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Optimal design and operation of the preparative chromatography of bioproducts

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OPTIMAL DESIGN AND OPERATION OF THE PREPARATIVE CHROMATOGRAPHY OF BIOPRODUCTS

Dissertation submitted to the

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DOCTOR OF SCIENCES

presented by

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A mis abuelos
Abstract

The Simulated Moving Bed (SMB) is a continuous countercurrent chromatographic separation process, that allows to split a feed mixture into two fractions. The principle of SMB is the simulation of the continuous movement of the solid by periodically switching the inlet and outlet ports of the unit; this is equivalent to the True Moving Bed (TMB) when a set of relationships is fulfilled. SMB yields to larger productivities and lower solvent consumptions when compared to column chromatography. Furthermore, high purities may be achieved even at relatively low resolution. As a result, SMB has become an established technology for the separation of hydrocarbons, sugars and enantiomers.

This thesis explores the use of the SMB technology in the bioseparations field where it has been considered only rarely up to the current time; indeed, column (batch) chromatography is extensively used for the downstream processing of biocompounds. In bioprocesses, the target species is generally contained in a complex mixture of many species having a broad range of physical and chemical properties; in this mixture, the target species may elute from the selected chromatographic media at an intermediate time and therefore, a central cut of the chromatogram is required. However, SMB has been traditionally used to split feed mixtures into two fractions, often pure, and this has been one of the main concerns regarding the application of SMB in biotechnology. Another important open question is related to cleaning issues, which are crucial in bioprocessing. In batch processes, a cleaning in place (CIP) step is performed after every cycle and often several columns are used in parallel. This is a crucial step in bioseparations and therefore, the use of SMB's in the field is compromised by the need of an adequate solution for continuous (online) CIP.

The first part of this work presents solutions that enable the use of the SMB technology in bioseparations. Namely, the Equilibrium Theory Model is used to analyze new SMB configurations that allow to recover the target species in a central cut, and to perform online cleaning. These are the three fraction SMB (3F-SMB) and the integrated cleaning in place SMB (CIP-SMB); the third SMB configuration is the enriched extract SMB (EE-SMB), which is indicated for binary separations. In the second part of the work, the theoretical findings described above are validated experimentally for a bioseparation of interest. The application investigated concerns the purification of plasmid DNA (pDNA) which is of interest in the areas of gene therapy and nucleic acid vaccination. The experimental CIP-SMB unit used is a modified version of the commercial unit ÄKTA Explorer from GE Healthcare. Finally, the third
part of this work covers the comparison of the optimized SMB and column chromatography processes for the pDNA purification step investigated experimentally. The list below provides some details about these investigations:

**Enriched Extract SMB.** A variation of the SMB operation called Enriched Extract SMB (EE-SMB) is investigated, in which part of the extract product is concentrated and the resulting enriched stream is re-injected into the SMB at that same point, i.e. at the inlet of section 2. This operation has been recently patented (M. Bailly, R.-M. Nicoud, A. Philippe, and O. Ludemann-Hombourger. Patent No. WO2004039468, 2004). In the study here presented, the Equilibrium Theory is used to obtain the constraints on the operating parameters and to identify the operating regimes for the EE-SMB operation. This analysis is carried out for a binary mixture whose adsorption behavior is described by a Langmuir isotherm. The operating regimes are translated into regions on the \((m_2, m_3)\) plane representation, that can be easily compared with the corresponding ones of the standard SMB given by the Triangle Theory. In particular, for the EE-SMB operation, the region of complete separation turns out to be a line segment, i.e. a one dimensional locus. The theoretical findings are confirmed through detailed simulations of the process, and the value of the new EE-SMB operation mode is assessed.

**Three fraction SMB and cleaning in place.** A Three Fraction Simulated Moving Bed (3F-SMB) unit for separating feed mixtures into three fractions is investigated; this responds to the requirement of many bioseparations which involve complex multicomponent feed mixtures (in opposition to the usual two component SMB feed mixtures, e.g. racemic mixtures). At linear chromatographic conditions, criteria for the selection of the flow rate ratios in the four sections of the unit are developed. These criteria translate into a region of three fractions complete separation in the \((m_2, m_3)\) plane. The performance of the 3F-SMB unit is investigated through detailed simulations using as a model system the separation of the four nucleosides: 2'-Deoxyadenosine, 2'-Deoxythymidine, 2'-Deoxyguanosine and 2'-Deoxycytidine. At non-linear chromatographic conditions, simulations on the same model system allow to draw qualitative conclusions on the performance of the 3F-SMB. Further on, the possibility of using the 3F-SMB unit to implement a cleaning in place step (CIP) into the standard SMB is shown and validated through simulations, using as a model system a mixture of two nucleosides and one highly retained impurity. This leads the so-called CIP-SMB configuration.

**Purification of plasmid DNA in a CIP-SMB unit.** Interest in producing large amounts of plasmid DNA (pDNA) has increased in the last decade as a result of the advances in gene therapy and DNA vaccines. The last stage of
the manufacturing process involves a purification scheme guaranteeing a safe and stable product, considering the authorities recommendations. This part of the work investigates the first polishing step of plasmid DNA carried out in a CIP-SMB unit based on size exclusion chromatography. The feed mixture consisted on a clarified cleared lysate, and the experiments were designed using the simple approach of Triangle Theory. The results indicate that total recovery of pDNA is possible and that the productivity can be tuned considering the trade-off with purity. Detailed simulations are used to validate these results, after establishment of a cleared lysate model.

Optimization and comparison of column chromatography and SMB. In this part of the work, the performances of the SMB and the column chromatography processes for two different case studies are analyzed: the first stage of the plasmid DNA polishing, and the Tröger’s base enantiomer separation, in which the adsorption isotherms are linear and non-linear respectively. Simulation tools are used together with an optimization routine (Non-Sorting Genetic Algorithm (NSGA)) in order to find the optimum operating conditions leading to maximum productivity and minimum solvent consumption; the optimum solution for each of the processes is a curve on the productivity-solvent consumption plane, the so-called Pareto set. The comparison between the column and the SMB processes is based on the relative position of the two Pareto sets calculated at equal conditions and for the same final purity and recovery of the target species. The results show that SMB is superior to column chromatography in the two case studies investigated, i.e. in the case of the linear isotherm (plasmid DNA), the productivity gain is up to a factor two for a given value of the solvent consumption. Furthermore, the flexibility of the SMB operation is larger, since the Pareto sets are flatter and they prolong into regions of the productivity-solvent consumption plane that are not accessible with the column chromatography process.
Résumé

La technique du Lit Mobile Simulé ou Simulated Moving Bed (SMB) est un procédé de séparation continue par chromatographie qui permet de fractionner un mélange de deux ou plusieurs espèces en deux courants de produit. Le principe du SMB consiste à simuler un contact à contre-courant entre la phase solide (le sorbant) et la phase fluide (le solvant). Pour ce faire, les courants d’entrée (alimentations) et de soutirage (produits) sont périodiquement déplacés d’une position dans le sens de l’écoulement du solvant, ce qui équivaut à déplacer les colonnes dans le sens inverse du solvant. Ce procédé est équivalent au procédé True Moving Bed (TMB)- dans lequel la phase solide s’écoule effectivement - à condition qu’une série de relations d’équivalence entre les variables opérationnelles se vérifie. La technique du SMB atteint des valeurs de productivités plus élevées que celles obtenues en batch (colonne unique), tout en diminuant la consommation de solvant. Cette technique est d’autant plus intéressante que la pureté des produits peut être assurée même dans les cas de faible résolution des espèces, grâce au contact à contre-courant. Ces avantages ont fait le succès de cette technique qui est couramment utilisée pour la séparation des hydrocarbures, des sucres et, plus récemment, dans le domaine de la purification d’enantiomères.

Dans ce travail de thèse, on étudie la possibilité d’utiliser la technologie SMB dans le domaine des bioséparations où jusqu’à présent, elle n’a que rarement été prise en considération. En effet, les bioséparations sont généralement accompagnées par des procédés discontinus, i.e. par chromatographic dans une colonne unique. Dans le domaine des bioséparations, l’espèce cible se trouve généralement dans un mélange complexe contenant beaucoup d’autres espèces qui ont des propriétés chimiques et physiques variées. L’espèce cible est donc probablement éluée de la colonne à un temps intermédiaire. Or, le SMB a été traditionnellement utilisé pour séparer des mélanges binaires en deux fractions, souvent pures, ce qui constitue un désavantage dans le domaine des bioséparations. Une deuxième limitation importante est liée aux protocoles de nettoyage des colonnes, qui sont cruciaux dans ce domaine et moins importants dans les applications traditionnelles du SMB. Dans le procédé chromatographique batch (à colonne unique), ce nettoyage généralement appelé CIP (Cleaning in Place) est réalisé une fois par cycle; parfois plusieurs colonnes sont utilisées en parallèle. Afin de pouvoir utiliser le SMB pour les bioséparations, il est nécessaire de trouver une solution technique qui puisse (permettre de) reproduire le CIP du procédé batch de façon continue (online).

La première partie de ce travail présente trois configurations différentes du
SMB, qui rendent possible son utilisation dans le domaine des bioséparations. La théorie de l'équilibre est l'outil adéquat pour l'analyse théorique de ces nouvelles configurations qui permettent de collecter l'espèce cible dans un mélange complexe, même quand son temps de rétention est intermédiaire, et qui offrent également une solution pour effectuer le CIP online. Les nouvelles configurations SMB mentionnées sont la Three Fraction SMB (3F-SMB) et la Integrated Cleaning in Place SMB (CIP-SMB). La troisième configuration appelée Enriched Extract SMB (EE-SMB) est plutôt indiquée dans les cas de séparations binaires, par exemple dans la purification d'énantiomères. Dans la deuxième partie de ce travail de thèse, la configuration CIP-SMB est validée expérimentalement pour la purification de plasmides d'ADN (pDNA), produit qui a un fort potentiel dans les thérapies géniques et la préparation de nouveaux vaccins. L'unité expérimentale de CIP-SMB utilisée est une version modifiée du produit commercial ÄKTA Explorer (GE Healthcare). Dans la troisième partie de cette thèse, le procédé continu SMB et le procédé discontinu à colonne unique sont comparés en termes de productivité et de consommation de solvant pour le cas de la bioséparation de pDNA étudiée expérimentalement.

Les domaines de recherche approchés sont présentés plus en détails ci-dessous:

**Enriched Extract SMB (EE-SMB).** Dans cette nouvelle opération SMB, une partie du courant de produit appelé Extrait (qui contient l'espèce avec le temps de rétention le plus élevé) est concentrée à la sortie de la zone 1 du SMB, avant d'être réinjectée à l'entrée de la zone 2. Cette opération a été récemment brevetée (Bailly, M. et al, WO2004039468, 2004). Dans ce travail de thèse, une étude de ce procédé a été menée dans le cadre de la théorie de l'équilibre afin de déterminer les critères de sélection des variables opérationnelles. Ces critères peuvent par exemple être utilisés pour obtenir les espèces purses respectivement dans l'Extrait et dans le Raffinat. Cette opération est intéressante pour la séparation de mélanges binaires dont l'interaction avec la colonne peut être décrite par une isotherme d'adsorption du type Langmuir. Les différents régimes opérationnels du EE-SMB peuvent être représentés dans le plan (m2, m3) pour comparaison avec ceux du SMB (Triangle Theory). En particulier, on montre que la région de séparation complète des deux espèces dans le procédé EE-SMB est un segment, c'est à dire un locus unidimensionnel. Des simulations détaillées du procédé confirmant ce résultat théorique, et aident d'autre part à quantifier l'intérêt de cette opération en termes de productivité.

**Three Fraction SMB (3F-SMB) et CIP-SMB.** L'opération appelée 3F-SMB permet de séparer un mélange d'espèces en trois fractions (ou courants de produits). Ceci est intéressant dans le domaine des bioséparations où très sou-
vent, les courants d’alimentation du procédé de séparation chromatographique sont très complexes et contiennent beaucoup d’espèces. Dans cette étude, des critères de sélection des variables opérationnelles sont dérivés et représentés dans le plan \((m_2,m_3)\) pour le cas d’une isotherme d’adsorption du type linéaire. La performance du 3F-SMB est étudiée à l’aide de simulations détaillées du procédé pour le cas de la séparation de quatre nucléosides : 2’-Deoxyadenosine, 2’-Deoxythymidine, 2’-Deoxyguanosine et 2’-Deoxycytidine. Le même système de nucléosides est utilisé dans des simulations avec des conditions non linéaires pour lesquelles il n’existe pas de solution analytique. On montre enfin que la configuration 3F-SMB peut être utilisée pour appliquer le CIP au SMB (CIP-SMB). Cette possibilité est aussi étudiée par simulations. Le mélange considéré contient deux nucléosides et une troisième espèce très fortement retenue dans la colonne, jouant le rôle d’impureté.

Purification de plasmides dans un CIP-SMB. Les succès de la recherche dans les domaines de la thérapie génique et des vaccins ADN génèrent un intérêt grandissant pour le développement d’un procédé de production performant pour les plasmides d’ADN (pDNA). La dernière étape de la production de pDNA est le procédé de purification. Celui-ci doit garantir le maintien des propriétés du produit tout en assurant que ses spécifications soient en accord avec celles préconisées par les agences régulatrices (e.g. FDA). Dans cette partie du travail, la première étape de la purification du pDNA est accomplie dans un CIP-SMB. On étudie la technique Size Exclusion, le mélange d’alimentation du SMB étant une solution cleared lysate. La conception de l’opération est accomplie en utilisant la théorie de l’équilibre. Les résultats montrent que le pDNA peut être totalement récupéré dans le raffinat, et que les principales impuretés peuvent être éliminées dans l’extrait (ARN, protéines, cDNA, etc). La productivité peut être contrôlée en manipulant les variables opérationnelles et en tenant compte du fait que la pureté diminue lorsque la productivité augmente. Des simulations détaillées permettent de vérifier ce résultat expérientiel ; dans ces simulations le mélange à séparer a été assimilé à un mélange ternaire.

Optimisation et comparaison des procédés discontinu et SMB. La performance du procédé SMB est comparée à celle du procédé à colonne unique pour deux cas différents : d’une part la première étape de la purification des pDNA étudiée expérimentalement, d’autre part la séparation des deux enantiomères de la base de Tröger. Ces deux cas sont différents en plusieurs aspects : tout d’abord, ces systèmes sont respectivement linéaire et non linéaire en ce qui concerne l’isotherme d’adsorption ; en outre, le premier est un exemple de bioséparation alors que le deuxième est un cas de séparation d’enantiomères.
et donc un exemple typique d’application du SMB. La routine d’optimisation (Non-Sorting Genetic Algorithm (NSGA)) est couplée avec les codes de simulation des deux procédés (continu et discontinu) pour obtenir les conditions optimales d’opération, c’est-à-dire celles qui entraînent des valeurs de productivité maximales et des valeurs de consommation de solvant minimales. Les solutions optimales de chaque procédé pour les deux cas étudiés peuvent être représentées dans les coordonnées productivité-consommation de solvant, pour obtenir des courbes de Pareto. La comparaison est basée sur la position relative des courbes de Pareto correspondant aux deux différents procédés et calculées dans des conditions équivalentes, c’est-à-dire pour la même pureté finale des produits et le même rendement (degré de récupération des espèces dans les produits par rapport à la quantité traitée). Le résultat démontre que le SMB est supérieur au procédé à colonne unique pour les deux cas étudiés. Dans le cas du pDNA (linéaire), le gain en productivité peut être le double pour une valeur donnée de la consommation de solvant. En outre la flexibilité du SMB est supérieure comme le démontrent les courbes de Pareto qui sont plus longues et plates, c’est-à-dire que d’avantage de valeurs de productivité ou de consommation de solvant sont atteignables.
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Chapter 1

Introduction

The project presented in this thesis had the initial aim of positioning the Simulated Moving Bed (SMB) technology [1] in the field of bioseparations where it had been considered only rarely up to that time; indeed, in the biotechnology field, most of the processes are carried out in a batch mode. In academic research, only few experimental studies had been carried out, mostly based on mixtures of pure proteins, antibodies, and other species [2-4]; however, very often these "synthetic" feed mixtures can hardly compare with the crude mixtures that are encountered in the downstream processing. An important characteristic of these complex mixtures is that the species have a broad range of physical and chemical properties; in this mixture the target species may elute from the selected chromatographic media at an intermediate time and therefore, a central cut of the chromatogram is required. SMB has been traditionally used to split feed mixtures into two fractions, often pure, and this has been one of the main concerns regarding the application of SMB to bioseparations. The second important open question was related to the cleaning issues, which are of smaller importance in other fields of application, but are crucial in bioprocessing as a consequence of the feed mixture characteristics and the final product applications (e.g pharmaceutical products). The batch process cleaning protocols are generally based on rules of thumb: the chromatographic media is regenerated by
applying a cleaning solution and allowing for a given contact time. In other cases, the chromatographic material is simply disposed off after a given number of cycles. In SMB there was no ready solution for cleaning online that could be used in order to reproduce the batch protocols.

In order to consider the SMB technology as an option for bioseparations, the uncertainties mentioned above had to be cleared. Namely, an effort was required in the direction of developing new SMB configurations that could cope with the need of recovering the target species in a central cut, and of performing online cleaning in SMBs. This theoretical work constitutes the first part of this thesis. The SMB process is analyzed by using the Equilibrium Theory, which constitutes a very powerful tool for studying the dynamics of chromatographic columns and therefore, of SMBs. This enabled the theoretical study of the so called Three Fraction SMB (3F-SMB) configuration which had been demonstrated experimentally shortly before for the separation of nucleosides [2], and earlier for the separation of cyclosporins [5]. In this configuration, the feed mixtures can be split in three fractions by letting the more retained group of molecules arrive in section 1, and desorbing them during the next cycle while the column is taken out of the loop. Other authors have put effort in extending the use of SMB to multicomponent mixtures as it can be appreciated in the literature of recent years [3, 6–8]. In the frame of this project, the issue of online cleaning has been solved by using a similar configuration to the 3F-SMB, where one column is taken out of the loop during two cycles: during the first cycle a cleaning agent is used, e.g. sodium hydroxide, while during the second cycle the column is equilibrated with the running eluent. This is the so-called CIP-SMB configuration. Finally, the use of the Equilibrium Theory to investigate new SMB techniques and to characterize the design of these operations was also applied to the so-called Enriched Extract SMB operation [9–11]. This technique is interesting for the separation of mixtures in two fractions, particularly enantiomers separations.

In the second part of the work, the theoretical findings described above have been validated experimentally for a biseparation of interest. The application investigated concerns the plasmid DNA (pDNA) purification. Plasmid DNA is of interest in the areas of gene therapy and nucleic acid vaccination; with respect to other DNA vectors, they are considered
safer, though possibly less efficient. In order to produce large amounts of pDNA fulfilling the strict health guidelines set by the regulatory agencies (such as FDA), there is a need of establishing an efficient large scale pDNA manufacturing process. The separation investigated in this thesis focuses on the first purification step of pDNA starting from a bacterial broth (the global purification scheme requires at least two more purification steps; Appendix B gives an overview of the pDNA downstream processing). An experimental CIP-SMB unit having the appropriate scale and functionalities has been built. This consists of a modified version of the commercial unit ÅKTA Explorer from GE Healthcare, which is rather flexible and easy to control thanks to the corresponding upgrade of the Unicorn software (GE Healthcare) (Appendix D contains a flow-sheet of the unit).

Experimental validation of SMB bioseparations like the one for pDNA discussed in this work are necessary but cannot by themselves encourage the use of the SMB technique; the complexity of the SMB operation when compared to the batch operation has to be compensated by an improvement in performance. This improvement has been shown in other fields of applications, e.g. lately for enantiomers separations [12–14]. Therefore, it is necessary to produce quantitative data about the SMB performance in bioseparations and to establish fair comparisons with the corresponding column chromatography alternatives. The comparison of the optimized SMB and column chromatography processes for the pDNA case investigated experimentally is covered in the third part of this thesis.

The thesis is organized as follows: the publications produced in the frame of this thesis are mentioned in the first footnote of every corresponding chapter. A general list of references is included at the end of the thesis, together with the list of symbols. In the next three sections of this introductory chapter the principle of the Simulated Moving Bed technique is presented, followed by a simple method to design the operation (in the case of a competitive Langmuir isotherm), and by the model equations describing the chromatographic columns. In the next two chapters the EE-SMB (Chapter 2), and the 3F-SMB and CIP-SMB configurations (Chapter 3) are analyzed extensively through detailed simulations, with application to a bioseparation in the later case, namely the separation of nucleosides on reversed phase, and to an enantiomers separation in
1. Introduction

the former. The CIP-SMB configuration is used in Chapter 4 to perform the first purification step of plasmid DNA from a cleared lysate. The comparison of SMB with column chromatography is presented in Chapter 5, where optimum operating conditions for the two processes are obtained for the case of the same plasmid DNA purification step investigated experimentally. The same comparison work is done for the case of an enantiomers separation, where the SMB technology is generally preferred. This serves to draw a parallel between the two fields of application and to prove that there are no significant differences between the two with respect to the performance gain of SMB when compared to column chromatography.

1.1 Principle of the Simulated Moving Bed Technology

The Simulated Moving Bed [1] (SMB) is a continuous countercurrent chromatographic separation process, that allows to split a feed mixture into two fractions, which are called Extract and Raffinate. An SMB unit consists of a number of fixed bed chromatographic columns connected in a loop, where the desorvent and feed, and the product streams are continuously feed or collected respectively. The principle of this technology is the simulation of the continuous movement of the solid by periodically switching the inlet and outlet ports of the unit. The real countercurrent flow pattern of the solid and the fluid phases leads to the so-called True Moving Bed technology (TMB) which yields better performances than the ones obtained in fixed bed chromatography; furthermore, total recoveries and high purities can be achieved even when the resolution between the species is not total. However, letting the solid phase flow has many disadvantages and consequently, the TMB is not feasible in practice. The Simulated Moving Bed technique, where the movement of the solid is just simulated, is equivalent to the TMB when a set of relationships is fulfilled; thus, SMB has the same advantages over column chromatography than the conceptual TMB process, provided that the equivalence relationships are fulfilled.
Figure 1.1 illustrates the scheme of a closed loop SMB unit separating the binary mixture of 1 and 2 (these are the less and more retained species, respectively), and working under complete separation conditions. In the closed loop TMB unit working under complete separation conditions, the ratio between the fluid and the solid flow rates in the four sections is chosen so that the more retained adsorbate, i.e. species 2, is conveyed by the solid phase towards the extract outlet, while the less retained adsorbate, i.e. species 1, is conveyed by the fluid phase and ends up in the raffinate stream. The nature of the continuous process requires the regeneration of the solid and the fluid phases (in sections 1 and 4, respectively) to prevent contamination of the products, e.g. the solid phase exiting section 1 should be free of species 2 so that this stream can be recycled into section 1 without causing pollution of the extract. In order to guarantee the same separation performance both in the conceptual TMB process and in the SMB process, the following equivalence relationships must be fulfilled:

\[ \frac{Q_s}{t^*} = \frac{Q_s}{1 - \varepsilon} \]  

where \( V \) is the volume of an SMB column, \( \varepsilon \) its bed void fraction and \( t^* \) is the switching time, that is the time period between two successive switches of the inlet and outlet ports. Equations 1.1 and 1.2 constitute the equivalence relationships between the flow-rates of the two units, i.e. \( Q_s^{SMB} \) and \( Q_s^{TMB} \), and between the switch time of the SMB, \( t^* \), and the volumetric solid flow-rate of the TMB unit, \( Q_s \).

The design of an SMB unit for a given separation performance consists in selecting the net flow-rate ratios, \( m_j \), in the different sections \( (j = 1, 2, 3, 4, \text{typically}) \), a section being each group of chromatographic columns located between two inlet or outlet ports (see Figure 1.1). The flow-rate ratio, \( m_j \), is a dimensionless quantity relating the net fluid
flow-rate and the solid flow-rate in section $j$:

$$m_j = \frac{Q_{jMB}^T - Q_s \varepsilon_p}{Q_s(1 - \varepsilon_p)} \quad (j = 1, \ldots, 4) \quad (1.3)$$

using equations 1.1 and 1.2, equation 1.3 can be written in the following equivalent form:

$$m_j = \frac{Q_{jMB}^T \varepsilon^* - V \varepsilon^*}{V(1 - \varepsilon^*)} \quad (j = 1, \ldots, 4) \quad (1.4)$$

where $\varepsilon_p$ is the intra-particle void fraction, and $\varepsilon^*$ is the overall bed void fraction, i.e. $\varepsilon^* = \varepsilon + (1 - \varepsilon)\varepsilon_p$.

### 1.2 Design of SMB operating conditions

In order to design an SMB operation, it is useful to exploit the equivalence between the SMB and the TMB units described above. A simplified model is used where mass transfer resistance and axial dispersion are neglected, i.e. the Equilibrium Theory model. This approach yields the so-called Triangle Theory, that provides a good prediction of the optimal operating conditions and the purity trends with respect to the operating parameters [15-17]. More precisely, it gives necessary and sufficient conditions for complete separation of the two components in the feed mixture, that correspond to an operating region in the operating parameter space, which is usually projected onto the $(m_2, m_3)$ plane.

Thanks to the understanding provided by the Equilibrium Theory and to the relative simplicity of the analysis, this approach is used both in academy and in industry for getting preliminary operating conditions that can be then refined and optimized with the help of detailed simulations [18].

\[1\] It is worth noting that $\varepsilon_p$ will be written as $\varepsilon_{pi}$ when it comes to bioseparations where the species in the mixture may have a broad range of molecular sizes and consequently may experience different pore accessibilities.
1.2 Design of SMB operating conditions

Figure 1.1: Scheme of a closed loop 2-2-2-2 simulated moving bed unit.
1.2.1 Langmuir isotherm

Let us consider the design of SMB operating conditions for the separation of mixtures that are characterized by a competitive Langmuir isotherm:

\[ n_i = \frac{H_i c_i}{1 + K_1 c_1 + K_2 c_2} \] (1.5)

where \( H_i = K_i N_i \) is the Henry constant of the species \( i \), \( K_i \) and \( N_i \) are its equilibrium constant and saturation loading capacity, respectively, and \( n_i \) and \( c_i \) are the adsorbed phase concentration and the fluid phase concentration.

Criteria for complete separation are obtained by using the Equilibrium Theory and writing the corresponding material balances. The criteria consist on the following set of constraints on the flow-rate ratios in the SMB sections:

\[ H_2 < m_1 < \infty \] (1.6)
\[ m_{2,\text{min}}(m_2, m_3) < m_2 < m_3 < m_{3,\text{max}}(m_2, m_3) \] (1.7)
\[ \frac{-\varepsilon_p}{1 - \varepsilon_p} < m_4 < m_{4,\text{max}}(m_2, m_3) \] (1.8)

where \( m_{2,\text{min}}, m_{3,\text{max}} \) and \( m_{4,\text{max}} \) constitute the boundaries of the complete separation region, which are explicit functions of the isotherm parameters and of the feed composition [17].

Figure 1.2 shows the complete separation region defined by the two inequalities of equation 1.7; the other operating regimes are also indicated (e.g. the region of operating conditions leading to pure a extract stream). The upper bound of the complete separation region (the curve \( wa \) in Figure 1.2) is made up of of two portions, one being a straight line segment and the other a curve. Above this upper bound, the extract stream is pure; that is, species 2 is recovered alone in the extract but some of it ends up in the raffinate. On the other hand, in the region located to the left of the triangle (i.e. to the left of the line segment \( bw \), the raffinate
1.2 Design of SMB operating conditions

Stream is pure; that is species 1 is recovered alone in the raffinate, but some of it ends up in the extract. Inside the triangle there is complete separation and thus, total recovery of both species. It can be demonstrated that the productivity of the SMB operation is proportional to the distance of the selected operating point to the diagonal [15], meaning that point w in Figure 1.2 leads to the best theoretical productivity (this issue is discussed again in Chapter 2, section 2.4.1).

The Equilibrium Theory solution for Langmuir isotherms is presented in detail in several publications [15, 17, 19 21]. In Chapter 2, section 2.3, two different methodologies for obtaining the region of complete separation are discussed in detail.

Figure 1.2: Complete separation region and SMB operating regimes for the binary separation of species 1 and 2 described by a Langmuir isotherm.

1.2.2 Linear isotherm

When the feed mixture is infinitely diluted, the competitive Langmuir isotherm of equation 3.14 approaches the non-competitive linear
isotherm:

\[ n_i = H_i c_i \]  \hspace{1cm} (1.9)

and the constraints on the flow-rate ratios (equations 1.6–1.8) reduce to the following set of decoupled equations [1, 17, 22]:

\[
\begin{align*}
H_2 &< m_1 < \infty \quad (1.10) \\
H_1 &< m_2 < m_3 < H_2 \quad (1.11) \\
\frac{-\varepsilon_p}{1 - \varepsilon_p} &< m_4 < H_1 \quad (1.12)
\end{align*}
\]

It follows that in the linear case, the complete separation region is a square triangle in the \((m_2, m_3)\) representation, as illustrated in Figure 1.3. It is worth noting that, contrary to the Langmuir case, the complete separation region given by equations 1.11 is independent of the feed concentration.
1.2 Design of SMB operating conditions

Figure 1.3: Complete separation region and SMB operating regimes for the binary separation of species 1 and 2, having linear adsorption behavior.
1.3 Detailed SMB Model

All the simulations carried out in the frame of this work have been performed using a detailed one-dimensional model of the SMB unit. This model accounts for all the phenomena taking place in the column: accumulation in the fluid and in the solid phases, convection and axial dispersion in the fluid phase, and mass transfer through a linear driving force model assuming that solid diffusion is the rate limiting step [23]. The detailed column model consists on the following material balance equations [24]:

\[ \varepsilon D_{li} \frac{\partial^2 c_i}{\partial z^2} = \varepsilon^* \frac{\partial c_i}{\partial t} + (1 - \varepsilon^*) \frac{\partial n_i}{\partial t} + u \frac{\partial c_i}{\partial z} \]  \hspace{1cm} (1.13)

\[ \frac{\partial n_i}{\partial t} = a_p k_i (n_i^* - n_i) \] \hspace{1cm} (1.14)

\[ n_i^* = f_i(\xi) \] \hspace{1cm} (1.15)

where \( t \) and \( z \) are the time and space coordinates respectively, \( a_p \) is the specific surface of the adsorbent particles, \( u \) is the superficial velocity, and \( k_i \) and \( D_{li} \) are the mass transfer coefficient and the axial dispersion coefficient of species \( i \), respectively. The function \( f_i \) in equation 1.15 corresponds to the adsorption isotherm of component \( i \) and \( n_i^* \) represents the adsorbed phase concentration at equilibrium with the fluid-phase composition \( \xi \).

