Doctoral Thesis

Antibody purification with ion-exchange chromatography

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Antibody Purification With Ion-Exchange Chromatography

A dissertation submitted to ETH ZURICH

for the degree of Doctor of Sciences

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Abstract

In this thesis a detailed characterization of the behavior of a polyclonal IgG on a preparative strong cation exchanger is discussed. The polyclonal mixture is studied using chromatographic and non chromatographic methods. The study showed that the mixture is composed by a very large number of components with different isoelectric points and, therefore, can be separated by ion-exchange chromatography.

The adsorption of the polyclonal mixture is studied using different approaches. First, the mixture is simplified by lumping the different components of the IgG into two "macro-components", referred to as pseudo-variants in the following. An analytical method for the determination of the concentrations of the two pseudo-variants is developed. Based on this, the mass transport and the adsorption isotherm parameters are determined experimentally using only well known sort-cut methods. This analysis is evidencing the difficulty of the determination of these parameters for proteins. The mass transport is very limited and the isotherm strongly dependent on the operating conditions.

In a second approach, the adsorption is characterized using a detailed multi-component pore model, while still considering two pseudo-variants only. This model is explicit in all transport parameters and includes salt dependent isotherms. Linear gradient experiments are used to fit the salt dependent adsorption isotherms and the mass transport parameters for the two pseudo-variants. Using the model, breakthrough curves are predicted with good accuracy. The model is also implemented to visualize the axial and radial concentration profiles of the two pseudo-variants in the column.

The mixture simplification with only two components was, in some cases, not able to reproduce the mixture profile. Therefore a more precise approach is used. The mixture is approximated with a larger number of pseudo-variants. An analytical protocol is proposed, which is able to differentiate between the pseudo-variants without the need of complete resolution on an analytical column. Gradient elution experiments are used to fit the adsorption parameters and breakthrough curves for the six components are predicted using the model.
Porosity strongly affects the mass transport and the adsorption isotherms. This parameter is studied in detail in the last part of this thesis. Changes in pore size distribution are first studied as a function of the salt concentration. Then a new technique to measure on-line the column porosity at different loading conditions is discussed.
Riassunto

In questa tesi il comportamento di un anticorpo policlonale su una resina a scambio ionico è discusso nel dettaglio. La miscela policlonale è studiata utilizzando metodi cromatografici e non cromatografici. Lo studio evidenzia che la miscela è composta da un gran numero di componenti aventi punti isoelettrici diversi e che quindi possono essere separati utilizzando cromatografia a scambio ionico.


In un secondo approccio, l’adsorbimento delle due pseudo-varianti viene caratterizzato utilizzando un modello matematico (“pore model”). Il modello è esplicito riguardo a tutti i parametri di trasporto di massa e include isoterme di adsorbimento dipendenti dalla concentrazione del sale. Gradienti lineari sono utilizzati per la determinazione dei parametri di trasporto di massa e delle isoterme di adsorbimento per le due pseudo-varianti. Utilizzando il modello, curve di sfondamento sono predette con notevole precisione. Il modello è successivamente utilizzato per visualizzare i profili di concentrazione delle due pseudo-varianti all’interno della colonna.

La semplificazione della miscela con solo due componenti è, in alcuni casi, risultata imprecisa. Quindi, un metodo più preciso è utilizzato in seguito. La miscela è approssimata con un numero maggiore di pseudo-varianti. Un protocollo analitico, capace di distinguere le pseudo-varianti senza che esse siano completamente separate su una colonna analitica è presentato. Gradienti lineari sono utilizzati per la determinazione dei parametri d’adsorbimento e curve di sfondamento sono predette utilizzando
La porosità ha un’influenza enorme sul trasporto di massa e sulle isoterme di adsorbimento. Questo parametro è studiato nel dettaglio nella parte finale della tesi. Variazioni della distribuzione dei pori sono studiate inizialmente in funzione della quantità di sale. Successivamente, un nuovo metodo è proposto tramite in quale è possibile misurare "on-line" la porosità in funzione della quantità di proteina caricata sulla resina.
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Chapter 1

Introduction

Target specific drugs like monoclonal antibodies (MAb), are gaining increasing interest for the treatment of different diseases, e.g. cancer and arthritis. As a consequence, their market is steadily increasing. In 2004, approximately 200 antibodies and their derivatives were in clinical trials, whereas 13 had already reached the market [1]. In 2005, 31 monoclonal antibody-based products have been approved for therapeutic or in vivo diagnosis purposes [2]. The total market for the monoclonal antibodies in 2010 is estimated to be of 25 billion dollars [3].

Nowadays, MAbs are mainly produced by mammalian cells. The production process is nowadays highly efficient and titers as high as 5-10 g/L can be reached. As a consequence, the downstreaming part had become the cost determining step, contributing to 50-80 % of the total production costs [1]. The purification is mainly based on chromatographic techniques and, in particular, on the use of protein A affinity resins. The major advantage of this resin is its high selectivity: in fact it specifically binds the constant part of the antibodies. This advantage is however balanced by the very high price of the resin.

A significant amount of work has been done to find alternative methods to protein A. The complex properties of the antibodies, e.g. its charge and hydrophobicity, can be exploited to achieve separation in cation exchange chromatography [4] and hydrophobic interaction chromatography [5], respectively. Both previous properties of the antibody can be used together in the so-called mixed-mode resins. The most promising resins of this family is the hydrophobic charge induction material [6, 7, 8]. Different supports
have been used in addition to the polymer based ones. Native silica [9], silica coated with thiophilic ligands [10], silica coated with ion-exchange ligands [11] and hydroxyapatite [12] have successfully been applied. Finally, it is worth mentioning that the complete purification is typically comprising of different chromatographic steps, where previously mentioned techniques must be combined in order to obtain a complete purification. For instance, a combination of ion exchange and hydrophobic interaction was successfully applied for the purification of a monoclonal antibody from host cell proteins [13, 14]. The removal of host cell proteins was comparable to a protein A process with polishing step [14]. Among the different stationary phases mentioned above, the use of cation exchange chromatography (CIEX) seems particularly interesting due to (i) the cost, (ii) the large chemical stability of the stationary phase and (iii) the possibility of efficiently separate different proteins based on their charge distribution [15].

Due to their large size, the mass transport in conventional stationary phases is particularly hindered, thus often producing very broad peaks and small dynamic binding capacities [16, 17, 18]. In addition to this, the adsorption mechanism of antibody molecules is particularly complex, due to the their equally complex structure. When dealing with ion-exchange chromatography, the adsorption behavior of these molecules can dramatically change as a consequence of small changes in either the ionic strength or the pH of the eluent. Under these conditions, the determination of the isotherm becomes particularly difficult and these difficulties are further increased by the hindered mass transport mentioned before. All these reasons are contributing in explaining the poor knowledge of monoclonal antibodies on ion-exchange columns.

The main objective of this work is then to clarify both the mass transport and the adsorption isotherm of antibodies on ion-exchange resins. This is particularly important for the development of appropriate numerical models for the simulation of these systems. In fact, mathematical modelling is becoming more and more important due to different reasons. The launch of the PAT (Process Analytical Technology) initiative [19] by the FDA (Federal Drug Administration) is forcing biopharmaceutical producers to move towards a more model-based control and monitoring of the production process and therefore also of the purification. Moreover, model based approaches can be effectively employed in the design of purification processes to shorten the design procedure
and to decrease material consumption, thus cutting down the costs. Mathematical modelling is also a very powerful tool for the understanding and the analysis of the adsorption process, as it will be extensively discussed in the Thesis.

