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Batch and Multi Column Chromatography for the Large Scale Production of Biologicals, API's and Food Ingredients

Preparative chromatography processes already implemented at large scale or considered as a first choice technology for the near future are discussed. Bulk antibiotics, amino acids, peptides and proteins, sugars and oligosaccharides, chiral and non chiral API's are the products taken as application examples

Keywords: Chromatography Batch Continuous Varicol® SMB BioSC

INTRODUCTION

Preparative chromatography is the working horse in the development and commercial production of molecules of biological interest. Since the first selective adsorption experiments achieved by Mikhail Tswett in the early 20th century for the separation of leaf pigments, preparative chromatography has quickly evolved from a simple technique using glass column packed with calcium carbonate and alumina, then paper sheets or thin layers as planar adsorbent, to a medium/high pressure column based technology that is suitable for scaling-up the separations to industrial scale production. The volumes to be purified by a single preparative chromatography system at commercial scale can be as low as few kg's/year for some highly potent Active Pharmaceutical Ingredients (Prostaglandin derivatives, taxanes…), or as high as 100,000 tons/year in food industry (sugar derivatives, amino-acids…). Between these two scales, all kind of molecules of biological interest are produced nowadays by means of preparative chromatography.

PREPARATIVE CHROMATOGRAPHY: PROCESS AND TECHNOLOGY

The classical process, known as *batch chromatography*, involves a single column filled with a solid adsorbent and percolated by a mobile phase under liquid state for most applications, but also supercritical state and even gas for some specific applications. This process has been widely used for the commercial production of Active Pharmaceutical Ingredients (API's) and advanced intermediates, and it is a must-have tool in the Biopharma Industry for the downstream purification of proteins or other complex molecules, produced by fermentation/cell culture or extracted from a biological source. In order to adapt the equipment to the small particle sizes of the stationary phases that become progressively available, column manufacturers have implemented technological improvements allowing to operate thick-walled columns at high pressure. Dynamic Axial Compression (DAC) was introduced in the 80's [1], allowing to stabilize the chromatography bed and keep the robustness required over the

long periods of production campaigns. New optimized fluid distribution systems were also developed to minimize dispersion effects in the column, and thus taking the full benefits of the narrow particle size distributions that could be supplied by the stationary phase manufacturers. With such optimized systems, the columns are operated at medium/high pressure and the method is called High Performance Preparative Liquid Chromatography (HPPLC) when the mobile phase is liquid, which is the most common situation.

As far as large scale production volumes are concerned, attention has focused on the choice of the operating mode in order to minimize the consumption of mobile phase and to maximize the productivity which is of key importance when large volumes of stationary phase are involved or when expensive adsorbents are used, the basic incentive being to minimize the purification costs. Among the alternatives to the classical batch chromatography process, much attention was paid to Multi-Column Chromatography (MCC) processes like Simulated Moving Bed (SMB) [2-4], a continuous process more adapted to a large scale production and involving a counter-current contact between the mobile liquid phase and the stationary solid phase.

Initially developed for the petroleum industry in the 60's, SMB has become after batch chromatography an established manufacturing tool in the Sugar industry for cane or beet molasses separation or for the production of High Fructose Corn Syrup. It has become a standard for this application in the 80's and a second generation of continuous processes like Sequential Simulated Moving Bed (SSMB) [5] have been introduced then, progressively replacing SMB for the existing applications and the new ones to come in the food industry.

A similar development was observed in Pharmaceutical Industry for the production of single enantiomer API's by chiral preparative chromatography, where the enantioselectivity required for the separation is brought by using costly chiral stationary phases of adequate particle size. MCC processes of first generation like SMB and of second generation like VARICOL® [6-7] brought the crucial economical advantage of minimizing the amount of stationary phase required to separate multi-tons or multi-dozen-

tons / year of pure single enantiomer, allowing to reach in a single chromatography step optical purities as high as > 99.0 % starting from the racemate mixture.

A TOOL FOR ALL CLASSES OF MOLECULES

Preparative chromatography has thus established itself over the last 40 years as a powerful production tool by the industries requiring purification of products aimed at human consumption for nutritive of therapeutic purpose.

Stationary phase suppliers have been able to develop the matrices and the surface chemistries required to create a selectivity in the interaction between the molecules to separate and the matrix surfaces, interactions based on Van Der Waals forces, hydrogen or ionic bonding or a combination of all, and allowing to separate most of the products according to their molecular structures.