The model equations are integrated using the method of lines. Space discretization is based on finite differences, and a sufficient number of points per column has been determined for every specific case after checking that this eliminates the error due to numerical dispersion. Integration in time is carried out using a commercial integrator for stiff equations, as appropriate when simulating the steep concentration profiles occurring in high performance liquid chromatography.
Chapter 2

Enriched Extract SMB

2.1 Introduction

This chapter aims at investigating a modification of the SMB technique, where part of the extract stream is concentrated continuously and re-injected at the same point of the unit, i.e. at the inlet of section 2, as shown in Figure 2.1. We call the corresponding process Enriched Extract SMB, or EE-SMB in short. This process has been recently patented [10, 11, 25].

The advantage of this approach with respect to the standard SMB stems in principle from the possibility of changing the nature of the composition fronts or transitions occurring in section 2. In the case of a Langmuir isotherm (see equation 1.5), that will be considered here, or anyhow of a favorable isotherm, the simple wave transition (present in section 2) of a standard SMB, is replaced in the EE-SMB by a shock transition. This occurs at enriched extract concentrations larger than a minimum, which in the case of a Langmuir isotherm corresponds to the watershed concentration [26].

Figure 2.1: Scheme of a four section enriched extract simulated moving bed (EE-SMB) unit used for the continuous separation of species 1 and 2. The open circle at the Extract port represents the enrichment step.
The use of the Equilibrium Theory yields constraints on the flow-rate ratios that have to be fulfilled in order to get complete separation in an EE-SMB unit. For the sake of comparison and completeness, section 2.3 summarizes the corresponding derivation of complete separation conditions for the case of a standard SMB. The validity of the theoretical conclusions of this study is assessed through detailed simulations; and the potential of the EE-SMB operation as compared to the standard SMB is analyzed and discussed. Section 2.2 provides some background information about the Equilibrium Theory.

2.2 Background on the Equilibrium Theory

2.2.1 One to one mapping between the \((c_1, c_2)\)-plane and the hodograph plane

It can be demonstrated using the Equilibrium Theory, that there exists a one-to-one mapping between the space of fluid or adsorbed phase concentrations and the \(\omega\) components in a related space (a 2D plane in case of a binary system) \([26, 27]\). The two \(\omega\) values associated with a given equilibrium composition of solid and fluid phase \((n_i, c_i)\) for \(i = 1, 2\) are called \((\omega_1, \omega_2)\), and are the positive roots of the following equation

\[
\frac{K_1 n_1}{H_1 - \omega} + \frac{K_2 n_2}{H_2 - \omega} = 1
\]  

(2.1)

where \(K_1, K_2, H_1\) and \(H_2\) are the adsorption Langmuir isotherm parameters (see section 1.2.1 and equation 1.5). The roots \((\omega_1, \omega_2)\) fulfill the fundamental rule given by equation 2.2:

\[
0 \leq \omega_1 \leq H_1 \leq \omega_2 < H_2
\]  

(2.2)

The mapping equation 2.1 may be inverted in order to get the concentrations as a function of the \(\omega\) values \((\omega_1, \omega_2)\). The following equations result from this rearrangement:
From the above equations it can be readily shown that the denominator of the Langmuir isotherm may be written as:

\[ \delta = 1 + K_1 c_1 + K_2 c_2 = \frac{H_1 H_2}{\omega_1 \omega_2} \]  

(2.5)

and that the solid phase concentrations are given by:

\[ K_1 n_1 = \frac{(H_1 - \omega_1)(\omega_2 - H_1)}{H_2 - H_1} \]  

(2.6)

\[ K_2 n_2 = \frac{(H_2 - \omega_2)(H_2 - \omega_1)}{H_2 - H_1} \]  

(2.7)

When one of the two species, i, is absent from a specific stream, j, i.e. \( c_i^j = n_i^j = 0 \), then one of the \( \omega \) values is given by equation 2.1, whereas the other equals \( H_i \).

### 2.2.2 Relation between the steady state and the operating conditions in a given SMB section

Let us consider a generic section of the countercurrent unit with inlet states a and b, corresponding to the fluid entering at the left end and the solid entering at the right end of the section, respectively. At steady state, three states may prevail in the column, these being states a, b and the intermediate state defined by the two, I. Every possible steady-state is separated from the others by transitions which are either a continuous simple wave when \( \omega_a^b < \omega_a^a \), or a shock when the opposite occurs.
Which state prevails in the section depends on the operating conditions, i.e. on the flowrate ratio $m$, and can be expressed in the following manner:

- **State a**, $(\omega_1^a, \omega_2^a)$ if $\max\{\omega_2^a, \omega_2^b\} \leq m^a$  \hspace{1cm} (2.8)
- **State I**, $(\omega_1^b, \omega_2^b)$ if $\max\{\omega_1^b, \omega_2^b\} \leq m^I \leq \min\{\omega_1^a, \omega_2^a\}$  \hspace{1cm} (2.9)
- **State b**, $(\omega_1^b, \omega_2^b)$ if $m^{b} \leq \min\{\omega_1^b, \omega_2^b\}$  \hspace{1cm} (2.10)

Once the composition of the constant state attained at steady state in the column is known, based on the composition of the inlet streams and on the value of $m$, the composition of the outlet streams can be calculated through mass balances at the column ends.

### 2.3 Design criteria for SMB separation: Triangle Theory

Let us consider the two section SMB unit shown in Figure 2.2a working at complete separation conditions.

The feed mixture, $F$, comprises species 1 (less retained component, or weak) and species 2 (more retained component, or strong) in an inert solvent, and the interaction with the chromatographic stationary phase is described by the binary Langmuir isotherm of equation 1.5, whose denominator is given by equation 2.5.

According to their retention properties species 2 is recovered in the extract, $E$, whereas species 1 is recovered in the raffinate, $R$, as shown in Figure 2.1. It has been proved elsewhere [28] that the results obtained for this simplified two section SMB unit in terms of complete separation region in the $(m_2, m_3)$ plane are valid also for the general configuration that includes sections 1 and 4, provided sections 1 and 4 are properly operated to regenerate the stationary phase and the mobile phase, respectively. For the sake of simplicity, $\varepsilon_p$ will be considered to be 0 throughout this study; however, the results may be extended easily to account for the case of $\varepsilon_p \neq 0$, as presented in previous publications.
Figure 2.2: Scheme of a two section countercurrent SMB unit with indication of the states associated to each stream and yielding complete separation. In Figure a) states are characterized by their composition, whereas in Figure b) the corresponding $\omega$ values are indicated.
2.3 Design criteria for SMB separation: Triangle Theory

[28]. At complete separation, species 1 and 2 cannot be present in sections 2 and 3 respectively, hence the following steady state mass balance equations must be fulfilled:

- mass balance equations through the cross-section labelled $A$ in Figure 2.2a

\[ \begin{align*}
m_2 c_1^\alpha &= n_1^\gamma \\
m_2 c_2^\alpha &= n_2^\gamma + (m_2 c_2^\beta - n_2^\gamma)
\end{align*} \quad (2.11) \quad (2.12) \]

where $c_i^j$ and $n_i^j$ are the fluid and solid phase concentrations of species $i$ in the corresponding stream (labelled $\alpha$, $\beta$ or $\gamma$ as in the figure) or section ($j$). Equation 2.11 implies that the net flux of species 1 through section 2, $(7\gamma 2 - \gamma f)$, is equal to zero, which is a necessary condition for the complete separation;

- mass balance equations through the cross-section labelled $B$ in Figure 2.2a

\[ \begin{align*}
m_3 c_1^\delta &= n_1^\gamma + (m_3 c_1^\delta - n_1^\gamma) \\
m_3 c_2^\delta &= n_2^\gamma
\end{align*} \quad (2.13) \quad (2.14) \]

Equation 2.14 indicates that the net flux of species 2 through section 3, $(7\gamma 3 - \gamma 2)$, is equal to zero;

- mass balances at the feed node for species 1 and 2

\[ \begin{align*}
m_3 c_1^\mu &= m_2 c_1^\alpha + (m_3 - m_2)c_1^\delta \\
m_3 c_2^\mu &= m_2 c_2^\alpha + (m_3 - m_2)c_2^\delta
\end{align*} \quad (2.15) \quad (2.16) \]

For a given set of operating conditions ($m_2$ and $m_3$), and once the states prevailing at steady state in sections 2 and 3 are defined (e.g. complete
separation), the system of equations 2.11 to 2.16 is a linear system in the following 6 unknowns: \( c_1^e, c_2^e, n_1^e, n_2^e, c_1^f \) and \( c_2^f \).

The following two overall mass balance equations around the control volume defined by the rectangular dotted envelope in Figure 2.2a can be obtained as a linear combination of equations 2.11 to 2.16 under complete separation conditions:

\[
(m_3 - m_2)c_1^e = m_3 c_1^3 - n_1^3 ;
(m_3 - m_2)c_2^e = -(m_2 c_2^2 - n_2^2); \tag{2.17}
\]

\[
(m_3 - m_2)c_1^f = m_3 c_1^3 - n_1^3
\]

\[
-(m_2 c_2^2 - n_2^2) = n_2^{ES} ; \tag{2.18}
\]

these two balances may replace two equations, e.g. equations 2.12 and 2.13. The following additional mass balance equations may be written at the raffinate and the extract nodes in order to calculate the product concentrations (i.e. \( n_2^{ES} \) and \( c_1^R \)):

\[
m_3 c_1^3 - n_1^3 = m_3 c_1^R ; \tag{2.19}
\]

\[
-(m_2 c_2^2 - n_2^2) = n_2^{ES} ; \tag{2.20}
\]

At complete separation conditions, the steady state concentrations in sections 2 and 3 are univocally defined and may be calculated using the Equilibrium Theory [26, 27]. As already presented in section 2.2, equation 2.1 is needed to calculate \( \omega_1 \) and \( \omega_2 \) for a given composition state, whereas equations 2.3 and 2.4 are used to calculate the composition corresponding to a given pair of \( \omega \) values.

In Figure 2.2b the states corresponding to the inlet and outlet streams are indicated as \( E(\omega_1^E, \omega_2^E) \) for the extract outlet, \( F(\omega_1^F, \omega_2^F) \) for the feed inlet, and \( R(\omega_1^R, \omega_2^R) \) for the raffinate outlet. In a complete separation regime the extract stream is free of species 1; it can be proved that in this case, one of the \( \omega \) values characterizing the extract state must be equal to \( H_1 \). Likewise, a pure raffinate stream (no species 2 is present) requires \( H_2 \) to be one of the corresponding \( \omega \) values. As a consequence, a pure solvent stream is associated to the \( \omega \) pair \((H_1, H_2)\).
2.3 Design criteria for SMB separation: Triangle Theory

Figure 2.3: Transition paths in the \((c_2, c_1)\) plane (hodograph plane) for the two section TCC unit shown in Figure 2.2, working at optimum operating conditions. The model system parameters used in this example are reported in Table 2.1.
The problem can be illustrated in the hodograph plane, \((c_1, c_2)\), as shown in Figure 2.3.

The desorbent stream as well as the pure solid phase stream correspond to the origin; states \(\beta\) and \(\gamma\) are both binary mixtures and correspond to points within the first quadrant. It has been proved elsewhere that at optimum operating conditions states \(\gamma\) and \(\beta\) coincide \[28\], as it is the case in the example of Figure 2.3. The intermediate states connecting the origin with state \(\beta = \gamma\), which are labelled P and Q, are necessarily located on one of the two axes, as only one species is present. The character of the transition paths connecting the states mentioned above, i.e. shocks or waves (see section 2.2.2), is indicated in Figure 2.3 by the symbols \(\Sigma\) and \(\Gamma\), respectively.

Connecting the inlet fluid state to the inlet solid state in section 2 yields the following sequence of states: \((H_1, H_2) \rightarrow (H_1, \omega_1^2) \rightarrow (\omega_1^2, \omega_2^2)\). According to 2.2.2, both transitions are simple waves since \(\omega_1 < H_1\). The intermediate state \(P(H_1, \omega_1^2)\) contains the more retained component exclusively, hence it is compatible with complete separation. This prevails at steady state if:

\[
H_1 \leq m_3 \delta_2 \leq \omega_2^2
\]  
(2.21)

Accordingly, in section 3 we shall consider the sequence of states \((\omega_1^3, \omega_2^3) \rightarrow (\omega_1^3, H_2) \rightarrow (H_1, H_2)\), where the corresponding transitions are shocks. Again the intermediate state \(Q(\omega_1^3, H_2)\) shall prevail at steady state for complete separation, and this requires that:

\[
H_1 \leq m_3 \delta_3 \leq \omega_2^3
\]  
(2.22)

In the equations above, \(\delta_j\) is the denominator of the Langmuir isotherm to be calculated at the corresponding steady state composition in section \(j\). It follows that \(\delta_3 = H_2/\omega_1^3\) and \(\delta_3 = H_1/\omega_1^1\), due to equation 2.5.

It is worth noting that also the transition state \((H_1, \omega)\), with \(\omega_2^3 < \omega < H_2\), corresponding to one of the composition states along the segment between the state O and the intermediate state P in the hodograph plane
(see Figure 2.3), would fulfill the complete separation condition. This is obtained at steady state if and only if $m_2 = \omega$, i.e. if and only if $m_2 = \omega^3/H_2$. As discussed elsewhere [15, 21], this condition does not add any additional set of operating points to those determined through equation 2.21, and can therefore be ignored.

The six equations 2.11, 2.14, 2.15, 2.16, 2.17 and 2.18 may also be recast in terms of the $\omega$ values, and for a given set of operating conditions ($m_2$ and $m_3$) and given states in the columns (e.g. the ones corresponding to complete separation) one obtains a non-linear system in the unknowns $\omega_1^3$, $\omega_2^3$, $\omega_3^3$, $\omega_1^2$, $\omega_2^2$ and $\omega_3^2$. In particular, the overall mass balances 2.17 and 2.18 would read:

\[
(\omega_1^3)^2 - \omega_1^3 (m_3 + H_1 + (m_3 - m_2)K_1c_1^F) + m_3 H_1 = 0 \quad (2.23)
\]

\[
(\omega_2^3)^2 - \omega_2^3 (m_2 + H_2 - (m_3 - m_2)K_2c_2^F) + m_2 H_2 = 0 \quad (2.24)
\]

Two of the six equations can be used to demonstrate that the relations $\omega_1^3 = \omega_1^2$ and $\omega_2^3 = \omega_2^2$ hold, thus reducing the number of equations and unknowns to four. It can also be proved that the relevant solution of equation 2.23 is the smaller root, whereas that of equation 2.24 is the larger root.

In previous publications, the Equilibrium Theory solution of this problem has been presented in detail [17, 19]. Usually it is solved by enforcing the upper and lower constraints given by equations 2.21 and 2.22, and by obtaining the boundary of the complete separation region in the form of constraints that involve $\omega_2^3$ and $\omega_3^3$. Another approach consists of selecting a pair $(m_2, m_3)$ and of solving the mass balance equations in sequence: equation 2.23 gives $\omega_1^3 = \omega_1^2$, equation 2.24 serves to calculate $\omega_2^3 = \omega_2^2$, and equations 2.11 and 2.14 are used to calculate the last two unknowns: $\omega_1^3$ and $\omega_3^3$. Finally, the constrains 2.21 and 2.22 are tested in order to conclude whether the selected pair $(m_2, m_3)$ belongs to the solution, i.e. to the complete separation region. Therefore, once the problem is defined in the way described above, all unknowns may be calculated and the system is perfectly defined for a given pair $(m_2, m_3)$. 

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2.4 Design criteria for EE-SMBs

Let us now consider the EE-TMB unit shown in Figure 2.4a. This is similar to Figure 2.2a except for the concentration of the fluid stream entering section 2 which is now the enriched extract, $E$, containing only species 2, but at higher concentration than that of the stream leaving section 1.

![Diagram](image)

Figure 2.4: Scheme of a two section countercurrent EE-SMB unit with indication of the states associated to each stream and yielding complete separation. In Figure a) states are characterized by their composition, whereas in Figure b) the corresponding $\omega$ values are indicated.

The mass balance equations written in the previous section hence apply, but the steady state concentration prevailing in section 2 of the unit achieving complete separation is different because of the enriched extract inlet. The enriched extract stream does not contain species 1, and therefore one of the $\omega$ values characterizing this stream must be equal...
2.4 Design criteria for EE-SMBs

to $H_1$. The second component, $\omega^E_2$, depends on the level of enrichment with respect to the watershed concentration. This corresponds to a particular point of the hodograph plane for Langmuir isotherms in which both families of characteristics (usually called $\Gamma_+$ and $\Gamma_-$) have slope 0. Indeed the envelope of the characteristics is tangent to the $c_2$ axes at the watershed point of coordinates:

$$
c_{1w}^w = 0
$$

$$
c_{2w}^w = \left(\frac{H_2}{H_1} - 1\right) \frac{1}{K_2}
$$

All points of the $c_2$ axis located to the left of the watershed point belong to a $\Gamma_-$ characteristic, whereas all points located to the right belong to a $\Gamma_+$ characteristic [27].

When the final enriched extract concentration, $c_2^E$, is smaller than the watershed concentration, $c_2^w$, the inlet state to section 2 of the EE-TMB corresponds to the pair $(H_1, \omega^E_2)$. Using the Equilibrium Theory, the steady state $S_2$ to guarantee complete separation operation can be defined; this corresponds to the intermediate state $(H_1, \omega^E_2)$ (also state $E$ guarantees complete separation, however, this case does not add any operating point to the region of complete separation obtained when the intermediate state is considered). Connecting the inlet fluid state to the inlet solid state in section 2 yields the following sequence of states: $(H_1, \omega^E_2) \rightarrow (H_1, \omega^E_2) \rightarrow (\omega^E_1, \omega^E_2)$. Since the conditions in section 3 do not change with respect to a standard SMB, the same sequence of states takes place: $(\omega^E_1, \omega^E_2) \rightarrow (\omega^E_1, H_3) \rightarrow (H_1, H_3)$, where the corresponding transitions are shocks. It is readily seen that this case ($c_2^E < c_2^w$) is for both sections identical to the one of the standard SMB and therefore leads to the same final solution, namely the complete separation criteria of the Triangle Theory for a standard SMB.

Let us assume on the other hand that $c_2^F$ is larger than $c_2^w$, which is the case considered in Figure 2.4b. In this case, the fluid inlet state to section 2 corresponds to the pair $(\omega^F_1, H_1)$; connecting this state to the inlet solid state (in section 2) yields the following sequence of states: $(\omega^F_1, H_1) \rightarrow$
(ω₁^E, ω₂^E) → (ω₁^γ, ω₂^γ). According to section 2.2.2, the first transition is a shock as expected, whereas the second can be either a shock or a simple wave depending on the concentration achieved in state γ. Only the fluid inlet state (ω₁^E, H₁) contains the more retained species 2, but not the less retained species 1, hence this is the state that must prevail in section 2 in order to get complete separation (see Figure 2.4b). Note that the behavior of section 3 is the same as in the standard SMB also in this case. It follows that the conditions to achieve complete separation in an EE-SMB unit where c₂^E > c₁^E are:

\[
\begin{align*}
ω₂^γ & \leq m_2 δ_2 \leq \infty \quad (2.27) \\
H₁ & \leq m_3 δ_3 \leq ω₂^β \quad (2.28)
\end{align*}
\]

where δ₂ = H₂/ω₁^E and δ₃ = H₁/ω₁^γ (equation 2.28 is identical to equation 2.22). The mapping equations 2.3 and 2.4 yield the relationship between c₁^E and ω₁^E in the enriched extract stream, and c₁^β and ω₁^β in the state prevailing in section 3:

\[
\begin{align*}
ω₁^E &= \frac{H₁}{1 + K₂ c₂^E} \quad (2.29) \\
K₁ c₁^β &= \frac{H₁ - ω₁^β}{ω₁^β} \quad (2.30)
\end{align*}
\]

The problem is illustrated in the hodograph plane in Figure 2.5, where (as it was the case for SMB in Figure 2.3) the transition paths are the ones corresponding to the optimum conditions of the particular example studied and defined in the caption, for which states γ and β coincide.

As already mentioned, equations 2.11 to 2.18 apply also in the case of the EE-SMB unit. Equations 2.11, 2.14, 2.15, 2.16, 2.17 and 2.18 are independent and contain all the unknowns of the design problem. This set of equations may be transformed into the equivalent form involving the ω values, i.e. the unknowns of the problem (for a given pair of m₂
2.4 Design criteria for EE-SMBs

Figure 2.5: Transition paths in the \((c_2, c_1)\) plane (hodograph plane) for the two section TCC unit shown in Figure 2.4, assuming \(m_3 = m_3^{\text{max}}\). The model system parameters used in this example are reported in Table 2.1.
and $m_3$ and once the prevailing states in the sections are defined): $\omega_1^e$, $\omega_1^o$, $\omega_2^e$, $\omega_2^o$ and $\omega_2^r$. When doing so, the overall mass balance of component 1 (equation 2.17) results to be the same quadratic expression in $\omega_1^o$ obtained in the case of the SMB and given by equation 2.23, whereas the overall mass balance of component 2 (equation 2.18) takes the following form

$$m_3 = \frac{H_2 c_2^F}{c_2^o (1 + K_2 c_2^E)} - \left( \frac{c_2^E}{c_2^o} - 1 \right) m_2$$

(2.31)

or in terms of $\omega$ values

$$m_3 = \frac{(H_2 - \omega_1^E)(H_2 - H_1) \omega_2^F \omega_2^o}{(H_2 - \omega_2^F)(H_2 - \omega_2^o) H_1} m_2 \left[ \frac{(H_2 - \omega_1^E)(H_2 - H_1) \omega_2^F}{(H_2 - \omega_2^F)(H_2 - \omega_2^E)} H_1 \right] - 1$$

(2.32)

Either equation of these does not involve any of the unknowns, but represents a linear relationship between $m_2$ and $m_3$ that constrains the choice of operating conditions and confines the region of complete separation in the $(m_2, m_3)$ plane onto a line, i.e. a segment of the line of operation given by equations 2.31 or 2.32.

As a consequence, the system to be solved is constituted of five equations in the six unknowns already mentioned. One of the five equations may be used to prove that $\omega_1^e = \omega_1^o$, reducing the number of equations to four and the number of unknowns to five. Since the number of unknowns is larger than the number of equations, there exists an infinite number of solutions fulfilling the system of equations 2.11, 2.14-2.18, for a given $(m_2, m_3)$ pair selected according to equation 2.32 and for the selected steady states in the sections. This is a remarkable result indicating that under the conditions to achieve complete separation in an EE-SMB unit, the composition states inside the unit are not univocally defined, even though the outlet compositions are fixed, being defined by the overall material balances.
2.4 Design criteria for EE-SMBs

2.4.1 Region of complete separation

Even if not all the unknowns can be calculated using the Equilibrium Theory, the region of complete separation for the EE-SMB operation is well determined. Figure 2.6 shows an example corresponding to the case study that will be analyzed below. Equation 2.31 is a straight line with negative slope in the \((m_2, m_3)\) plane. The segment \(e\gamma\) belonging to this line is the locus of operating points leading to complete separation. The triangle-shaped complete separation region, \(b\gamma\), corresponding to the same separation performed in a standard SMB unit is also shown.

In order to better specify the position of the line of equation 2.31 where complete separation is achieved, let us consider the constraints defined by equations 2.27 and 2.28 and replace the expressions for \(\delta_2\) and \(\delta_3\):

\[
m_2 > \frac{\omega_2^2}{H_2} \tag{2.33}
\]

\[
m_3 < \frac{\omega_2^3}{H_1} \tag{2.34}
\]

These two equations together with the mass balance of species 1 through the cross-section \(A\) (equation 2.11) can be combined to prove that \(\omega_2^0 \leq \omega_2\). Then equations 2.33 and 2.34 may be recast as:

\[
\frac{m_3H_1}{\omega_1^2} < \omega_2^o \leq \omega_2 \tag{2.35}
\]

because \(\omega_1^2 = \omega_1^o\). It can be observed that the term \(m_3H_1\) in equation 2.35 is equal to the last term of the quadratic equation 2.23. As a consequence, equation 2.35 may be re-written in the following form:

\[
\frac{\omega_1^{(0)} \omega_1^{(0)} \omega_1^{(0)}}{\omega_1^2} < \frac{m_2H_2}{\omega_1^F} \tag{2.36}
\]

where \(\omega_1^{(0)}\) and \(\omega_1^{(0)}\) are the larger and smaller roots of equation 2.23, respectively. As already mentioned in section 2.3, the relevant root of
Figure 2.6: Regions of complete separation in the \((m_2, m_3)\) plane obtained through the Equilibrium Theory, for the EE-SMB operation (segment \(cy\)) and the standard SMB operation (triangular region delimited by the lines connecting points \(a\), \(b\) and \(w\)), for the model system parameters and the conditions specified in Tables 2.1 and 2.2. In the case of the standard SMB, the region above the curve \(aw\) leads to pure extract, whereas the region on the left of line \(bw\) leads to pure raffinate; for any other operating point neither outlet stream is pure. In the case of the EE-SMB, the region located on the right of segment \(cy\) leads to pure extract, whereas the region on the left of this same segment leads to pure raffinate. The square triangle shows, for comparison, the region of complete separation in the case of infinite dilution of the feed mixture (linear range) for the standard SMB.
2.4 Design criteria for EE-SMBs

equation 2.23 is the smaller one, i.e. $\omega_1^{\beta \theta}$. Since $\omega_1^{\beta}$ must correspond to the actual solution of equation 2.23, the left hand side of equation 2.36 turns out to be $\omega_1^{\beta \theta}$. Solving equation 2.23, substituting $\omega_1^{\beta \theta}$ in equation 2.36 and reorganizing leads to the following constraint involving $m_2$ and $m_3$

$$m_3 \left\{ H_1 - \frac{m_2 H_2}{\omega_1^{\beta}} (1 + K_1 c_1^F) \right\} > \frac{m_2 H_2}{\omega_1^{\beta}} (H_1 - m_2 K_1 c_1^F) - \left( \frac{m_2 H_2}{\omega_1^{\beta}} \right)^2 \quad (2.37)$$

The equation of the curve defined by the last equation restricts the choice of $m_2$ and $m_3$ to a given region of the $(m_2, m_3)$ plane. Since the linear relationship of equation 2.32 (the overall mass balance of species 2) must apply as well, the complete separation locus is reduced to the segment of this line fulfilling equation 2.37, as well as the obvious condition $m_3 \geq m_2$. In Figure 2.6, this is the segment $e_3$. The end point of the segment, point $y$, leads to maximum productivity, as it will be discussed below. The coordinates of this point, $(m_2^{opt}, m_3^{opt})$, may be expressed in terms of $\omega_1^{\beta}, \omega_1^{F}$ and $\omega_2^{F}$ as:

$$m_2^{opt} = \frac{\omega_1^{F} \omega_2^{F}}{H_2} \quad (2.38)$$

$$m_3^{opt} = \frac{(H_2 - H_1) \omega_1^{F} \omega_2^{F} + (H_1 - \omega_1^{F}) \omega_1^{F} \omega_2^{F}}{H_1 (H_2 - \omega_1^{F})} \quad (2.39)$$

It is worth noting that the optimum point coincides with the intersection between the line of equation 2.31 and the prolongation of the straight line constituting the upper side of the complete separation triangle of the equivalent standard SMB operation (line $wy$), as can be seen in Figure 2.6 and proven mathematically.

With the help of the model developed above, several effects may be studied that allow gaining deeper understanding about the potential of the EE-SMB operation.
The performance of any chromatographic operation aims at maximizing the productivity of the operation while keeping the solvent consumption as low as possible. The productivity of the SMB or EE-SMB operation (or in general, of any continuous chromatographic process) is generally defined by the following expression:

\[ P = \frac{Q_F c_T^F}{n_c V} = \frac{(m_3 - m_2)(1 - \varepsilon^*)c_T^F}{n_c t^*} \]  

(2.40)

where \( P \) is the productivity of the complete separation operation (i.e. the two species are recovered pure in the extract and raffinate respectively), \( c_T^F \) is the total feed concentration and \( n_c \) is the total number of columns in the unit. Therefore, \( P \) is proportional to the product \((m_3 - m_2)c_T^F\), where \((m_3 - m_2)\) is the distance between the operating point and the diagonal in the \((m_2, m_3)\) plane. As a consequence, in the case of the SMB operation, the optimum operating point (in terms of productivity) for a given total feed concentration corresponds to the vertex of the triangular region of complete separation; in a similar way, the end point of the complete separation segment (point \( y \) in Figure 2.6) leads to the maximum productivity in the EE-SMB. It is worth noting that this remark assumes implicitly that the switch time, \( t^* \), is always the same. This is of course not the case in SMB applications. However, the choice of \( t^* \) depends on column efficiency and pressure drop in a way that is not captured by the Equilibrium Theory [19].

Contrary to the productivity, the solvent consumption in the open loop configuration is inversely proportional to the product \((m_3 - m_2)c_T^F\) according to the following expression:

\[ S = \frac{Q_D}{F c_T^F} = \frac{m_1(1 - \varepsilon^*) + \varepsilon^*}{(m_3 - m_2)(1 - \varepsilon^*)c_T^F} \]

(2.41)

Since this has to be minimized, point \( y \) is optimal also in terms of solvent consumption.
2.4 Design criteria for EE-SMBs

The effects of the overall feed concentration, $c_T^F$, and of the enriched extract concentration, $c_E^F$, are analyzed in the next two subsections by considering how the complete separation locus changes when one of these two parameters is varied. The results are compared with the corresponding effects on the standard SMB performance. It is worth noting that in the EE-SMB case, the cost of the additional enrichment operation has to be taken into account in the final performance comparison, since there exists a trade-off between the productivity gain of the EE-SMB with respect to SMB and the additional operating cost related to the enrichment.

