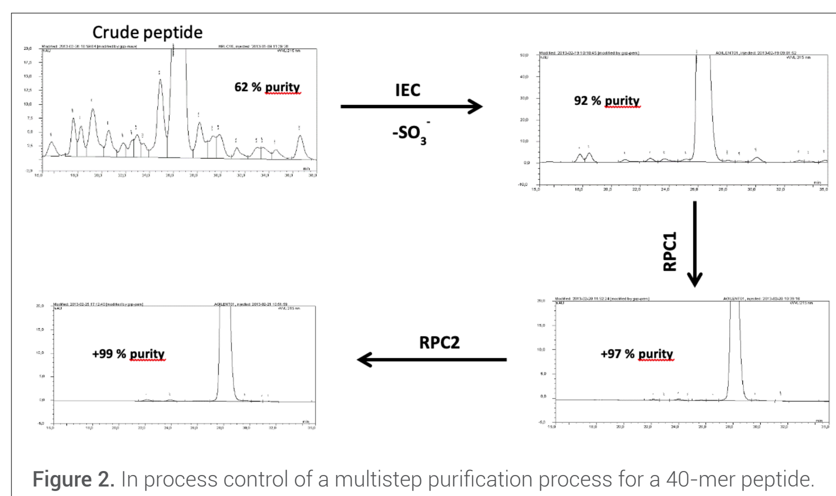


Figure 2. In process control of a multistep purification process for a 40-mer peptide.

The phase system in preparative chromatography

There are many different aspects to consider when deciding which buffer and solvent system to be used:

- Selectivity aspects
- Adsorption isotherm aspects (band broadening)
- Peptide and stationary phase stability
- Type of buffer, buffer capacity, pH, ion strength
- Pore size of the adsorbent, particle size
- Type of organic solvent
- Viscosity aspects (buffer/solvent) - influencing mass transfer and pressure drop.
- Peptide solubility aspects
- Peptide aggregation aspects

Often, only a small set of different buffers are required to solve most of the selectivity problems in RP chromatography. Solutions containing additives like 0.1-0.5% acetic acid or 0.1% TFA without any cations will not act as true buffers. These solutions will normally cause distorted and unfavorable adsorption isotherms, ending up with a large band broadening. This is especially pronounced when the peptide structure contains basic amino acids (e.g., arginine and lysine). The presence of cations in the mobile phase will suppress non-linear silanophilic interaction, resulting from heterogeneous surfaces. This will result in more favorable adsorption isotherms in mass overloaded chromatography.

Based on the need for new and improved RPC stationary phases and the concept of orthogonality, an innovative reversed-phase based on ion exchange doped mixed mode material was developed a few years ago (4). Using these mixed mode phases in a repulsive mode, improve the separation performance and selectivity significantly.

However, electrostatic repulsion can also be introduced by regulating the buffer system in the mobile phase, in order to improve the discrimination of peptide related impurities. This concept has been utilized for many years within the PolyPeptide Group and is illustrated by the following chromatograms (Figure 3 : analysis of the crude, Figure 4 : preparative separation using electrostatic repulsion, Figure 5 : analysis of the collected fractions).

DSP PROCESS LATEST TRENDS

In the synthetic peptide world focus is on synthesis; efforts are often put on upstream, not on downstream. Thus, purification process is often relegated to a secondary position in development process and can be resumed in "inject the maximum and keep the best fractions". This approach has its own limits in an industrial and regulated environment.

Chromatographic theories have mainly been spread out from academic labs for 35 years, everyone has heard about the adsorption isotherms widely described and used by Prof. Georges Guiochon to optimize the separation conditions of a preparative chromatography system. The practical application can be summarized by the fact that there is a limit regarding the injected quantity of a mixture due to the non-linear and competitive effects in the column, with a loss of either the obtained purity or the obtained yield of the purified product recovered.

To overcome this difficulty, the loading must be kept in a "reasonable" range.

A still common practice in peptide purification is to perform a quite massive loading on the preparative column with a long gradient, and to collect multiple fractions, analyzed and pooled according to the achieved purity. Some of the collected fractions, enriched but still below the expected purity, are then recycled to get an acceptable yield of the process.