1.1 State of the Art

In chromatography, different mechanisms are contributing in determining the overall mass transport of the solutes. Axial diffusion, film mass transport, pore and surface diffusion all contribute to the overall mass transport. However, for large molecules like antibodies, the mass transport is mainly determined by the effective pore diffusivity. The effective pore diffusivity can be determined in two ways: using macroscopic and microscopic methods [20]. The first are based on the direct determination of mass transfer rates from the macroscopic concentration profiles using a model. These methods are easy to implement, but provide only average values for the effective diffusivity. Within the macroscopic methods, isocratic pulse response is the most easy one to implement. This method is based on the assumption that the adsorption isotherm is linear and that the injection pulse is infinitesimal. The effective diffusivity can be calculated from the moments of the elution peak. This method has, however, different limitations: small deviations from linearity of the isotherm can produce large tailing, thus affecting the mass transport determination [21]; the method is very sensitive to the accuracy of the buffer used; and, finally, since the moment determination is affected by baseline drift, experiments should be done under conditions were the elution peaks are fairly symmetrical (i.e. low flow rates). Gradient elution response is also often used to estimate the effective diffusivity. This method has the advantage that also non pure samples can be applied directly. Two sets of experiments are needed: variation of the gradient slope (retention factor determination) and variation of the mobile flow rate. The effective pore diffusivity can be determined from the HETP defined for gradient elution [22, 23]. Frontal analysis experiments is another popular macroscopic method. Here the mass transport is measured in high loading conditions and the results can directly be used for scale-up purposes. It has the disadvantage of requiring large amounts of protein and of being very time consuming. Moreover, an
appropriate chromatography model and the knowledge of the adsorption isotherm are needed. The effective pore diffusivity can be then determined from the solution of the mass balance equations under the assumption of a constant pattern behavior and a constant separation factor isotherm [22].

Batch adsorption experiments can also be applied for the determination of the effective diffusivity. Stirred batch adsorption [24, 25, 26, 27, 28, 29] is one of these methods. Here the effective diffusivity is determined from uptake curves. Shallow bed adsorption is another alternative. Here the same procedure as for the frontal analysis method is used, but using a column containing only few adsorbent particles. The advantage is the smaller consumption of proteins. In general, batch methods suffer from the disadvantage that the effective diffusivity is measured in a different hydrodynamic environment than in the chromatography column.

In the case of microscopic methods, the effective pore diffusivity is determined from the intraparticle concentration profiles. The advantage is that the effective diffusivity can be determined directly as a function of protein concentration and that no adsorption mechanism has to be assumed. However, complicated equipments are needed, the analysis is limited to optical clear matrix and fluorescent labelling is often required. In addition to this, impurities or preferential adsorption of native proteins can cause interferences. The visualization of the concentration profile can be done either by confocal microscopy [30, 31, 32, 33, 34, 35] or by light microscopy [36, 37, 38, 39, 40].

The determination of the effective pore diffusivity is made even more complex by the fact that it strongly depends on the operating conditions. Different results show the influence of the protein concentration on the effective pore diffusivity. Chang and Lenhoff determined the effective diffusivities of lysozyme in a set of preparative strong cation-exchange stationary phases based on different base matrices [27]. In their study, the experimental data from batch uptake data in a stirred vessel were fitted with either the pore diffusion model or the homogeneous diffusion model. The estimated pore diffusivities decreased with increasing protein concentration. The authors proposed that protein-protein interactions and pore constrictions resulting from protein adsorption contribute to this effect. Other authors have also reported diminishing diffusivity with increasing protein concentration on different stationary phases [18, 41, 42]. Melter
et.al. used a regression technique to determine the effective diffusivity of a monoclonal antibody on a weak cation exchanger [15]. They also found a decreasing diffusivity for increasing protein loading.

Controversial results have been reported with respect to the effect of ionic strength on pore diffusion. Axelsson et al. investigated protein diffusion in agarose gel and reported a decrease of the pore diffusion coefficient based on the neutralization of the electrostatic forces between protein molecules, which results in a shielding effect of the protein charges [43]. This agrees with the results of protein diffusion in solution [44, 45]. The opposite effect, i.e. an increase in diffusivity with increasing salt concentration was measured in other studies [27] and it is probably due to the increase of the pore size with increasing ionic strength.

The determination of the effective diffusivity for multicomponent protein system has been investigated only by few authors. Two-component adsorption kinetics on the agarose based ion-exchanger SP-Sepharose-FF was investigated by Skidmore and Chase [24]. Carta et al. [46] studied the adsorption kinetics of mixtures of cytochrome C and lysozyme on the same resin by spectrophotometry. Two component protein adsorption on a different resin, HyperD-M, was investigated by Lewus and Carta [47]. The resin consisted in porous silica particles filled with a gel. Smooth intra-particle concentration profiles were observed in all those gel-type structures for single and multi-component systems [48]. Melter et.al. studied the multi-component competitive adsorption of three monoclonal antibody variants on the preparative cation-exchanger, Fractogel EMD COO− (S) [49]. Despite these contributions, the measurements of the effective pore diffusivity for proteins remain a very difficult task and the understanding of the mass transport process of large molecules is still very poor.

Different experimental techniques for the determination of the adsorption isotherm can be found in literature [50]. The batch method is the most well known among these methods. A known amount of adsorbent is equilibrated in a closed vessel with a solution possessing a known initial concentration of solute. At equilibrium, the solute concentration in the liquid phase is determined and from a mass balance, the adsorbed amount is calculated. To construct the adsorption isotherm, several experiments have
to be performed changing the initial solute concentration and the adsorbent quantity. The advantage of this method is, however, the low solute consumption. The drawbacks are the high amount of work needed and the low accuracy. Another well known method is frontal analysis. In frontal analysis, the column is loaded with a protein solution with known concentration, until the feed concentration is reached at the outlet of the column. From the time at which the adsorption front reaches the column outlet and the solute concentration, the amount adsorbed on the column can be calculated. An identical result can be obtained by regenerating the equilibrated column and collecting the elution fraction. In order to save solute, frontal analysis can be applied also to very small column. This method is often referred to as shallow bed adsorption [51]. With successive frontal analysis experiments, the complete isotherm can be determined. Frontal analysis can be applied also for the determination of multi component adsorption isotherms [52], but this method is very time and solute consuming. Perturbation method is also frequently applied [53, 54, 55, 56]. The column is equilibrated with different solute concentrations. Then, a small perturbation is introduced in the system. The retention time of the perturbation gives information about the local total derivative of the isotherm ($\partial q_i / \partial c_i$). Applying the method to different feed concentrations, a set of derivatives can be determined. From these derivatives the isotherm can be found by integration. The main advantage of this method is that no detector calibration is needed, but it has the same drawbacks as frontal analysis. Elution by characteristic point can also be applied to determine the adsorption isotherm [57]. This method is limited to very efficient columns, thus exhibiting very fast mass transfer. For these columns, equilibrium theory can be used to analyze the dispersed fronts in overloaded chromatograms [50]. However, this method can be seldom applied to proteins due to their slow mass transport. The last methods discussed here is peak fitting [58, 59, 60, 61, 62]. In this method a mathematical model is used to fit experimental profiles under overloaded conditions. The isotherm model has, however, to be known a priori.

It is important to note that for each method described above, the specific porosity of the solute investigated has to be known. Errors in the porosity determination leads to erroneous isotherm determination [50]. The determination of the porosity is
in principle a trivial experiment: in the so-called inverse size exclusion chromatography (iSEC), a tracer has to be injected under non adsorption conditions and from its retention time the porosity can be calculated. The selection of the tracer is instead not trivial. This has not to adsorb under the experimental conditions studied, has to be easily detectable and must have a well defined size. Polymer tracers are usually applied, for which correlations between mass and size have been developed.

Different isotherms have been proposed to describe the adsorption of proteins in ion exchange resins [63]. The most important ones are the Langmuir isotherm [64] and the steric mass action isotherm [65]. The mass action isotherm takes into account the competition for adsorption between the solute and the salt and is subject to the following assumptions: (i) the multipointed nature of the protein can be reduced to a single characteristic charge; (ii) competitive binding can be expressed by a mass action equilibrium where electro-neutrality on the stationary phase is maintained; and (iii) the binding of large molecules causes steric hindrance of salt counter ions bound to the surface. These sites are then unavailable for adsorption. The effect of co-ions is neglected and the isotherm parameters are assumed to be constant. The Langmuir isotherm assumes a monolayer adsorption on an energetically homogeneous surface. Moreover, it is assumed that adsorbed molecules are not affecting the adsorption of other molecules. This adsorption isotherm is very commonly used to describe the adsorption behavior of simple molecules under isocratic conditions. Many authors have shown that for constant pH and salt concentration, the adsorption behavior of proteins is well described by Langmuir-type isotherms [40, 66, 67, 68, 69]. Moreover, it has been demonstrated that the mass action law in its simplest form can be reduced to linear adsorption equilibrium if the salt concentration is much larger than that of the solute. In this case, a salt dependent expression of the Henry coefficient can be derived [70]. Both isotherms have successfully been applied for single components.