**Effect of feed concentration**

The effect of varying the total feed concentration while keeping the relative feed composition, i.e. $(c_2^F/c_f^F)$, and the enriched extract concentration constant is shown in Figure 2.7.

The slope of the complete separation line, $(1 - c_2^F/c_f^F)$, increases (decreases in absolute value), and the line bends counterclockwise when the total feed concentration increases, being the intersection with the diagonal the same (i.e. $m_2 = m_3 = H_2/(1 + K_2 c_2^F) = \omega_F^E$). It is readily seen that, for constant values of $t^*$, the productivity given by equation 2.40 is subjected to two opposite effects, since the term $(m_3 - m_2)$ decreases when the total feed concentration, $c_T^F$, increases. It can be shown that productivity increases when going to larger total feed concentrations (i.e. the influence of $c_T^F$ is more significant), as it is the case for the standard SMB, where the triangle bends counterclockwise making the region of complete separation smaller, as it is shown in Figure 2.7 [15]. It can be proved that the productivity of the EE-SMB operation is in any case larger than that of the SMB; however, the relative productivity gain of EE-SMB with respect to SMB remains constant when changing the feed concentration.

In the case of EE-SMB, it is easy to prove that the solvent consumption given by equation 2.41 decreases when increasing the total feed concentration, while keeping $m_1$ and the enriched extract concentration constant. The same trend is observed in the case of the SMB, and the
Figure 2.7: Effect of the total feed concentration on the regions of complete separation for the EE-SMB and the standard SMB operations for the model system parameters and the column characteristics shown in Tables 2.1 and 2.2 ($c_{f}^c = c_{f}^p = 2-10$ mg/ml, $c_{f}^w = 18$ mg/ml). The dashed lines correspond to infinite dilution of the feed.
2.4 Design criteria for EE-SMBs

relative position of these two curves (two polynomial of fourth degree in $e_r^F$) is such that the curve corresponding to EE-SMB is located below the corresponding curve for SMB. The relative gain of EE-SMB with respect to SMB in terms of solvent consumption remains constant when increasing the total feed concentration, as it is the case for productivity.

When the feed mixture entering the standard SMB unit is infinitely diluted, the competitive Langmuir isotherm approaches the non-competitive linear isotherm ($n_i = H_i c_i$) and the constraints given by equations (2.21) and (2.22) reduce to the expression $H_1 \leq m_2 < m_3 \leq H_2$ corresponding to the dotted square triangle in Figure 2.7. In the case of the EE-SMB operation, the limit of infinitely diluted feed mixture leads to a complete separation segment having infinite slope. It is worth noting that the limit of infinite dilution does not lead to the linear case solution (the square triangle), since the enriched extract inlet keeps the concentration level in the unit high, thus imposing a non-linear behavior even when the feed concentration is very small.

**Effect of the enriched extract concentration**

Let us consider now Figure 2.8, where the enriched extract concentration is varied while the total feed concentration is kept constant. When the enriched extract concentration is equal to the watershed concentration, the complete separation segment coincides with the left-hand side boundary of the complete separation triangle of the corresponding SMB operation, as shown in Figure 2.8.

This means that the productivities of both operations coincide. At higher extract concentrations, the segment shifts downwards and leftwards in the $(m_2, m_3)$ plane at the same time as the slope increases (decreases in absolute value). This leads to a productivity increase, since the optimum moves further away from the diagonal (see equation 2.40). As soon as the concentration of the enriched stream is larger than the corresponding watershed concentration, the EE-SMB gives better performance in terms of productivity. This result is shown in Figure 2.9, where the relative productivity of the EE-SMB operation versus the SMB operation,
Figure 2.8: Effect of the enriched extract concentration on the locus of complete separation of the EE-SMB operation for the model system and column characteristics shown in Tables 2.1 and 2.2 ($c_2^E = c_2^F = 4$ mg/ml, $c_2^E = 10.4 - 18$ mg/ml). At the watershed concentration, $c_2^E = 10.4$ mg/ml, the line segment of complete separation coincides with the left-hand side boundary of the triangular region of complete separation of the corresponding SMB operation, i.e. segment $bw$. 
both at optimum conditions (i.e. point $y$ in Figure 2.6), is plotted against the enriched extract concentration for a case in which the watershed concentration equals 10.4 mg/ml (both productivities are calculated using equation 2.40; the details are given in the figure caption).

![Figure 2.9: Relative productivity of the EE-SMB operation with respect to the standard SMB operation at increasing enriched extract concentrations for the model system and column characteristics shown in Tables 2.1 and 2.2. Equation 2.40 has been used to compute the productivities.](image)

Below the watershed, the nominal productivity of both operations is equal (i.e. $P^* = 1$ in Figure 2.9) since the complete separation regions coincide; however the purity achieved in the standard SMB unit (operated at optimum conditions) is smaller than the purity achieved in the EE-SMB unit, for the same $c^*_F$ and $m_j (j = 1, \ldots, 4)$, making the comparison of the two productivities inconsistent. In order to get the same purity with the standard SMB operation, the operating point has to be chosen inside the complete separation triangle, leading to a lower productivity value. This issue will be discussed more in detail in section 2.5.2 and in the conclusions.
It can be proved that the solvent consumption of the EE-SMB operation decreases when going to enriched extract concentrations larger than the watershed point, for a given total feed concentration. In conclusion, the EE-SMB operation is at least as good as the SMB operation when the enriched extract stream concentration equals the watershed point, and it is better whenever higher extract concentrations are adopted.

2.5 Detailed simulations

The SMB and EE-SMB simulations were carried out using the detailed SMB model presented in Chapter 1. This model accounts for convection and axial dispersion in the fluid phase, and mass transfer, through a linear driving force model, assuming that solid diffusion is the rate limiting step. The model equations consist of material balances that are integrated using the method of lines. Space discretization is based on finite differences, and a minimum of 400 points per column is used. Integration in time is carried out using a commercial integrator for stiff equations. The equations 1.13-1.15 shown in section 1.3 of Chapter 1 are coupled with mass balances at the nodes, and the switching mechanism is implemented. The enriched extract operation is simulated in the following way: the concentrations of species 1 and 2 at the outlet of section 1 are averaged over the switch period (in the standard SMB, this corresponds to the average extract concentration, i.e. a constant value at cyclic steady state); the enrichment factor is calculated as the ratio between the enriched extract concentration selected and the average concentration of species 2 at the outlet of section 1 and; this factor is then used to calculate the concentration of species 1 (the impurity) in the enriched extract inlet stream. All simulations were run long enough to reach the cyclic steady state (the number of cycles required to reach steady state depends very much on the operating conditions, and ranges between 40 and 90 cycles).

The model system corresponds to two enantiomers having the properties described in Table 2.1. In this case, the watershed concentration is 10.4 mg/ml. The SMB and EE-SMB units considered have four sections; the
2.5 Detailed simulations

relevant column properties are given in Table 2.2, together with those operating conditions that remain constant in all simulations.

<table>
<thead>
<tr>
<th>Component</th>
<th>$H_i$ ($\ell$)</th>
<th>$K_i$ (ml/mg)</th>
<th>$k_i$ (1/s)</th>
<th>$D_{L,i}$ (cm/s²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.7025</td>
<td>0.0209</td>
<td>0.45</td>
<td>$4.0 \times 10^{-3}$ (cm/s)</td>
</tr>
<tr>
<td>2</td>
<td>6.0075</td>
<td>0.0267</td>
<td>0.45</td>
<td>$4.0 \times 10^{-3}$ (cm/s)</td>
</tr>
</tbody>
</table>

Table 2.1: Adsorption isotherm parameters and transport parameters for the model system under investigation in this study.

<table>
<thead>
<tr>
<th>$L$</th>
<th>10 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S$</td>
<td>0.785 cm²</td>
</tr>
<tr>
<td>$\varepsilon = \varepsilon^*$</td>
<td>0.32</td>
</tr>
</tbody>
</table>

| $c^E_F = c^F_F$ | 4 mg/ml |
| $c^E_S = c^F_S$ | 18 mg/ml |

<table>
<thead>
<tr>
<th>config.</th>
<th>2-2-2-2 open loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t^*$</td>
<td>120 s</td>
</tr>
<tr>
<td>$m_1$</td>
<td>8.0</td>
</tr>
<tr>
<td>$m_4$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2.2: SMB configuration, column and packing characteristics, feed and enriched extract concentrations, and SMB operating conditions that are constant for all simulations reported in Table 2.3.

Under the conditions selected here, the transition I $\rightarrow \gamma$ described before in Figure 2.5 corresponds to a shock. This representation assumes that states $\beta$ and $\gamma$ in Figure 2.4b coincide (i.e. the value of $m_3$ is selected to be maximum). However, this is not the case for the simulations presented in section 2.5.1.

It is worth noting that the multiplicity of solutions encountered in the Equilibrium Theory analysis, does not apply in the case of the detailed simulation results. Indeed, the introduction of the axial dispersion and mass transfer effects selects a unique concentration profile in the unit among the infinite possibilities offered by Equilibrium Theory.

EE-SMB simulations were run at the operating conditions shown in Figure 2.10 and summarized in Table 2.3, reported at the end of this chapter.
(series A, B, C, D and E). Standard SMB simulations were also run at the conditions of series F. These simulations have the aim of confirming the theoretical findings presented above and moreover, of giving an insight about the robustness and the practical application of the EE-SMB operation when accounting for axial dispersion and mass transfer effects, i.e. under more realistic process conditions.

The effect of the enriched extract concentration is also analyzed with the help of detailed simulations in order to complement the theoretical background presented in subsection 2.4.1. This analysis includes the cases where the extract is enriched at concentrations below the watershed point, where the region of complete separation calculated through Equilibrium Theory is the same triangular region obtained for the corresponding standard SMB operation, as discussed in subsection 2.4.

The simulation results are analyzed in terms of extract and raffinate purities, and productivity. The values of purities \( X \) and productivities \( P \) for species 1 and 2 in the raffinate \( R \) and extract \( E \) streams, respectively, are calculated using the following expressions:

\[
X_1^R = 100 \frac{c_1^R}{c_1^R + c_2^R}
\]
\[
X_2^E = 100 \frac{c_2^E}{c_1^E + c_2^E}
\]
\[
P_1 = \frac{Rc_1^R}{n_eV}
\]
\[
P_2 = \frac{Ec_2^E}{n_eV}
\]

2.5.1 Modelling results

Simulations were run along the lines A, B, C, D, E and F shown in Figure 2.10 (the actual operating points are not shown in this figure for the sake of clarity). Line A coincides with the locus of complete separation conditions, i.e. segment \( A_1A_7 \). Lines B, C, D and E are parallel to the
diagonal and cross the regions of the \((m_2, m_3)\) plane located to the left and to the right of the segment \(A_1A_7\), which are the regions of only pure raffinate and only pure extract, respectively. Simulations were also run beyond the optimum point (which corresponds to point \(A_7\)), in the region of both polluted extract and raffinate. The results of the simulations expressed in terms of purities are shown in Table 2.3 (reported at the end of this chapter) and plotted in Figures 2.11, 2.13 and 2.15. The values of productivity are calculated using equations 2.44 and 2.45 and are also reported in Table 2.3.

Figure 2.11 shows the extract and raffinate purity trends along line \(A\). The results of this series of simulations show pure extract and raffinate from \(A_1\) to \(A_6\), with a drop of the raffinate purity when coming to point \(A_7\), in which maximum productivity is predicted by the Equilibrium Theory. This is due to the ideal character of the Equilibrium Theory prediction, which indeed neglects the effects of mass transfer resistance and axial dispersion. For a point immediately above the predicted optimum, i.e. \(A_8\), a significant purity drop is observed.

Figure 2.11 also shows that the raffinate purity decreases faster than the extract purity. This effect can be analyzed in Figure 2.12 where the concentration profiles of species 1 and 2 along the unit at cyclic steady state are shown for the cases of runs \(A_5\) and \(A_8\). The dotted profile corresponds to the operating point \(A_5\) where the Equilibrium Theory model predicts complete separation, whereas the solid profile is the one of run \(A_8\), i.e. the operating point beyond the predicted optimum. It can be observed that the decrease of \(m_2\) when going from \(A_5\) to \(A_8\) allows the concentration profile of species 1 to broaden in section 2 of the unit, approaching the extract port and therefore causing some pollution in the extract. In section 3, the concentration profile of species 2 changes significantly from \(A_5\) to \(A_8\), and explains the pollution of the raffinate.

The purity trends corresponding to the operating points along lines \(B\), \(C\), \(D\) and \(E\) are shown in Figure 2.13 and reported in Table 2.3. The trend predicted by the Equilibrium Theory is confirmed, namely the points located on the left of the segment \(A_1A_7\) exhibit pure raffinate and polluted extract, and the opposite occurs for the points located on the right of this segment. Again the effect of the operating conditions on the raffinate purity is more significant than that of the extract purity due
Figure 2.10: Operating lines A, B, C, D, E, and F containing the operating points listed in Table 2.3. Line A coincides with the segment of complete separation; the points corresponding to the simulation runs $A_1$ to $A_6$ in Table 2.3 are also plotted. Lines B, C, D, E and F are parallel to the diagonal and the operating points are not plotted for the sake of clarity. The model system parameters are shown in Table 2.1, and the column characteristics and the common operating conditions are reported in Table 2.2.
2.5 Detailed simulations

Figure 2.11: Extract and raffinate purity trends calculated for operating points along line A (see Figure 2.10 and Table 2.3).
Figure 2.12: Concentration profiles along the EE-SMB unit corresponding to runs $A_5$ (dashed) and $A_6$ (solid) immediately before the switch (see Figure 2.10 and Table 2.3).
to the large concentration of species 2 in the unit (consequence of the enrichment) and therefore also in section 3.

Figure 2.13: Extract and raffinate purity trends for operating points along lines B, C, D and E, obtained by detailed simulations of the process (see Figure 2.10 and Table 2.3).

Figure 2.14 shows the two concentration profiles along the EE-SMB unit for the steady states of runs E7 and E10 reported in Table 2.3. The two profiles are very different due to the steady state change happening in section 3. In the case of run E7, the steady state corresponds to the intermediate state in which only species 1 is present, whereas in the case of run E10, the steady state corresponds to state , where both species are present and end up together in the raffinate. It can be noticed that even when a small portion of the concentration profile of species 2 is present at the level of the raffinate port, the result in terms of purity drop may be very significant due to the very high relative concentration of species 2 with respect to species 1 in the EE-SMB operation mode.

The extract and raffinate purity trends obtained along line E in the
Figure 2.14: Concentration profiles along the EE-SMB unit corresponding to the transition happening from run $E_7$ (dashed) and $E_{10}$ (solid) immediately before the switch (see Figure 2.10 and Table 2.3).
case of the EE-SMB (see Figure 2.13) may be compared with the corresponding SMB purity trends along the same line crossing the triangular complete separation region (series F), as shown in Figure 2.15. In the case of a standard SMB, the behavior is rather smooth and the trends are quite symmetric when coming from the complete separation region into a point outside, both in the region of pure extract and in the region of pure raffinate. This is in contrast with the asymmetric behavior observed in the case of the EE-SMB, where the raffinate purity profile as a function of $m_2$ is very steep.

![Figure 2.15: Extract and raffinate purity trends for operating points along line F in a standard SMB unit obtained by detailed simulations of the process (see Figure 2.10 and Table 2.3).](image)

As a consequence, it can be concluded that under the operating conditions of the complete separation locus derived in section 2, and especially under the optimal operating conditions, the performance of the unit is very sensitive to any disturbance (e.g. perturbations of the operating conditions, changes in the feed mixture, perturbations in the enrichment
step, etc.) and also very much affected by the accuracy of the isotherm parameters. This is equivalent to saying that the operation is not robust, i.e. small disturbances are sufficient to cause a qualitative and quantitative performance change in the unit, eventually bringing the system to an operating regime in which the specifications are not met. The robustness of the EE-SMB operation is significantly lower than the one of the SMB operation, as it can intuitively be understood by considering the fact that the complete separation region is a two dimensional region of the \((m_2, m_3)\) plane in the case of the standard SMB, whereas it is reduced to a one dimensional line segment in the case of the EE-SMB.

2.5.2 Effect of the enriched extract concentration

Let us now consider EE-SMB separations carried out at different enriched extract concentration levels with operating conditions corresponding to the optimal ones for the specific \(c_F^E\) value. The same model system as above is used, where the watershed concentration is 10.4 mg/ml. The results of the simulations in terms of purities of the outlet streams and of recovery of the target compound are illustrated in Figures 2.16 and 2.17, respectively. It should be kept in mind that when operating an SMB unit or when running detailed simulations at the optimum operating conditions calculated using the Equilibrium Theory, one does not expect to achieve complete separation, since the optimal operating point is the least robust and the most sensitive to model inaccuracies, e.g. the lack of column efficiency effects in the Equilibrium Theory model.

With reference to Figure 2.16 both purity curves exhibit a salient point at the watershed concentration. At enriched extract concentrations below the watershed, namely from \(c_F^E\) equal to 2 mg/ml up to 10.4 mg/ml, the extract purity increases significantly as the enriched extract concentration increases, whereas above the watershed, the purity decreases slightly and then stabilizes at around 99.8%. Concerning the raffinate purity, the trends are inverted as expected, but the purity change in the raffinate for \(c_F^E > c_F^E\) is slightly more pronounced. Considering the Equilibrium Theory, the optimal operating conditions are always the same below the watershed concentration regardless of the value of \(c_F^E\), since the complete separation region does not change; these conditions are identical to the
2.5 Detailed simulations

Figure 2.16: Extract and raffinate purity in the EE-SMB unit as a function of the enriched extract concentration. The model system parameters are shown in Table 2.1, and the column characteristics and the common operating conditions are reported in Table 2.2 ($c^E_f = c^R_f = 4$ mg/ml).
optimal operating conditions for the SMB. Above the watershed, the optimal operating conditions change with $c_2^E$, as the segment of complete separation moves on the $(m_2, m_3)$ plane, and the productivity increases, as explained above (see Figures 2.8 and 2.9).

Figure 2.17: Recovery of species 2 in the extract stream of the EE-SMB unit as a function of the enriched extract concentration. The model system parameters are shown in Table 2.1, and the column characteristics and the common operating conditions are reported in Table 2.2 ($c_1^F = c_2^F = 4$ mg/ml).

Figure 2.17 shows the recovery of species 2 in the extract as a function of $c_2^E$, which has been calculated using the following expression:

$$Y_2^E = 100 \frac{c_2^E Q_E}{c_2^F Q_F} = 100 \left[ 1 - \frac{c_2^R (m_3 - m_4)}{c_2^E (m_3 - m_2)} \right]$$ (2.46)

Above the watershed, the recovery of species 2 in the extract increases with $c_2^E$ and at the same time the raffinate purity improves. The minimum recovery (97.9%) is in fact achieved at the watershed.
2.6 Conclusions

Let us compare this with the standard SMB separation carried out at the same operating conditions \((m_1\text{ and feed concentration})\) as all the EE-SMB separations at \(c_{2f}^F < c_{2f}^R\). Under these conditions, the SMB operation achieves the following values of purity and recovery: \(X_{2f}^E = 96.7\%\), \(X_{1f}^R = 96.3\%\), \(Y_{2f}^E = 96.3\%\) and \(Y_{1f}^R = 96.7\%\). These values are worse than the ones obtained in all the EE-SMB cases, both below and above the watershed concentration.

The detailed simulation results presented in Figure 2.16 show that even below the watershed, where the nominal productivities of the SMB and EE-SMB processes given by equation 2.40 at optimal conditions are the same \((P* = 1)\), the SMB leads to lower purity values, thus reducing the effective productivity with respect to the theoretical value of one \((i.e. P* < 1)\).

2.6 Conclusions

Equilibrium Theory has been used to derive complete separation criteria for the EE-SMB operation [11]. It was found that these correspond to a one dimensional locus in the \((m_2, m_3)\) plane, i.e as compared to the two dimensional triangle-shaped region of the standard SMB operation. However, Equilibrium Theory does not allow to fully resolve the problem in the sense that this has multiple solutions for a given pair of \((m_2, m_3)\) values. However, knowing the complete separation region, it is easy to select operating points in a systematic way in which detailed simulations can be run. The introduction of the dispersive effects defines the solution in a univocal way, so that the internal concentration profiles can be determined. The detailed simulations allow to validate the model and study the performance of the EE-SMB operation. From this study, it follows that the performance of the EE-SMB operation is always at least as good as the one of SMB when the enriched extract concentration is equal to the watershed point, and it is better for any enriched extract concentration above this point. Due to the asymmetric character of the extract and raffinate purity trends (shown in Figures 2.11 and 2.13), the operation is particularly advantageous to recover species 2 in the extract. Figure 2.9 shows how the productivity of EE-SMB may be 1.5 times
larger than that of SMB, for a sufficient enrichment of the extract stream. Furthermore, at these convenient larger enrichments, the raffinate purity increases, as shown in Figure 2.16, at the same time as the recovery of component 2 in the extract increases (see Figure 2.17). Comparing the SMB purity values with the ones shown in Figure 2.16 for the EE-SMB operation at $c_2^R > c_2^E$, it results that the extract purity in the last case is significantly higher (up to 99.9%). In terms of recovery of species 2, the minimum value achieved in the EE-SMB, i.e. the one corresponding to $c_2^E = c_2^R$, is larger than the one corresponding to the standard SMB operation. This confirms the attractiveness of the EE-SMB operation whenever the target compound in the mixture to be separated is the more retained species and it has to be recovered at high purity.
### 2.6 Conclusions

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Table 2.3: Operating conditions and separation performance of the simulation runs corresponding to operating points along the straight lines shown in Figure 2.10. The resulting purities and the productivities are calculated using equations 2.42-2.45.

Subscripts and superscripts used in this chapter

1 less retained species
2 more retained species
\( D \) desorbent
\( E \) extract
\( ES \) index related to the solid phase of the extract state
\( F \) feed
\( i \) species, \((i = 1, 2, ..., c)\)
\( I, P, Q \) intermediate state
\( j \) stream/section index, \((j = 1, 2, ...)\)
\( opt \) optimum
\( R \) raffinate
\( S_2 \) steady state in section 2 of the SMB
\( T \) total
\( TMB \) True Moving Bed
\( \otimes, \odot \) related to the larger and smaller root
Chapter 3

Three-fraction SMB / CIP-SMB

3.1 Introduction

With reference to Figure 3.1a) and 3.1b), the SMB is in principle equivalent to the True Moving Bed (TMB) process, where the adsorbent particles and the fluid move countercurrently. In the SMB, the continuous movement of the solid is simulated by periodically switching the inlet and outlet ports of the unit. Accordingly, complete separation requires that proper relationships between flow rates and switching time, $t^*$, are fulfilled (see equations 1.1-1.4 in Chapter 1). For a binary mixture of B and C in a TMB under complete separation conditions, the ratio between fluid and solid flow rates in the four sections of the unit is chosen so that the more retained adsorbate B is carried by the solid phase to the extract outlet, while the less retained adsorbate C is conveyed by the mobile phase to the raffinate outlet. The regeneration of the solid and fluid phase, required by a continuous process, is carried out in sections 1 and 4, respectively.

Figure 3.1: Scheme of (a) a True Moving Bed unit with four sections and (b) a four section simulated moving bed unit (open-loop) for the binary separation of B and C, B being the more adsorbable component.
3.1 Introduction

There are however situations where more than two species have to be separated, e.g. the four diastereoisomers of a molecule with two chiral centers, or the nucleosides that will be discussed in the following, or the case of a main product that has to be purified by eliminating impurities that are eluted both before and after it. In the last case in particular, a three-fraction separation process would be required. For this the TMB process in Figure 3.1a) can be extended as shown in Figure 3.2a) by adding the new section zero and by collecting a third fraction, where the more retained impurities are conveyed.

This is common practice also in continuous distillation, but has the intrinsic limitation that the extract product will always be polluted by the more retained impurities, to an extent that cannot be reduced at will; the more retained impurities will always show up at the extract port on their way to the third fraction port, because of the very nature of the countercurrent contact between fluid and solid phase.

On the contrary column (batch) chromatography is ideally suited for multilaction separation tasks, since species with different retention clute at different times and can be collected separately. The price to be paid is the batch nature of this technique, and the associated difficulty to achieve high purity separation with high efficiency.

Separating a mixture in three fractions, each with as high a purity as required, is possible in the SMB unit with the equivalent configuration as the TMB in Figure 3.1a) [2, 24, 29]. In this chapter, the behavior of the three-fraction SMB (3F-SMB) unit is analyzed and criteria to evaluate when and how the three fraction separation is possible are provided.

The key concept behind this application of the SMB technology is that the countercurrent contact between fluid and solid is only simulated in an SMB. This allows exploiting its advantages, particulary high efficiency, but at the same time avoiding pollution of the extract stream as in the TMB case. This can be achieved by trapping the more retained species (impurities) in the column while the main product is collected pure from the extract port, and by switching the SMB ports just before these undesired species reach the column end. This is illustrated in the process scheme of Figure 3.2b). A similar configuration was reported in the patent literature [5, 30]. This concept has also been demonstrated experimentally [2].

We believe that the new SMB operation that is presented here is valuable not only from the application perspective, but it is also remarkable from a conceptual viewpoint. In fact, the unit drawn in Figure 3.2b) operates at the
Figure 3.2: Scheme of (a) a True Moving Bed unit with four sections and (b) a four section simulated moving bed unit (open-loop) for the binary separation of B and C, B being the more adsorbable component.
3.2 Design criteria for 3F-SMBs: linear isotherms

same time as a countercurrent and a batch unit. More specifically, the central sections, i.e. sections 2 and 3 between the extract and the raffinate ports, behave like countercurrent units, though in a simulated way, and develop concentration profiles of the main product B and of the weak component C that are typical of countercurrent contact. On the contrary sections 0 and 1 where the strong impurity A is separated from the main product behave like batch columns, and in fact the concentration profile of A is pulse-like, as in single column chromatography. We are not aware of other units in chemical engineering that share this peculiarity.

3.2 Design criteria for 3F-SMBs: linear isotherms

Let us consider the ternary mixture constituted of the species A, B and C under linear chromatographic conditions where $H_C < H_B < H_A$, i.e. B can be viewed as the main product, C as the less retained impurity, and A as the more retained impurity. The SMB configuration to be considered is shown in Figure 3.3. This 3F-SMB scheme has two columns in sections 1 and 3, and 1 column in section 2; there is no conventional section 4, and section 0 allows to recover A. The wash step carried out in the section identified with the label r, i.e. the re-equilibration section, is only necessary when a different solvent or mobile phase composition is used in section 0 in order to desorb A. The objective is to obtain C, B and A pure in the three product streams, i.e. Raffinate (R), Extract (E) and Third fraction (T), respectively.

With reference to Figure 3.3, the conditions for the complete separation of B and C into the Extract and Raffinate are given by the fulfillment of the inequalities 1.10 to 1.12, presented in Chapter 1. As far as component A is concerned, a more thorough analysis of its propagation through the SMB columns is required; this propagation is illustrated in Figure 3.4. Under linear conditions, this is independent of the other two species. Let us consider the amount of A feed to the unit, specifically to the inlet of section 3 during a switch period of duration $t^*$.

Whatever its concentration, A propagates along a chromatographic column at the speed given by:

$$w_j = \frac{u_j}{\varepsilon^* + (1 - \varepsilon^*)H_A}$$  \hspace{1cm} (3.1)
Figure 3.3: Scheme of a five section Simulated Moving Bed unit with configuration 1-2-1-2-1 for the separation of three components A, B and C, being A and C the most and the least retained species, respectively. The so-called third component, A, is recovered at the outlet port of section 0.
3.2 Design criteria for 3F-SMBs: linear isotherms

where \( u_j \) is the superficial velocity in section \( j \), and \( j = 3 \) in this case. Assuming no \( A \) is present in section 3 immediately after the port switch, at the end of the switch period \( A \) will occupy only a portion of section 3, whose length is called \( S_3 \) and is given by the propagation velocity \( w_3 \), times the switch time:

\[
S_3 = w_3 t^*
\]  

(3.2)

We are assuming here that \( S_3 \) is smaller than the column length, \( L \), so as component \( A \) is necessarily trapped in the first column of section 3.

Upon port switching, the first column of section 3 where \( A \) has been injected, i.e. column \( \delta \) in Figure 3.4, switches back to the position of the last column of section 2. Therefore, although feed is continuous, only a finite amount of \( A \) is trapped into this column, and this will now be followed in its different positions along the SMB unit. In the \( n_2^2 \) columns, which constitute section 2, \( A \) propagates at a rate \( w_2 \) for an overall distance given by:

\[
S_2 = n_2^2 w_2 t^*
\]  

(3.3)

Therefore just before the port switch that brings the column to occupy the position of the last column of section 1, \( A \) occupies a portion of column \( \delta \) comprising the interval between \( S_2 \) and \((S_2 + S_3)\). During the next switching period, while the outlet of the column is connected to the extract stream where the main product \( B \) has to be collected pure, the pulse of \( A \) travels a distance \( S_1 \) that is required to be:

\[
S_1 < L - S_2 - S_3
\]  

(3.4)

where \( S_1 = w_1 t^* \).

At the end of such switching interval, the column is switched to a position further upstream of the extract port, and \( A \) occupies a portion of it between \((S_1 + S_2)\) and \( S \), where:

\[
S = S_1 + S_2 + S_3 = t^*(w_1 + n_2^2 w_2 + w_3)
\]  

(3.5)

It is rather clear that if the distance covered by \( A \) is larger than the length of the column, i.e. \( S > L \), then the extract is polluted with \( A \), and the process specification is not fulfilled. On the contrary, the main product in the extract
Figure 3.4: Schematic representation of the behavior of the first four sections in a 3F-SMB unit, with one column per section. The vertical axis is time. The dashed area indicates the portion of column occupied by the pulse of component A, which is initially fed at the beginning of section 3, at the end of the first, second, third and fourth switch periods (t*, 2t*, 3t*, 4t*). The propagation velocity is indicated by \( w_j \) and the distance covered by the A pulse by \( S_j \) in the \( j \)-section. The Greek letters identify the columns so that the position of one specific column can be followed in time.
3.2 Design criteria for 3F-SMBs: linear isotherms

is pure provided \( S < L \), which is indeed the design constraint specific for the 3F-SMB operation. This can be usefully recast in terms of the flow rate ratios, \( m_3 \), using the expression of \( m_3 \) given by equation 1.4 together with the equation 3.1:

\[
m_1 + n_f^2 m_2 + m_3 < H_A - \frac{\varepsilon^*}{1 - \varepsilon^*} (1 + n_f^2)
\]

(3.8)

The last equation sets actually an upper bound for \( m_1 \) that has to be combined with the relevant inequalities in equations 1.10 to 1.12 to guarantee separation of \( B \) and \( C \) in sections 2 and 3, complete removal of \( B \) but no removal at all of \( A \) from section 1, and removal of \( A \) from section 0, thus yielding the following complete separation constraints for a 3F-SMB:

\[
H_A^0 \leq m_0
\]

(3.7)

\[
H_B \leq m_1 \leq H_A - \frac{\varepsilon^*}{1 - \varepsilon^*} (1 + n_f^2) - n_f^2 m_2 - m_3
\]

(3.8)

\[
H_C \leq m_2 \leq H_B
\]

(3.9)

\[
H_C \leq m_3 < H_B
\]

(3.10)

\[
0 \leq m_r
\]

(3.11)

where \( m_r \) is calculated from equation 1.4 using the flow-rate \( Q_r \) in the re-equilibration section.