The choice of the most suitable technology/process to a specific application depends on several factors. Batch chromatography or MCC, liquid mode or supercritical mode, high pressure or low pressure, using organic solvents or 100 % aqueous mobile phase, pure or as mixtures are critical choices that are sometimes made on the basis of technical constraints related to the molecular structure and size, solubility, stability of the product to be purified. Some regulatory constraints may also be critical like the reluctance in food industry to use organic solvents beside ethanol, or the difficulties to obtain the agreements from local authorities to operate high pressure equipment in the case of large scale SFC systems.

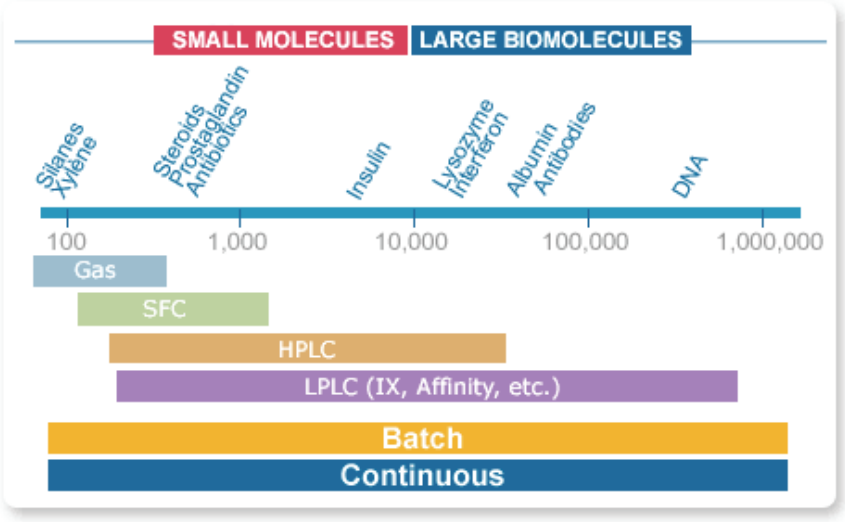


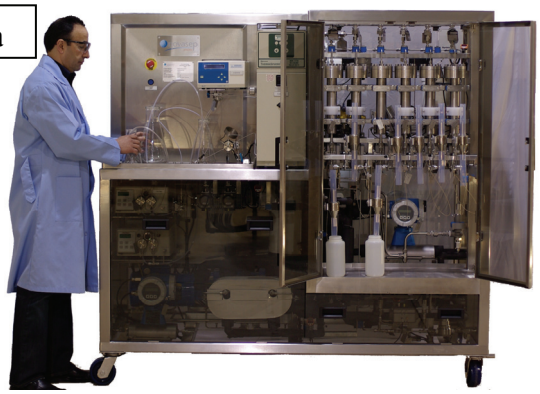
Figure 1: Preparative chromatography techniques and products molecular weight

As shown in **Figure 1**, Gas chromatography or Supercritical Fluid Chromatography is applied to molecules of small molecular weight that are soluble in organic solvents. Due to the favourable properties of diffusivity of the solute in the mobile phase, some baseline separations can be achieved in very short times leading to very high productivity.

Productivities as high as several kg product/kg stationary phase/24h can be achieved and multi-tons/year commercial scale productions can be performed using SFC equipment fitted with columns of 200 or 300 mm internal diameter (ID) operated at typically 200 bar.

F**igure 2** below shows a small industrial scale SFC equipment. The picture on the right side shows the largest HPLC column in the world, of 1600 mm ID and 40 bar pressure rating. On each picture an operator is shown to help appreciate the scale.

2a



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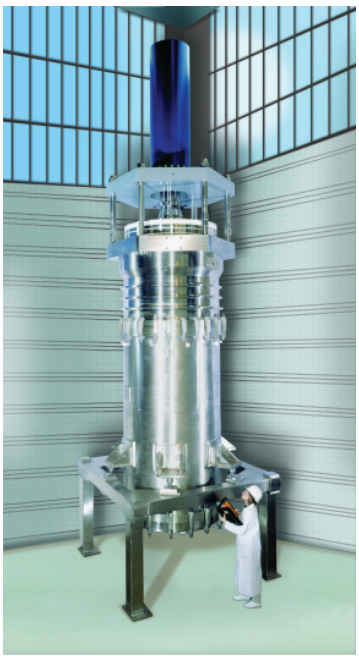

Figure 2: Prochrom® Supersep 400 (2a) and Prochrom® LC.1600.4000.VE40 (2b)

HPPLC is certainly the most versatile preparative chromatography process. By opposition to MCC processes suitable mainly for binary separations, the separation of multi-component mixtures is allowed using HPPLC by collecting the column outlet stream in several fractions.