The determination of multi-component adsorption equilibrium isotherm is more complicated. It typically includes three steps, namely (i) the determination of the adsorption isotherm of the single components, (ii) the extension to the multi-component case and (iii) the choice of suitable techniques to regress and validate the developed
multi-component adsorption isotherm. One possibility when characterizing multi-component systems is in fact to first determine single-component adsorption isotherms and then derive from these the behavior of the multi-component system. For this, some assumptions about the competitive behavior of the various components is needed in order to define the multi-component equilibrium models. In the case of thermodynamically consistent systems, this operation can lead to a precise characterization of the adsorption behavior of the mixture [71]. However, in general this procedure may result in predictions only of limited accuracy [50]. There are a number of models to describe the complex behavior of multi-component adsorption isotherms, but the determination of the corresponding parameters still remains laborious [72]. For instance, Hashim et al. [73] could only obtain an accurate prediction of the adsorption equilibrium data of two solutes using empirical parameters, because the amount of available adsorption sites depended on the feed composition. For some systems, the competitive Langmuir isotherm [74] is a simple and valid alternative to the complex isotherm described above. This isotherm considers only the diminishing of the available adsorption sites due to the presence of the other components, but neglects all other interactions (e.g. solute-solute). The multi component Langmuir isotherm is valid only for very similar components with almost equal saturation capacities.

Different models have been proposed for the description of a chromatographic column [63]. The most comprehensive model present in literature is the so-called general rate model (GRM) [75, 76, 77, 78]. In this model, the concentration profile along the column axis is coupled with the description of the profile along the particle radius. Accordingly, it is possible to detaily describe the mass transport in the particles due to liquid and solid diffusion, as well as the adsorption kinetics. The model is comprising of two sets of partial differential equations (PDEs) for the concentration profiles in the liquid phases and one ordinary differential equation (ODE) for the mass balance in the solid. A first simplification of the model can be obtained by neglecting pore diffusion inside the particles. The resulting model is often referred to as lumped pore model [79] and the concentration profile along the particle radius is substituted by an average particle concentration and a linear driving force for mass transport across the
particle surface is assumed. Therefore, the PDE corresponding to the particle mass balance is substituted by an ODE. A further simplification is obtained by lumping all non-equilibrium mechanisms inside the particle due to both transport and kinetics into a single kinetic coefficient. In addition to this, all the different porosities are grouped into a single total accessible porosity of the solute. The corresponding model is referred to as kinetic model [75] and it involves the solution of a PDE (concentration profile along the column axis) and an ODE (concentration in the solid phase). If the kinetics in the kinetic model is sufficiently fast, a further simplification can be introduced to obtain the so-called equilibrium-dispersive model [80, 81, 82]. Here, equilibrium is assumed between the liquid and the solid phases and all transport resistances are lumped into a single apparent axial dispersion. A single set of PDEs is then obtained. In all previous cases, only numerical solutions are possible, with the exception of the moment analysis, for which analytical solutions are available (for diluted conditions). If also the remaining axial dispersion is neglected, thus obtaining an ideal column behavior, the so-called ideal model [83, 84, 85] is obtained, for which analytical solutions can be obtained with the method of characteristics [86, 87]. All previous models, with the exception of the more general one (GRM), cannot be applied in general to proteins, where pore diffusion is playing a primary role in determining the column behavior [88].

Polymer based ion-exchange resins are often the only choice in the separation of biomolecules. In fact, GMP rules require column sanitization with sodium hydroxide, which is not practical in silica based columns [89, 90]. In this work, Fractogel SE HiCap is considered. This resin is made of a methacrylate based polymeric resin with an hydrophilic surface. The functional sulfoethyl-groups are located on so-called tentacles, that is long polymer chains bound to the surface [91]. The high selectivity and capacity of this material, where non-specific solute-matrix interactions are minimized, makes it promising with respect to the separation of very similar compounds with very small charge differences.

In this work, monoclonal antibodies are replaced by a polyclonal antibody mixture. Polyclonal antibodies are produced by the B-cells (a kind of white blood cell) in the
blood. Each B-cell clone produces a different antibody. Since in the human body there are thousands of B-cell clones, a polyclonal antibody is a mixture of many, slightly different, antibodies. Immunoglobulin G, which is the most abundant antibody present in the plasma, is composed by a constant part, called Fc-domain, and a variable part, called Fab-domain (refer to Figure 1.1. The Fc-domain represents the bottom of the Y shaped IgG molecule and it is identical in all antibodies. The Fab-domain is the upper part of the molecule and its structure changes depending on the target antigen. Polyclonal IgG are used against immunodeficiency and to treat autoimmune and inflammatory diseases [92]. They are industrially obtained from human blood plasma by ethanol precipitation or by chromatography separation [93]. The main advantage of polyclonal antibodies is that they are much cheaper than monoclonal antibodies and are easily available (several tons of them are produced every year). This makes them very suited for adsorption studies. In addition to their use as model system for MAbs, the polyclonal IgG mixture is a very challenging system for chromatography.
1.2 Outline

In the following the structure of the Thesis is outlined. In Chapter 2 the adsorption of the polyclonal IgG mixture on a strong cation exchanger column is characterized experimentally using analytical ion exchange and size exclusion columns. Following this analysis, the mixture has been approximated by considering two pseudo-variants only. An analytical procedure for the determination of the concentration of the two pseudo-variants is developed. The general behavior of the PAb mixture on the preparative IEX column is described. Shortcut methods are utilized for a first determination of the isotherm and the mass transport parameters.

In Chapter 3 the experiments of previous chapter are utilized for the determination of the adsorption parameters by peak fitting. A two-component pore model is presented. The model considers explicitly all contributions to mass transport. Moreover, salt dependent adsorption isotherm are used. Isocratic experiments under non adsorption condition and at different flow rates are used for the determination of the column porosity and mass transport parameters. Diluted and overloaded gradients are then used for the determination of the salt dependent isotherms. Using the regressed parameters, breakthrough curves are predicted. Moreover, the concentration profiles of the two pseudo-variants along the column axis and the particle radius are discussed.

Chapter 4 presents a more detailed analysis of the adsorption of the polyclonal mixture. Six pseudo-variants are used to reproduce the mixture profiles. A method is developed to determine the concentration of the six pseudo-variants without the need of complete resolution on the analytical column. A multi-component pore model is used to fit the single profiles of the six components. Linear and overloaded gradients are used to determine the six salt dependent isotherms and the mass transport parameters. Breakthrough curves of the single components are predicted using the model.

The effect of the operating conditions on porosity is studied in detail in Chapter 5. The change in porosity during linear gradient elution experiments and during loading experiments is studied. Polyvinylpyrrolidone tracers are used to measure the porosity as a function of the salt concentration in the buffer and as a function of the adsorbed amount of two model proteins.
Chapter 2

Experimental Characterization of the Adsorption

2.1 Introduction

In general, large proteins like antibodies often exhibit peculiar behaviors on conventional stationary phases for chromatography. Due to their large molecular size, a large fraction of the total column porosity is not accessible to these molecules. This has a large impact on the transport inside the particles, which, as a consequence, is typically very slow, and on the total capacity of this columns, which is then small. In turn, these two effects are influencing typical chromatographic operations, such as loading and separation. In the first case, the loading can be very inefficient and the so-called dynamic binding capacity very small compared to the static capacity of the column. In the second case, the purification may also be very inefficient, since the severe transport limitations of such columns are broadening the outlet peaks and reduce peak resolution.

The thermodynamic behavior of mono- and polyclonal antibodies is also very peculiar. The competition for adsorption with salts is very strong and the antibody affinity towards the stationary phase can dramatically change as result of little variations in the salt content. In addition to this, the protein net charge can considerably change in response to pH variations, thus also affecting the affinity of the antibody. The characterization of this behavior is generally very complex and made even more problematic due to two additional facts: (i) the presence of strong transport limitations, which are
considerably deforming the shapes of the peaks and make the application of typical methods for isotherm characterization very problematic; (ii) the presence of several variants in the same clone or, as in the case of this work, the presence of different clones, that is of a broad range of molecules with different adsorption behaviors.