A few remarks are worthwhile. The Henry constant of \( A \) in section 0, i.e. \( H_A^0 \), might be different from that of sections 1 to 3, \( H_A \), if a different mobile phase from that in the other sections is used. This might be necessary whenever the more retained impurity is very strongly bound to the stationary phase, i.e. \( H_A \) is very large, and a strong solvent must be used to make regeneration of the stationary phase in section 0 at all possible. In this case the re-equilibration section is necessary and its flow rate has to be chosen so as to fulfill equation 3.11.

It is worth noting that, while there is always a feasible range of \( m_0, m_2, m_3 \) and \( m_r \) values (with \( m_3 > m_2 \)), which satisfy the constraints 3.7, 3.9, 3.10 and 3.11, the constraint given by equation (3.8) on \( m_1 \) is of a different nature. The upper bound depends in fact on \( m_2 \) and \( m_3 \), as well as on \( H_A \) and \( n_f^2 \), hence a feasible range of \( m_1 \) values exists only when the upper bound in equation 3.8
Three-fraction SMB / CIP-SMB

is larger than the lower bound, i.e. in the region of the \((m_2, m_3)\) plane defined by the linear inequality:

\[
m_3 \leq H_A - H_B - \frac{\varepsilon^*}{1 - \varepsilon^*} (1 + n_c^2) - n_c^2 m_2
\]

These results can be illustrated graphically in the \((m_2, m_3)\) plane (see Figure 3.5), where the classical right triangle for the complete separation of B and C (equations 3.9 and 3.10) is shown together with the critical straight line with negative slope defined by equation 3.12. Below this line, there exists a range of feasible \(m_1\) values according to equation 3.8.

Therefore, in a 3F-SMB unit, in the frame of the Equilibrium Theory, only the points of the triangle that lie below the critical line allow to achieve the separation of the feed mixture in three pure fractions, provided the flow rate ratios \(m_2, m_1\) and \(m_r\) fulfill the corresponding constraints 3.7, 3.8 and 3.11. In reality, as the complete separation triangle shrinks under the effect of dispersion processes [23], also the effective position of the critical line shifts towards smaller \(m_2\) and \(m_3\) values, thus making the attainment of complete separation conditions in real 3F-SMBs more difficult. It is worth noting that the feasible region becomes larger, the larger the difference in retention between A and B, and the smaller the number of columns in section 2. Note that Figure 3.5 has been drawn with reference to the nucleosides separation that will be discussed later. In this case the model parameters take the following values: \(H_A=27.7\), \(H_B=7.40\), \(H_C=3.15\), \(n_c=1\) and \(\varepsilon^*=0.8\).

In order to better clarify the situation as far as the flow rate ratio \(m_1\) is considered, let us take a line crossing the complete separation region in the \((m_2, m_3)\) plane of Figure 3.5, as well as a set of operating points along it. A very easy way to obtain such a line experimentally is to keep the flow rates constant in equation 1.4 and let \(t^*\) vary. By taking in particular the flow-rate values \(Q_2=0.31\) and \(Q_3=0.35\), the column volume \(V=2.49\) ml and the other model parameters values as indicated above, the nine operating points a to i correspond to \(t^*\) values from 650 to 990 seconds. It is rather evident that points b to e belong to the complete separation region for the 3F-SMB, whereas point a fails to fulfill the constraint on \(m_2\) of equation 3.9, and points f to i do not admit any feasible operating range for \(m_1\). This is better illustrated in the \((m_2, m_3)\) diagram of Figure 3.6. The same operating points from a to i have been represented by taking the \(m_1\) value equal to the arithmetic average between the lower and the upper bounds of equation 3.8. The thick horizontal line represents the constant lower bound in equation 3.8 \(m_1 = H_B\), below which
3.2 Design criteria for 3F-SMBs: linear isotherms

Figure 3.5: Region of complete separation for A, B and C in the \((m_2, m_3)\) plane. This diagram is obtained from the region of complete binary separation of B and C (calculated through the Equilibrium Theory), and the critical line defined by equation 3.12. The region inside the triangle and below the critical line allows complete separation of the three compounds in three fractions (A in T, B in E, and C in R), while above this line, either the extract is polluted by A or the third fraction is polluted by B or both.
the third fraction is polluted by the species B that has not been removed completely from section 1. The thick line with negative slope represents the upper bound of equation 3.8, above which A pollutes the extract. This is obtained by substituting in the right hand side of equation 3.8 the expression of \( m_2 \) as a function of \( m_2 \), which represents the operating points from \( a \) to \( i \). The intersection point of these two straight lines defines the critical \( m_2 \) value, which equals 4.46 in this case, and corresponds to the intersection of the critical line with the straight line going from \( a \) to \( i \) in Figure 3.5. Beyond this \( m_2 \) value, no feasible range for \( m_1 \) is available; here the upper bound in equation 3.8 is in fact smaller than the lower bound. Besides the line at the critical \( m_2 \) value, two more vertical dotted lines defined by the binary region of complete separation of species B and C are shown in Figure 3.6. These represent the \( m_2 \) values where the straight line going from \( a \) to \( i \) intersect the boundaries of the complete separation region of the B-C binary system. The lower bound of \( m_2 \), coincides with the Henry constant of component C \( (H_C = 3.15) \), while the upper bound leads for this particular set of operating conditions to \( m_2 = 6.08 \).

These lines divide the plane in eight regions achieving different purity performance [29]. Starting at low values of \( m_2 \) (i.e. \( m_2 < 3.15 \)) the raffinate purity is ensured, while the extract is polluted with the less retained component C. Above the upper bound for \( m_1 \) (the solid line with negative slope), the extract is also polluted by component A, while raffinate and third fraction are pure. On the contrary, below the lower bound (the horizontal solid line), the third fraction is polluted by component B and only the raffinate is obtained as a pure stream. For intermediate \( m_1 \) values only the extract is polluted by C, while the other two streams are pure. Between \( m_2 = 3.15 \) and \( m_2 = 4.46 \), the separation of species B and C is guaranteed. Three regions can again be identified depending on the selected \( m_1 \) value. Between the two boundaries of \( m_1 \), the three fraction are obtained pure, while above and below these boundaries, the extract or the third fraction are polluted, respectively. At values of \( m_2 \) between 4.46 and 6.08, the binary separation of B and C is still guaranteed (operating points are inside the binary triangle in Figure 3.5), and the raffinate is thus pure. Once the critical \( m_2 \) value 4.46 is exceeded only one of the two products, i.e. extract or third fraction, can be recovered pure. The general rule is that larger flow rates in section 1, i.e. higher \( m_1 \) values, tend to pollute the extract with the more retained component, A, whereas lower \( m_1 \) values tend to pollute the third fraction with B. At values of \( m_2 \) larger than 6.08 the performance as far as extract and third fraction are concerned is similar to the previous case, but now the raffinate is always polluted with B.
3.2 Design criteria for 3F-SMBs: linear isotherms

Figure 3.6: Critical upper and lower bounds of $m_1$ as a function of $m_2$ for the separation of a feed mixture in three fractions under linear conditions. The dashed vertical lines represent the boundaries of the regions in terms of $m_2$. The solid lines are the upper and lower bounds of $m_1$ defined by equation 3.8. The composition of the three fractions is indicated in every region, where the underlined species is the one expected in the given stream, and the others are impurities.
Finally, it is worth noting that in the presence of significant extra-column dead volume between the chromatographic columns of the SMB unit, two modifications to the equations above should be made. First, the modified flow rate ratios should be used, which are obtained from those in equation 1.4 by subtracting the dead volume associated to section $j$, $V_j^{D}$, in the numerator of the right hand side [16]. Secondly, the upper bound of $m_1$ in equation 3.8 is modified by subtracting the term $\phi$, defined as follows:

$$\phi = \frac{V_1^D + n_2 V_2^D + V_3^D - (V_{in}^D + V_{out}^D)}{V(1 - \varepsilon)} \quad (3.13)$$

where $V_{in}^D$ and $V_{out}^D$ are the extra-column dead volumes between the inlet port and the column inlet, and between the column outlet and the outlet port, respectively.

### 3.2.1 Cleaning in place

Cleaning in place (CIP) is a rather common practice, particularly in chromatographic bioseparations, where the stationary phase experiences some kind of modification due for instance to chemisorption of impurities present in the feed mixture. Such modification leads to physical and chemical degradation of the stationary phase and to loss of performance. The regeneration of the column, to be performed in the CIP step, requires the adoption of harsher conditions, typically through the use of a proper solvent, e.g. a sodium hydroxide solution that brings the column back to its original performance. The 3F-SMB operation presented here can easily accommodate such a CIP step. Such irreversible column modification can in fact be viewed as the effect of the adsorption of an impurity, $A$ in our nomenclature, whose retention is so strong that its Henry’s constant becomes practically infinity. The operating conditions for section 1 given by equation 3.8 reduce to those applicable to a binary SMB, i.e. equation 1.10, since $H_A$ in the right hand side goes to infinity and the dependence on $m_2$ and $m_3$ becomes immaterial. As a consequence, the critical line in the $(m_2, m_3)$ plane of Figure 3.5 moves upwards and does not intersect the binary complete separation triangle any more. For the sake of simplicity, in this work the action of the CIP solvent will be characterized only by the reduced value of the Henry’s constant of the strong impurity $A$ in section 0, i.e. $H_A^*$ in equation 3.7.
3.2 Design criteria for 3F-SMBs: linear isotherms

3.2.2 Design criteria for 3F-SMBs: non-linear isotherm

Let us consider now the case of a ternary system when competitive adsorption occurs, which can be described through a multicomponent non-linear competitive isotherm, \( n_i = f_i(c_A, c_B, c_C) = f_i(c) \), of a favorable type, e.g. the Langmuir isotherm:

\[
\begin{align*}
    n_i &= \frac{H_i c_i}{1 + \sum_j K_j c_j} \quad i = A, B, C \\
    &\text{(3.14)}
\end{align*}
\]

The system analyzed in the previous section can be viewed as the limiting case of such a system at very high dilution, where the non-linear isotherm can be approximated by a linear one.

In order to determine operating conditions leading to complete separation in a nonlinear 3F-SMB, the same procedure as above should be followed. However, a number of difficulties arise due to the fact that in multicomponent chromatography the adsorption of all species is coupled, and the adsorption and desorption fronts of each species are faster or slower depending on the concentration of the other species. First of all the region in the \((m_2, m_3)\) plane for complete separation of species B and C, the so called complete separation triangle, is not the same as in the binary case, since it is affected by the presence of A and depends on its upstream concentration in a way that is rather difficult to predict (see [19] for the extension of the Triangle Theory to multicomponent Langmuir systems). Secondly the adsorption of A takes place through a shock front whose propagation velocity depends also on the concentration levels of B and C. The propagation velocity of the shock in the different sections is given by \(\tilde{w}_j\), that can be written as follows:

\[
\begin{align*}
    \tilde{w}_j &= u_j \left( \varepsilon^* + (1 - \varepsilon^*) \frac{f_A(c_A, c_B, c_C)}{c_A^*} \right)^{-1} \\
    &\text{(3.15)}
\end{align*}
\]

where \(c_i^*\) is the concentration of species \(i\) upstream of the given shock. These concentrations are not known a priori, hence the velocity of the shock cannot be calculated. As a result, the position of the critical curve, given by equation 3.12 in the linear case, cannot be calculated a priori under non-linear conditions. Comparing equations 3.1 and 3.15, it is readily seen that the velocity of the shock, \(\tilde{w}_j\), is larger than \(w_j\), so that the critical boundary is shifted downwards in the \((m_2, m_3)\) plane. This implies that the region of complete separation is smaller under non-linear conditions than in the linear case.
3. Analysis of the performance of 3F-SMBs

The results presented in the previous section will now be assessed through simulations, using data from a real chromatographic system and a detailed model of the SMB unit.

3.3.1 Separation of nucleosides on reversed phase

Nucleosides molecules consist of a purine or a pyrimidinic base linked to a pentose. The phosphate esters of nucleosides are called nucleotides, and are the building blocks of DNA and RNA chains. The major DNA nucleosides are: 2'-Deoxyadenosine (dA), 2'-Deoxythymidine (dT), 2'-Deoxyguanosine (dG) and 2'-Deoxycytidine (dC). This is the model system chosen for our simulations, and the relevant experimental data have been reported in a companion experimental work [2]. From these the values of the model parameters summarized in Table 3.1 have been obtained. The Henry constants for the nucleosides have been measured experimentally using an AKTA Explorer system (Amersham Biosciences, Uppsala, Sweden). Columns were packed with the reversed phase media Source 30RPC from Amersham Biosciences and had length of 15 cm and inner diameter of 4.6 mm. The overall bed void fraction was $\epsilon^*=0.8$, with a bed void fraction $\epsilon =0.4$. The mobile phase is water with 4 vol% of ethanol as modifier. The number of ideal stages in the column and the mass transfer coefficients were estimated, together with the axial dispersion coefficient, using the classical procedure based on the Van Deemter plot. The adsorption behavior was found to be linear in the concentration range of total solubility. As a model system, the three fraction separation of the four nucleosides mentioned above will be considered, where dT and dG will be collected in the extract, dC in the raffinate and dA, the most retained nucleoside, in the third fraction.

3.3.2 Model equations

The simulations have been carried out using the detailed one-dimensional model of the SMB unit presented in section 1.3 of Chapter 1. This model accounts for all the phenomena taking place in the column: accumulation in the fluid and in the solid phases, convection and axial dispersion in
3.3 Analysis of the performance of 3F-SMBs

Component | $H_i[-]$ | $a_i k_i[1/s]$ | $D_{li}[cm^2/s^2]$  
--- | --- | --- | ---  
$dA$ | 27.7 | 0.1 | $3.75 \times 10^{-2}$  
$dT$ | 9.60 | 0.5 | $3.75 \times 10^{-3}$  
$dG$ | 7.40 | 0.5 | $3.75 \times 10^{-3}$  
$dC$ | 3.15 | 1.0 | $3.75 \times 10^{-3}$  

Table 3.1: Adsorption isotherm parameters, mass transfer and axial dispersion coefficients for the nucleosides $dA$, $dT$, $dG$ and $dC$.

the fluid phase, and mass transfer through a linear driving force model assuming that solid diffusion is the rate limiting step [23]. A minimum of 450 points per column are used after checking that this eliminates the error due to numerical dispersion.

3.3.3 Linear chromatographic conditions

The nucleosides separation mentioned above is studied in a four section SMB, with column configuration 2-1-2-2. Section 0 and the re-equilibration section in Figure 3.3 are not simulated, but it is assumed that they behave ideally, i.e. the solid phase in the last column of the re-equilibration section contains no solute adsorbed before the port switch. With respect to the nomenclature adopted in the general treatment presented in the previous sections, the least adsorbable component C, is $dC$, the strongest one A is $dA$, while the intermediate component B is $dT+dG$. Since two species have to be collected in the extract, the less retained of the two, i.e. $dG$, is the key species as far the separation from $dC$ and the behaviour of sections 2 and 3 are concerned, whereas the more retained species, i.e. $dT$, is the key component when the regeneration of section 1 is considered.

Accordingly the constraints 3.8 to 3.10 and 1.12 on the operating parameters $m_j$ modify as follows:
Three-fraction SMB / CIP-SMB

\[ H_{dT} \leq m_1 \leq H_{dA} - \frac{\varepsilon^*}{1 - \varepsilon^*} (1 + n_c^2) - n_c^2 m_2 - m_3 \] (3.16)

\[ H_{dC} \leq m_2 \leq H_{dG} \] (3.17)

\[ H_{dC} \leq m_3 \leq H_{dG} \] (3.18)

\[ \frac{-\varepsilon_p}{1 - \varepsilon_p} \leq m_4 \leq H_{dC} \] (3.19)

As shown before, the equation of the critical line can be derived from the inequality 3.16, leading to:

\[ m_3 = H_{dA} - H_{dT} - \frac{\varepsilon^*}{1 - \varepsilon^*} (1 + n_c^2) - n_c^2 m_2 \] (3.20)

In order to make the constraint arising from the upper bound in equation 3.16 less restrictive, we consider in the following the case where only one column is used in section 2, i.e. \( n_2^2 = 1 \). The corresponding region of complete separation is shown in Figure 3.5.

Figure 3.7 shows the concentration profiles in a 3F-SMB unit at cyclic steady state, immediately after the switch of the inlet and outlet ports, for the operating conditions corresponding to run c in Figures 3.5 and 3.6. The species dC, dG and dT exhibit the typical non-monotonic profiles observed in a SMB operated under linear conditions [31]. On the contrary, the most retained species dA is distributed in a series of pulses, that correspond to the implementation of the concept illustrated in an idealized way in Figure 3.4, i.e. without considering axial dispersion and mass transfer resistance. In particular, we see that in this case two pulses are present in section 1 instead of only one as in Figure 3.4. This is due to the particular operating conditions adopted, which imply that some of the dA transported from the second to the first column of section 1 at the end of a switching interval is able to break through again in the second column in the next interval. This obviously does not occur if section 1 has only one column. It is worth noting that the presence of the small tail of dA at the beginning of the second column of section 1 (immediately after the switch) causes pollution of the extract stream with dA.
3.3 Analysis of the performance of 3F-SMBs

Figure 3.7: Cyclic steady-state concentration profiles of dA, dT, dG, dC in a 3F-SMB unit at the operating conditions corresponding to point c in Figures 3.5 and 3.6 (see also Table 3.2). The profiles are drawn immediately after the port switch.
The separation performance of the different runs is quantified in terms of the purity of the outlet streams (third fraction, extract and raffinate), which are defined as follows:

\[ X^T = \frac{c^T_{dA}}{c^T_{dA} + c^T_{dT} + c^T_{dC} + c^T_{dG}} \times 100 \]  

\[ X^E = \frac{c^E_{dG} + c^E_{dT}}{c^E_{dA} + c^E_{dT} + c^E_{dC} + c^E_{dG}} \times 100 \]  

\[ X^R = \frac{c^R_{dC}}{c^R_{dA} + c^R_{dT} + c^R_{dC} + c^R_{dG}} \times 100 \]

Let us first consider runs a, b, c, d, e, f, g, h and i, whose operating conditions are defined in Table 3.2. The corresponding operating points are shown in the \((m_2, m_3)\) and in the \((m_2, m_1)\) planes in Figures 3.5 and 3.6, respectively. It is worth noting that in all these runs the \(m_1\) value has been chosen as the arithmetic average of the upper and lower bound in equation 3.16. This applies to the runs where a feasible \(m_1\) range exists, i.e. runs a to e, and also to those where such a range does not exist (see Figure 3.6). The purity values in the three product streams are shown in Figure 3.8 as a function of \(m_2\), together with the upper and lower bounds of \(m_2\) defining the Equilibrium Theory conditions to achieve complete three fraction separation.

<table>
<thead>
<tr>
<th>Run</th>
<th>(t^* ) [s]</th>
<th>( m_1 )</th>
<th>( m_2 )</th>
<th>( m_3 )</th>
<th>( m_4 )</th>
<th>( X^T ) [%]</th>
<th>( X^E ) [%]</th>
<th>( X^R ) [%]</th>
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<tbody>
<tr>
<td>a</td>
<td>650</td>
<td>11.5</td>
<td>2.74</td>
<td>3.61</td>
<td>2.50</td>
<td>100</td>
<td>82.0</td>
<td>100</td>
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<tr>
<td>b</td>
<td>720</td>
<td>10.7</td>
<td>3.47</td>
<td>4.43</td>
<td>2.50</td>
<td>100</td>
<td>97.0</td>
<td>100</td>
</tr>
<tr>
<td>c</td>
<td>745</td>
<td>10.4</td>
<td>3.73</td>
<td>4.73</td>
<td>2.50</td>
<td>100</td>
<td>97.2</td>
<td>100</td>
</tr>
<tr>
<td>d</td>
<td>770</td>
<td>10.1</td>
<td>3.99</td>
<td>5.02</td>
<td>2.50</td>
<td>100</td>
<td>97.3</td>
<td>100</td>
</tr>
<tr>
<td>e</td>
<td>795</td>
<td>9.87</td>
<td>4.25</td>
<td>5.31</td>
<td>2.50</td>
<td>100</td>
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<td>100</td>
</tr>
<tr>
<td>f</td>
<td>850</td>
<td>9.26</td>
<td>4.82</td>
<td>5.96</td>
<td>2.50</td>
<td>93.5</td>
<td>97.2</td>
<td>100</td>
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<tr>
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<td>890</td>
<td>8.82</td>
<td>5.23</td>
<td>6.43</td>
<td>2.50</td>
<td>84.9</td>
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<td>100</td>
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<tr>
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<td>925</td>
<td>8.43</td>
<td>5.60</td>
<td>6.84</td>
<td>2.50</td>
<td>77.5</td>
<td>97.3</td>
<td>100</td>
</tr>
<tr>
<td>i</td>
<td>990</td>
<td>7.71</td>
<td>6.27</td>
<td>7.60</td>
<td>2.50</td>
<td>64.0</td>
<td>97.3</td>
<td>75.7</td>
</tr>
</tbody>
</table>

Table 3.2: Operating conditions and separation performance of the simulation runs a to i in Figures 3.5 and 3.6.
3.3 Analysis of the performance of 3F-SMBs

Figure 3.8: Third fraction, extract and raffinate purity values plotted versus $m_2$ for the operating points a to i (see Figure 3.5 and Table 3.2). The first two vertical straight lines indicate the lower and upper boundaries of the three fraction complete separation region of dA, dT, dG and dC in terms of $m_2$, and the third vertical line indicates the upper boundary of the binary complete separation region in terms of $m_2$. 
The nine runs above can be divided in four groups, as indicated by the position of the corresponding operating points in the \((m_2, m_3)\) plane in Figure 3.5. The first group contains run \(a\) only, and is characterized by pure raffinate and third fraction, whereas the extract is not pure due to the presence of the weakest species, i.e. \(dC\). The second group consists of runs \(b, c, d\) and \(e\), where complete three fraction separation should be achieved according to the criteria defined above. The third group is constituted of runs \(f, g\) and \(h\), and is characterized by pure raffinate and either pure extract and third fraction polluted by \(dT\) and \(dG\), or pure third fraction and extract polluted by \(dA\), depending upon the value of \(m_1\). Finally, the fourth group consists only of run \(i\), where none of the product streams is pure, since also the raffinate is polluted with \(dT\) and \(dG\).

These observations, based on the criteria developed in the previous section, are fully consistent with the purity values reported in Table 3.2 and in Figure 3.8, as long as the raffinate and the third fraction are considered. The observed behavior of the extract purity is less obvious and requires a deeper analysis. Run \(a\) exhibits very low extract purity as expected, whereas all the other runs achieve a similar purity value, slightly above 97%. This is different from expectations, particularly for runs \(b\) to \(e\) where complete separation should be achieved based on the ideal criteria above. This is consistent with the observation that in the concentration profiles corresponding to run \(c\) in Figure 3.7 (immediately after the port switch) it is apparent that a certain amount of \(dA\) leaves the unit in the extract stream. This is due to the broadening of the pulse of \(dA\), while it travels along the column where it has been injected. The criterion that issues from equation 3.5 and the analysis in Figure 3.4 applies only in the absence of band broadening, and therefore it can fail in the case under examination.

In order to vary the extract purity reached in run \(c\), one can modify the value of \(m_1\). To this aim, a series of simulations have been carried out by considering the runs \(j, k, m, n, o, p, q\) and \(r\) in Figure 3.6. These runs share the same values of the flow rate ratios of run \(c\) with the exception of \(m_1\), whose values are summarized in Table 3.3 and in Figure 3.9, together with the corresponding performance parameters. As expected from the position of the operating points in Figure 3.6, decreasing \(m_1\) leads to
better values of $X^{E}$, while increasing it yields lower $X^{E}$ values. The value of $m_{1}$ does not affect the raffinate purity, since before recycling every column is regenerated in section 0 and in the re-equilibration section. On the contrary, the purity of the third fraction follows a pattern that is consistent with the position of the operating point in Figure 6. All runs but $m$, $k$ and $j$ achieve in fact 100% $X^{T}$. In these runs some portion of $dT$ is taken to the third fraction outlet stream and therefore also the extract recovery falls below 100% as apparent in Figure 3.9. It is worth noting that none of the runs considered allows reaching complete separation in all three outlet streams. This is an intrinsic limitation of this unit which, depending on the relative adsorptivity of the heavy component, may or may not achieve complete separation.

![Figure 3.9: Purity values of the third, extract and raffinate fractions and extract recovery versus $m_{1}$. The operating conditions corresponding to the points $j$, $k$, $m$, $n$, $o$, $p$ and $r$ (see Figure 3.6) are summarized in Table 3.3.](image)

The effect of $m_{1}$ is well illustrated in Figure 3.10, where the concentration
### 3. Three-fraction SMB / CIP-SMB

#### Table 3.3: Influence of \( m_1 \) on the performance of the 3F-SMB unit at the conditions of run \( c \), i.e. \( m_2 = 3.73, \frac{m_3}{m_2} = 4.73, m_4 = 2.50 \) and \( t^* = 745s \) (refer to Figures 3.5 and 3.6 and to Table 3.2).

<table>
<thead>
<tr>
<th>Run</th>
<th>( m_1 )</th>
<th>( X^T ) [%]</th>
<th>( X^E ) [%]</th>
<th>( X^R ) [%]</th>
<th>( Y^E ) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( j )</td>
<td>7.00</td>
<td>65.2</td>
<td>99.6</td>
<td>100</td>
<td>73.5</td>
</tr>
<tr>
<td>( k )</td>
<td>8.00</td>
<td>78.4</td>
<td>99.3</td>
<td>100</td>
<td>86.5</td>
</tr>
<tr>
<td>( m )</td>
<td>9.00</td>
<td>90.6</td>
<td>98.7</td>
<td>100</td>
<td>95.1</td>
</tr>
<tr>
<td>( n )</td>
<td>9.80</td>
<td>100</td>
<td>98.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( o )</td>
<td>10.0</td>
<td>100</td>
<td>97.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( p )</td>
<td>10.4</td>
<td>100</td>
<td>97.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( q )</td>
<td>10.8</td>
<td>100</td>
<td>96.7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( r )</td>
<td>11.5</td>
<td>100</td>
<td>95.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( t )</td>
<td>12.5</td>
<td>100</td>
<td>94.5</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Profiles at steady state are shown after the port switch in the case of run \( j \). When comparing these with those curves corresponding to run \( c \) in Figure 3.5, it is evident on the one hand that the extent of dA pollution in the extract is less significant, since the amount of dA at the beginning of the second column of section 1 before the port switch is much smaller. The extract purity is in fact 98.7% instead of 97.2%. On the other hand, since \( m_1 \) is smaller than its lower bound, the species dT is not eluted in section 1 and part of it is carried over to section 0 where it is eluted together with dA in the third fraction.

#### 3.3.4 Cleaning in place

In order to simulate the situation where a continuous CIP step is added to a SMB operation, we consider the separation of dT (more retained component, \( H_{dT} = 9.60 \)) and dG (less retained component, \( H_{dG} = 7.40 \)), in the presence of impurities (species A) that have a very high adsorptivity, that we simulated with \( \lambda = 100 \). Under these circumstances the requirement of a complete three fraction separation does not add additional constraints of practical relevance, since the critical line corresponding to the equation 3.12 is far away from the triangle of complete separation of dG and dT, which is defined by \( H_{dG} \leq m_2 \leq m_3 \leq H_{dT} \).

Two runs are considered here, both under linear chromatographic conditions and with the same operating parameters (see Table 3.4), but with
3.3 Analysis of the performance of 3F-SMBs

Figure 3.10: Cyclic steady-state concentration profiles of dA, dT, dG, dC in a 3F-SMB unit at the operating conditions corresponding to point m in Figure 3.6 and Table 3.3. The profiles are drawn immediately after the port switch.
Table 3.4: Performance of the CIP step when purifying a mixture of dT, dG and impurities A with $H^g = 100$, each at $c_f^E = 2$ mg/ml. The operating conditions are $m_2 = 8$, $m_3 = 8.5$ and $m_4 = 4$.

3.3.5 Non-linear chromatographic conditions

Let us analyze now the separation performance of a 3F-SMB unit when the mixture to be separated is characterized by the non-linear competitive Langmuir isotherm 3.14. Since the exact criteria derived for the linear case cannot be extended to the non-linear case, we limit our analysis to the effect that changes on the feed concentration of the four nucleosides to be separated have on the product purities when the 3F-SMB is operated with the same operating conditions, namely those corresponding to point $c$ in Figures 3.5 and 3.6. In order to use the Langmuir isotherm given by equation 3.14, the value $K = 0.1$ ml/mg has been used for all involved components (the Henry constants, axial dispersion and mass transfer parameters are kept the same, as reported in Table 3.1).