Most classes of molecules can be purified by HPPLC provided the right stationary phase and mobile phases are selected. However, as shown in **Figure 1**, the applications of HPPLC stay limited to molecules of less than few 10's thousands Dalton. One of the reasons to this is coming from the fact that some of the chromatography supports used for separation of large proteins are soft gels that cannot be operated under high flow or under high mechanical pressure. Rigid matrices based on silica or synthetic polymers (PS/DVB, PolyAcrylates…) are more suitable to HPPLC (or medium pressure) conditions, but they often require the utilization of solvents that can affect the biological activity of proteins by changing their tertiary structures.

Smaller molecules are commonly purified by HPPLC in Pharmaceutical and Biopharmaceutical industry for all therapeutic applications. Steroids, prostaglandin derivatives, peptides and polypeptides like Insulin and Insulin analogues, are just few of the numerous examples of molecules that are produced by means of HPPLC.

However this technology was found less useful to the food industry where solvents other than ethanol are not welcome. The product is to be consumed in large quantities by the final consumer who would have to absorb the residual amounts of toxic solvents. Nevertheless, some class of molecules classified as food supplements are consumed at lower daily doses, for a disease treatment or for prevention purposes. They are sometimes commercialized at very high chemical purity (and sold at rather high prices) and are produced using HPPLC technology. One example is the family of anti oxidants that can be extracted from vegetal oils (Tocopherols, beta carotenes), from animal oils or even produced by fermentation (poly insaturated fatty acid esters like EPA and DHA, for instance).

It is shown in **Figure 1** that a batch process or continuous process can be applied to all classes of molecules, whatever their size. Even though there are many examples of separation of proteins by continuous chromatography at laboratory scale, their implementation at industrial scale has been limited so far to the purification of small molecules for Food Ingredients and Pharmaceutical Industries. However, it will be seen later in this article that a multi-columns technology (different from SMB) is getting mature now for the purification of proteins at commercial scale. As a matter of fact, it has been adapted to the conditions of purification of proteins that involve for many purification steps, a sequence of elution with different buffers instead of continuous isocratic conditions.

CHIRAL CHROMATOGRAPHY: A PHARMACEUTICAL APPLICATION

In the pharmaceutical field, more and more chemists are concluding that preparative chiral chromatography is a viable option to reduce time-to-market in early and late development phases and to obtain robust and cost-effective routes for both late and commercial production. The choice for the best chromatography process should often be made on a case-by-case basis, however general rules can easily be applied.

Usually SFC and HPPLC are the methods of choice in early development when time is of utmost importance and for production quantities ranging from grams to several tens of kilograms.

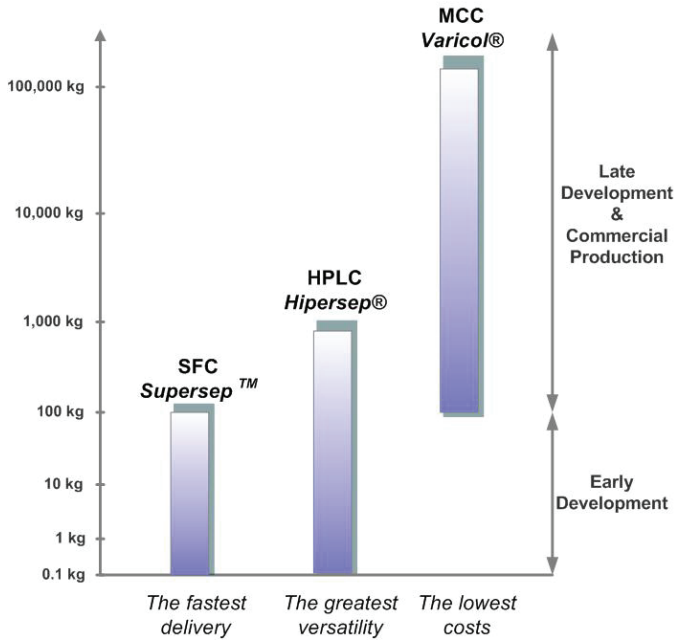

Figure 3: Preparative chromatography processes in chiral separation applications.