In this chapter a detailed characterization of the behavior of a commercially available PAb on a commercial cation exchange chromatographic column is discussed. In particular, the characterization will proceed in different steps: first, a characterization of the PAb and of the column structure is presented. Then, it will be studied the mass transport and the adsorption of the PAb. In addition to this work, techniques to reduce the complexity of the system will be also discussed, where the different clones and variants composing the PAb are lumped into a limited number of pseudo-variants, i.e. in homogeneous pseudo-components.

2.2 Materials

2.2.1 Stationary Phase and Columns

The resin is packed, following the instruction of the manufacturer, into an Unicorn (50x5 mm) glas column (GE Healthcare Bio-Science AB, Sweden). The packed column resulted in a bed height of 4.3 cm, which corresponds to a volume of 0.83 mL (20 % resin compression). The small volume of the column is needed to run loading experiments without using too much protein. The main properties of Fractogel SE HiCap, as given by the manufacturer, are summarized in Table 2.1.

An analytical weak cation exchanger column (100x4 mm, ProPac WCX-10 column from Dionex) and a size exclusion column (300x78 mm, TSK-GEL G3000SWXL column from Tosoh bioscience) with guard column (40x6 mm, SWXL) are used for the analysis of the polyclonal IgG mixture. Isoelectric focusing experiments are performed using a PhastGel IEF 3-10 gel (GE Healthcare Bio-Science AB, Sweden) stained with PhastGel Blue R stain (GE Healthcare Bio-Science AB, Sweden).
2.2. Materials

<table>
<thead>
<tr>
<th>column</th>
<th>Fractogel EMD SE HiCap (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>matrix</td>
<td>crosslinked PMA</td>
</tr>
<tr>
<td>functional group</td>
<td>sulfoethyl group</td>
</tr>
<tr>
<td>mean particle size</td>
<td>65 µm</td>
</tr>
<tr>
<td>pore size</td>
<td>800 Å</td>
</tr>
<tr>
<td>binding capacity (lysozyme)</td>
<td>150 mg/ml</td>
</tr>
</tbody>
</table>

Table 2.1: Properties of the strong cation exchange resin used in this work.

2.2.2 Mobile Phase and Chemicals

Experiments under no adsorption conditions are run using 50 mM phosphate buffer at pH = 7. The phosphate buffer is prepared mixing sodium dihydrogen phosphate (Fluka, Switzerland), disodium hydrogen phosphate (Lancaster, England) and sodium chloride (J.T. Baker, USA). Each component of the buffers is exactly weighted using a precision balance (METTLER AT250, Mettler-Toledo, Switzerland). Adsorption experiments are run using a 20 mM sodium acetate buffer at pH=5. This buffer was made mixing sodium acetate (Merck, Germany) and acetic acid (Carlo Erba reagents, Italy). Sodium chloride was used as modifier changing the solution ionic strength. Inverse size exclusion experiments are carried out using dextran standards (Sigma-Aldrich, Switzerland). The mass transport coefficients are measured using: the polyclonal IgG mixture (Gammanorm, Octapharma, Switzerland), human serum albumin (Sigma, Switzerland), myoglobin (Sigma, Switzerland) and acetone (J.T. Baker, Holland). Water is filtered through a Millipore Synergy system before use. All chemicals are "pro analysis" grade and all solutions are degassed and filtered through a 45 µm filter before use.

2.2.3 Chromatography Equipment

The experiments are performed using an Agilent 1100 Series HPLC, equipped with a quaternary gradient pump, an autosampler and a temperate two column switch. The detection is done by a diode array detector and a refractive index detector. The column outlet can be fractionated using a Gilson FC203B fraction collector.
2. Experimental Characterization of the Adsorption

2.3 Methods

2.3.1 Column Porosity

The knowledge of the column porosity is essential for the prediction of the solute elution times and to understand the origin of the mass transport limitations. The complete column pore size distribution is measured by inverse size exclusion chromatography (iSEC) [94]. According to this procedure, tracers of different molecular weight are injected under non retention conditions and the total liquid accessible volume, \( V_{t,i} \), is measured. The total porosity is then defined as [95]:

\[
\varepsilon_{t,i} = \frac{V_{t,i}}{V_c}
\]  

(2.1)

where \( V_c \) is the volume of the column. The average retention volume \( V_{t,i} \) is calculated from the first order moment of the elution peak. The particle volume accessed, \( V_{p,i} \), with respect to the particle volume, \( V_p \), is referred to as particle porosity:

\[
\varepsilon_{p,i} = \frac{V_{p,i}}{V_p} = \frac{V_{p,i}}{(1 - \varepsilon_b) V_c}
\]  

(2.2)

where \( \varepsilon_b \) is the bed porosity, that is the total porosity accessed by a molecule entirely excluded from the particle pores. Therefore, the total and the particle porosity are correlated to each other by the following equation:

\[
\varepsilon_{t,i} = \varepsilon_{p,i} \cdot (1 - \varepsilon_b) + \varepsilon_b
\]  

(2.3)

Using tracers with different dimension, the so called inverse size exclusion chromatography curve is constructed, where the total porosity is plotted against the logarithm of the hydrodynamic diameter of the different tracers [94]. Two limits can be identified: the column free volume, \( \varepsilon_t \), that is the volume accessed by a tracer so small to enter every pore; and the bed volume, \( \varepsilon_b \), that is the volume accessed by those tracers entirely excluded by all particle pores.

2.3.2 Mass Transport

Mass transport in the particle pores can be very slow for large molecules, as antibodies, and has a very strong influence on the shape of the eluting peaks. As discussed in the
2.3. Methods

Introduction, different methods have been developed for the determination of the pore diffusivity. These methods can be divided into two classes: off-line and on-line methods. Off-line methods, e.g. confocal (or light) microscopy or uptake experiments [32, 40, 96], use unpacked resin. The on-line methods are instead applied directly on the packed chromatography column [46]. These have the advantage of measuring the parameters in the same hydrodynamic conditions as during the chromatography experiment. Note that in case of adsorption (even in the simple case of linear adsorption), the mass transport determination requires the knowledge of the adsorption isotherm. As discussed later, this is difficult to be precisely measured, due to the complexity of the PAab mixture. Therefore, it resulted convenient to carry out experiments under non-adsorbing conditions, so to not introduce an additional degree of uncertainty in the estimation of the mass transport parameters.

A popular method to characterize mass transport limitations inside a packed bed of porous particle relies on the measure of the so-called height equivalent of a theoretical plate (HETP). This procedure is based on the simplified description of a column into a number of identical equilibrium plates, introduced by Synge et. al. in 1941 [97]. The HETP is representative for the separation capacity of a chromatographic column and it is providing a normalized measure of the elution peak variance. The HETP can be calculated from the characteristics of the elution peak as in the following:

\[
HETP = \frac{\mu_2}{(\mu_1)^2} L
\]

where \(\mu_1\) and \(\mu_2\) are the first and the centered second moment of the elution peak, i.e. the retention time and the peak standard deviation respectively.

The HETP value can be conveniently expressed in terms of the physical parameters governing the mass transport inside the particles. This can be done by considering the solution of the general rate model (GRM), which is the most comprehensive model for chromatography and which accounts explicitly for all different contributions to the mass transfer resistance. From the first two moments of the solution of the GRM equations developed by Kubin and Kucera [98, 99], the HETP can be written for conditions of no adsorption as [63]:

\[
HETP = \frac{2D_{ax}}{u_{int}} + \frac{2d_p}{F} \left( \frac{F\varepsilon_p}{1 + F\varepsilon_p} \right)^2 \left[ \frac{1}{6k_f} + \frac{d_p}{60D_{eff}} \right] u_{int}
\]
where $D_{ax}$ is the axial diffusion coefficient of the column, $u_{int}$ the interstitial velocity, $\varepsilon_p$ the particle porosity, $d_p$ the particle diameter, $F = \varepsilon_b/(1 - \varepsilon_b)$ the phase ratio, $\varepsilon_b$ the bed porosity, $D_{eff}^p$ the effective diffusion coefficient in the pores and $k_f$ the film mass transport coefficient.