In the case of a two fraction SMB separation, it is well known that in-

<table>
<thead>
<tr>
<th>Run</th>
<th>config.</th>
<th>$m_1$</th>
<th>$X^T$ [%]</th>
<th>$X^E$ [%]</th>
<th>$X^R$ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-1-2-1</td>
<td>10</td>
<td>100</td>
<td>99.6</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>2-2-2-1</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
3.3 Analysis of the performance of 3F-SMBs

<table>
<thead>
<tr>
<th>$c^F$ [mg/ml]</th>
<th>$X^T$ [%]</th>
<th>$X^E$ [%]</th>
<th>$X^R$ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>100</td>
<td>97.2</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>96.8</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>96.1</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>95.2</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>94.3</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.5: Effect of the feed concentration on the performance of a 3F-SMB under non linear conditions, i.e. feed concentration of 10 mg/ml for each component. Henry constants, axial dispersion and mass transfer parameters are reported in Table 3.1 and $K_i=0.1$ ml/mg. The operating conditions correspond to run c in Table 3.2, i.e. $m_1 = 10.4$, $m_2 = 3.73$, $m_3 = 4.73$, $m_4 = 2.50$ and $t^* = 745$s.

Increasing feed concentration for an operating point belonging to the linear complete separation region, e.g. point c in Figure 3.5, yields slightly increasing $X^E$ and strongly decreasing $X^R$ values [15, 17]. In the case of a three fraction SMB separation, this is combined with the effect described in section 3.2 above, i.e. the more stringent character of the upper bound on $m_1$ with respect to the linear constraint given by equation 3.8.

The effect of changing the feed concentration on the product purities for the operating point c is illustrated in Figure 3.11 and the operating conditions are reported in Table 3.5 together with the corresponding performance parameters. As expected, raffinate purity drops beyond a certain feed concentration level. Contrary to the normal behavior for binary SMB’s, extract purity decreases steadily as $c^F$ increases. This is due to pollution of the main product in the extract by the most retained species, i.e. dA in this case. Increasing $c^F$ leads in fact to greater difficulty in the non linear case than in the linear case to fulfill the criteria for complete three fraction separation. In other words, the region of complete separation of dG and dC shrinks [15], and the curve ensuring the separation of dA and dT moves towards smaller values of $m_2$ and $m_3$, i.e the chosen operating point lies above it at high feed concentration.

As in the linear case, it is also worth analyzing how the value of $m_1$ can be tuned in order to improve the extract purity. This is illustrated in Figure 3.12 and reported in Table 3.6, where the results obtained by varying $m_1$ for constant values of $c^F = 10$ mg/ml, and of $m_2$, $m_3$ and
Figure 3.11: Purity values of the third, extract and raffinate fractions at different feed concentration values $c_f^*$ for a Langmuir adsorption isotherm. The composition of dA, dT, dG and dC in the feed is always 1:1:1:1. All the operating parameters, i.e. $t^*$, $m_1$, $m_2$ and $m_3$, correspond to those of point c in Table 3.2, except for the feed concentration that varies here from 2 to 20 mg/ml.
3.3 Analysis of the performance of 3F-SMBs

t* are given. A similar effect as in the linear case is observed, namely that extract purity can be improved by decreasing m1, and the price to pay is the larger amount of dT in the third fraction, and the lower value of the recovery of the components to be collected in the extract. However, also in this case no complete separation for the three outlet streams is obtained, i.e. while improving extract purity by decreasing m1, the purity of the third fraction worsens rather sharply.

Figure 3.12: Third fraction, extract, raffinate purity values and recovery of the extract as a function of m1 under non linear conditions, i.e. feed concentration of 10 mg/ml for each component. Henry constants, axial dispersion and mass transfer parameters are reported in Table 3.1 and K1=0.1 ml/mg. The operating conditions correspond to run c in Table 3.2, i.e. m1 =10.4, m2 =3.73, m3 =4.73, m4 =2.50 and t* =745s.
Table 3.6: Effect of $m_1$ on the performance of a 3F-SMB under non linear conditions, i.e. feed concentration of 10 mg/ml for each component. The Henry constants, axial dispersion and mass transfer parameters are reported in Table 3.1 and $K_i=0.1$ ml/mg. The operating conditions are $m_2 = 3.73$, $m_3 = 4.73$, $m_4 = 2.50$ and $t^* = 745$ s.

### 3.4 Concluding remarks

In this chapter, we have introduced a special simulated moving bed operation that allows for the continuous chromatographic separation of mixtures in three fractions, i.e. the 3F-SMB unit [2, 5, 24, 29].

Under linear chromatographic conditions, and using equilibrium theory, we identify criteria to achieve complete three fraction separation. These can be formulated in terms of the flow rate ratios $n_{ij}$, i.e. the key operating parameters for SMB units. These criteria represent an extension of the so-called Triangle Theory, and show that the possibility of achieving complete three fraction separation depends on the retention behavior of the components to be separated and on the unit configuration. In particular, a significant selectivity between the strongly retained species collected in the third fraction, and those collected in the extract, is required to achieve the goal.

The behavior of 3F-SMB units has been analyzed extensively through detailed simulations, with application to the separation of nucleosides on reversed phase. The results confirm the expected theoretical trends, but indicate also that the practical implementation of the 3F-SMB concept might fail in cases where the selectivity is not high enough. This is particularly true in the case of mixtures, where the adsorption behavior is non linear.

A special case, where the proposed technique is indeed very effective,
3.4 Concluding remarks

is when a cleaning in place (CIP) step is required. In this case in fact, the selectivity between the impurities and the main product is virtually infinitely large, and the criteria for complete three fraction separation may always be fulfilled, as clearly shown by both the theory and the simulations.

Subscripts and superscripts used in this chapter

\[ A \text{ more retained species} \]
\[ B, C \text{ less retained species} \]
\[ dA 2'-\text{Deoxyadenosine} \]
\[ dC 2'-\text{Deoxycytidine} \]
\[ dG 2'-\text{Deoxyguanosine} \]
\[ dT 2'-\text{Deoxythymidine} \]
\[ D \text{ desorbent} \]
\[ D_0 \text{ desorbent in section 0} \]
\[ E \text{ extract} \]
\[ F \text{ feed} \]
\[ i \text{ component index, } (i = A, B, ...) \]
\[ j \text{ section index, } (j = 1, 2, ...) \]
\[ r \text{ reequilibration section} \]
\[ R \text{ raffinate} \]
\[ T \text{ third fraction} \]
\[ u \text{ upstream} \]
Chapter 4

SMB Operation for pDNA Size Exclusion Purification

4.1 Introduction

Interest in plasmid DNA has been increasing during the last decade as a result of the developments in gene therapy and DNA vaccines. Although its application has been in many cases more difficult than expected, gene therapy remains a very promising therapeutical approach for sicknesses like cancer, diabetes and certain inherited diseases [32]. Moreover, a new and powerful method of vaccination involving the administration of pathogen's genes is emerging to deal with new disease threats [33, 34].

Plasmid DNA (pDNA) vectors are preferred over viral based vectors because they minimize the risk of viral infection. However, targeting of pDNA vectors...
is less efficient, meaning that larger amounts of pharmaceutical-grade plasmid are required for a treatment. These amounts will likely be in the order of milligrams [35]. A large scale pDNA production process needs to be established in order to satisfy the demand of pharmaceutical grade pDNA for clinical trials and eventually for commercialization. The manufacturing process must fulfill all the requirements dictated by the international agencies (namely the FDA). Summarizing, gene therapy research needs to proceed together with process development, based on GMP considerations as well.

The production of pDNA starts with the fermentation of the selected bacterial strain. In order to harvest the plasmid that is contained in the cells, a lysis is performed to destroy the wall and cellular membrane. Lysis yields an heterogeneous mixture containing solids that are separated by centrifugation. Most of the chromosomal DNA (cDNA) is separated with the cell debris during centrifugation [36]. Even in the case of high copy number plasmid, pDNA represents less than 3 (w/w)% of the cleared lysate [37]. The main contaminants to be eliminated are RNA, cDNA fragments, proteins and endotoxins. The supercoiled covalently closed form of pDNA (ccc) is thought to be therapeutically more effective than the open-circular, linear, or multimeric forms [36], and therefore these forms have to be separated from the final product. The classical lab protocols use extraction with organic solvents (such as phenol), toxic reagents (such as ethidium bromide) and animal derived enzymes (like lysozyme or RNase) [38]; since such protocols are not scalable or suited for clinical production they do not constitute a basis for large scale process development. Chromatographic techniques are preferred [38-40], either alone or in combination with other techniques, generally related to membrane technology [41, 42]. Plasmid purification schemes include typically several chromatographic steps, often called capture, intermediate purification steps and polishing [39]. In the past, some works have focused on one of these steps [43, 44], while other tried to describe the downstream processing more in detail by considering the whole sequence of steps [37, 38, 45]. Apart from the classical packed bed, expanded bed chromatography has also been investigated [35], aiming at reducing back pressure and hence at increasing the productivity. In the same direction of increasing productivity, monoliths are being studied because of their larger binding capacities when compared to particulate packing [46]. A number of review papers concerning plasmid DNA manufacturing processes have been published since 1999 [35, 37, 40, 45]. The main properties of plasmid DNA and some details about its downstream processing are summarized in Appendixes A and B, respectively.

The Simulated moving bed (SMB) technology [1] could play an important role
4.1 Introduction

in the upscaling of biomolecules purification processes. It is known from other fields of application that when compared to column chromatography SMB allows to increase productivity while reducing the solvent consumption [14]. SMB was first used in the petrochemical industry, and then successfully downscaled to be used in the food industry and lately in the purification of chiral molecules [13]. In the new field of bioseparations the challenges to be faced are related to the differences with traditional SMB applications: the nature of the molecules and often the high density of the samples, the requirement of aqueous mobile phases, typically buffer solutions, and the compressible chromatographic media typically used in bioseparations [47]. SMB was initially conceived to separate a feed mixture of two components into two pure streams, and was recently extended to the separation of multicomponent mixtures in three fractions [2, 6, 7, 24]. Its role in a bio-purification process may be that of accomplishing a group separation, or a polishing, step in which only one of the fractions, namely the one containing the target compound, is of interest.

Only little attention has been directed so far to the establishment of cleaning protocols based on experimental data rather than undocumented rules of thumb. In some cases, disposal of the chromatographic media is recommended, but it is clear that no conclusions can be drawn about this point without an optimized cleaning protocol that would compare cleaning in place (CIP) costs and chromatographic media expenses. The technical implementation of a CIP step into the SMB operation has been addressed recently [24, 48, 49]. The so-called CIP-SMB scheme is illustrated in Figure 4.1. The standard SMB corresponds to the central sections (1 to 3) where the separation is achieved. The fourth zone, which contributes to enrich the raffinate stream in the classical SMB configuration, is not used here. The feed is split into two fractions, extract and raffinate, containing the more and the less retained species respectively.

The columns are switched backwards one position in the opposite direction of the fluid flow every switch period \(t^*\) in order to simulate a countercurrent movement between the solid and the fluid phase. The column arriving to the position corresponding to the first column in section 1 is sent to the cleaning section during the next switch period. There, the cleaning is performed with the selected cleaning agent. During the next switch period, the column arrives to the re-equilibration section where it is equilibrated to the running buffer conditions, getting ready to be introduced again into the SMB loop. If only one column is placed in the cleaning section, the contact time is limited to the switch time interval; if more columns are placed in the cleaning section the contact time is given by the corresponding multiple of the switch time. The development of a cleaning protocol should identify the best cleaning agent, the
frequency and the contact time required. Appendix C contains details about the cleaning protocol, references and definitions concerning CIP.

The work presented in this chapter studies the first purification step of plasmid DNA, i.e. a group separation step, based on size exclusion chromatography (SEC) and using the simulated moving bed technology (SMB). The idea of starting the purification scheme by a size exclusion step has already been proposed in the frame of column chromatography processes [37, 44], and is exploited here as a proof-of-concept for the SEC-SMB. SMB ensures a continuous and fast processing of the cleared lysate from which the pDNA has to be purified. A fast group separation step is convenient because it guarantees the stability of the sample. The main objective is to eliminate the RNA, the proteins and most of the cDNA fragments. Some impurities may be left in the product in order to guarantee complete recovery of the plasmid. Additionally, a size exclusion step in front of the purification may reduce the level of endotoxins by as much as 8000 times [50]. Later purification steps will involve complete elimination of RNA, cDNA and proteins, further reduction of the endotoxin levels and separation of the pDNA forms other than ccc.

The operating conditions selected in this work to run the SMB experiments are not optimum from the productivity point of view, but these serve to elucidate the interdependence between purity and productivity that will help to select optimal operating conditions once the overall purification scheme is considered. The optimization of the overall process requires the achievement of certain purity levels at the end of each purification step; each of these steps needs to be optimized in turn to maximize productivity and minimize solvent
4.2 Materials and Methods

The experiments were run with pDNA samples having two different origins. The first was produced in house and had a plasmid size of 10 kilobases (the details are given in section 4.2.1 below). Starting from the frozen cells and in order to harvest the plasmid contained in them, the lysis was performed in different batches short before the corresponding SMB experiment. The second source of material was Strathmann Biotec AG (Dengelsberg, Germany) that provided directly the cleared lysate, with a plasmid size of 8 kilobases. All pDNA samples were filtered and concentrated before use as described in section 4.2.2. In the following sections, the origin of the samples and the lysis batch are specified for each SMB run.

4.2.1 Bacterial growth and cell lysis

Recombination deficient Escherichia coli JM109 was chosen as host strain to avoid the production of multimeric forms by homologous recombination. The chosen plasmid, pSPZ2Not, has been described previously [51].

Media composition

The defined mineral medium used for the preculture and fed-batch fermentation contained on a per liter basis: glucose 10 g (5 g for the preculture medium); yeast extract 5 g, (NH₄)₂HPO₄ 3 g; MgSO₄(7H₂O) 0.7 g; KH₂PO₄ 10 g; Na₂HPO₄ 1.5 g; CaCl₂(2H₂O) 0.03 g; citric acid 1 g; thiamine 0.02 g; trace element solution 7 ml; ampicillin 0.1 g. The pH was adjusted to 7.0 by addition of 10M NaOH. The trace element solution contained on a per liter basis: CaCl₂ (2H₂O) 2.28 g; FeSO₄ (7H₂O) 10 g; ZnSO₄(2H₂O) 1.4 g; MnSO₄(2H₂O) 0.38 g; CuSO₄(5H₂O) 1 g; MoO₃Na₂(2H₂O) 0.5 g; Na₂B₄O₇(10H₂O) 0.23 g. The feeding solution contained on a per liter basis: glucose 500 g; MgSO₄(7H₂O) 20 g; thiamine 0.2 g and 6 ml trace element solution.
Fed-batch fermentation

E. coli JM109 (pSPZ2Not) was taken from a glycerol stock stored at -80°C and plated on LBamp agar. A LBamp liquid medium, 4 ml, was then inoculated with a single colony and cultivated for 12 h at 37°C. With 2 ml of this culture, a 100 ml seed culture was inoculated and incubated for 11 h at 30°C and 230 rpm. The fermentation was carried out in a 5 L Biostat bioreactor (B. Braun Biotech International GmbH, Melsungen, Germany) containing 3 l of initial medium. As the glucose in the medium was almost depleted, the feeding solution was added exponentially following the equation:

\[
Feed(t) = \left( \frac{\rho}{Y_{X/S}} + m \right) \frac{c_{X0}V_0}{c_s} \exp(\rho t)
\]

where \(Feed(t)\) (l/s) is the feeding rate, \(\rho\) (1/s) is the desired specific growth rate (set to 0.17 l/h), \(Y_{X/S}\) (g/g) is the cell yield on carbon substrate (0.5 for glucose), \(m\) (g/(g s)) is the specific maintenance coefficient (0.04), \(c_{X0}\) (g/l) is the cell dry weight concentration at the beginning of the feed, \(V_0\) (l) is the volume in the reactor at start of the feed, \(c_s\) (g/l) is the concentration of glucose in the feeding solution, \(t\) is the elapsed time since start of the feed.

The feed was set linear after 23 hours of fermentation to maintain sufficient oxygen supply. The temperature was kept constant at 30°C throughout the fermentation. The pH was controlled at 7.0 by the automatic addition of 25\% (v/v) NH₄OH or 1M H₂SO₄. Polypropylenglycol (PPG 2000) was used as antifoam agent. To maintain the pO₂ value above 20% saturation, the stirrer speed and the aeration rate were adapted. The cell concentration during growth was monitored by measuring light absorbance of diluted samples at 600 nm in an Ultraspec 100Pro spectrophotometer (Amersham Biosciences). At the end of the fermentation, cells were harvested by centrifugation at 10000 g for 20 minutes at 4°C and stored at -20°C. The Micro DCU 300 control unit and the MFCS/win 2.0 software package of B. Braun Biotech were used to control the fermentation parameters.

Cell lysis

The cell lysis protocol was adopted from the standard alkaline lysis method [52]. Cells were resuspended in an ice-cold solution of 1.1 % glucose (w/v), 10 mM Tris, 50 mM EDTA at pH 8.0 with a ratio of 7.8 ml per gram cell wet weight and kept on ice. Cells were then lysed by the addition of 2 volumes
of 1% SDS (w/v), 0.2M NaOH, kept on ice for 10 minutes and mixed gently a few times. 1.5 volumes of an ice-cold solution of 60% (v/v) 5M potassium acetate and 11.5% glacial acetic acid were added, mixed and stored on ice for 20 minutes. The suspension was decanted through a sieve, centrifuged for 20 minutes at 4°C at 13400 g. The supernatant was filtrated through a 1.2 μm filter; the DNA was concentrated again and stored at 4°C until further processing.

4.2.2 Sample concentration

After centrifugation, the sample was pumped through a 1.2 micron polypropylene SCF capsule filter (CUNO SFC, Mainz, Germany). In order to concentrate the sample the filter was put inline with an ultra-filtration unit (GE Healthcare, Uppsala, Sweden) equipped with a hollow fiber membrane of 300 kDa NMWC (nominal molecular weight cutoff). Two ultrafiltration units were alternatively used, one of 3.3 m² and the other of 290 cm².

4.2.3 Size Exclusion columns and running buffer

Considering the sizes of the plasmid DNA and the impurities, the Sepharose 6 FF size exclusion media was selected (GE Healthcare, Uppsala, Sweden). This media has an exclusion limit of 300 kDa. Nine columns (HR 10/10 column, GE Healthcare, Uppsala, Sweden) were packed with this media, of which eight were used in the SMB and the ninth was used for analysis. The packing quality was assessed through acetone and potassium chloride injections. The column characteristics are summarized in Table 4.1. All experiments and analysis were performed in 2M NH₄SO₄, 100 mM TRIS, 10 mM EDTA (pH 7). Under these conditions good separation between pDNA and RNA is achieved [37, 48, 49]. Chemicals were purchased from Fluka (Buchs, Switzerland).

4.2.4 Analytical methods

In order to assess the separation performance of the experimental runs, the purity of the extract and raffinate streams has to be evaluated. To this aim 500 μl of each stream were injected into the analytical column at the buffer conditions indicated in 4.2.3. The corresponding chromatogram was compared with the one of the pDNA sample used as
4. SMB Operation for pDNA Size Exclusion Purification

<table>
<thead>
<tr>
<th>media</th>
<th>Sepharose 6 FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_p$</td>
<td>90 µm</td>
</tr>
<tr>
<td>$L$</td>
<td>10 cm</td>
</tr>
<tr>
<td>$d$</td>
<td>1 cm</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>0.32</td>
</tr>
<tr>
<td>max $Q$</td>
<td>7 ml/min</td>
</tr>
</tbody>
</table>

Table 4.1: Column characteristics (SMB and analysis).

feed during the experiment. However a calibration curve relating UV absorbance at 254 nm and concentration is not available and would be difficult to determine, particularly in the case of the mixture of RNA and proteins, when also considering that different starting materials from different sources are used. These difficulties motivate us to use a pragmatic approach, which is based on the following definition of area percent:

$$A_i[\%] = \frac{100 \times (\text{Area } i\text{th peak})}{\text{Total area}} \quad (4.2)$$

These quantities are certainly related to weight or mole percent, though not being exactly equivalent. Knowing its limitations, this method is used for practical reasons, since it simplifies the analysis and gives anyhow sufficient qualitative information about the SMB separation performance, particularly for comparative purposes.

Some samples were also desalted on HiTrap desalting columns (GE Healthcare, Uppsala, Sweden) prior to agarose gel electrophoresis according to standard laboratory techniques using a 0.8% agarose gel [52].

4.2.5 Experimental setup

The analytical injections were carried out in an ÄKTA Purifier unit (GE Healthcare, Uppsala, Sweden). The SMB experiments were carried
4.3 Characterization of the sample

4.3.1 Sample composition

Figure 4.3 shows the chromatogram obtained by injecting into the column used for analysis 500 µl of one of the pDNA samples used in this study (in particular, the one named as 96). Based on this, as well as on similar chromatograms of other pDNA samples, we can identify two main peaks, i.e. the first constituted of pDNA which is completely excluded from the pores of the media, and the second that corresponds to the group of many different species (mainly RNA and proteins) that access the pores and are pollutants as far as the pDNA is concerned.
4. SMB Operation for pDNA Size Exclusion Purification

Figure 4.2: Modified version of the ÄKTA Explorer unit (GE Healthcare, Uppsala, Sweden).
4.3 Characterization of the sample

Figure 4.3: Chromatogram of a 500 μl injection of plasmid DNA sample #6 at $Q = 0.8$ ml/min; the vertical dashed lines indicate the retention times of the main peaks.
In order to design the pDNA purification, e.g. using an SMB unit, we need to characterize qualitatively the species retention leading to the chromatogram in 4.3. To this aim, we exploit the classical relationships for the retention time of a species $i$ in a chromatographic column:

$$t_{RI} = V(\varepsilon + (1-\varepsilon)\gamma_i)/Q$$

(4.3)

Where $\varepsilon$ is the bed void fraction and the parameter $\gamma_i$ depends on the pore accessibility of species $i$, $\varepsilon_{pi}$, and its retention in the chromatographic media according to the relationship:

$$\gamma_i = \varepsilon_{pi} + (1-\varepsilon_{pi})K_i$$

(4.4)

where $K_i$ is the Henry constant accounting for any kind of possible linear interaction with the media.

For the sake of simplicity, we described the multicomponent mixture eluting in the second broad peak as constituted of two pseudo-species only, namely the main part of the peak, labeled RNA, eluting between about 7 minutes and about 12 minutes in Figure 4.3 and some smaller molecules, e.g. proteins, labelled n, that constitute the tail of the peak and elute after 12 minutes in Figure 4.3. We then assign $\gamma_i$ values to the pDNA and to the two pseudo-species by using the mid point of the corresponding elution time interval in equation 4.3. The values are shown in Figure 4.3. From these, we obtain $\varepsilon$ of 0.32 by assuming $\gamma_{pDNA} = 0$, as well as $\gamma_{RNA} = 0.95$ and $\gamma_n = 1.7$.

It is worth noting that the mathematical description of the broad peak shown in Figure 4.3 is not aimed; indeed, only the elution time range of the peak is needed for a good SMB design. The retention time of the early impurity (mostly RNA) defines the selectivity between this and pDNA and is used to design the central sections of the SMB unit, whereas the retention time of the later impurities is required to properly design section 1 of the SMB. This is discussed in detail in chapter 4.4.

There exists a certain sample variability leading to different characteristic $\gamma_{RNA}$ and $\gamma_n$ values for different samples, and this variability has
4.3 Characterization of the sample

to be taken into account during the design of the experiments. Values of $\gamma_r$ larger than one are obtained in some cases indicating some kind of interaction with the chromatographic media. This may be due to some hydrophobic interaction effect appearing as a consequence of the high salt concentration in the mobile phase during the experiments.

The value of $\gamma_r$ characterizing the operation in the cleaning and re-equilibration section of the SMB unit corresponds to the value of 1.7 [48, 49]. This value was obtained by considering the times required to saturate one column with NaOH 1M and to regenerate it back to the usual buffer conditions described in 4.2.3.

4.3.2 Sample Fractionation

The fractionation experiment illustrated in Figure 4.4 aims at characterizing the sample and at validating the assumption of linear retention behavior, i.e. that the elution of every component is indeed independent of the elution of the other species. In the column used for analysis, 2 ml of a pDNA sample were injected at 0.8 ml/min. Since the conductivity of the sample is below that of the buffer, a negative conductivity peak elutes at the time corresponding to the bed void fraction volume. Samples were taken continuously at the outlet of the column during elution; their sampling periods are indicated by vertical lines in Figure 4.4.

The extra-column dead volume, the dead volume between the UV and the conductivity cells and between the conductivity cell and the sampling port were measured by injecting potassium chloride and blue dextrane mixtures, and were taken into account during the analysis of the results. All fractions were analyzed in the analytical column by injecting 500 $\mu$l at the same velocity and buffer conditions. For a given fraction, the retention time of the characteristic peaks obtained during analytical re-injection indicates the presence of pDNA (peak eluting at about 4 min) or other species (longer retention times). This information serves to understand which species is present in which fraction of the fractionation chromatogram, and to clarify its properties, particularly the composition of the overloaded peaks. These data are reported in the bar chart of
4. SMB Operation for pDNA Size Exclusion Purification

Figure 4.4: Absorbance at 254 nm (solid line) and conductivity (dashed line) during the injection of 2 ml of a plasmid DNA sample in the analytic column ($Q = 0.8$ ml/min). The fractions collected are indicated by vertical solid lines.
4.4 SMB Design Criteria

Figure 4.5 that confirms the nature of the peaks as discussed in section 4.3.1.

Figure 4.5: Bar chart of the area of the peaks in the chromatograms of each of the fractions collected in the fractionation experiment shown in Figure 4.4. Note the different scale for the two bar types. It is evident that no pDNA is present in late fractions.

Figure 4.6 shows the analytical chromatograms (500 µl) of fractions 3, 9 and 14, that have been chosen as representatives of the three types of chromatograms obtained. It is worth noting that no pDNA is found in any fraction after the fifth (the pDNA peak in the fifth fraction is so small that is not evident in Figure 4.5) i.e. in any RNA-rich fraction, and all retention times are consistent, with a linear non competitive retention behavior.

4.4 SMB Design Criteria

Making use of triangle theory [15, 17] that was briefly introduced in section 1.2, the conditions for complete separation and proper regeneration in the CIP-SMB are written as follows [48, 49]:

101
Figure 4.6: Analytical chromatograms (500 µl) corresponding to fractions 3, 9 and 14 of the fractionation experiment shown in Figure 4.4 (Q = 0.8 ml/min).
The conditions given by equations 4.7 and 4.8 translate into the region of complete separation shown in Figure 4.7. In the area defined by the triangle, the two streams (extract and raffinate) are pure. For the group separation considered here, complete separation means that 100% of the pDNA fed during operation is recovered in the raffinate at 100% purity. In the region above the triangle, complete recovery of pDNA is again expected, but the purity values are below 100%. Productivity increases with increasing distance of the operating point (of coordinates $m_2$, $m_3$) from the diagonal. Therefore, the optimum point for complete separation is the vertex of the triangle. At these optimal conditions, pDNA in the raffinate is not more diluted than in the feed, since $m_2$ is equal to $\gamma_{p\text{DNA}}=0$, or anyhow very small, and the mass balance for the plasmid imposes that $c_{p\text{DNA}}^R \sim c_{p\text{DNA}}^F$. The overall mass balance for the impurities eluted in the extract stream indicates that the extract dilution is controlled by the selectivity between pDNA and RNA.

The flow rate in every section has to be maintained below the packing flow rate, adding an additional constraint to the operation. The total pressure drop in the SMB was not limiting in our experiments. It is worth noting that the inequalities 4.5 and 4.9 apply only at the conditions used for these experiments (1M NaOH, running buffer conditions) as described earlier in section 4.3.1.
4. SMB Operation for pDNA Size Exclusion Purification

4.5 Experimental Results

4.5.1 Operating conditions

The operating conditions (flow rate ratios) used during the SMB runs are reported in Table 4.2. The selected conditions are also shown in the $(m_2, m_3)$ plane of Figure 4.7. The differences in the values of $\gamma_{RNA}$ and $\gamma_n$ among different samples were taken into account during the design of the experiments, but for the sake of simplicity only one region of complete separation is shown in Figure 4.7, which was calculated using an average value of $\gamma_{RNA}$. All the experiments were performed with a switch time of 5 minutes. The cleaning in place was carried out with NaOH 1M at the conditions indicated in Table 4.2 ($m_0$ and $m_r$). The values of productivity were calculated using the expression:

$$P = \frac{100 \times F_{c_{pDNA}}^F}{8V_c}$$ \hspace{1cm} (4.10)

and considering a reference pDNA concentration in the feed mixture of 1 mg/ml. Equation 4.10 assumes complete recovery of pDNA in the raffinate, and as a result, it is only applicable inside and above the complete separation region. The values of productivity obtained are used to compare the runs. As indicated before, the productivity increases when getting further away from the diagonal in the $(m_2, m_3)$ plane.
<table>
<thead>
<tr>
<th>Run</th>
<th>pDNA sample</th>
<th>Origin</th>
<th>$\gamma_{DNA}$</th>
<th>$\gamma_n$</th>
<th>$m_0$</th>
<th>$m_1$</th>
<th>$m_2$</th>
<th>$m_3$</th>
<th>$m_r$</th>
<th>$P$ [mg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>ST</td>
<td>1.17</td>
<td>2</td>
<td>2.71</td>
<td>2.94</td>
<td>0.439</td>
<td>0.762</td>
<td>0.651</td>
<td>0.316</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>ETH</td>
<td>0.92</td>
<td>1.7</td>
<td>2.71</td>
<td>2.94</td>
<td>0.439</td>
<td>0.762</td>
<td>0.651</td>
<td>0.316</td>
</tr>
<tr>
<td>C</td>
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<td>ETH</td>
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<td>0.651</td>
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<td>0.271</td>
<td>0.762</td>
<td>-0.005</td>
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</tr>
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<td>1.18</td>
<td>4.02</td>
<td>1.00</td>
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<td>3.27</td>
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</tr>
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<td>ETH</td>
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<td>1.5</td>
<td>2.52</td>
<td>2.52</td>
<td>0.271</td>
<td>1.26</td>
<td>3.27</td>
<td>0.700</td>
</tr>
<tr>
<td>H</td>
<td>8</td>
<td>ETH</td>
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<td>2.34</td>
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<td>3.04</td>
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</tr>
<tr>
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<td>9</td>
<td>ETH</td>
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<td>2.34</td>
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<td>3.04</td>
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</tr>
<tr>
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<td>2.34</td>
<td>2.80</td>
<td>0.270</td>
<td>1.47</td>
<td>3.04</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Table 4.2: Operating conditions of the SMB experimental runs. ST indicates that the cleared lysate was provided by Strathmann Biotec AG. ETH means that the cleared lysate was prepared in our labs following the procedure discussed in section 4.2.1. pDNA sample 10 is a mixture of pDNA samples 8 and 9 ($\gamma_{pDNA}$ is always 0). All experiments have been carried out with a switch time ($t^*$) of 5 minutes.
Figure 4.7: Region of complete separation for pDNA and RNA in the \((m_2, m_3)\) plane, calculated from equations 4.7 and 4.8 \((\gamma_{pDNA} = 0, \gamma_{RNA} = 0.95)\). Provided that the constraints given by equations 4.5 and 4.9 are also fulfilled, complete separation is achieved in the region inside the triangle and therefore, pDNA is completely recovered. In the region above the triangle the pDNA (raffinate) is completely recovered but polluted with RNA. The experimental runs at the conditions reported in Table 4.2 are also indicated.