For late development and commercial production, when robustness of the process and its cost-effectiveness are key, MCC processes such as Varicol® are the solution of choice. Unlike SFC and HPPLC, MCC is a continuous process. It is typically two to ten times more productive than a batch process. MCC is designed for the separation of binary mixtures and is thus perfectly adapted to the resolution of racemates. In this process, one long column is replaced by a loop of four to six shorter columns, optimising the usage of chiral stationary phase and the mobile phase. Only pure fractions are collected, leaving mixed fractions to re-circulate through the columns. Although the development time is longer than with a batch process, everything is designed to minimize production and operating costs, and to optimize both robustness and productivity. Integrating automated solvent recycling and process analytical technologies (PAT), MCC makes the perfect tool for late development and commercial production. It is important to mention, however, that in most cases, only isocratic conditions can be used since the mobile phase is recirculated in the system.

Leviteracetam (Keppra®), Escitalopram (Cipralex®/Lexapro®), Levocetirizine (Xyzal®), Armodafinil (Nuvigil®), Sertraline (Zoloft®) are examples of drugs produced using SMB/Varicol® technologies. It

was estimated in 2005 that the total dedicated chiral capacity production was exceeding 1500 Metric tons of racemate / year.

Keppra® (Levetiracetam), UCB's top-selling drug with sales over € 1 billion in 2007, is a well-known success story with more than 100 tons per year of the API produced by chiral chromatography. Indeed, the chiral separation process allows a decrease in production costs of 50 % compared to asymmetric synthesis and a decrease of 76 % compared to diastereoisomeric salt formation. In the case of Keppra®, the solvent recycling rate reached 99.97 % leading to the introduction of only 180 mL of fresh solvent per kilogram of pure enantiomer obtained !.

Cephalon's Nuvigil® (Armodafinil) is a good example of how chromatography can help to reduce time-to-market [8]. Armodafinil, a wakefulness-promoting agent, is the single enantiomer of Modafinil, which has already been approved for several indications, including sleep disorders and narcolepsy. Cephalon first used a preferential crystallization process for pre-clinical and initial clinical trials supply. However, this process was neither efficient nor economical. Consequently, Cephalon decided to try chiral chromatography and from a simple analytical separation a process for multi-ton production was developed and implemented in less than two years. The chromatographic route allowed Armodafinil® to be quickly produced for clinical trials. The process was then implemented first at a CMO's site and then transferred to two other production sites for commercial scale production. Moreover, fast chromatographic development allowed Cephalon to free up resources to evaluate other options which led to the development of a second generation process via an asymmetric synthesis route. This new process proved to be more cost-effective, especially since the racemization of the non-desired enantiomer was not possible.

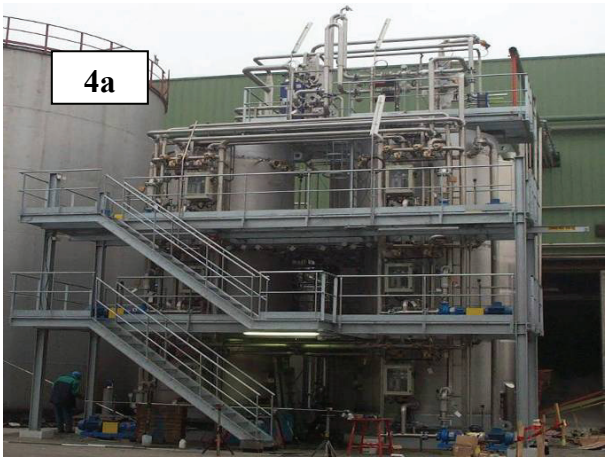

Figure 4a: Varicol® 6-1000 (50 bar)

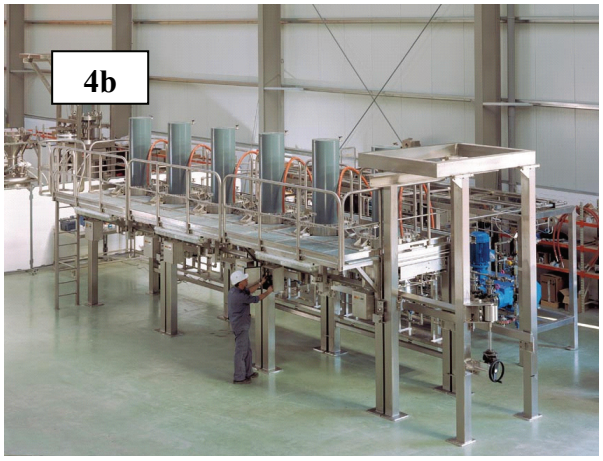

Figure 4b: SSMB 6-3200 (5 bar)