The axial diffusion can be calculated assuming that hindered molecular diffusion and eddy diffusion are additive [80]:

$$D_{ax} = 0.7D_m + u_{int}R_p$$

(2.6)

As it will be discussed in the following, the first term in the r.h.s. of Equation 2.5, accounting for the axial dispersion, is negligible and the HETP value becomes then linearly dependent on the interstitial velocity $u_{int}$. The slope is comprising of two terms. The first one is expressing the transport limitations in the external laminar diffusion layer of the particle. The corresponding film mass transport coefficient can be estimated from the equation of Wilson and Geankoplis [100]:

$$k_f = \frac{D_m 1.09 \left( \frac{u_s d_p}{D_m} \right)^{1/3}}{d_p \varepsilon_b}$$

(2.7)

where $u_s$ is the linear velocity and $D_m$ is the molecular diffusion coefficient. This can be calculated from the equation by Young et al [101]:

$$D_m = 8.31 \times 10^{-8} \frac{T}{\eta_b M^{1/3}}$$

(2.8)

where $T$ is the absolute temperature, $\eta_b$ the solvent viscosity and $M$ the molecular weight of the solute.

Film resistances are generally negligible with respect to those inside the particle pores, due to the very low effective pore diffusivity, $D_{eff}^p$. This is correlated to the molecular diffusion coefficient by the following equation:

$$D_{eff}^p = \frac{K_p \varepsilon_p D_m}{\tau}$$

(2.9)

where $K_p$ is the hindrance factor and $\tau$ the tortuosity.

### 2.3.3 Adsorption Isotherm

Different approaches have been used for the description of the adsorption isotherm of proteins on ion-exchange resins [70]: the law of mass action [65], the Donnan potential [102] and different others empirical correlations [103, 104, 105, 106]. Although some
of these isotherms are describing the adsorption in a correct mechanistical way, they require the determination of many physical parameters. The Langmuir isotherm \[64\] represents a convenient alternative. Even if it has no mechanistical justification outside of the linear region of the isotherm \[70\], this isotherm is frequently used because it needs the determination of only two parameters for each component. The multi-component competitive Langmuir isotherm can be written as \[63\]:

\[
q_{ieq} = \frac{H_i c_{ieq}}{1 + \sum_{j=1}^{n} \frac{H_j q_{ieq}}{c_{eqj}}}
\]  

\(q_{ieq}\) and \(c_{ieq}\) are the equilibrium concentrations of the i-th component in the solid and in the liquid phase, respectively. \(H_i\) is the Henry coefficient and \(q_{i\infty}\) the saturation capacity \[50, 63\]. These two parameters are strong function of the ionic strength in ion-exchange. Here, the following dependencies are assumed:

\[
H_i = \alpha_i I_m^{\beta_i} \\
q_{i\infty} = \gamma_i - \delta_i I_m
\]  

The parameters \(\alpha_i, \beta_i, \gamma_i\) and \(\delta_i\) are constants. The expression for the Henry coefficient (Equation 2.11) can be theoretically justified from the mass action law imposing electroneutrality and very diluted conditions \[70\]. The use of a ionic strength dependent saturation capacity, instead, has no theoretical justification and is used here to account for the changes in saturation capacity observed by many authors for different ionic strengths values \[35, 40, 107, 108, 109, 110\]. The values of \(\alpha_i\) and \(\beta_i\) can be determined from isocratic experiments at different ionic strengths or from linear gradient elution experiments applying the method of Yamamoto \[111\]. For many proteins the isocratic experiments are very difficult to apply due to the very strong dependence of the Henry constant from the ionic strength. The method of Yamamoto is therefore very often used. For the determination of \(\gamma_i\) and \(\delta_i\), the saturation capacities of the two pure components have to be determined as a function of the salt concentration. This is done by frontal analysis of the pure components.

**Yamamoto method**

Yamamoto and co-workers \[106, 111\] presented a simple graphical method for the determination of the peak elution time under linear gradient elution chromatography. As-
2. Experimental Characterization of the Adsorption

Assuming a power dependence between the distribution coefficient and the ionic strength, the parameters $\alpha_i$ and $\beta_i$ can be graphically determined by running LGE experiments with different gradient steepnesses [106]. This procedure becomes particularly simple if it is assumed that the solute Henry coefficient is tending to that of the salt for large salt concentrations. In this case, if the logarithm of the conductivity at the peak maximum and the normalized gradient slope are plotted together, the two parameters can be determined from the slope and the intercept of the line regressing the experimental data [70]. The logarithm of the gradient slope is related to the logarithm of the conductivity by the following equation:

$$\log(GH) = \log\left(\frac{I_R^{(\beta+1)}}{\alpha(\beta+1)}\right)$$

(2.13)

$GH$ is the gradient slope normalized with respect to the column stationary phase volume: $GH = g(V_t - V_0)$, $g$ the gradient slope in concentration/volume, $V_t$ the total column volume and $V_0$ the column void volume; $I_R$ is the conductivity at the peak maximum. According to Yamamoto, the parameter $\beta$ can be related to the number of charges involved in the adsorption and $\alpha$ to the ion exchanger capacity. The function relating the Henry constant to the ionic strength can then be calculated from:

$$H_i = \alpha_i I_m^{-\beta_i} + H_s$$

(2.14)

$H_s$ is the Henry constant of salt. This parameter is usually much smaller than the Henry constant of the protein and is therefore neglected in the following.

**Equilibrium capacity**

The equilibrium capacity of a chromatographic resin can be measured by frontal analysis. The column is loaded with the mixture until a constant concentration of all components is reached at the column outlet. The equilibrium capacity can then be estimated in two ways: by integration of the breakthrough curve (BTC) [95] or by eluting the adsorbed amount and determining the concentrations of each component. In this work, the second approach is preferred. In fact, the elution concentration is often slowly reaching a plateau, making it difficult to estimate the value of the signal at complete breakthrough. Due to the long duration of the breakthrough experiments, small mistakes in the estimation of the plateau-value can turn into large mistakes in the
evaluation of the equilibrium capacity. The elution fraction is collected and the concentration of the components determined. Knowing the volume of the elution fraction, the masses of all the components, adsorbed on the column \( (m_{ads,i}) \) can be calculated. The equilibrium capacity of component \( i \) can then be calculated as:

\[
q_{eq,i} = \frac{m_{ads,i} - (c_{f,i}V_c\varepsilon_i + c_{f,i}V_d)}{V_c(1 - \varepsilon_{tot})}.
\] (2.15)

The term \( (c_{f,i}V_c\varepsilon_i + c_{f,i}V_d) \) represents the amount of component \( i \) present in the liquid phase in the column and in the apparatus respectively.

In the case of a multi-component isotherm, the equilibrium capacities are function of the ionic strength and of the concentrations of all components present in the mixture. In general, the saturation capacity of each component has to be determined. In our case, it is not practical to fractionate the polyclonal mixture and measure the single saturation capacities independently. Since the clones are very similar to each other, it is reasonable to assume that they have the same saturation capacity.

### 2.4 Results and Discussion

#### 2.4.1 Column characterization

The pore size distribution of the column plays a determinant role in defining the column behavior and performance. In fact, it is determining the pore accessibility of the solute and, in turn, both the effective diffusion rate and the surface available for adsorption. The pore size distribution of the material was measured by inverse size exclusion chromatography, using dextrans standards at two different ionic strengths. This is shown in Figure 2.1, where the total porosity of the tracers is plotted versus the logarithm of the corresponding hydrodynamic radius. Tracers characteristics are summarized in Table 2.2. All measurements were performed under non adsorption conditions using a 50mM phosphate buffer, pH=7 and at two salt concentrations. From Figure 2.1 the bed porosity and the column total porosity, corresponding to the two asymptotes of the S-curve, can be observed (0.39 and 0.83, respectively, at \( I_m = 0.52 \text{ M} \)). The total porosity for IgG, HSA and myoglobin has been extrapolated from the iSEC curve at \( I_m = 0.52\text{M}: 0.56, 0.66 \text{ and } 0.80 \), respectively. The following diameters were assumed:
<table>
<thead>
<tr>
<th>Tracer</th>
<th>Molecular Weight [Da]</th>
<th>Hydrodynamic diameter * [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran 1</td>
<td>1200</td>
<td>1.8</td>
</tr>
<tr>
<td>Dextran 6</td>
<td>6000</td>
<td>4.1</td>
</tr>
<tr>
<td>Dextran 9</td>
<td>9300</td>
<td>5.1</td>
</tr>
<tr>
<td>Dextran 40</td>
<td>40000</td>
<td>10.6</td>
</tr>
<tr>
<td>Dextran 56</td>
<td>56000</td>
<td>12.5</td>
</tr>
<tr>
<td>Dextran 70</td>
<td>70100</td>
<td>14.4</td>
</tr>
<tr>
<td>Dextran 110</td>
<td>110000</td>
<td>17.5</td>
</tr>
<tr>
<td>Dextran 200</td>
<td>200000</td>
<td>23.7</td>
</tr>
<tr>
<td>Dextran 380</td>
<td>380000</td>
<td>32.6</td>
</tr>
<tr>
<td>Dextran 710</td>
<td>710000</td>
<td>44.5</td>
</tr>
</tbody>
</table>

Table 2.2: Dextran tracers used for the iSEC experiments. *: calculated correlating the data from DePhillips et al [94].