With reference to Figure 4.7, it can be noticed that the operating points in the \((m_2, m_3)\) plane are located in the region of complete separation and in that where the extract is pure, i.e. it contains no pDNA; and the raffinate is polluted, i.e. the pDNA-rich product stream contains also some RNA and other species. In the latter region one can achieve a higher productivity as defined in equation 4.10 than in the complete separation region, but the price to be paid is a lower purity of the raffinate. The further from the diagonal the operating point, the higher the productivity and the lower the raffinate purity. In both regions considered in this study the recovery of pDNA is complete. Such a choice of operating conditions is justified when considering the overall purification process.
4.5 Experimental Results

of plasmid DNA, of which the CIP-SMB step based on size exclusion chromatography is just the first one. Therefore, the goal is to guarantee complete recovery at a high productivity, whereas one can compromise on purity, since the following purification steps will allow achieving its specified value.

4.5.2 Discussion

During the SMB experiments the UV monitor was placed at the extract port. The cyclic steady-state (periodic UV pattern) was reached before the third cycle in all cases (the cycle duration is the number of columns times the switch time, 40 min in this case), but the experiments were continued up to 4-7 cycles.

The re-equilibration of the media after CIP was monitored by measuring the pH at the outlet of the re-equilibration section, i.e. section r. At the selected conditions shown in Table 4.2 (rnr), the pH in the column was back to the operating value of 7 from the high level achieved during CIP within the switch period (5 min).

The analysis of the extract and the raffinate corresponding to the last cycle was carried out following the method described in chapter 2.4. Figure 4.8 shows some of the resulting chromatograms, namely the ones corresponding to runs H, G, J and K. The location of point H in Figure 4.7 (see Table 4.2), indicates that complete separation is to be expected, as confirmed by Figure 4.8a); indeed, no impurities of RNA can be detected, and no pDNA is present in the extract (or it is below the detection limit, as shown in Figure 4.8 right). For runs G to K, decreasing raffinate purity is expected, and this trend is confirmed by Figures 4.8b-4.8d (left). The extract analysis shown in Figures 4.8b-4.8d (right) shows that no pDNA is present in the extract. The negative peak in the extract of run H (Figure 4.8a -right) is probably related to the salt concentration change that may affect the UV cell function.

For samples having the same origin, it is possible to observe the increase in productivity by the increase of the area (or the height) of the pDNA peak. In fact, Runs J and K in Figure 4.8, show how the pDNA peak
Figure 4.8: Chromatograms of 500 μl injections of extract and raffinate (Q = 0.8 ml/min) for runs E, G, J and K.
height increases from 1070 to 1200 mAU (254 nm), when the productivity goes from 1.55 to 1.75 mg/h ml (see Table 4.2).

<table>
<thead>
<tr>
<th>Run</th>
<th>$m_2$</th>
<th>$m_3$</th>
<th>$P$ [mg/h ml]</th>
<th>$A_{pDNA}$ [%] (exp.)</th>
<th>100–$A_{pDNA}$ [%] (exp.)</th>
<th>$X_{pDNA}$ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.439</td>
<td>0.762</td>
<td>0.316</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
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<td>0.763</td>
<td>0.316</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
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<td>0.762</td>
<td>0.487</td>
<td>100</td>
<td>+</td>
<td></td>
</tr>
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<td>0.915</td>
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<td>++</td>
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</tr>
<tr>
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<td>0.157</td>
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<td>1.00</td>
<td>100</td>
<td>++</td>
<td></td>
</tr>
<tr>
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<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
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<td>1.41</td>
<td>70</td>
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<td></td>
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<tr>
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<td>1.55</td>
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<td>50</td>
<td>32.4</td>
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<tr>
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<td>1.75</td>
<td>50</td>
<td>50</td>
<td>30.5</td>
</tr>
<tr>
<td>L</td>
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<td>0.93</td>
<td>80</td>
<td>20</td>
<td>45.8</td>
</tr>
<tr>
<td>M</td>
<td>0.270</td>
<td>1.47</td>
<td>1.21</td>
<td>50</td>
<td>50</td>
<td>38.1</td>
</tr>
</tbody>
</table>

Table 4.3: Experimental separation performance (Area fraction at 254nm) and simulated separation performance (purities) for some of the runs. The symbol "+" indicates that traces (not quantifiable amounts) of RNA are found in the sample; the number of "+" symbols gives a qualitative assessment of the amount of RNA.

The raffinate purity estimates given by $A_{pDNA}$ -see equation 4.2-, is reported in Table 4.3. As expected, there exists a trade-off between purity and productivity, i.e. the increase of productivity is obtained at the cost of a lower purity. Notice that runs J, K and L were performed with pDNA sample 28, whereas run N used pDNA sample 210.

The productivity and solvent consumption calculated here do not take into account the shutting down period (the amount of pDNA to be produced during a given campaign, i.e. the amount of feed stock to be processed, needs to be larger than a certain critical value, so that the shutting down period does not affect significantly the productivity).
4. SMB Operation for pDNA Size Exclusion Purification

4.6 Modelling Results

4.6.1 Modelling the cleared lysate

The pDNA sample can be modelled considering the mixture of three components having the molecular sizes (retention times) corresponding to the $\gamma$ values shown in Table 4.4. Estimates of the axial dispersion and mass transfer parameters are also reported in this table. Figure 4.9 compares an elution profile obtained by detailed simulation of the cleared lysate, with the experimental chromatogram of pDNA sample 5.

![Figure 4.9: Comparison between the experimental chromatogram of plasmid DNA sample 5 (dashed line) ($Q$ = 0.8 ml/min, 500 µl injection) and the modelled chromatogram (solid line) obtained by detailed simulation with the parameters reported in Table 4.4.](image)

The model is able to describe the main retention features of the sample; pDNA and RNA are simulated by species 1 and 2 of Table 4.4 respectively. The correct description of the tail is crucial for the design of SMB, particularly of section 1, and is simulated by a third component, as it appears in Figure 4.9 and Table 4.4.
4.6 Modelling Results

<table>
<thead>
<tr>
<th>Component, $i$</th>
<th>$\gamma_i$</th>
<th>$D_{Li} \text{ [cm}^2\text{/s]}$</th>
<th>$k_i \text{ [1/s]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDNA</td>
<td>0</td>
<td>0.015</td>
<td>10</td>
</tr>
<tr>
<td>RNA</td>
<td>0.95</td>
<td>0.045</td>
<td>0.35</td>
</tr>
<tr>
<td>$n$</td>
<td>1.7</td>
<td>0.045</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 4.4: Cleared lysate model parameters. The velocity, $u$, is in cm/s.

In the model, the stationary phase is assumed to be non porous and the retention is characterized by the values (see equations 4.3 and 4.4). Since pDNA is in reality excluded from the pores (not retained), mass transfer resistance does not play a role for it. In the model this is handled by choosing a very large mass transfer coefficient (meaning that the mass transfer is very fast and therefore not limiting). This allows to model correctly large volume injections such as for instance, the one shown in Figure 4.4. For RNA and $n$, the mass transfer coefficients were just estimated from the experiments.

### 4.6.2 Model Equations

The column chromatography and SMB simulations were carried out using the detailed one-dimensional model presented in Chapter 1 (equations 1.13–1.15). This model accounts for convection and axial dispersion in the fluid phase, and mass transfer, through a linear driving force model, assuming that solid diffusion is the rate limiting step [23]. A minimum of 300 points per column was used.

### 4.6.3 Modelling results

Detailed SMB simulations were run using the three component system shown above at the conditions of some of the experimental runs, namely Runs C, E, J, K, L and N (see Table 4.2). The simulated raffinate purities are reported in the last column of Table 4.3, where they can be compared with the experimental values reported in the fifth column (area fraction). Calculated values and experimental results exhibit a similar trend, although as expected they do not coincide, since the experimental...
data refer to the area fraction defined in equation 4.2 and this does not necessarily correspond quantitatively with the calculated purity, which is based on calculated outlet concentrations. These results confirm the trade-off between productivity and raffinate purity that was expected, and demonstrate that the model can be used as a tool to design SEC-SMB operation for pDNA purification.

4.7 Conclusions

The CIP-SMB configuration proposed can be used to accomplish the size exclusion group separation step of pDNA. Using the simple approach of triangle theory, the experiments can be easily designed in order to achieve the 100% recovery required at this early stage of the purification process. The implementation of the CIP step into the SMB is technically possible. The details of the cleaning procedure (frequency, cleaning agent, contact time, etc) require the establishment of the so-called cleaning protocol (see Appendix C). Thanks to the CIP, the pressure in the unit was kept stable during operation, and no change of performance in time was noticed.

Subscripts and superscripts used in this chapter

- $^0$ initial
- $^A$ more retained species
- $^B$ less retained species
- $^i$ component index ($i = 1, 2, ..., c$)
- $^j$ stream/section index ($j = 1, 2, ...$)
- $^\text{max}$ maximum
- $^\text{min}$ minimum
- $^n$ very retarded (and possibly retained) impurities
- $^{pDNA}$ plasmid DNA
- $^R$ raffinate
- $^{RNA}$ retarded impurities (mainly RNA)
- $^S$ relative to the mass of carbon substrate
- $^X$ relative to the mass of cells
Chapter 5

Optimization of Column Chromatography and SMB Processes

5.1 Introduction

Chromatographic separations can be carried out either in the batch operating mode, using one single column (or several columns working in parallel) packed with a particulate or monolithic stationary phase (alternatively fluidized beds are sometimes used), or in the continuous mode in a multicolour setup adopting the Simulated Moving Bed (SMB) process or one of its modifications (Varicol, Powerfeed...). In the later case, several fixed bed columns are used to separate continuously a binary feed mixture into two fractions; multicomponent mixtures can also be split in two or more fractions containing species having similar retention properties, as discussed in the previous chapter and in recent literature [2, 5, 8, 24, 53]. Closed-loop recycling in column chromatography constitutes another process option [54–56], which is less common in practice, and will not be considered in this work.

5. Optimization of Column Chromatography and SMB Processes

Column chromatography is extensively used in bioseparations due to its simplicity when compared to multicolumn processes. The batch mode allows for cleaning in place after every cycle and often a few columns are used in parallel. Several studies have reported procedures to find optimum operating conditions or minimum production costs for several modes of the column chromatography process [57-61]. SMB has not been used so far in the field of bioseparations; however, and despite the differences with respect to the traditional SMB applications, there is no fundamental barrier that prevents the use of SMB in bioseparations. It is established that for tasks such as chiral separations SMB allows in most cases to increase productivity and reduce the solvent consumption, when compared to the same process carried out by column chromatography; furthermore, due to its working principle, SMB may achieve total recovery in situations where the column chromatography recovery is limited by low selectivities. The challenges in bioseparations are related a) to the nature of the molecules and often to the high density of the samples, b) to the need for cleaning in place, c) to the use of aqueous mobile phases (usually buffer solutions) and of gradients obtained using modifiers, and d) to the use of compressible chromatographic media. As discussed in Chapters 3 and 4, it is possible to perform an online CIP step in SMBs [48, 49]; the CIP operation has to be designed and possibly optimized, i.e. number of columns in the cleaning and in the regeneration sections, and adequate flow-rates.

In order to introduce the SMB technique in the bioprocess field, it is necessary to provide quantitative data about the SMB performance and to establish a fair comparison with the corresponding column chromatography alternatives. The aim of the work reported in this chapter is to make such a comparison at isocratic conditions, and for the cases of two model systems of interest requiring two different types of stationary phases: a compressible media, typically used in bioseparations, and a rigid media used in chiral separations.

For a given application, the comparison between the optimum SMB operation and the optimum column chromatography operation is the only way to draw fair conclusions about the relative performance of the two [62-64]. The performance of any chromatographic process is described by two variables, or performance indicators, related by a trade-off: productivity and solvent consumption. These are to be maximized and minimized respectively, while taking into account the product quality requirements (e.g. purities) and the technical limitations of the operation (e.g. pressure drop). The constrained optimization of productivity and solvent consumption for a given chromatographic process constitutes a multiobjective optimization problem, namely a two objective optimization. The decision variables of the optimization problem are typically
related to the size of the unit and to the operating conditions of the process. Looking for a global optimum for such a multiobjective optimization problem would require making a choice about the relative importance of the different performance indicators; such a choice might be based on contingency and therefore not general enough. A better approach, that will be followed here, is to search for a set of optimal solutions, which are equally good with respect to all performance indicators at the same time in a sense that will be clarified shortly. This is the so-called Pareto set, which in the case of a two objective optimization results to be a curve (in the productivity, solvent consumption plane). To make this clear let us consider a general two objective optimization problem in which $I_1$ is to be maximized and $I_2$ has to be minimized, subject to a number of constraints such that maximum pressure drop, minimum product purity, etc. The solution of this constrained optimization problem is the Pareto set shown in Figure 5.1.

Comparing points $p$ and $r$ located on the Pareto curve and above it respectively, it is readily seen that the performance is certainly worse in $r$, since $I_1$ is smaller and $I_2$ is larger; this implies that decision variables leading to point $p$ constitute better operating conditions than the ones corresponding to $r$. Let us now consider point $q$ which is located above the Pareto set and to the right of point $p$. Contrary to $r$, point $q$ is better than $p$ when $I_1$ is considered, but it is worse when $I_2$ is considered; in principle, point $p$ could be considered better than $q$. However, comparing the performance of point $q$ with a point located on the Pareto set else than $p$, e.g. point $s$, it is readily seen that $q$ is worse than $s$, hence it is excluded from the optimum solution. This finding can be generalized by saying that any point above the Pareto set has worse performance than the points constituting the Pareto set. Comparing now points $p$ and $s$, both located on the Pareto set, one point cannot be considered better than the other since $I_1$ is better in $s$ whereas $I_2$ is better in $p$. This is the characteristic feature of any point on the Pareto set and it is generally referred to as a non-dominance condition; when moving along the Pareto set, each of the points (corresponding to a specific set of decision variables) is non dominant with respect to the others. The points located below the Pareto set are not feasible because the product quality requirements are not fulfilled (e.g. the purity of the target species is too low). The Pareto set may therefore be considered as the boundary of the domain of feasibility. The final choice of optimum operating conditions, i.e the choice of a specific point on the Pareto set, depends upon additional considerations such as equipment costs, equipment availability, stability of the sample, costs of the precursors, value of the products, etc. As shown schematically in Figure 5.1, a Pareto curve exists only in a finite domain of the $(I_1, I_2)$ plane, and its extension is a measure of the versatility of
Figure 5.1: Scheme of a Pareto set in the plane of the performance indicators \((I_1, I_2)\); the process aims at maximizing \(I_1\) and minimizing \(I_2\).
5.2 Background on the model systems

In this work, two chromatographic operations are considered. The first corresponds to the first step of the plasmid DNA (pDNA) polishing stage described in Chapter 4 and in the literature [48, 49]. At this early stage of the purification cascade, high recovery of pDNA is required, at purity levels which are less critical, since these can be improved during the next purification stages. The second case considered in this work is the separation of the Tröger's base (TB) enantiomers [65].

The choice of the first model system is motivated by the interest in the high added-value pDNA product that has grown over the last years, as the research on gene therapy and vaccination with nucleic acids moves forward. Plasmids are generally produced in Escherichia Coli by fermentation and then harvested after cell disruption. In order to satisfy the strict relevant regulations, the plasmid must be highly purified and in its supercoiled form (ccc pDNA); the main impurities are RNA, proteins, chromosomal DNA fragments and endotoxins. A large scale pDNA manufacturing process needs to be established in order to satisfy the demand of pharmaceutical grade pDNA for clinical trials and eventually for commercialization. The downstream processing of pDNA aims at recovering the plasmid at the purity levels required by regulatory agencies, such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA). It is unlikely that only one purification step serves to achieve the high purity specifications required by the authorities. Indeed, most often a combination of purification steps is considered in the literature, and these are grouped in three stages: primary recovery, intermediate recovery and final purification (or polishing). The chromatographic step investigated here corresponds to the first step of the polishing stage, and it is based on a size exclusion (SE) mechanism, which implies a linear chromatographic behavior. At the conditions selected, there exists a baseline separation between pDNA and the main impurity, i.e. RNA [48, 49]. The description of the SMB process and some experimental results (at suboptimal conditions) are contained in Chapter 4. The main properties of plasmid DNA and some
5. Optimization of Column Chromatography and SMB Processes

Details about its downstream processing are summarized in Appendices A and B, respectively.

In order to extend the comparison results of column chromatography and SMB to a non-linear chromatographic problem, the separation of the Tröger's base (TB) enantiomers is chosen as a second case study. Over the last decades it has been recognized that the two enantiomers of a chiral drug may have different biological activity; as a consequence, producing enantiomerically pure substances has become a crucial issue [13]. The enantioselective synthesis is not always possible or even not profitable, and the authorities responsible for drug administration impose high purity requirements [66]; the industry is thus faced with the challenge of searching innovative separation processes with high yields. Chromatography is one of the key operations in this field [67], and the simulated moving bed technique is generally preferred due to the larger productivities achieved and the reduced solvent consumption obtained in most of the cases, when compared to column chromatography. Nowadays this is a rather accepted statement when it comes to enantiomer separations; in this chapter it is demonstrated for the case of the TB enantiomers, which are frequently used as model system in various fields, including chiral chromatography [65, 68, 69].

5.2.1 Plasmid DNA purification

In the purification step considered here, the pDNA is purified from the so-called cleared lysate, which is a rather complex mixture of many components, namely RNA, chromosomal DNA fragments, proteins and endotoxins. At the conditions selected during the experiments reported in Chapter 4, the pDNA (i.e. the ccc pDNA together with its isoforms) is completely excluded from the pores and elutes from the column as a single peak. The other species that can access the pores, are thus retarded and elute from the column later. In order to model the chromatographic behavior of this mixture, a rather simple model system has been selected, which is still able to capture the key features of the retention behavior of the cleared lysate. This is done by considering a three component mixture (labelled pDNA, RNA and n) whose components exhibit linear retention behavior characterized by the following isotherm:

\[ n_i = \gamma_i c_i = \left[\varepsilon_{pi} + (1 - \varepsilon_{pi}) K_i\right]c_i \]  \hspace{1cm} (5.1)

where \( c_i \) and \( n_i \) are the fluid and the solid phase concentrations of species \( i \), and \( \varepsilon_{pi} \), \( K_i \) and \( \gamma_i \) are the accessible intraparticle porosity, the adsorption

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5.2 Background on the model systems

Figure 5.2: Simulated overloaded chromatogram of cleared lysate. The species are labelled pDNA (target), and RNA and n (impurities). $t_a$ and $t_b$ are the breakthrough time of pDNA and the elution time of the injected volume, respectively; the cycle time, $t_c$, is defined as $t_b - t_a$. Collecting the pDNA between times $t_a$ and $t_Y$ guarantees the minimum recovery ($Y_{min}$).
equilibrium constant and the linear retention parameter (pseudo Henry constant) of species \(i\), respectively. The first term of the second equation, \(\varepsilon_i \rho_i c_i\), accounts for the retardation of species \(i\) due to its characteristic pore accessibility, whereas the second term, \(\varepsilon_i (1 - \varepsilon_i) K_i c_i\), accounts for the adsorption on the stationary phase; this last term is zero, i.e. \(K_i = 0\), for a purely size exclusion based mechanism. In Chapter 4 (section 4.6.1), Table 4.4 reports the model parameters for the three species considered here, namely the \(\gamma_i\) values used in equation 5.1, and the axial dispersion and mass transfer coefficients, estimated from experiments. From equation 5.1, the total concentration of species \(i\) is given by:

\[
c_i = \varepsilon_i c_i + (1 - \varepsilon_i) n_i = [\varepsilon + (1 - \varepsilon) \gamma_i] c_i
\]

where \(\varepsilon\) is the intraparticle bed void fraction. Using the last equation with \(\gamma_i\) given by equation 5.1 allows using a standard force model for the chromatographic column where the total porosity \(\varepsilon^*\) is given by \(\varepsilon\). Figure 5.2 shows a simulated chromatogram of the cleared lysate using the model parameters reported in Table 4.4.

The CIP and regeneration steps are not considered in this work. In order to include them in the performance analysis, equivalent cleaning protocols for the column and the SMB processes have to be defined. This is a critical issue in chromatographic bioseparations, whose clarification is beyond the scope of this work.

### 5.2.2 Tröger's base enantiomer separation

The competitive adsorption of the (+)-TB and the (-)-TB enantiomers in ethanol on microcrystalline cellulose triacetate at 50°C can be rather well described by the following binary Langmuir isotherm, at least for the purpose of this study [70]:

\[
n_i = \frac{\gamma_i c_i}{1 + K_A c_A + K_B c_B}
\]

where \(A\) and \(B\) are the more (the (+) form) and the less (the (-) form) retained species respectively. The values of the parameters used in this study are shown in Table 5.1; Figure 5.3 shows the corresponding simulated chromatogram of the TB racemic mixture.
5.2 Background on the model systems

Figure 5.3: Simulated chromatogram of the Tröger’s base enantiomers in microcrystalline cellulose triacetate (CTA) (ethanol) at 50°C. The species are labelled A and B. \( t_a \) and \( t_b \) are the breakthrough time of B and the elution time of the injected volume, respectively; the cycle time, \( t_c \), is defined as \( t_b - t_a \). Collecting A and B between the times \( t_a \) and \( t_{X1} \) and between \( t_{X2} \) and \( t_c \) guarantees the purities of the two fractions, \( X_{A_{min}} \) and \( X_{B_{min}} \).
5. Optimization of Column Chromatography and SMB Processes

Table 5.1: Physical properties of the TB system

<table>
<thead>
<tr>
<th>Component</th>
<th>( \gamma_i ) [-]</th>
<th>( K_i ) [ml/mg]</th>
<th>( D_{Li} ) [cm²/s]</th>
<th>( k_i ) [1/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.45</td>
<td>0.39</td>
<td>0.01 ( u_{cm/s} )</td>
<td>0.09</td>
</tr>
<tr>
<td>B</td>
<td>2.18</td>
<td>0.065</td>
<td>0.01 ( u_{cm/s} )</td>
<td>0.15</td>
</tr>
</tbody>
</table>

5.3 Modelling and Optimization

5.3.1 Chromatographic Model

The column chromatography and SMB simulations were carried out using the detailed one-dimensional model presented in chapter 1, that accounts for convection and axial dispersion in the fluid phase, and mass transfer through a linear driving force model, assuming that solid diffusion is the rate limiting step. Thirty points per centimeter of column were used after verifying that this gives a good enough numerical accuracy. Integration in time is carried out using a commercial integrator for stiff equations, as appropriate when simulating the steep concentration profiles occurring in high performance liquid chromatography. In the case of the SMB, the mass balance equations 1.13-1.15 reported in Chapter 1 are coupled with mass balances at the nodes, and solved in time by accounting for the switching mechanism, as explained in Chapter 1.

5.3.2 Optimization

Decision variables and performance indicators

The performance of SMB and of column chromatography is measured in terms of productivity and solvent consumption, i.e. good performance means large productivities and low solvent consumptions. These are the so-called performance indicators, \( I_i \), of the optimization problem. The operating variables constitute the set of decision variables, i.e. the parameters to be varied in order to optimize the process; these depend upon the nature of the problem considered, e.g. the injected volume belongs to the set of decision variables in the column chromatography case, but not...
in the case of the SMB operation, where the feed is continuous; on the contrary, the flow-rate ratios, \( m_{ij} \), belong to the set of decision variables of the SMB operation and not of the column chromatography.

In some cases, the space of decision variables may be reduced by fixing some parameters or by restricting the optimization to a given region of this space; this is used to simplify the optimization problem in the case where many decision variables are involved.

The productivity \( (P) \), is defined as mass of product recovered per unit volume of stationary phase and time. Thus, it provides information about the amount produced per unit time, with respect to the cost of the equipment required; indeed, the amount of stationary phase is proportional to the size of the unit required, which determines in turn the cost of the equipment. In the context of this work, the solvent consumption \( (S) \) is defined as the amount of solvent used per unit time and mass of product recovered; this quantity is multiplied by the feed concentration of the target species in order to get a dimensionless solvent consumption. The optimization requires \( I_1 = P \) to be maximum and \( I_2 = S \) to be minimum. The mathematical expressions of the performance indicators depend upon the decision variables corresponding to the specific problem studied, as it is explained in detail below.

The concentration factor, \( \alpha \), achieved for a given set of optimum decision variables, is defined as the concentration of the target species in the product fraction divided by the concentration of the target species in the feed mixture, i.e. \( c_i/c_f \). For a given pair \( (P, S) \) the value of the concentration factor is fixed, and as expected from the definition, this takes values smaller or equal to 1. Another important parameter is the throughput, \( T \), which is defined as the amount of product processed per unit of time, which can also be calculated from the optimum solution. It is important to notice that the column section, being a scaling factor, is kept constant in all our computations.

**Constraints**

The objective functions are optimized subject to two kind of constraints: constraints on the product quality, i.e. purity \( (X) \) and recovery \( (Y) \) of the
target compounds, and constraints on the operation, i.e. arising typically from considerations about the chromatographic media stability, or the maximum pressure drop in the unit, or the minimum switch time, in the SMB case.

In the column chromatography case, it is necessary to fix the times at which the fraction collection of a given species starts and ends in order to calculate the purity and the recovery; purities are based on the relative amounts collected during this period. In the case of the SMB, the purities are calculated considering the average concentrations of the species over one cycle at cyclic steady state (a cycle is the time interval in which one column goes through all positions in an SMB unit, i.e. \( n_c t^* \), where \( n_c \) is the total number of columns).

The constraint on the pressure drop plays a role whenever the chromatographic media is rigid; indeed, overcoming the maximum allowable pressure drop would cause destruction of the beads constituting the bed and would lead to unacceptable degradation of performance. The pressure drop, \( \Delta p \), is calculated applying Darcy’s law

\[
\frac{\Delta p}{L} = \varphi Q
\]  

where \( L \) is the length of the bed, \( Q \) is the fluid-flow rate and \( \varphi \) is a parameter depending on permeability of the bed and viscosity of the mobile phase that can be easily measured. In the case of SMB, since the flow rates vary along the unit, Darcy’s law is applied to each section \( (Q = Q_j \) with \( j = 1, 2, 3, 4 \)) and then the terms are summed up in order to get the total pressure drop in the unit.

Most of the preparative chromatographic media available and used in bioprocesses are compressible. For a given media, column diameter, column length, solvent nature and temperature, there exists a critical velocity above which the properties of the bed change significantly, namely, the bed is compressed and the pressure drop becomes infinite; this is the so-called critical velocity \([47]\). It is evident that the packing velocity for a given column, which represents the maximum allowable operating velocity, has to be chosen below the corresponding critical value, but not...
5.3 Modelling and Optimization

far from it since high velocities lead to larger productivities and throughput. Overcoming the packing velocity, \( u_{\text{max}} \), leads to compression of the column and the subsequent degradation in performance and increase in pressure drop. The critical velocity, \( u_c \), may be calculated through the following correlation [47]:

\[
u_c \text{L}_o = \eta \frac{\text{L}_o}{d} + \theta
\]  
(5.5)

where \( \text{L}_o \) is the gravity settled bed height, \( d \) is the column diameter and \( \eta \) and \( \theta \) are empirical constants that depend upon the stationary phase, the mobile phase used, the temperature and the column section. In this work, \( u_{\text{max}} = 0.8 u_c \) will be considered. In the case of SMB, this condition must apply in section 1 of the unit, where the flow is always larger than in the other sections, i.e. \( u_1 = u_{\text{max}} \).

It can be proved that in the case-studies analyzed in this work, the flow-rate in the unit at optimal operating conditions corresponds to the maximum flow-rate allowed. This is given by the maximum pressure drop constraint in the case of rigid media, and by the critical flow-rate in the case of compressible media. This rule holds only if the efficiency of the columns is high enough and the performance is only limited by the velocity at which the process is run. In all the optimizations carried out in this work, the maximum flow-rate condition is used after verifying that this is indeed the one that applies for the two cases considered. The mathematical expression of the \( \Delta p \) or \( u_{\text{max}} \) constraint constitutes a relationship between the decision variables, which reduces the number of degrees of freedom in the system.

For SMB, there exists an additional operation constraint, which requires the switch time to be larger than a certain minimum value, typically of the order of 25 to 30 seconds. Below this value of the switch time the disturbances associated to port switching would occur so frequently with respect to the switch time to hamper the possibility of achieving a stable and steady SMB operation.
Optimization algorithm, penalty functions and objective functions

The optimization consists of the following basic steps: for a given set of decision variables selected in the defined ranges or windows (i.e. the subset of the space of the decision variables where optimum conditions are searched) the simulation of the chromatographic process is carried out, and the performance indicators \( I_i \) and performance parameters (purities and/or recoveries of the target species) are calculated. The values of the constraints are taken into account during the optimization by using a penalty function, as it is typically the case in constrained optimization problems. Whenever the constraints are not met, penalty functions are applied to the performance indicators, so that this values are worse to an extent that is a function of the deviation from the constraints. The general expression of the penalty function used here takes the following form:

\[
J_l = F_l \left[ 1 - \mu \sum_{i=1}^{c} \left( \frac{|C_i^* - C_i|}{C_i^*} \right)^q \right] \quad l = 1, 2 \quad (5.6)
\]

where \( c \) is the number of constraints that have to be tested, \( C_i \) are the values of the constrained quantities and \( F_l \) depend on the performance indicators; \( C_i \) and \( F_l \) are calculated by the model; \( C_i^* \) are the relevant values of the upper or lower bounds given by the constraints, \( \mu \) is the parameters tuning the penalization algorithm, and finally, \( J_l \) are the objective functions, i.e. the quantities to be optimized. The \( J_l \) values are used by the optimization algorithm during the selection of a new decision variables set, i.e. the optimizer uses \( J_1 \) and \( J_2 \) to assign a performance index to the specific set of decision variables, and this performance index is utilized in the selection of the new decision variables.