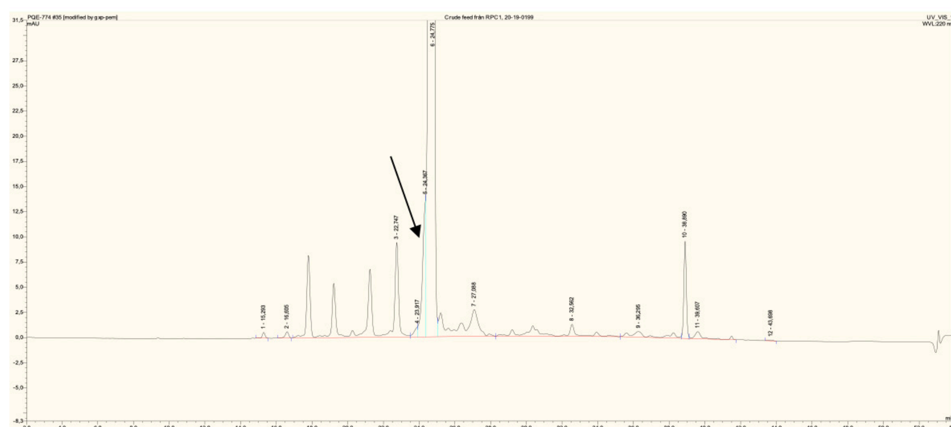


Figure 3. Analytical control of a crude feed solution of a commercial peptide. All the impurities are isomers. The in-process control is performed using a 4.6x 150 mm, 2.5 um, C18, 0.1 % TFA and a shallow acetonitrile gradient. The arrow shows a partly separated isomer in the front.

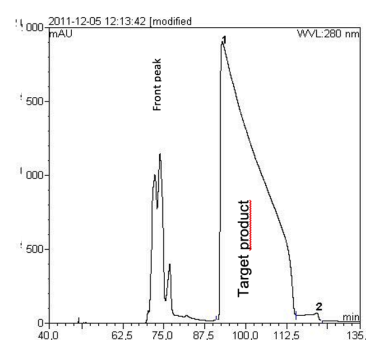


Figure 4. Preparative separation and mass overloaded injection (75 g/ liter CV) by using a high-quality standard silica (Osaka Soda, 15 um-C18-RPS) and a buffer system inducing electrostatic repulsion. The target product is collected without any peak shaving as well as the front peak (eluting with the void).

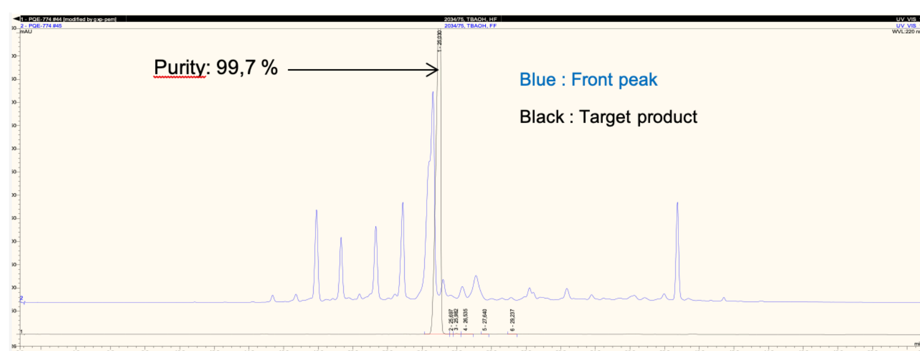


Figure 5. In process control. Overlay chromatograms of the front and target product fractions. As can be observed from the chromatogram below- all impurities (late and early eluting) will elute with the column void in figure 4.

The overall purification cost can be depicted by the following formula (5) combining the purification operating cost with the cost of the crude:

$$\frac{€}{kg} = \frac{a\,(€/h)}{\Phi} + \frac{b(€/kg)}{X_i.\Theta}$$

Φ : productivity (kg of product purified / hour)
Θ : Yield
X_i : Crude Purity

To recycle or not to recycle

We could intuitively think that a massive loading is favorable to improve the process performance. However, a too high loading either kills the yield, which is critical when the crude cost is high (as it is often the case for synthetic peptides manufactured at industrial scale), or required fractions recycling with additional injection, which then reduced the productivity and increased the purification cost. The right compromise should be carefully considered in this multi-parameter optimization by considering the cost contributors of the developed application.

The logistics required to handle side fractions and recycling should be taken into account in the process design, as it can represent a significant additional operating cost at an industrial scale.

Automation

Automation is available on modern preparative chromatography devices for few decades. It is widely applied for the purification of small molecules but remains relatively uncommon for the purification of peptides and other biomolecules.

In order to use automation most efficiently, the way to develop the process should be slightly revised, with the use of small injection on efficient columns with a fast gradient. Multiple injections can be completely driven by automation. An analogy with (the trendy) flow chemistry can be attempted, rather than loading massive amount of product on oversized column with a methodology copied from the lab-scale operations like manual fractioning.

Implementation of automation in the chromatographic process should ideally be fully integrated from the development stage.