Figure 2.1: Total porosity vs logarithm of the hydrodynamic diameter. The open symbols represent the measurements at $I_m = 0.52M$, whereas the full symbols measurements at $I_m = 0.02M$. The extrapolated porosity for IgG, HSA and myoglobin (from right to left) are plotted as open triangles.
### Table 2.3: Bed porosity, column total porosity and extrapolated total porosities for the proteins at two ionic strengths.

<table>
<thead>
<tr>
<th>Porosity</th>
<th>( I_m = 0.02\text{M} )</th>
<th>( I_m = 0.52\text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \varepsilon_b )</td>
<td>0.37</td>
<td>0.39</td>
</tr>
<tr>
<td>( \varepsilon_t )</td>
<td>0.81</td>
<td>0.83</td>
</tr>
<tr>
<td>( \varepsilon_{t,\text{acetone}} )</td>
<td>-</td>
<td>0.83</td>
</tr>
<tr>
<td>( \varepsilon_{t,\text{myoglobin}} )</td>
<td>-</td>
<td>0.80</td>
</tr>
<tr>
<td>( \varepsilon_{t,\text{HSA}} )</td>
<td>-</td>
<td>0.66</td>
</tr>
<tr>
<td>( \varepsilon_{t,\text{IgG}} )</td>
<td>-</td>
<td>0.56</td>
</tr>
</tbody>
</table>

IgG \( d=11\text{ nm} \) [112], HSA \( d=7\text{ nm} \) [113] and myoglobin \( d=3.8\text{ nm} \) [114]. For acetone the total porosity is assumed to be equal to the column total porosity. The porosity values are summarized in Table 2.3. From the Figure 2.1 and the Table 2.3 it can be seen that the porosity is only slightly affected by the ionic strength. This effect will be discussed in detail in Chapter 5. From the total porosity, the particle porosity, i.e. the accessible particle void fraction, can be calculated from Equation 2.3. HSA and IgG can access only 44 and 28% of the particle volume, respectively. This means that only a small part of the particle surface is effectively used for IgG adsorption. Moreover, from the relatively large exclusion of IgG from the particle pores it can be already foreseen that the transport inside the pores of this solute will be particularly hindered as discussed in the following.

#### 2.4.2 Polyclonal IgG Mixture Characterization

The undiluted polyclonal IgG mixture contains 95% of human IgG with in a concentration of 165 mg/ml [115]. Four IgG subclasses are present in the mixture in the following percentages: IgG1: 59%, IgG2: 36%, IgG3: 4.5% and IgG4: 0.5%. IgA is also present, but in very small amounts (0.05%). Some excipient are also present in the mixture: glycine, sodium chloride, sodium acetate and water. Due to the simultaneous presence of four subclasses, each of which is likely to contain many variants, a very large number of components is expected in the original mixture.
In order to better characterize the polyclonal mixture, this has been analyzed by chromatography, using size exclusion and weak cation exchanger analytical columns. The first experiment is aimed to reveal the presence of dimers or fragments of IgG, whereas the second allows us to see if the different subclasses and variants can be separated by their charge. In addition to this, isoelectric focusing (IEF) has been carried out to reveal the presence of variances with different pI values.

**Size exclusion chromatography**

The size exclusion experiment of the PAb mixture was run using a 25mM sodium phosphate buffer with 0.1M sodium sulfate at pH=7. This is shown in Figure 2.2, where it can be observed that two components of different size are present in the mixture. From the calibration of the SEC column (not shown), it can be estimated that the two peaks in the chromatogram correspond to IgG monomer and dimer respectively and that around 20 % of dimers are present in the mixture.

![Figure 2.2: Polyclonal IgG mixture injected on the size exclusion column.](image-url)
2.4. Results and Discussion

Figure 2.3: Isoelectric focusing of the polyclonal mixture. Lane 1: pI marker (IEF Calibration Kit Board pI 3-10), Lane 2: polyclonal mixture.

**Isoelectric focusing**

The PAb mixture has been analyzed by IEF in order to analyze the pI of the different components of the mixture. This is shown in Figure 2.3. It can be observed that Gammanorm is comprising of a continuous range of components whose pI value is ranging in between about 6.5 and 10. As discussed by Melter et al. [116], who has studied the separation of three monoclonal antibody variances on both analytical and preparative columns, cation exchange resins can provide an outstanding resolution power in the presence of even smaller pI differences. Therefore, it is expected that the use of ion-exchange chromatography can be very effective in separating the Gammanorm variances.

**Analytical cation exchanger column**

The analytical cation exchanger analysis aims to differentiate between molecules with different charge. The column used is described in the Experimental part. The mixture was eluted with a linear gradient, where the ionic strength was changed from 0.07M to 0.52M in 40min (25 CV). Fractions have been taken every minute, reinjected and eluted with the same protocol. Note that different injections volumes have been tested in order
Figure 2.4: Linear gradient elution of the polyclonal IgG mixture on the analytical weak cation exchanger column. The elution profile was fractionated in 1 minute intervals and the fractions reinjected. The dashed curve represents the concentration of the elution buffer. The ionic strength is constant at 0.07 M for 5 minutes then increases to 0.52 M in 40 min.

to determine the injection limits under which the peak is eluted under linear adsorption conditions, i.e. where no peak shift is observed. The elution profile of the mixture and of all the fraction on the analytical column are shown in Figure 2.4. From this figure it can be seen that the peak width of each fraction (solid curves) is much narrower than that of the original mixture (dashed curve). This is confirming the presence of a large number of components, as already evidenced by the IEF experiment (cf Figure 2.3). Moreover, it is showing the outstanding resolution power of the analytical column. By decreasing the fractionation interval, even more peaks can be separated. Clearly, the separation of the variants is limited by the resolution power of the column (that is, by the peak width), which is not infinite, and it is reasonable to assume that each peak of Figure 2.4 also contains many variants.
Even if the complexity of the original mixture is reduced to a limited number of pseudo-variants (i.e., a collection of IgG variants that, due to the similarity in behavior, can be assimilated to a single homogeneous component) as shown in Figure 2.4, the analytical burden remains considerable. However, looking at the shape of the main peak of Figure 2.4, it is possible to observe a shoulder in the peak front. This allows us to suppose that the overall mixture is made of two main pseudo-variants, corresponding to the shoulder and to the main peak, respectively, which in the following will be considered single components. In order to verify this possibility, fractions 1 to 4 were merged together producing the first pseudo-variant, whereas fractions 5 to 14 were merged producing the second pseudo-variant. In Figure 2.5, the outlet concentration profile on the analytical column corresponding to the polyclonal mixture and the two pseudo-variants is shown. The merged fractions correspond with a good approximation with the shoulder and the main peak of the polyclonal mixture, respectively. Figure 2.6 shows the size exclusion chromatogram of the two pseudo-variants. As it can be seen, both pseudo-variants contain both monomers and dimers. This demonstrates that the pseudo-variants are not corresponding to dimer and monomer. On the other hand, it can be observed in Figure 2.7, where the IEF of the two pseudo-variants is shown, that the two pseudo-variants are instead characterized by clearly pI ranges (see lanes 2 and 3), thus explaining the different elution time on the IEX column. For the characterization of the elution profiles on the strong cation exchanger column used in this work, an analytical procedure, allowing us to simply resolve the two pseudo-variants is needed. From the Figure 2.5 it can be seen that the first pseudo-variant elutes after about 17 min. This elution time corresponds to an ionic strength in the outlet of 0.18 M. The second pseudo-variant elutes at higher ionic strength. In order to separate the two pseudo-variants the following elution protocol is then used: an isocratic phase at an ionic strength of 0.18 M lasting for 4 min (2 CV) is followed by a gradient going from 0.18 M to 0.82 M in 9 min (5 CV). The corresponding elution profiles are shown in Figure 2.8. As it can be seen from Figure 2.8, the two pseudo-variants can be resolved by the proposed elution protocol. The second pseudo-variant (gray curve) elutes almost completely (more that 90 %) during the gradient, whereas the first variant (black curve) instead elutes mostly (more than 80 %) during the
Figure 2.5: Linear gradient elution of the polyclonal IgG mixture on the analytical weak cation exchanger column. The elution profile of the pseudo-variant 1 (black curve) and 2 (gray curve) are shown. The dashed curve represents the concentration of the elution buffer. The ionic strength is constant at 0.07 M for 5 minutes then increases to 0.52 M in 40 min.
2.4. Results and Discussion