The functions \( F_l(I_i) \) are generally equal to the performance indicators; however, in the case studied here, the two functions \( F_1 \) and \( F_2 \) are selected such that \( F_1 = I_1 = P \) and \( F_2 = 1/(1 + I_2) = 1/(1 + S) \). This is convenient because, by doing so, both objective functions have to be maximized. \( S \) may always be back calculated and indeed, during the
5.4 Plasmid DNA purification

analysis of the results presented below, the physically meaningful indicator, $S$, is used. The formal definition of the two objective optimization problem solved in this work is the following:

$\max_{d_1,d_2,...} J_1$ \hspace{1cm} (5.7)

$\max_{d_1,d_2,...} J_2$ \hspace{1cm} (5.8)

where $d_1,d_2,...$ are the decision variables, which are defined below for each case. The optimization routine used successfully in this work is a Non-Sorting Genetic Algorithm (NSGA) [71]. This method has already been used in the frame of chromatographic process optimization and SMB technology [18, 70].

5.4 Plasmid DNA purification

5.4.1 Definition of the column chromatography optimization problem

Figure 5.2 shows a simulated overloaded chromatogram of the mixture with properties given in Table 4.4 in a 10 cm column at the conditions described previously in Chapter 4. The first peak corresponds to the pDNA which is completely excluded from the pores. The times $t_a$ and $t_b$ are the breakthrough time of pDNA, and the total elution time of the injected sample, respectively. The first is defined as the time at which 0.25% of the injected mass of pDNA is eluted, and the second as the time at which 0.25% of the mass of species $n$ injected is not yet eluted. The cycle time, $t_c$, is defined as the minimum time interval required between two consecutive injections, i.e the difference $t_b - t_a$.

The search of the optimum operating conditions expressed by equations 5.7 and 5.8 is carried out in the space of the decision variables, these being the length of the column, $L$, the flow-rate, $Q$, and the injected volume, $V_i$ (refer to Table 5.2 for other details).
The operation constraint is given by the maximum flow rate, since the media used for this application is compressible. As a consequence, the choice of the decision variable \( Q \) is subject to an upper constraint \( Q_{\text{max}} = 0.8 Q_c \); in this particular case, \( Q_c \) is given by the expression:

\[
Q_c [\text{ml/s}] = 0.1055 + 0.6940 \frac{1}{L [\text{cm}]} \tag{5.9}
\]

The other two constraints are related to the quality of the product: these are the pDNA purity \( X \) and recovery \( Y \). The purity and the recovery constraints are fixed by the nature of the purification step. In this work, the choice of minimum recovery is kept constant at \( Y_{\text{min}} = 0.98 \), and different minimum purities \( (X_{\text{min}}) \) are investigated.

The minimum recovery constraint, \( Y_{\text{min}} \), is used to determine the time at which the collection of the pDNA fraction ends; this is the time \( t_Y \) shown in Figure 5.2, which is obtained by integrating the pDNA concentration profile and by applying the following equation:

\[
Y_{\text{min}} = 0.98 = \frac{Q}{V_T c_{pDNA}^{F}} \int_{t_a}^{t_Y} c_{pDNA} dt \tag{5.10}
\]

where \( c_{pDNA}^{F} \) is the concentration of pDNA in the feed mixture and \( V_T \) is the injected volume. Once \( t_Y \) is calculated using equation 5.10, the recovery constraint is necessarily fulfilled and the purity of the pDNA fraction, \( X \), is calculated as:

\[
X = \frac{\int_{t_a}^{t_Y} c_{pDNA} dt}{\int_{t_a}^{t_Y} (c_{pDNA} + c_{RNA} + c_n) dt} \tag{5.11}
\]

Whenever the selected operating conditions lead to baseline separation between the pDNA and the RNA peaks shown in Figure 5.2, \( t_Y \) is set equal to the total elution time of pDNA and both, recovery \( (Y) \) and purity \( (X) \), are equal to 1. It follows that in our optimization procedure \( Y \) may be either 0.98 or 1.
Productivity is calculated according to the definition given in section 5.3.2, which in the case studied here corresponds to the following expression:

\[ P = \frac{YV_t c_{pDNA}}{t_c AL} \]  

where \( Y \) is the recovery of pDNA (0.98 or 1), \( t_c \) is the cycle time and \( AL \) is the volume of the column. The productivity is therefore expressed in terms of two of the three decision variables, i.e. \( L \) and \( V_t \), where \( L \) is a function of the maximum flow-rate through equation 5.9.

According to the definition given in section 5.3.2, \( S \) is written in the following dimensionless form:

\[ S = \frac{Qt_c}{YV_t} \]  

In order to write the penalty function, the corresponding parameters shown in the first column of Table 5.4 are used in the general expression of equation 5.6 (this table is shown at the end of the chapter). The two penalty functions, acting on \( F_1 = P \) and \( F_2 = 1/(1+S) \) respectively, have only one term that depends on the single constraint \( C_1 = X \); the penalty functions are applied whenever \( C_1 < C_1^* \) and the exponent of these terms in equation 5.6 is \( q = 2 \) in both cases. As mentioned above, the second product quality constraint, i.e. the recovery, is used to determine the time at which the collection of the pDNA fraction ends, and therefore it is always fulfilled by definition.

### 5.4.2 Definition of the SMB optimization problem

The decision variables of the SMB optimization problem are the flow-rate ratios in the sections, i.e. \( m_1, m_2, m_3 \), the switch time, \( t^* \), and the column length, \( L \). In order to reduce the complexity of the problem, the flow-rate ratio in section 4 is kept constant at \( m_4 = -0.05 \). The feed concentrations and the characteristics of the columns are reported in Table 5.2. It is
important to highlight that, similarly to the column chromatography case, the column section is kept constant for this analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$ [cm²]</td>
<td>0.785</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>0.32</td>
</tr>
<tr>
<td>$m_4$</td>
<td>-0.05</td>
</tr>
<tr>
<td>Configuration</td>
<td>1-2-2-1 closed loop</td>
</tr>
</tbody>
</table>

Table 5.2: Column characteristics, constant process parameters and concentration of the cleared lysate feed mixture used for the optimization problems

As explained in section 5.3.2, the maximum flow rate constraint applies to section 1, hence $Q_1 = Q_{max} = 0.8Q_c$, thus implying that the columns are not compressed.

In the SMB case, pDNA is to be recovered in the raffinate stream whereas the impurities end up in the extract. The raffinate purity is defined as:

$$X = \frac{c_{pDNA}^R}{c_{pDNA}^R + c_{RNA}^R + c_{n}^R}$$  (5.14)

where $c_{i}^R$ is the concentration of the species $i$ in the raffinate averaged over one cycle:

$$c_{i}^R = \frac{1}{n_c t^*} \int_{t}^{t+n_c t^*} c_{i}^R dt$$  (5.15)

where the average can be calculated from any time $t$ after reaching cyclic steady state.
5.4 Plasmid DNA purification

The recovery is given by the expression:

\[ Y = \frac{R_{pDNA}^R}{F_{pDNA}^F} \]  \hspace{1cm} (5.16)

where \( F \) and \( R \) are the feed and the raffinate flow-rates.

The performance indicators are defined as:

\[ P = \frac{R_{pDNA}^R}{n_cAL} \]  \hspace{1cm} (5.17)
\[ S = \frac{D + F}{FY} \]  \hspace{1cm} (5.18)

where \( n_c \) is the total number of columns in the unit and \( D \) is the desor- bent flow-rate.

Contrary to the column chromatography case where the constraint on \( Y \) is always fulfilled since it is used to determine the collection period for pDNA, in the SMB case the constraints on \( X \) and \( Y \) may be fulfilled at the same time, or not. As a result both constraints have to be tested and their fulfillment has to be accounted for in the penalty functions. These are obtained by using the parameters reported in the second column of Table 5.4 in the general expression of equation 5.6 (this table is shown at the end of the chapter). The two penalty functions, acting on \( F_1 = P \) and \( F_2 = 1/(1+S) \), may have two terms which are dependent on the two constraints \( C_1 = X \) and \( C_2 = Y \); these are applied whenever \( C_i < C_i^* \). As indicated in Table 5.4, a "soft" penalty is also applied in the cases where \( C_i > C_i^* \) since the optimum is always expected at \( C_i = C_i^* \), due to the trade off existing between the fulfillment of the constraints and the optimization of the objective functions; the exponents of these terms are \( q = 2 \) in both cases.
5.4.3 Results

Column chromatography

Figure 5.4 shows the Pareto sets obtained for several purity values ranging from 0.85 to 0.99, and at a constant recovery of 0.98. As expected, the larger the purity, the larger the solvent requirements for a given productivity. At $X = 0.99$, the optimum operation cannot be run at $P$ larger than about 1.7 mg/(h ml), and a small increase in $P$ affects strongly the solvent requirements, as indicated by the steepness of the 0.99 purity Pareto set in the region of maximum $P$. At $X = 0.85$ on the contrary, maximum productivity is as large as about 0.43 mg/(h ml).

Figure 5.4: Column chromatography, pDNA case study. Pareto sets for $X = 0.85-0.99$ and $Y = 0.98$. On the $X = 0.95$ Pareto set the decision variables and concentration factor corresponding to the larger and the smaller $P$ respectively are: $L = 11.2$ cm, $V_f = 2.6$ ml, $Q = 0.13$ ml/s, $\alpha = 0.78$ and $L = 36$ cm, $V_f = 12$ ml, $Q = 0.10$ ml/s, $\alpha = 0.90$ (refer to Tables 4.4 and 5.2 for more details).
Figure 5.5: Column chromatography, pDNA case study. Trends of the decision variables along the Pareto set at $X=0.95$ and $Y=0.98$ (refer to Table 5.2 for more details).
Figure 5.6: Column chromatography, pDNA case study. Trade-off between purity and productivity, and between purity and solvent consumption, for a column length of about 16.5 cm; $X = 0.95, Y = 0.98$ (refer to Table 5.2 for more details).
5.4 Plasmid DNA purification

How the decision variables change along the Pareto set is illustrated in Figure 5.5, where $P$ and $S$ are plot against the three decision variables for the case where $X = 0.95$ and $Y = 0.98$. In order to get large productivities the flow-rate must increase, whereas the length and the injected volume have to be kept small. Similar trends are observed in the case of the solvent consumption (as expected from the trade off relating $P$ and $S$): large flow-rates, and smaller column lengths and injected volumes lead to larger solvent consumptions. As expected, the concentration factor, $\alpha$, increases when going to smaller solvent requirements. The fact that performance can be improved when lower purity values are specified is illustrated from a different point of view in Figure 5.6. In this Figure, the values of $P$ and $S$ corresponding to the points on the Pareto sets of Figure where $L = 16.5$ cm are plotted against purity, thus showing that $P$ decreases and $S$ increases when $X$ increases.

Figure 5.7 shows the relation between $V$ and $V_I$ for the points constituting the 0.85 and 0.99 purity Pareto sets. The two variables are linearly related and these linear relationships may be used together with the pressure drop constraint in order to select the optimum operating conditions for a given column.

**SMB**

Figure 5.8 shows the SMB Pareto sets corresponding to $Y = 0.98$ and $X = 0.95$, and to $Y = 0.98$ and $X = 0.90$, together with the corresponding column chromatography Pareto sets (for the same values of $X$ and $Y$). From this figure, it is clearly seen that the SMB operation is superior to the column chromatography operation since the SMB Pareto sets are far below those corresponding to column chromatography. For instance, in the case of $X = 0.95$ and $Y = 0.98$, and taking $S = 4$, the productivity is more than doubled when using SMB. Furthermore, in the column chromatography case, it is not possible to access the region of high $P$ even by increasing $S$. This shows how the SMB is more versatile than the column chromatography process.

In Figure 5.8, the trends of $L$, $t^*$, $\alpha$ and $Q_j$ with $P$ and $S$ are also indicated by arrows: larger productivities require smaller values of $L$
Figure 5.7: Column chromatography, pDNA case study. Linear relationship between the injected volume and the column volume. The linear regression parameters in the equation $V_I [ml] = a + bV [ml]$ are the following: $X=0.85$, $a = 0.6987$, $b = -2.376$; $X=0.90$, $a = 0.6678$, $b = -2.6289$; $X=0.95$, $a = 0.628$, $b = -2.9819$; $X=0.97$, $a = 0.6108$, $b = -3.3013$; $X=0.99$, $a = 0.573$, $b = -3.7411$ (refer to Table 5.2 for more details).
5.4 Plasmid DNA purification

Figure 5.8: SMB and column chromatography Pareto sets corresponding to the first purification step of pDNA in Sepharose 6FF for $X = 0.95, 0.90, Y = 0.98$. On the $X = 0.95$ Pareto set the operating conditions and concentration factor corresponding to the larger and the smaller $P$ respectively are: $t^* = 20$ s, $L = 3.2$ cm, $Q_1 = 0.22$ ml/s, $Q_2 = 0.044$ ml/s, $Q_3 = 0.036$ ml/s, $Q_4 = 0.036$ ml/s, $\alpha = 0.89$ and $t^* = 65$ s, $L = 7.2$ cm, $Q_1 = 0.16$ ml/s, $Q_2 = 0.027$ ml/s, $Q_3 = 0.08$ ml/s, $Q_4 = 0.025$ ml/s, $\alpha = 0.94$ (2M AS, 100mM Tris buffer and 10mM EDTA, refer to Table 5.2 for more details).
and \( t^* \), and larger values of \( Q_j \), as expected. Again, the concentration factor increases at higher solvent requirements; it is worth noting that \( \alpha \) is larger in the case of the SMB process (the values are given in the figure captions).

In terms of throughput, with reference to the column cross section considered here, i.e. \( A = 0.785 \text{ cm}^2 \), and taking for instance the case of \( X = 0.90 \), the values range from 80 to 96 mg/h, while the maximum value achieved in column chromatography (at this purity) is 36 mg/h.

**Discussion**

The optimization problem for column chromatography involves three decision variables, namely column volume \( V \), flow rate \( Q \), and volume injected \( V_I \). The solution of the optimization problem defines a Pareto set, on each point of which the three decision variables are univocally defined and the separation performances are given. Incidentally in the case considered here the optimal flow rate corresponds always to the maximum flow rate allowed by packing stability considerations. This is due to the fact that column efficiency is high in this case and packing stability constraints are controlling over column efficiency limitations.

With reference to model equations 1.13-1.15 and considering a specific system, i.e. assigned adsorption isotherms and packing characteristics (in terms of bed void fraction, mass transfer coefficients, and axial dispersions), it is quite clear that the three decision variables are not correlated. In other words, there is a one-to-one mapping between triplets of decision variables and parameters in the model equations, thus making each triplet of decision variables associated in a unique way to specific separation performances, in terms of productivity and solvent consumption.

Previous literature reports different conclusions, namely that only two independent variables exist for column chromatography [55, 62]. One of these is the number of theoretical stages, which is defined as \( N = V/h(Q) \), where the function \( h(Q) \) expresses the dependence of the HETP on the flow rate, e.g. through the van Deemter equation. The other is the loading factor, \( L_f \), which is by definition proportional to the ratio \( V_I/V \). The cited authors base their conclusions on the use of an equilibrium
dispersive model, consisting of only equation 1.13, where the adsorbed phase concentration is directly given by the adsorption isotherm and the axial dispersion coefficient lumps the effect of mass transfer resistance, axial mixing and axial molecular diffusion into a single parameter. In the frame of this model and based on the definitions above, it is clear that there are infinite combinations of triplets of decision variables $V$, $Q$, and $V_f$ yielding the same values of $N$ and $L_f$, hence the same separation performances.

Nevertheless, there is no contradiction between our analysis and the previous literature that we have just mentioned [55, 62]. In fact, it is well known that the equilibrium dispersive model is only a good approximation of the detailed model used in this work, in the limit of columns with a large number of stages [23]. Simulations carried out with the detailed model of equations 1.13-1.15 (not reported here) confirm that choosing different triplets of the decision variables that correspond to the same values of $N$ and $L_f$ lead to performances, which are indeed different, but only slightly; the best performances are achieved for the triplet of decision variables in the calculated Pareto set. Thus concluding, the correct number of decision variables depends on the model adopted; the larger the level of detail in the description of the process, the more the decision variables. In the case of column chromatography using efficient columns two decision variables, i.e. $N$ and $L_f$, are enough to capture most of the important effects using a lumped description of mass transfer and axial dispersion. Three decision variables are however required when the more detailed description of these mechanisms such as that provided by the model used in this work is preferred.

A related observation can be made with reference to the optimization results obtained for the SMB process. Simulations not reported here show that if the maximum flow rate in section 1 is constrained to be 60% of the critical flow rate, instead of 80% as in the results illustrated in Figure 5.8, the Pareto set (calculated for a minimum purity of 0.90) is only slightly worse than that shown in Figure 5.8. This result can be explained as follows: at a smaller flow rate, the column length corresponding to optimal operating conditions is downsized in such a way that the productivity, i.e. the amount of pDNA purified per unit time and unit volume of the SMB unit, changes only to a small extent. Of course in this case,
if columns of the same cross-section are adopted the throughput of the SMB unit will be significantly smaller where the maximum flow rate is 60% of the critical one than when it is 80% of it.

5.5 Tröger’s base enantiomer separation

5.5.1 Definition of the column chromatography optimization problem

Figure 5.3 shows a simulated chromatogram of the Tröger’s base enantiomers, (+)-TB and (-)-TB, which are referred to as species A and B, respectively. The times $t_a$ and $t_b$ are defined as in the case of the pDNA purification, i.e. the breakthrough time of the first eluting species (time for the elution of 0.25% of the mass of B injected), and the total elution time of the injected sample (time at which 99.75% of the mass of species A injected is eluted), respectively. The cycle time, $t_c$, is defined as the difference $t_b - t_a$.

The search of the optimum operating conditions is carried out in the same space of decision variables, as in the case of pDNA: length of the column, $L$, flow-rate, $Q$, and injected volume, $V_i$. The feed concentrations and the characteristics of the columns are reported in Table 5.3. As in the pDNA case, the column chromatography case, the column section is kept constant in this analysis.

Since the microcrystalline cellulose triacetate is a rigid stationary phase, the operation constraint is given by the maximum pressure drop. The parameter $\varphi$ in equation 5.4 is $2.34 \times 10^6 \, \text{g/(s cm$^5$)}$, and the maximum pressure drop is 40 bar, as to previous literature [70]. The product quality specifications are the purities of the two enantiomers, $X_A$ and $X_B$, and their recoveries, $Y_A$ and $Y_B$. In order to have data consistent with those reported in the literature, the purity and recovery constraints investigated are $X_A = X_B = Y_A = Y_B = 0.97$.

For a given set of decision variables, the values of $X$, $Y$, $P$ and $S$ are obtained by adopting the following procedure. The purity of B, $X_B$,
5.5 Tröger's base enantiomer separation

<table>
<thead>
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<th>Parameter</th>
<th>Value</th>
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<td>( c_f^A = 0 )</td>
</tr>
<tr>
<td></td>
<td>( c_f^B = 0 )</td>
</tr>
</tbody>
</table>

Table 5.3: Concentration of the racemic mixture, column characteristics and constant process parameters used during the optimization problems

is calculated and forced to be equal to the specific value \( X_{\text{min}} \), thus determining the time \( t_{X_1} \), at which the fraction collection of species B ends, as shown in Figure 5.3:

\[
X_B = X_{\text{min}} = 0.97 = \frac{\int_{t_{t_a}}^{t_{X_1}} c_B \text{d}t}{\int_{t_a}^{t_{X_1}} (c_A + c_B) \text{d}t} \tag{5.19}
\]

The purity of the A enantiomer is calculated for the fraction collected between \( t_{X_1} \) to \( t_b \):

\[
X_A = \frac{\int_{t_{X_1}}^{t_b} c_A \text{d}t}{\int_{t_{X_1}}^{t_b} (c_A + c_B) \text{d}t} \tag{5.20}
\]

If this purity is below the minimum purity required, i.e. \( X_A < X_{\text{min}} \), a shorter time interval has to be selected, i.e. the collection of the A fraction has to be started later than \( t_{X_1} \). This is done by integrating the concentration profiles from \( t_b \) back to a certain time \( t_{X_2} \), with \( t_{X_2} > t_{X_1} \), such that the purity requirement is fulfilled:

\[
X_A = X_{\text{min}} = 0.97 = \frac{\int_{t_{X_2}}^{t_b} c_A \text{d}t}{\int_{t_{X_2}}^{t_b} (c_A + c_B) \text{d}t} \tag{5.21}
\]

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Using this procedure to find adequate collection time intervals for both fractions, the purity constraints are always fulfilled. The recoveries of the two species can then be computed using the following expressions:

\[ Y_A = \frac{Q}{V_i c_A^F} \int_{t_x^1}^{t_x^2} c_A dt \quad (5.22) \]

\[ Y_B = \frac{Q}{V_i c_B^F} \int_{t_x^2}^{t_x^1} c_B dt \quad (5.23) \]

The values of \( Y_A \) and \( Y_B \) are used to compute the productivity, \( P \), as:

\[ P = \frac{V_i (Y_A c_A^F + Y_B c_B^F)}{t_c AL} \quad (5.24) \]

The productivity is therefore expressed in terms of two of the three decision variables, i.e. \( L \) and \( V_i \), and these two are in this case related to the flow-rate \( Q \) by the maximum pressure drop given by Darcy's law, equation 5.4. The dimensionless form of the solvent consumption \( S \) is given by the following relationship:

\[ S = \frac{Q t_c (c_A^F + c_B^F)}{V_i (Y_A c_A^F + Y_B c_B^F)} \quad (5.25) \]

The penalty functions are obtained using the parameters shown in the third column of Table 5.4 (with \( q = 2 \)) in the general expression of equation 5.6 (this table is shown at the end of the chapter). The two penalty functions, acting on \( F_1 = P \) and \( F_2 = 1/(1 + S) \), may have four terms which are functions of the four constraints: \( C_1 = X_A \), \( C_2 = X_B \), \( C_3 = Y_A \), \( C_4 = Y_B \); these terms are considered whenever \( C_i < C_i^* \). As indicated in Table 5.4, a "soft" penalty is also applied in the cases where \( C_i > C_i^* \).
5.5.2 Definition of the SMB optimization problem

In the case of the SMB process, the decision variables are the flow-rate ratios in the sections, i.e. \( m_1, m_2, m_3, m_4 \), the switch time, \( t^* \), and the column length, \( L \), which are the same ones considered for SMB optimization of the pDNA case, plus \( m_5 \) which is now included whereas it was kept constant in section 5.4.2. Table parameters

As in the column chromatography case studied before, the constraints are the purities and the recoveries of both enantiomers, \( X_A, X_B, Y_A \) and \( Y_B \). It can be easily proven through simple mass balances that for an equimolar feed mixture of \( A \) and \( B \) and identical purity constraints on both the extract and the raffinate fractions, the SMB operation leads to recovery values such that \( Y_A = Y_B = X_A = X_B \). Therefore, once the purity values are selected as \( X_A = X_B = 0.97 \), the recovery constraints are fulfilled and the basis for comparison between the SMB process studied in this section and the column process studied in section 5.5.1 are well established.

The purities are defined as usual, i.e. the average concentration of the target species (\( A \) in the extract, \( B \) in the raffinate) divided by the average total concentration. The expressions of the recoveries are also straightforward and similar to equation 5.16. The productivity and the solvent consumption are defined in the following way

\[
P = \frac{Ec^e + Rc^r}{n_eAL} \quad (5.26)
\]

\[
S = \frac{(D + F)(c^e_A + c^r_B)}{F(Y_Ac^e_A + Y_Bc^r_B)} \quad (5.27)
\]

where the concentrations are averaged over one cycle. The penalty function parameters of equation 5.6 are shown in the fourth column of Table 5.4 (with \( q = 2 \)) (this table is shown at the end of the chapter). The two penalty functions may have two terms which are functions of the two constraints: \( C_1 = X_A, C_2 = X_B \) and are applied when \( C_i < C_i^* \).

The results of this optimization have been reported elsewhere [70], and will be simply used in the following.
5. Optimization of Column Chromatography and SMB Processes

5.5.3 Results

Figure 5.9 shows the SMB and the column chromatography Pareto sets for the TB enantiomer separation at 0.97 purity and recovery of both enantiomers. Like in the linear pDNA separation case, the SMB Pareto set is far below the column chromatography case. Furthermore, the last one is rather short, indicating that only a narrow productivity range is accessible. As a result, it can be concluded that the SMB process achieves better performance and is more flexible than the corresponding column chromatography process.

Figure 5.9: SMB and column chromatography Pareto sets corresponding to the Tröger's base enantiomer separation in microcrystalline cellulose triacetate (CTA) (ethanol) at 50°C for $X_A = X_B = Y_A = Y_H = 0.97$ (refer to Tables 5.1 and 5.3 for more details.)
5.6 Concluding remarks

This work analyzes the performance of the SMB and the column chromatography processes for two different separation problems: the first stage of the polishing step within the downstream processing of pDNA, and the Tröger's base enantiomer separation, in which the adsorption isotherms are linear and non-linear, respectively. Simulation tools are used together with an optimization routine in order to find the optimum operating conditions leading to maximum productivity and minimum solvent consumption; the optimum solution for a given process is a curve on the productivity-solvent consumption plane \((P, S)\), called Pareto set. The comparison between the column and the SMB processes is based on the relative position of the two Pareto sets in this plane.

The optimization problems have been formulated very carefully so that the two processes are comparable and the conclusions meaningful, for each of the case studies. This procedure involves the selection of the decision variables, the definition of comparable objective functions and the consideration of equal operative and product quality constraints for the two processes.

For the column chromatography pDNA purification process, several purities are considered leading to different Pareto sets; this helps to confirm several effects, for instance the trade-off between purity and performance. In the SMB case, two purities are investigated. Comparing the position of two corresponding Pareto sets, the SMB process is found to outperform column chromatography as it may be seen in Figure 5.8 for two different purities \((X = 0.90, X = 0.95)\) and constant recovery \((Y = 0.98)\), i.e the productivity may be doubled for a given value of the solvent consumption. Furthermore, the region of high \(P\) cannot be achieved using the column process.

The same is observed in the TB case, where, as expected considering its non-linear adsorption behavior, the benefit provided by SMB is even larger than in the linear case. Indeed, one important feature of the SMB process is that high performances can be achieved even at rather low values of selectivity and at relatively low efficiencies. Figure 5.9 also shows that the versatility of the SMB operation is significantly larger,
as indicated by the long and flat SMB Pareto set when compared to the short and steep Pareto curve of column chromatography.

The investigations presented in this work confirm the potential of the SMB process based on performance, throughput and versatility. These advantages have to be weighed up against the higher complexity of the SMB operation and the larger investment effort required, as compared to column chromatography. This applies also to bioseparations, as it has been proved here for one case-study. It is worth noting that the case considered here where the retention behavior was linear is a rather simple one, but still SMB has proven to be better than column chromatography. We argue that in the case of more difficult bioseparations, e.g. nonlinear as the case of the enantiomers of the Tröger’s base, the improvement of performance using SMB would be even larger and more attractive.
Table 5.4: Penalty function parameters of equation 5.6 for the column chromatography and the SMB separation processes of the two case studies investigated in this work; the parameter $q$ is equal to 2 in all cases.
5. Optimization of Column Chromatography and SMB Processes

Subscripts and superscripts used in this chapter

- $A$: more retained species
- $B$: less retained species
- $i$: component index ($i = 1, 2, ..., c$)
- $j$: stream/section index ($j = 1, 2, ...$)
- $\max$: maximum
- $\min$: minimum
- $n$: very retarded (and possibly retained) impurities
- $pDNA$: plasmid DNA
- $R$: raffinate
- $RNA$: retarded impurities (mainly RNA)
Chapter 6

Conclusions

Preparative chromatography represents a key technology for biotechnology related separations and purifications. Classical single column preparative chromatography is now complemented by alternative operation modes that allow for significant improvements in terms of productivity and solvent consumption, particularly when the separation process is scaled up. The more important technology available is the Simulated Moving Bed (SMB) that is a continuous multi-column chromatographic technique that allows splitting mixtures into two fractions (e.g., two pure species). Both single column batch chromatography and SMB have gone through tremendous improvements during the last few years, particularly in the field of chiral and fine chemical separations. In the new field of bio-separations, the challenges to be faced are related to the differences with traditional SMB applications: the complex nature of the molecules involved, the high viscosity of the samples, the requirement of aqueous mobile phases, usually buffer solutions, and the compressible chromatographic media typically used in bio-separations.

The project has established new chromatographic separation methods based on SMB technology, new criteria for the identification and the selection of the best operation mode for a given bio-separation and new tools for its optimal design. Three new SMB operating modes have been developed. For all of them, complete separation criteria have been developed using the Equilibrium Theory, and these criteria have been then validated through detailed simulations of the process. These are the three fraction SMB (3F-SMB), the integrated
cleaning in place SMB (CIP-SMB) and the enriched extract SMB (EE-SMB) operations. The 3F-SMB allows to separate mixtures in three fractions and was analyzed by considering the separation of the nucleosides; the CIP-SMB solves the problem of the online cleaning in place which in the case of bio-separations is absolutely necessary and was so far unresolved for SMB; in the EE-SMB a concentration step is added between sections 1 and 2, leading to an increase in productivity and extract purity with respect to the standard SMB operation.

The plasmid DNA (pDNA) application investigated in this work constitutes a relevant example for a bioseparation; the high added value of pDNA requires a performing production and downstream processing in which SMB, in particular the CIP-SMB mode, can certainly play an important role. The results are indeed promising, namely the operating conditions can be selected by using the theoretical understanding developed in our research work, in order to tune the pDNA recovery and purity (which are related by a trade-off). The positive outcome of the experimental results is complemented by a quantitative comparison of the SMB and the column chromatography processes at their respective optimum operating conditions (i.e. two multiobjective optimization problems). The results show that CIP-SMB is superior to column chromatography for the particular pDNA purification step investigated here, as it happens in other fields of applications.