MCC: A STANDARD TOOL IN FOOD INGREDIENT INDUSTRY

Figure 4 shows two MCC systems used in Pharma and Food industries. The left picture is a Varicol® fitted with 6 columns of 1000 mm internal diameter, and operated at high pressure. The right side picture shows a SSMB system used for the production of Fructose 55. Almost all High Fructose Corn Syrup producers are familiar with such a multi-column process for enrichment application, which is used to separate glucose and fructose, and involves chromatography systems fitted with columns up to 5 m ID filled with up to 200 m^3 of resin per system.

This sugar separation is performed on cation exchange resin (calcium form) using warm water as eluent. The retention mechanisms are specific to this application whose principle is also called “ligand exchange chromatography”. Both fructose and glucose enter the media matrix but fructose forms a complex with Ca^{2+} ions of the matrix and is more retained. Fructose it is the slow component which can be separated from the glucose travelling faster. Large molecules like polysaccharides are excluded from the matrix and are eluted even faster, and recovered in the same fraction as glucose.

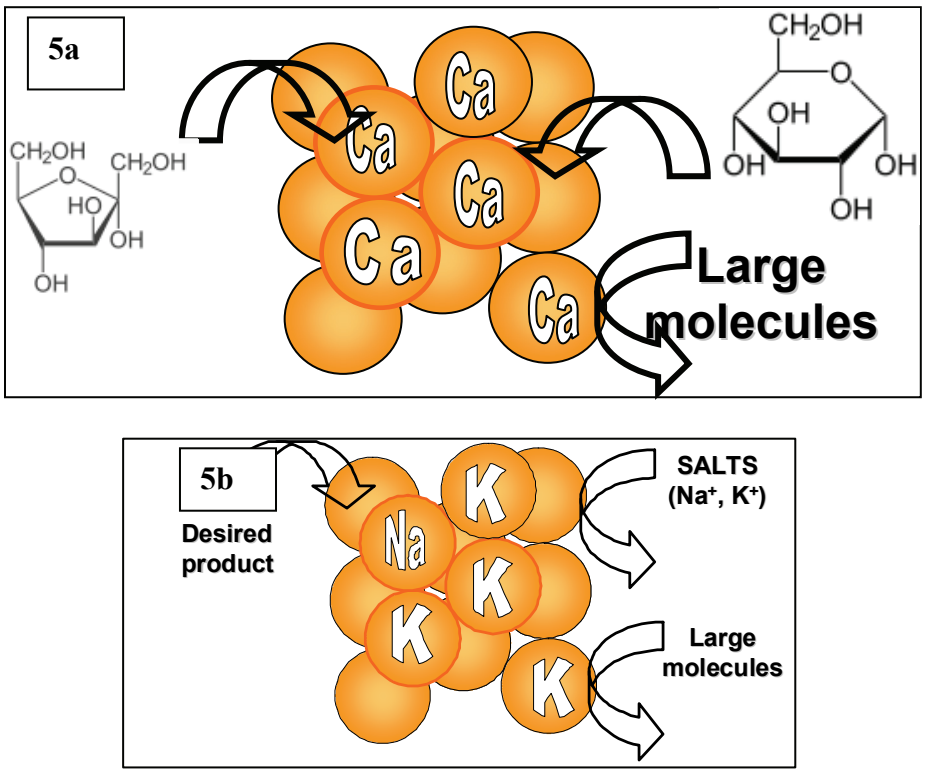

Figure 5: Retention mechanism for glucose-fructose separation (5a) and ion exclusion applications (5b)

It is worth mentioning that glucose is not retained by the matrix as it is eluted in the dead volume, and fructose is only slightly retained as it is typically eluted after elution of a volume of water 0.7 to 0.75 bed volume only. Such low conditions of retention still allow to reach high performances in terms of productivity, with up to 2 kg of feed injected per 1 liter of resin in 24 hour, this figure being comparable to a typical productivity value that can achieved in Pharmaceutical applications with SMB or Varicol® systems. However, the volumes of sugars purified every year on a single system are much larger, with as an example, 150,000 tons of fructose55 produced with a SSMB system fitted with columns of 5 m internal diameter, the biggest system installed so far (China). Such a large piece of equipment is obviously designed for an operation at low pressure, using stationary phases of large particle size.