Figure 2.6: Polyclonal IgG mixture (dashed curve), pseudo-variant one (black curve) and pseudo-variant two (gray curve), injected on the size exclusion column.

Figure 2.7: Isoelectric focusing of the two pseudo-variants. Lane 1: pI marker (IEF Calibration Kit Board pI 3-10), Lane 2: Pseudo-variants 1, Lane 3 pseudo-variant 2.
Figure 2.8: Analytical elution protocol applied to the polyclonal IgG mixture (dashed curve) as well as to the pseudo-variant one (black curve) and two (gray curve). The line represents the concentration of the elution buffer. The ionic strength is constant at 0.18 M for 4 minutes then increases to 0.82 M in 9 min.
isocratic phase.

The proposed analytical method is therefore able to fractionate the original mixture in two macro pseudo-variants with reasonable precision, small analytical effort and time consumption. The elution profile of polyclonal mixture is also shown in Figure 2.8 (dashed curve). According to this analytical tool the mixture contains 23% of pseudo-variant 1 and 77% of pseudo-variant 2. The analytical method was also applied to the monomer and dimers isolated from the SEC experiment as it is shown in Figure 2.9. It can be seen that both the monomer (gray curve) and the dimers (black curve) are made of variants eluting in the isocratic region and in the gradient, as it is the case for the original mixture (dashed curve). Comparing Figure 2.8 and 2.9, it is interesting to notice the different elution behavior in the isocratic region (i.e. for elution times shorter than 5 min). In Figure 2.8, where the two pseudo-variants are separately injected, two peaks always elute. In Figure 2.9, where monomers and dimers from the SEC column are separately injected, the dimer is completely in the second peak, whereas the monomer shows only a very small second peak. Accordingly, it can be concluded that the two peaks of Figure 2.8 in the isocratic region are corresponding to monomers and dimers respectively. No appreciable difference can be observed in the peak eluted during the gradient. In spite of this, the relative composition of monomer and dimer is not matching the one measured by SEC. Moreover, the presence of the small peak in the monomer injection seems to indicate that (i) monomers can also be eluted in the second peak or (ii) partial dimerization is taking place in the column under these conditions. This point is currently under further investigation. However, as also discussed in Figure 2.5 and 2.7, the dominant characteristic distinguishing the two pseudo-variants is the different pI value and not the size. Accordingly, the presence of dimers in the mixture will be ignored in the following. Figure 2.10 shows the elution profile of the polyclonal mixture as well as of the two pseudo-variants on the preparative Fractogel SE HiCap column. This experiment confirms that the elution order of the two pseudo-variants is the same as on the analytical column. The experimental protocol presented in this section is used as an analytical tool for the determination of the concentration of the two pseudo-variants in the further experiments.
Figure 2.9: Analytical elution protocol applied to the polyclonal IgG mixture (dashed curve) as well as to the dimers (black curve) and to the monomers (gray curve). The line represents the concentration of the elution buffer. The ionic strength is constant at 0.18 M for 4 minutes then increases to 0.82 M in 9 min.
2.4. Results and Discussion

Figure 2.10: Polyclonal IgG mixture (dashed curve) and pseudo-variant one (black curve) and two (gray curve) injected in the strong cation exchanger column. The dashed curve represents the concentration of the elution buffer. The ionic strength is constant at 0.07 M for 5 minutes then increases to 0.52 M in 40 min.
2.4.3 Mass Transfer Effects

Under non-adsorption conditions, the mass transport parameters can be determined from the shape of the elution profiles. It is assumed that in these conditions all the IgG subclasses and variants are behaving in the same way, so that the mixture can be treated as a single pure component. This is implying that all the species have the same dimension and that the effect of dimers on the estimated mass transport coefficients is negligible. The mass transport parameters are determined for the IgG mixture as well as for HSA. Isocratic elution under non adsorbing conditions are performed at different interstitial velocities (ranging from 0.02 to 0.31 cm/s) and are shown in Figures 2.11. It can be seen that, as the interstitial velocity increases (that is in the direction of the arrow), the peaks become more and more tailed and the peak maximum is moving to the left. This behavior is typical of system characterized by strong mass transport limitations. For large velocities, the peak front elutes at a volume corresponding to dead volume of the column. Under these conditions, in fact, the characteristic time for diffusion inside the particles becomes much larger than the characteristic time for convective transport along the column. This behavior is stronger for larger molecules, whose pore diffusion rate inside the particles becomes smaller.

The HETP was calculated from the first and the second statistical moments of the elution peak (Equation 2.4). This procedure is applied to the elution peaks of IgG, HSA, myoglobin, acetone. The corresponding HETP values are plotted as a function of the interstitial velocity in Figure 2.12. The slope of the HETP vs interstitial velocity curve is inversely proportional to the effective diffusivity in the pores. In fact, looking at Figure 2.12, we can see that the slope is increasing for increasing molecule dimension (acetone, myoglobin, HSA and IgG). By applying Equation 2.5 and estimating the film mass transfer coefficient from Equations 2.7 and 2.8, the effective diffusivity in the pores can be evaluated, which is summarized in Table 2.4. The effective pore diffusivity is decreasing for increasing dimension of the tracer following the expected behavior. Very small values have been estimated from Figure 2.12 for the two biggest solutes, HSA and IgG, which are about 10 and 20 times smaller than the estimated value of the molecular diffusivity, respectively. This is however expected for such large molecules and is also confirmed by other authors: Stone and Carta [40] found $D_{p}^{\text{eff}} = 4.7 \times 10^{-8}$ cm$^2$/s for BSA.
Figure 2.11: Polyclonal IgG (a) and HSA (b) injected under isocratic and non adsorbing conditions. The interstitial velocity was varied from 0.02 to 0.31 cm/s, the arrow shows the direction of increasing velocity.
36  2. Experimental Characterization of the Adsorption

Figure 2.12: HETP plotted as a function of interstitial velocity for IgG (full triangles), HSA (open triangles), myoglobin (full squares) and acetone (open squares).

on SP Sepharose FF and Boyer et al [16] measured $D_{\text{eff}}^p = 2.5 \times 10^{-7}$ cm$^2$/s for myoglobin, $D_{\text{eff}}^p = 5.6 \times 10^{-8}$ cm$^2$/s for HSA and $D_{\text{eff}}^p = 2.3 \times 10^{-8}$ cm$^2$/s for IgG on Sepharose CL-6B. These literature values are very close to the values found in this work and the difference is negligible considering the different materials used by the other authors.