The commercial workstation ÁKTA Explorer provided by GE Healthcare has been modified by adapting both the hardware and the software in order to accommodate the CIP-SMB configuration used during the experiments. The unit is rather flexible and may be used in different operating modes, although its flexibility is limited by the number of valves that can be controlled by the actual version of the software.

More bio-related SMB applications have to be investigated in the near future in order to convince both the industry and the authorities about the interest of using SMB in bioprocesses. This also requires developing understanding about the cleaning and validation issues. Finally, thorough comparisons of the SMB and the column chromatography processes are required; in this context, process optimization is an essential tool that provides deeper understanding of both operations and helps to assess the real advantages of one and the other techniques. In the future, the development of new stationary phases tailored for the separation of large molecules, the progresses made in SMB process control and the development of chromatographic media cleaning protocols will certainly have an impact on the use of SMB's in the large scale production of high added value products.
# List of Symbols

- $a_p$ specific surface of the adsorbent particles, [1/cm]
- $A$ column cross section, [cm$^2$]
- $c$ number of constraints, [-]
- $c_i, c_i'$ fluid phase concentration of species $i$, related to the stream $j$, [mol/l, mg/ml]
- $c_w$ watershed concentration, [mol/l, mg/ml]
- $C_1, C_2$ constraints, [-]
- $C_i, C_j$ minimum value of the constraints, [-]
- $d$ column diameter, [cm]
- $d_1, d_2, ...$ decision variables
- $D$ desorber flow-rate, [ml/s]
- $D_{1,4}$ axial dispersion coefficient of component $i$, [cm$^2$/s]
- $E$ extract flow-rate, [ml/s]
- $F$ feed flow-rate, [ml/s]
- $F_1, F_2$ functions of the performance indicators, i.e. $F_i(I_i)$
- $H_i$ Henry's constant of component $i$, [-]
- $I_1, I_2$ performance indicators
- $J_1, J_2$ objective functions
- $k_i$ mass transfer coefficient component $i$, [cm/s]
- $K_i$ Langmuir equilibrium constant of component $i$, [ml/mg]
- $L$ column length, [cm]
- $L_o$ gravity settled bed height, [cm]
- $L_f$ loading factor, [-]
- $m$ specific cell maintenance coefficient [g/g]
- $m_j$ flow-rate ratio in section $j$, [-]
- $n_r$ total number of columns, [-]
- $n_j$ number of columns in section $j$, [-]
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<th>Symbol</th>
<th>Definition</th>
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<td>$n_i, n_i^j$</td>
<td>adsorbed phase concentration of species $i$, related to the fluid stream $j$, [mol/l, mg/ml]</td>
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<td>time, [s]</td>
</tr>
<tr>
<td>$t^*$</td>
<td>switch time, [s]</td>
</tr>
<tr>
<td>$T$</td>
<td>throughput, [mg/s]</td>
</tr>
<tr>
<td>$u$</td>
<td>superficial velocity, [cm/s]</td>
</tr>
<tr>
<td>$u_c$</td>
<td>critical velocity, [cm/s]</td>
</tr>
<tr>
<td>$u_j$</td>
<td>superficial velocity in section $j$, [cm/s]</td>
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<tr>
<td>$V$</td>
<td>column volume, [ml]</td>
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<tr>
<td>$V_0$</td>
<td>initial volume, [ml]</td>
</tr>
<tr>
<td>$V_j$</td>
<td>injected volume, [ml]</td>
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<tr>
<td>$V_{j}^{D}, V_{in}^{D}, V_{out}^{D}$</td>
<td>dead volume associated to section $j$, inlet/outlet of a column [ml]</td>
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<tr>
<td>$w_j$</td>
<td>propagation velocity of component A in section $j$, [cm/s]</td>
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<td>$w_j$</td>
<td>shock velocity of component A in section $j$, [cm/s]</td>
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<td>$x$</td>
<td>dimensionless space, [-]</td>
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<td>$X_i^j$</td>
<td>purity of component $i$ in the stream $j$, [-, %]</td>
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<tr>
<td>$y$</td>
<td>dimensionless quantity defined by equation 5.6, [-]</td>
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<td>$Y_i^j$</td>
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<tr>
<td>$Y_{c/s}$</td>
<td>cell yield on carbon substrate [g/g]</td>
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<tr>
<td>$z$</td>
<td>space coordinate, [cm]</td>
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**Greek letters**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>$\alpha$</td>
<td>concentration factor, [-]</td>
</tr>
<tr>
<td>$\gamma_i$</td>
<td>Henry’s constant of component $i$, [-]</td>
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152
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>$\Gamma$</td>
<td>characteristic in the hodograph plane, i.e. image of a simple wave [\text{-}]</td>
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<td>$\delta_j$</td>
<td>denominator of the Langmuir isotherm in section j, [-]</td>
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<tr>
<td>$\varepsilon$</td>
<td>bed void fraction, [-]</td>
</tr>
<tr>
<td>$\varepsilon^*$</td>
<td>overall bed void fraction, [-]</td>
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<tr>
<td>$\varepsilon_{pi}$</td>
<td>intraparticle void fraction related to species $i$, [-]</td>
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<td>empirical constant in equation 5.5, [cm$^3$/s]</td>
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<td>$\theta$</td>
<td>empirical constant in equation 5.5, [cm$^2$/s]</td>
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<td>specific growth rate, [1/s]</td>
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<td>$\phi$</td>
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<td>penalty function parameter in equation 5.6, [-]</td>
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<td>$\Sigma$</td>
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<td>dimensionless time, [\text{-}]</td>
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<td>$\phi$</td>
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<td>$\varphi$</td>
<td>Darcy's law parameter in equation 5.4, [g/(s cm$^5$)]</td>
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<tr>
<td>$\omega^j$</td>
<td>characteristic parameter corresponding to the state $j$, [-]</td>
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Appendix A

Properties of pDNA

Prokaryotic cells, e.g. bacteria, have one single chromosome containing its genetic information. This is a double stranded DNA molecule called chromosomic DNA or cDNA, which is circular in most of the cases. Many bacteria have in addition one or several extrachromosomal genetic elements, called plasmids, that are not essential, but confer an advantage to the cell. The plasmids have autonomous replication capacity and every specific plasmid has a characteristic average number of copies per cell, going from a few in some cases to several hundreds in others. A plasmid can be genetically modified in such a way that an interesting gene can be inserted into it. A gene is a sequence of nucleotides (primary structural units of DNA and RNA) that codifies the necessary information to produce a certain protein, e.g. one having therapeutical interest. This possibility is at the basis of the plasmid-based therapies, where the plasmid is the so-called vector. It conveys the gene of interest to the cells and tissues of the patient, where its expression produces the required protein, exactly where it is needed [35].

Plasmids are covalently closed, double stranded DNA molecules, and like any other nucleic acid, they are formed by nucleotides (monomers) joined by phosphodiester bonds. Each of the phosphoric groups is ionized at pH values above 4, meaning that the net charge of pDNA is negative and
equal to the number of nucleotides constituting the molecule. The two anti-parallel DNA strands constitute the known double helix structure by establishing hydrogen bonds between the complementary nucleotides in each of the strands. The aromatic bases of the nucleotides are oriented towards the inner part resulting in a highly hydrophobic region. In addition, each phosphate residue may form multiple hydrogen bonds with water. The arrangement of the nucleotides leads to the formation of the so-called grooves, which may be crucial for instance in ligand-DNA binding since they enable solvent and ligand molecules to access the nucleotides [45]. The helix axis of pDNA can also be coiled in space constituting a higher ordered molecule rising to different isomers (topoisomers) of which the supercoiled (sc or ccc) pDNA and the open circle (oc) pDNA are the most common. Other variants such as linear, denatured and oligomeric may also be present in cell lysates.

Supercoiled pDNA is more efficient in terms of gene expression and therefore it is preferred for gene delivery [45]. sc pDNA is considered a very dynamic molecule because it can collapse or expand by increasing or decreasing the solvent ionic strength [72], or by changing the type of counter-ions. This dynamic behavior is very useful during the chromatographic purification steps. For instance, a compacting agent like spermidine or spermine [73] may be used to condensate the sc pDNA molecule, this increasing the accessibility of pDNA into the pores of the matrix, and therefore increasing the capacity of the media. Due to the double stranded circular shape, structuring salts affect less the pDNA molecule than the single strand RNA molecule, resulting in a different compacting effect. This feature is used to increase selectivity between pDNA and RNA in size exclusion chromatography [43, 49].

Typically, the physical and chemical properties of pDNA and proteins are compared because most of the macromolecule chromatographic media are designed for protein applications and nevertheless used for pDNA purification purposes [74]. Diffusion coefficients of pDNA in solution are significantly lower than the ones corresponding to proteins. This is due to the difference in mass, size and structure of the molecules. The typical diffusion coefficient of sc pDNA is of the order of $10^{-8}$ cm$^2$/s.

Proteins are not easily damaged by shear fields due to their size and globular shape. They are included in the Kolmogorov microscale of tur-
bulence, which is unaffected by the geometry of the external source providing the motion [40]. Indeed, the larger the size, the greatest the shear induced degradation potential. Protein sizes (i.e. equivalent sphere diameters) range between 2 and 10 nm whereas pDNA molecules are much larger, having an average hydrodynamic diameter of 150-250 nm (for pDNA of 5-10 kb, which is the accepted ideal range for therapeutical purposes). The high molecular weight chromosomal DNA (cDNA) is very sensitive to fluid mechanical forces due to its large size and to the effects of the lysis on its structure. This can be a serious disadvantage for the downstream processing because cDNA fragments of similar size to the pDNA may form due to shear forces. sc pDNA denatures above 12.5 pH units. At pH of around 12, cDNA denatures whereas pDNA remains in solutions [39], giving an ideal range of pH for the separation of cDNA and pDNA during cell lysis.

The high molecular weight of pDNA, and in general of the nucleic acids, yields very viscous solutions [75].
Appendix B

Downstream Processing of pDNA

B.1 Introduction

The unit operations conforming the downstream processing of biological molecules differ from their counterparts in the chemical industry, due to their specific physical and chemical properties. For instance, most bio-molecules, such as proteins, do not stand heat and therefore, cannot be distilled; they have a three dimensional structure which should be maintained along the purification scheme, since very often the activity of the bio-product depends strongly on this 3D structure, etc. The unicellular organisms used to produce bio-products typically range from 1 to 20 microns in size while the macromolecules and the small molecules produced have molecular weights from $10^3$ to $10^6$ and less than $10^3$ Daltons, respectively [76]. The downstream processing of bio-products involves three stages which are generally called primary recovery, intermediate recovery, and final purification or polishing. The first stage aims at separating the cells from the fermentation broth and at releasing the bio-product together with the rest of the cell content; the intermediate
recovery aims at concentrating and already purifying the bio-product; finally, during the polishing stage, several purification steps are used to reach the product quality specifications required.

B.2 Downstream processing

A large scale pDNA manufacturing process needs to be established in order to satisfy the demand of pharmaceutical grade pDNA for clinical trials and eventually for commercialization. The general production process scheme is described in the literature [35, 39, 40].

The downstream processing of plasmids is one of the last stages of the manufacturing process and aims at recovering the plasmid at the purity levels required by regulatory agencies, such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA). The yield of the operation should be as high as possible meaning that ideally 100% recovery of plasmid is achieved.

Usually two types of undesired substances may contaminate the final product. These are firstly contaminants which are external to the cleared lysate system, for instance ligands from chromatographic media, solvent residuals used in the downstream processing etc., and secondly pollutants, which are internal to the cleared lysate system. The main pollutants are RNA, DNA fragments, proteins and endotoxins. In fact, pDNA is only present at maximum 3 (w/w)% in the cleared lysate mixture [37]. In addition, since the supercoiled covalently closed form of pDNA (sc pDNA) is thought to be therapeutically more effective than the open-circular, linear or multimeric forms, all those are considered pollutants and have to be separated. More than 90% of the pDNA should be in the sc form [75]. Typically, a final pDNA product should be free from host chromosomal DNA (cDNA, <0.05 µg/µg pDNA), host proteins (undetectable), RNA (undetectable) and endotoxins (<0.1 EU/µg pDNA) [40].

It is unlikely that only one purification step serves to achieve the high purity specifications required by the authorities. Indeed, most often a combination of purification steps is studied in the literature. Once the
purification steps of the downstream processing are proved to be effective, the overall purification scheme can be optimized in order to maximize the purity and minimize the operation costs. The optimization of the overall purification process sets in turn the optimum operating conditions for each individual step.

B.2.1 Stages in the downstream processing of pDNA

The cell broth from fermentation of E. Coli constitutes the starting material for the downstream processing.

During the primary recovery, the cells are separated from the fermentation broth and plasmid DNA is released from the cells together with the cell content.

Cell harvesting is usually accomplished by centrifugation, filtration or microfiltration. Cells are then re-suspended in a buffer solution containing an agent having the ability of disrupting ionic or hydrogen bonds between lipids and proteins. Typically EDTA is used due to its chelating ability. EDTA removes cation Ca²⁺ and Mg²⁺ from the cell wall and membranes, destabilizing their structures and facilitating lysis. Glucose and Sucrose are often included in the buffer to protect plasmids against shearing [36]. The disruption of the cells is probably the most critical issue of all unit operations in the downstream processing. Cell disruption may be accomplished mechanically (sonication, bead milling, microfluidisation and homogenization), however this kind of techniques usually lead to low pDNA recoveries. Alkaline lysis is the most common procedure. An alkaline solution of a detergent is used, generally NaOH and SDS (sodium dodecyl sulphate). This solution solubilizes the cell membranes and leads to irreversible protein denaturation and lipid precipitation, while keeping the plasmid denaturation reversible. cDNA is partially attached to protein and membrane components of the cells; thus, the precipitation of these components tend to carry the cDNA into the solid phase. The resulting lysate is non Newtonian and highly viscous, making it difficult to handle at large scale. Mixing during lysis is a key issue: firstly because the plasmid denaturates at pH higher than 12.5, and therefore the uniform distribution of pH guarantees the preservation
of the plasmid; and secondly because at the same time the mixing should be gentle enough to avoid the fractionation of cDNA and RNA, since high molecular weight molecules have more ability to be precipitated. After neutralization of the alkaline lysate, the precipitated is formed, and the solids are separated by centrifugation, filtration or flotation followed by filtration.

Lysis may also be performed using a buffer containing detergent (e.g. Triton R) and lysozyme at high temperature (70-80°C) [45, 77]. The use of a continuous heat exchanger may be advantageous at large scale [78]. The presence of endogenous nucleases may be profitable since reduction of RNA with low pDNA losses may be obtained [79]. However, the use of animal derived exogenous enzymes, such as lysozyme and ribonuclease (RNase), is not encouraged by the regulatory agencies; as a consequence, other proteins which are not animal-derived are being investigated [80].

The intermediate recovery aims at concentrating and already purifying the pDNA contained in the stream coming from primary recovery. The intermediate recovery may be by-passed to directly go from the primary recovery to the final purification; however, the life time of chromatography media decreases as well as the column performance; the risk of column clogging increases and at the same time, the cleaning requirements rise.

Typical operations at this stage are the precipitation of pDNA with help of organic solvents or other agents (isopropanol, PEG, etc...), and the precipitation of impurities like proteins, endotoxins, and higher molecular weight RNA using salts such as ammonium sulfate, ammonium acetate, etc [81]. Every precipitation step requires a subsequent centrifugation or filtration step. Other unit operations may be used at this stage, like aqueous two-phase extraction with PEG/K2HPO4 [82] systems, PEG/(NH3)2SO4 systems [83] and others like EO50PO30/Dextran T-500 [41, 84]. Another approach is the use of tangential flow ultrafiltration (UF) in the form of a hollow fiber tube or an open flat plate [85]. The choice of the membrane material has to be made and the pore size, the dimensions of the unit and the operating conditions have to be established in order to optimize the operation and guarantee the integrity of the nucleic acids (that may degrade due to shear forces). A preparative
B.2 Downstream processing

scale Centrifugal Precipitation Chromatography (CPC) method is also reported in recent literature [86].

The scheme of the final purification or polishing, consists of several steps, that are based on chromatographic or non-chromatographic techniques, or a combination thereof [45, 87, 88]. Regardless the methods selected, the final purification ends with a sterilizing filtration of the plasmid DNA [89]. Among the non-chromatographic techniques, aqueous two-phase extraction [82, 84, 90] and separation by membranes [42] and tangential flow filtration [85] are the most popular. Among the chromatographic techniques, particulate fixed bed [37, 43, 91, 92], expanded bed column chromatography [35, 91] and annular chromatography [93] have been investigated, while multi-column processes such as SMB have not been studied extensively so far. Expanded bed chromatography has the advantage of reducing pressure drop and avoiding the clogging problems that may arise when by-passing the intermediate purification steps. The application of monolithic beds in pDNA purification has also been reported in recent literature [46, 94].

Most of the studies reported in the literature investigate one or more of the following chromatographic mechanisms: ion exchange, reversed phase, hydrophobic interaction, size exclusion and affinity chromatography. The separation principle of these techniques is summarized in the following sections.

Anion Exchange (AIEXC). This technique uses the interaction between negatively charged pDNA (the phosphate groups) and stationary phases carrying positively charged functional groups, such as tertiary or quaternary amines. Bound molecules are eluted using an increasing salt gradient in order of increasing charge density. Because the overall charge of nucleic acids depends upon the number of bases constituting the molecule, the expected elution profile follows the order of increasing molecular size. However inversions have been observed in the predicted retention behavior. This suggests that the separation is conformation dependent [35]. The flexibility of the nucleic acids promote a better fit within the pore curvatures enabling more charges to interact with the stationary phase, thus leading to higher retention times.
Some anion exchangers run under certain conditions are able to separate the pDNA topoisomers, due to their different charge density derived from the differences in structure (compaction). AIEX is ideal for removing RNA, oligonucleotides and proteins, whereas cDNA fragments, large RNA fragments and endotoxins [95] may co-purify with the pDNA. The similar binding behavior of these species reduces the capacity of the media. This technique has been investigated extensively in the literature, alone [92, 96] or in combination with other chromatographic mechanisms [37, 46, 77, 87, 94, 97-99].

**Reversed Phase (RPC).** Reversed Phase resins consist typically of alkyl chains of carbons bound to a silica or polymeric matrix. The separation is based on the interaction between the alkyl chains and the hydrophobic groups of the target molecule. Some RPC resins are able to bind pDNA molecules in the presence of inorganic salts. The secondary structure of nucleic acids influences the retention time. The torsion constraint arising from supercoiling makes the bases able to interact with the stationary phase, leading to increasing retention with increasing supercoiling [35]. However most often, the bond is not tight due to the highly charged hydrophilic character of pDNA [88]. This can be altered by adding an ion-pairing reagent as triethylamine. The positively charged amine interacts with the negatively charged pDNA phosphates, neutralizing the charge and coating the molecule with alkyl groups that bind tightly to the resin. This is called ion-pair chromatography. Plasmid is eluted with increasing concentrations of an organic solvent such as acetonitrile or an alcohol [88]. Endotoxins remain in the column and need to be removed during sanitation, typically with 1M NaOH [100]. The use of ion-pairing reagents and organic solvents is an important drawback in ion pair chromatography. Purification methods based on reversed phase and ion chromatography are reported in the literature [77, 100, 101].

**Hydrophobic interaction (HIC).** Matrices carry mildly hydrophobic ligands to separate plasmids from RNA, cDNA, denatured plasmid forms, oligonucleotides and endotoxins. HIC exploits the higher hydrophobicity of single stranded nucleic acids that show a higher exposure of the hydrophobic aromatic bases when compared with double stranded
nucleic acids [45]. The elution of bound species is achieved by decreasing the running buffer salt content (i.e. the conductivity). HIC constitutes a very promising technique for sc pDNA purification because it involves the use of safe reagents. Purification methods based on hydrophobic interaction can be found in the literature [45, 46, 74, 83, 87, 94, 99, 102].

**Size exclusion (SEC).** Molecules are separated on the basis of size or their apparent hydrodynamic radius. Elution is in reversed order of size, with the largest molecules eluting first. SEC is very effective at separating plasmids from RNA, nucleotides, proteins, etc, and may also be used to eliminate salts (buffer exchange). Typically the different pDNA forms exit the column as a broad non-Gaussian peak: large fragments of cDNA may elute as the leading edge, followed by oc pDNA and the sc pDNA [45]. Smaller solutes and salts are separated from the leading DNA peak. The use of high concentrated salt solutions contributes to increase selectivity, due to the larger compacting effect that the salt has on the single strand nucleic acids with respect to the double stranded [43]. Reduction of endotoxin levels during SEC operation has been proved [50]. Currently SEC resins are designed for protein purification, and only a few of them have pores large enough to retain plasmid. Purification methods based on size exclusion can be found in the literature [37, 43-45, 50, 87, 97, 99]

**Affinity (AC).** This technique is based on the recognition of a particular structure in the target plasmid molecule by an immobilized ligand. For instance, triple helix formation between oligonucleotides bound to the matrix and double stranded sequences in the pDNA have been studied [103, 104]. Others used a sequence-specific DNA-binding protein such as the *E.Coli* Lac repressor [105, 106]. These media have at the same time extremely high selectivities and very low versatility, since each ligand targets a specific sequence of nucleotides. The drawback in this case is the large cost of the media, which makes it difficult to upscale.
Appendix C

Cleaning and Sanitation of Chromatographic Media

C.1 Cleaning in place

Any chromatographic operation carried out in the frame of a bio-process requires a cleaning in place (CIP) step. This CIP step aims at maintaining the performance of the column and, in some cases, at guaranteeing the sanitation of the media. Sanitation refers to the elimination of microorganisms that affect the quality of the product. The performance of any chromatographic column can be diminished by clogging (accumulation of insoluble matter) and precipitation of solutes on the stationary phase (accumulation of denatured protein, lipids, etc). The CIP step can itself be responsible for diminishing the performance of the columns when the treatment is too harsh. It is important to follow the pH and solvent compatibility recommendations given by the manufacturer.
C. Cleaning and Sanitation of Chromatographic Media

C.1.1 Testing the performance

Typical performance tests serving to monitor clogging and precipitation of solutes on the stationary phase, are based on measurement of the following variables using a probe molecule:

- **Number of theoretical plates, \( N \)**

  \[
  N = 16 \left( \frac{t_r}{W_b} \right)^2
  \]
  \[
  \text{(C.1)}
  \]

  where \( t_r \) is the retention time of the probe molecule, and \( W_b \) is the width of the peak at the baseline.

- **Height Equivalent to the theoretical plate, \( \text{HETP} \)**

  \[
  \text{HETP} = \frac{L}{N}
  \]
  \[
  \text{(C.2)}
  \]

  where \( L \) is the length of the chromatographic bed.

- **Tailing factor, \( T \)**

  \[
  T = \frac{W_{0.05}}{2f}
  \]
  \[
  \text{(C.3)}
  \]

  where \( W_{0.05} \) is the width of the probe peak at 5% of full height, and \( f \) is the distance from the leading edge to the midpoint of the peak [107].

- **Asymmetry, \( A_s \)**

  \[
  A_s = \frac{b}{a}
  \]
  \[
  \text{(C.4)}
  \]

  where \( a \) is the distance from the leading edge of the peak to the midpoint of the peak, and \( b \) is the distance from the midpoint of the peak to the trailing edge; it is usually determined at 5 or 10% of maximum peak height.

- **Pressure drop, \( \Delta P \)**

- **Yield (in general for the processed target molecule).**
C.2 Sanitation of the media

These measurements are done in the frame of the so-called Reuse study, as defined in section C.4. The probe molecule has to be selected carefully, considering the nature of the stationary phase and the salt content of the buffer solution used to run the tests. The probe molecule may be an internal standard, selected to mimic the target species, or an external standard, which very often does not interact with the stationary phase (e.g. UV absorbing molecules such as acetone, p-aminobenzoic acid (PABA), or conductive species such as NaCl) [107].

In order to assess possible damage of the column during the CIP step, the next variables may be measured during the so-called Sanitation/storage study (whose characteristics are summarized in section C.4), using the feed mixture in which the target molecule is contained, or an internal standard:

- **Binding capacity, BC**

  \[
  BC = \frac{1}{V_c} \int_0^\infty (c_F - c_{out})dV
  \]  

  where \(c_F\) and \(c_{out}\) are the concentrations of solute in the feed solution and at the outlet of the column respectively, \(V\) is the volume of solvent, \(V_c\) is the column volume.

- **Selectivity, S**

  \[
  S = \frac{k_i}{k_j}
  \]  

  where \(k_i\) and \(k_j\) are the retention factors of components i and j.

- **Detection of leachables like hydrolyzed ligands (especially important for affinity or ion-exchange chromatography) [108, 109]**

- **Visual tests by chromatogram comparison: injection of the feed stream at analytical amounts before and after CIP [110].**

C.2 Sanitation of the media

In order to satisfy the product quality specifications, the presence of any contaminant should be avoided. This includes any kind of germ, that
C. Cleaning and Sanitation of Chromatographic Media

could accumulate in the column, grow and pollute the product at any instance during the process. To avoid this possibility, a sanitation step is required in order to regenerate the media. Contamination of the product by leachables issued from the chromatographic media is also possible and has to be investigated. The sanitation step may be accomplished during the CIP step, depending on the selected cleaning agent [110–112].

C.2.1 Type of tests

Sanitation tests include tests on virus clearance, endotoxins, etc., once the CIP step has been carried out. The resistance of the germs against the selected CIP/storage agent has to be tested. A sample of the chromatographic media may also be tested.

C.3 Cleaning and sanitation of the media in the pDNA case-study

C.3.1 Reuse study

Overloaded injections of filtered cleared lysate solution, were performed in one of the SMB columns in order to run a Reuse Study. The buffer conditions are the ones explained in Chapter 4; CIP was not performed at any instance during the study. Between two consecutive injections, the column was equilibrated with the buffer solution and a small volume of a KCl solution was injected. In order to test the performance of the column, the chromatograms (both the cleared lysate and the KCl peaks) were compared and the pressure drop was measured.

After 50 injections, no change on the chromatograms and on the pressure drop of the column was observed.
C.3.2 CIP with NaOH

Sodium Hydroxyde was selected as cleaning agent. Sepharose 6 FF belongs to the family of size exclusion media; thus, no capacity loss or leaking of ligands due to CIP can be expected. The specifications for this media provided by GE Healthcare indicate that the chemical stability of the agarose polymer is guaranteed during seven days at 40°C with 2M NaOH, 70% ethanol, 1% SDS, 8M urea; it is safe to work between 2 and 12 pH units.

The inversion of the flow during cleaning may be beneficial if the clogging or precipitation starts to happen in the column [113]; a specific study on this question could not be found in the literature. In general, if the flow-rate in section 1 of the SMB is properly chosen, the CIP should not be a way of eluting retained/retarded molecules; on the contrary, it should aim to dissolve the precipitated matter (in the former case, the flow reversal could be detrimental, since the trapped molecules could experiment a longer way out of the column than the one corresponding to normal flow direction). The inversion of the flow during CIP was not carried out during the experiments reported in Chapter 4 because this required significant changes in the set-up (namely, the replacement of the check-valves between the columns—see Appendix D—by on/off valves).

It has been reported elsewhere that NaOH or ethanolic acetic acid solutions may be insufficient to inactivate some bacterial strains, and only mixtures of these two are effective [112]. However, the incidence of this cases cannot be evaluated and therefore, for the sake of simplicity, no sanitation study was performed.

C.4 Definitions

- Validation: Establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality [114].
C. Cleaning and Sanitation of Chromatographic Media

- Validation protocol: Written plan stating how validation will be conducted, including test parameters, product characteristics, production equipment, and decision points on what constitutes acceptable test results [114].

- Validation of the performance of a chromatographic process: involves demonstrating that the chromatographic media can be reused, cleaned, and sanitized and that the process, as defined, is robust, and that clearance can be shown for both impurities and adventitious and endogenous viruses [111].

- Standard operation protocols (SOP's): unambiguous set of instructions describing a certain operation or process. Each column in a purification process needs to have SOP's for column packing and testing. Then, once the column is accepted, it needs SOP's for equilibration, load, wash, regeneration and cleaning [107].

- Qualification: is the part of process validation that ensures that equipment and protocols are capable fulfilling the specified requirements [107].

- Qualification of a chromatographic column: involves the characterization of the column and its performance. Can be divided into two parts: hardware qualification (size, manufacturer, resin qualification, work-flow, location in the plant, peripheral equipment, leachables, etc.) and column protocol qualification (stability of the resin during usage, i.e. under equilibration, load, wash, regeneration and cleaning conditions) [107].

- Column bed integrity: assessment on the bed quality. Column bed integrity is measured to confirm the quality and consistency of the chromatographic media. Several measures are commonly used: number of theoretical plates, number of plates per unit length, height equivalent to the theoretical plate (HETP) or reduced plate height, tailing factor and asymmetry [107].

- Column degradation: diminution of the column performance due to aging of the media. The two main sources of potential column degradation are load (precipitation, clogging) and regener-
C.4 Definitions

- Reuse study: (small-scale) series of tests carried out to verify the continued resin performance (column bed integrity) after multiple use [115].

- Characterization study: (small-scale) series of tests carried out to prove that a process is robust and to define acceptable limits of operation [115].

- Sanitation and storage study: (small-scale) series of tests carried out to demonstrate that regeneration is sufficiently sanitizing and that the storage solution is bacteriostatic and fungistatic [115]. May also include analysis of the process performance after sanitation treatment.

- Leachables: contaminants arising from the chromatographic medium itself [110]. Includes ligands and dissolved support molecules.

- Cleaning in place (CIP): is the process to ensure that process lines, vessels and reactors are free of organic contaminants and microorganisms. Involves the use of chemicals, high pressure, etc.

- CIP procedure (or protocol) in chromatography: series of measures adopted during a chromatographic step aiming to clean the columns in order to maintain the performance of the column and maximize or achieve the desired quality of the product eluted during the process (the product quality is defined with regard to sterility, endotoxin content and leachables) [110].

- Sanitation: inactivation of germs by chemical treatment or other [112, 116].

- Storage conditions: solution to which the media is exposed during the time it is not to use. This solution should also be sanitizer.
Appendix D

Flow-sheet of the Experimental CIP-SMB unit
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