Based on the same principle of a binary separation, a large number of separations has been developed for industrial scale production like polysaccharides enrichment (fructo oligo saccharides FOS and galacto oligo saccharides GOS), maltodextrines purification, specialty sugars (e.g. Tagatose, Mannose), high grade lactose, polyols (arabitol, xylitol, sorbitol, mannitol...) or betaïne recovery from beet. For

most of these applications, one fraction is eluted first and contains the non retained product(s) together with the materials excluded from the matrix by an ion or size exclusion process, and the second eluted fraction second contains the product(s) that is (are) slightly retained by the matrix. The mechanism is sometimes based only on an ion/size exclusion process and no interaction with the matrix surface is involved, with the more retained product entering the beads through the macropores while the less retained products are excluded.

FROM FOOD INGREDIENTS TO THERAPEUTIC PROTEINS

The next example of Multi-Column-Chromatography process whose principle is described in **Figure 6** has been developed first for Food Ingredient industry and applied at multi thousands tons scale (Amino-acid Lysine, mono nucleotides), but it is also gaining increasing interest from the Biopharmaceutical and Pharmaceutical companies for the downstream purification of proteins, monoclonal antibodies (MAbs), or antibiotics produced by fermentation.

This process, named Sequential Multi-Columns Chromatography (SMCC) is semi-continuous. It has been implemented at industrial scale for the production of several 10's thousands of Lysine every year on a single system. A sequence of several elution steps involving different buffers of different pHs or ionic strength is integrated in a process where the feed stream is supplied almost continuously in the system.

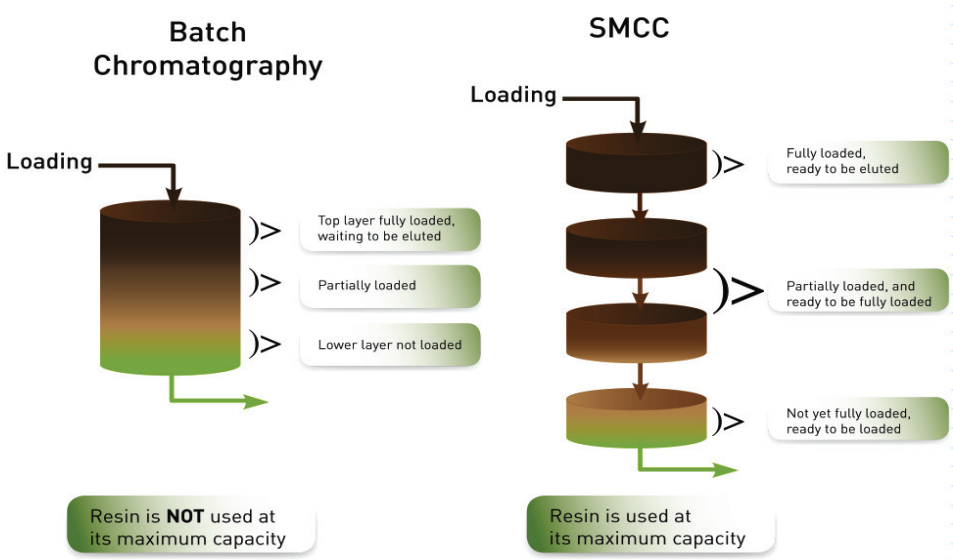

Figure 6: Process comparison batch chromatography and SMCC

SMCC, referred to as ionic "SSMB" in the case of Lysine and mono-nucleotides production, or "Bio-SC" for MAbs or other therapeutic applications, uses the same type of stationary phase and the same separation techniques as used in any given batch process. **Figure 6** contrasts a single batch column process with a four-column configuration SMCC process. The flow diagram shows that with SMCC, the single batch column is 'sliced' into four smaller columns. Three of the four columns are available for loading in linear sequence while the fourth column is eluted, regenerated and equilibrated. This happens asynchronously out of sequence with the continuing loading phase until it is ready to return to the linear sequence in its proper turn.