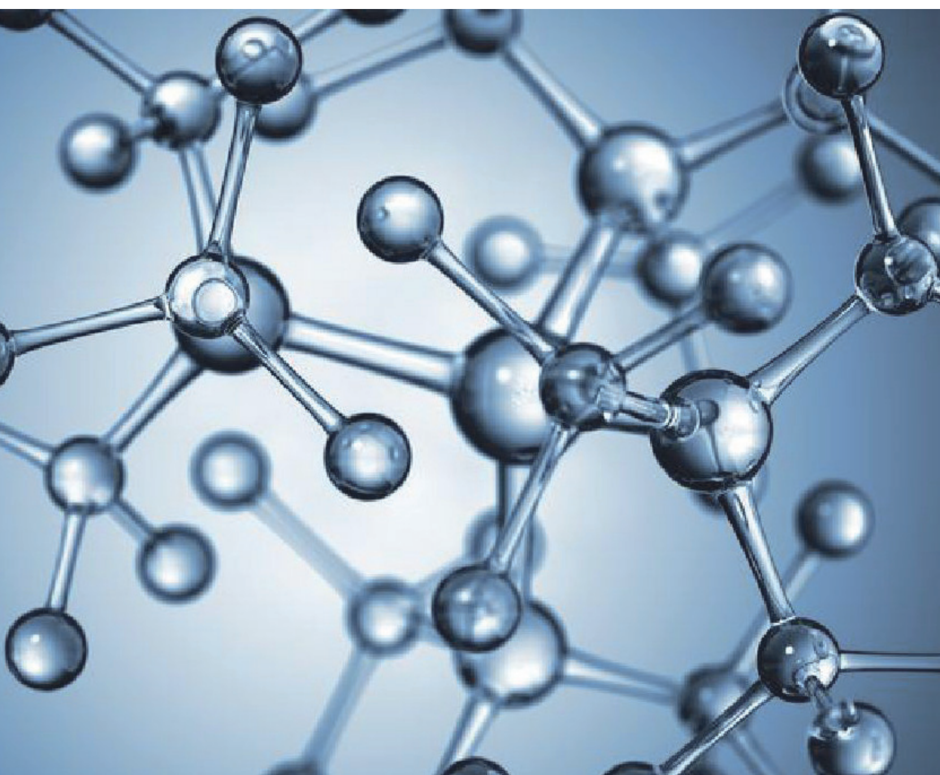
A few rules have to be fulfilled for a successful application; several aspects can be underlined:

The physico-chemistry and quality:

- A consistent quality of the feed, the feed is often obtained as several batches, the quality of these batches must be constant, this is, of course, the goal of the upstream development.
- Chemical and physical stability of the feed (crude) solution: the batch to be processed has to be chemically stable during the injection period, but also physically, no precipitation, jellification, aggregation. An in-depth study will make it possible to propose a valid solution based on solvent choice, concentrations, pH and, of course, filtration
- A chemical and physical stability of the fractionated solution, the pooled product and sometimes the reworked side fractions (same recommendations as for the crude solution).

The equipment and process design:

- Automation is basically implemented on the preparative chromatographic system with a feed pumping device and a fraction collection system.



The whole device is controlled by a PLC and thus allow to repeat the following sequence: injection of a dedicated quantity of feed, running out the gradient/isocratic sequence, collecting the fractions in dedicated containers.

- The reproducibility of the purification is naturally subordinate to the reliability of the system. This requires an adequate level of expertise of the operators and regular maintenance and qualification of the equipment.
- An additional critical point is related to the buffer tanks: for biomolecules and especially peptides, tight control of the buffer composition is required to obtain a reproducible separation, a variation in pH, a gradient or a temperature drift may have a huge impact on the obtained chromatogram. It is essential to maintain these values in an acceptable range during the whole process.
- The downstream process's efficiency is related to the purification step and the other operations required: IPC analytical capacities to manage the collected fractions, concentration/solvent removal capacity (evaporation, membranes) to treat the collected pools before the final isolation step…

With respect to those rules a stable process can be designed, running 24h a day with high productivity, operational efficiency and good ROI.

From batch chromatography to multi-column processes

Multi-column chromatography technologies were initially developed in the late '50s in the petrochemical industry. The concept was developed in the '90s in the pharmaceutical industry, with several large scale applications, mainly for chiral purifications.

The adaptation of the concept to the purification of complex mixtures under gradient conditions required for most of the peptide purification emerged more recently, based on different concepts.

The most advanced concepts are:

- The MCSGP process developed by YMC-Chromacon : this twin column concept is integrating the direct online reprocessing of the side fractions, which are directly reinjected without isolation, the system must run in a steady state to collect a reproducible purity and concentration of the desired product (6).
- The Geyser/GSSR process developed by Novasep: this multi-column device (3 or more) is using the multi-column concept used high overloaded conditions to isolate the expected pure peptide with a higher yield and productivity compared to a standard batch single-column process (7).

These two new multi-column concepts may open new perspectives for future industrial applications, provided:

- the control system is robust enough to secure the process robustness – the development of advanced control and PAT tools is clearly a direction to follow to ensure the industrial success of these concepts
- the generated savings pay for the cost of the technology and of the extra work required to develop the application

This will require even stronger expertise of the users to master and secure the process.

CONCLUSION

Purification is a critical unit operation in the manufacturing route of a peptide. Its optimization requires the expertise of the chromatographer together with the skills of process engineers to select the most suitable solution to produce peptide APIs. The selection of the chromatographic conditions is of paramount importance, and an appropriate expertise and methodology are crucial to propose the appropriate process development strategy throughout the clinical trials. Mastering the latest technologies also offers great opportunities for the implementation of an efficient and reliable process.

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