In a classic batch process, the column is loaded until the breakthrough threshold is reached at the outlet of the column. At constant bed height, as the loading flow rate increases, the amount of loaded product decreases as mass transfer phenomena prevent a fast capture of target product. If one considers the state of the column before product leakage is attained, the layers of stationary phase near the column inlet are more loaded than the layers close to the outlet of the column because of the slanted shape of the breakthrough curve. In the most inefficient processes, loading still has to be stopped even if a significant portion of the column is poorly loaded. In this scenario, the more completely loaded layers, perhaps

approaching the maximum capacity of the stationary phase, are ready to be washed, eluted, etc. However, in batch chromatography, all process steps must be applied to the whole column; therefore, the poorly loaded layers are washed, eluted and regenerated in spite of their low contribution to capture task, thus underusing the capacity of the stationary phase (and the mobile phase as well, as well as time).

THE ROLE OF CHROMATOGRAPHY IN THE PRODUCTION OF BIOPHARMACEUTICALS

Among the stages in a purification process, chromatography still remains the "work horse" with at least two orthogonal chromatographic steps. The purpose of these steps is to separate the target protein from product process related impurities and ultimately, purify the target protein to very high levels, often above 99% purity. This is achieved by selectively adsorbing and desorbing target proteins and contaminants while using time and different elution conditions. These conditions are based on the physico-chemical properties of the target product and impurities to ensure an effective separation.

The main types of chromatography currently used in bio-separations are affinity chromatography (AC), ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC), or mixed mode separations as in Hydroxyapatitechromatography. In separations using AC, IEX, HIC and Hydroxyapatite chromatography, products contained in a feed can be sorted into three main types: non-retained products (weak interaction with the stationary phase), the target product (interaction) and undesired products (partial or strong interaction). In the loading and washing steps, non-retained products flow through the column unhindered to waste.

Washing and regeneration steps remove non-target bound or unbound species and the target product is obtained during the elution step.

In capture steps, it is well known that batch column processes are limited and rarely, if ever, do they utilize the maximum capacity of a stationary phase during the loading step; the higher the loading flow rate, the lower the dynamic capacity. Dynamic Binding Capacity (DBC) is a commonly used term that

defines the available capacity of a stationary phase as a function of the loading flow velocity (**Figure 7**). Therefore, increasing flow velocity has a double effect: cycle time decreases but the available binding capacity decreases as well.

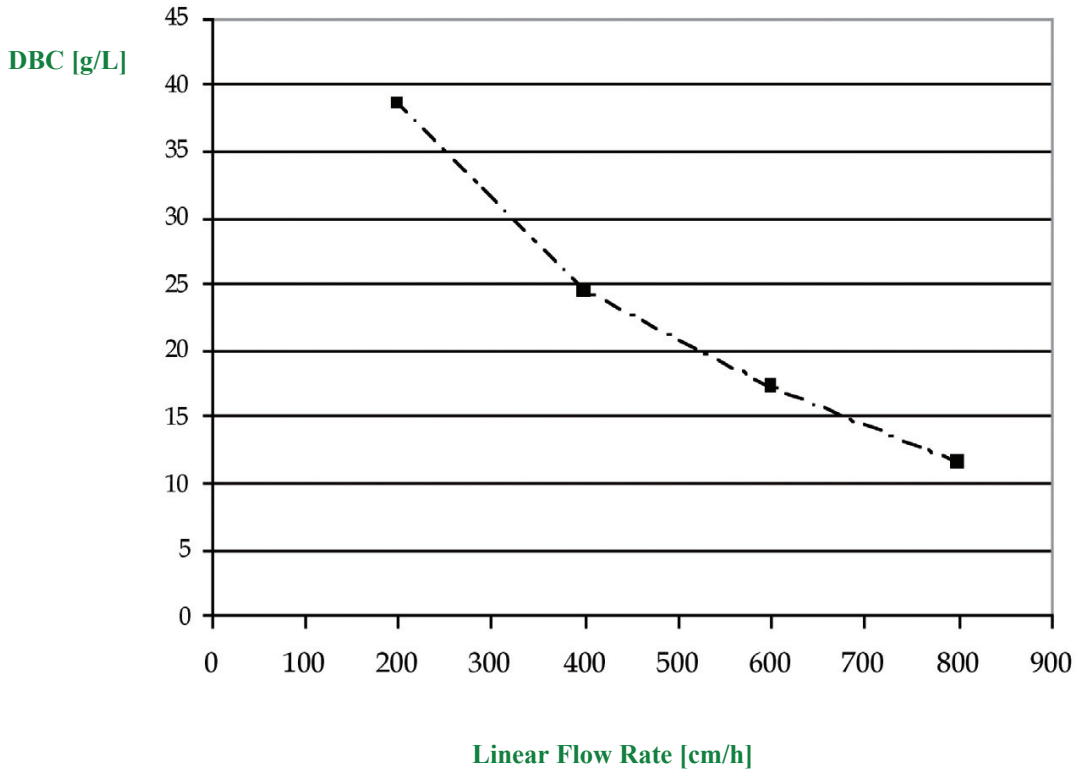

Figure 7: Dynamic Binding Capacity vs. Linear Flow Rate. **Dynamic Binding Capacities for hIgG (Cload = 1g/L) at 10% breakthrough at a column height of 103 mm using Protein A media**

In response, production processes are being driven to large volumes of stationary phase and buffers resulting in large column diameters in order to maximize both process speed and productivity. This, in turn, brings other difficulties from the cost of large volumes of resin and buffer to the difficulty of managing the flow distribution and packing of such large columns.

In order to limit or remove this constraint, stationary phase providers focus on increasing capacities, improving accessibility to specific binding sites and increasing mechanical stability especially at higher flow rates.

INCREASED FERMENTATION PRODUCTIVITY: HOW TO ADDRESS THIS IN DOWNSTREAM PROCESSING?

In defining the problem, the answer becomes apparent. As it happened in Petroleum, Food and Pharmaceutical Industries, the concept of using multiple columns for one separation step has gained interest of most all the Biopharmaceutical companies.

SMCC applied to MonoClonal Antibodies, other proteins or smaller molecules like antibiotics takes the multi-column concept to the next level. Full automation and flexible asynchronous scheduling of multiple columns (up to 6) enables processes to run at linear flow rates over 1000 cm/h while effectively utilizing almost 100% of the capacity of the resin. By approaching the maximum loading capacity while increasing process flow rates, the end result is a significantly improved productivity (g protein / L stationary phase / day) of 1.5 to 4 fold, as well as significant, 2 to 4 fold, reduction in wash, elution and regeneration buffers with much faster process rates. Due to the increased productivity (g protein / L stationary phase / day) the quantity of stationary phase can be reduced proportionally up to a factor of four. Thus, the use of several columns (typically 3 to 4) in the SMCC process significantly reduces the footprint of chromatographic columns (for example: from one 1.7 m column for batch process to 4 x 0.6 m columns with the SMCC process). Furthermore, several chromatographic and TFF steps can be integrated to make continuous Downstream Processing possible. In such a scenario, efficiency is gained from each process leading to significant savings and/or capacity increases. The SMCC technology offers significant efficiency to both single or multiple unit process chromatography steps in existing processes based on resin volume, manpower and buffer savings alone. In the case of a new facility design running Downstream Processing in a semi-continuous way, SMCC technology studies have shown that based on footprint, utility requirements, tanks and other hardware capital expenditure, savings can be expected to be in the region of 25-40% and COG's for Downstream Processing could be reduced up to 70%.

Figure 8: BioSC pilot system – SMCC process

CONLUSION

Preparative Chromatography has become a powerful production tool for the commercial purification of many products for human consumption, such as therapeutic products (MAbs, API's…), food ingredients (small organic acids, sugar and derivatives, amino acids…) or functional ingredients (vitamins, anti-oxidants…).

There is almost no limitation in the production scale, as very large demands can be fulfilled by using several units of the largest scale. As an example in sweeteners industry, huge capacities can be reached by operating in parallel several multi-column SSMB systems of 5 m ID for each column, with sometimes a total throughput of more than 500,000 metric tons of dried feed processed every year on the same single fructose manufacturing plant.

Economics are not always in favour of a chromatography route but there is almost no technical limitation related to the molecular structure of the product to be purified, as most of the products can be separated using one of the numerous stationary phases available as bulk for large scale production. Even

optical isomers of identical physical properties (beside rotation of polarized light) and identical chemical properties (at least in a non chiral environment) can be separated at large scale and it was estimated in 2005 that the total dedicated chiral capacity production was exceeding 1500 metric tons of racemate / year.

Over the last 50 years, hardware technologies have improved in parallel with the stationary phases technologies, and new chromatography products and processes are being made available to the chromatographers who are always reconsidering their approaches accordingly. In all industries, several generations of preparative chromatography solutions have been developed, the first ones addressing the technical needs related to the molecular structure of the product to purify, and the last ones addressing the financial constraints of a commercial scale production.

One of the current challenges of biopharmaceutical industry is to decrease the costs of the downstream processing, to adapt their purification lines to the high titers achieved in fermentation broths. It is clear that chromatography technologies will contribute to address this issue.

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