Using the calculated parameters, an analysis about the relative importance of the different contributions to the mass transport of IgG can be done. It is convenient to refer to the HETP equation (Equation 2.5). The HETP is composed by two terms: one accounting for axial diffusion and one for diffusion into the particle. The latter term is comprising of the film mass transport and the effective pore diffusion. In our case, it can be estimated from Equation 2.5 that the film mass transport term $(1/6k_f)$ is about 85 s/cm, whereas the effective pore diffusion term $(d_p/60D_{\text{eff}}^p)$ is about 4500 s/cm. This shows the governing role of the effective pore diffusivity with respect to the film mass transport. Then, the axial diffusion term can be compared to the pore diffusion term. At $u_{\text{int}} = 0.16$ cm/s, the first term $(2D_{\text{ax}}/u_{\text{int}})$ is in the order of $5 \cdot 10^{-3}$
2.4. Results and Discussion

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$D_m \text{ [cm}^2\text{/s]}$</th>
<th>$k_f \text{ [cm/s]}$</th>
<th>$D_{\text{eff}} \text{ [cm}^2\text{/s]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>$5.99 \times 10^{-6}$</td>
<td>$1.09 \times 10^{-2}$</td>
<td>$1.09 \times 10^{-6}$</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>$9.07 \times 10^{-7}$</td>
<td>$3.10 \times 10^{-3}$</td>
<td>$1.60 \times 10^{-7}$</td>
</tr>
<tr>
<td>HSA</td>
<td>$6.02 \times 10^{-7}$</td>
<td>$2.36 \times 10^{-3}$</td>
<td>$5.69 \times 10^{-8}$</td>
</tr>
<tr>
<td>IgG</td>
<td>$4.50 \times 10^{-7}$</td>
<td>$1.95 \times 10^{-3}$</td>
<td>$2.31 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

Table 2.4: Molecular diffusivity, film mass transfer coefficient and pore diffusivity calculated for the four molecules investigated. *: calculated at $u_{\text{int}} = 0.16 \text{ cm/s}$.

...cm, whereas the second about $5 \times 10^{-1} \text{ cm}$. The term accounting for the axial diffusion is therefore negligible, thus confirming that HETP is dominated by pore diffusion and, thus, proportional to the interstitial velocity, as discussed in the Methods section.

An interesting result is found plotting the effective pore diffusivity as a function of the molecular diffusivity multiplied by the particle porosity. As it can be seen from Figure 2.13, the experimental points fall on a straight line. Comparing this result with the expression of Equation 2.9, it can be inferred that the term $K_p/\tau$ is constant. For molecules with increasing diameter, the hindrance is expected to increase and, thus, the term $K_p$ to decrease. Accordingly, also the tortuosity must decrease with increasing molecular size. It can be speculated that this effect is due to strong pore exclusion measured for large molecules, i.e. by the fact that larger molecules cannot access all the smallest pores and, therefore, their path must be more ”regular”.

2.4.4 Isotherm Determination

As discussed above, the polyclonal mixture, can be approximated by a two-component mixture. For the two components, the Henry constants as a function of the ionic strength and the equilibrium capacities at three ionic strengths are measured in the following.

Linear gradient elution experiments

Figure 2.14 shows injections of the original polyclonal mixture (solid curves) as well as of the first pseudo-variant (dashed curve) on the strong cation exchanger column eluted...
by linear gradients with increasing slope (following the arrow). After the injection the ionic strength is kept constant at 0.07 M for 20 min (12 CV). Then a gradient is started, which reaches a final ionic strength of 1.02M. The duration of the gradient is varied between 120 (75 CV) and 240 min (150 CV). The first pseudo-variant has been obtained by fractionating the original PAb mixture by applying the analytical method described previously. The elution profiles of the mixture in Figure 2.14 exhibits the typical shoulder already observed on the analytical column. The steeper the gradient, the slimmer is the peak, due to the self-sharpening effect of the gradient [70]. The first pseudo-variant is always eluted under the shoulder of the main peak, confirming that a clear cut in the original PAb has been made.

**Henry vs ionic strength, Yamamoto method**

The parameters relating the Henry constant to the ionic strength can be determined by plotting the logarithm of the normalized gradient slope (GH) versus the ionic strength
Figure 2.14: Linear gradient elution experiments of the polyclonal IgG mixture (solid line) as well as of the first pseudo-variant (dashed line) on Fractogel SE HiCap. The calibrated UV signal is plotted against elution volume. The ionic strength is constant at 0.07 M for 20 minutes, then increased to 1.02 M in 120, 180, 200 and 240 minutes respectively (the arrow shows the direction of increasing gradient steepness).
Figure 2.15: Logarithm of the normalized gradient slope (GH) versus the ionic strength at the peak maximum ($I_R$) for the two pseudo-variants. Pseudo-variant 1: open symbols, Pseudo-variant 2: full symbols at the peak maximum ($I_R$). This is shown in Figure 2.15 for the two pseudo-variants of Figure 2.14. The second pseudo-variant is assumed to have the same peak maximum as that of the mixture. From the expression of the straight line regressing the experimental points, the parameters $\alpha_i$ and $\beta_i$ can be determined (see Table 2.5). The corresponding Henry coefficient as a function of the ionic strength can be calculated applying Equation 2.14 and are shown in Figure 2.16. Two things must be noted from this picture. First, the Henry coefficients of the two pseudo-variants are a strong function of the ionic strength of the eluent. For this reason, it is practically impossible to carry out isocratic experiments in order to estimate the Henry coefficients. In fact, small experimental errors in the eluent ionic strength would result in large errors in the Henry coefficient and, thus, in the elution time. In addition to this, the Henry coefficient becomes rapidly very large by decreasing the salt concentration and, therefore, the elution time becomes very soon very long. This effect, summed to the large
Table 2.5: $\alpha$ and $\beta$ parameters calculated with the Yamamoto method for the two components.

<table>
<thead>
<tr>
<th>Pseudo-variant 1</th>
<th>$\alpha_i$ [-]</th>
<th>$\beta_i$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudo-variant 1</td>
<td>$1.39 \times 10^{-2}$</td>
<td>3.63</td>
</tr>
<tr>
<td>Pseudo-variant 2</td>
<td>$3.30 \times 10^{-4}$</td>
<td>6.27</td>
</tr>
</tbody>
</table>

Figure 2.16: Henry constant as a function of the ionic strength for the two pseudo-variants. Pseudo-variant 1: dashed curve, Pseudo-variant 2: full curve
mass transfer resistances, which are becoming more important for large affinity values, is greatly deforming the elution peak and makes it very broad. Under such conditions, the errors in the estimation of the peak moments are too large.

The second observation is that the Yamamoto method is, as expected, predicting two different values of $\beta$, i.e. of the steepness of the two curves of Figure 2.16. As discussed above, this can be correlated to the presence of different net charges of the two pseudo-variants. This was also evidenced by the IEF experiment of Figure 2.7.

The diverging behavior of the two curves is important under loading conditions, i.e. at low salt concentrations. Under these conditions, the selectivity of the two pseudo-variants becomes very large and this should lead to a pronounced displacement effect in the presence of concentration shocks.

Frontal analysis

The non linear part of the PAb adsorption isotherm is investigated by carrying out frontal analysis. The column is loaded with the protein solution under isocratic conditions using three different ionic strengths. The liquid at the column outlet is fractionated and the concentration of the two components is measured using the described analytical method. Figures 2.17, 2.18 and 2.19 show the breakthrough curves measured at $I_m = 0.07$, 0.12 and 0.17 M respectively. The experiments were run at flow rate equal to 0.12 ml/min. After the loading, the column was eluted with a buffer containing 0.5 M NaCl and the elution fraction is collected. Before the next experiment, the column was cleaned in place with 10 column volumes of a 0.25 M NaOH/water solution. The column was successively equilibrated for 20 CV using the same buffer solution used for the following BTC experiment. At $I_m = 0.07$ (Figure 2.17) the two pseudo-variant are very strongly adsorbing (see the Henry function of Figure 2.16). Therefore, it can be supposed that the column is fully saturated. In spite of this, the two pseudo-variants have very different affinity, thus causing a strong displacement of the less retained one, as observed in Figure 2.17 by the presence of a maximum in the elution profile of the first pseudo-variant (open squares). Note that the overall outlet concentration (full triangles) is reaching a plateau value after about 50 CVs, whereas the competition between the two pseudo-variants is lasting till 100 CVs are eluted.
Figure 2.17: Breakthrough curve of the polyclonal IgG mixture on Fractogel SE HiCap. The ionic strength is equal to 0.07 M and the feed concentration to 3.2g/L. The open and the full squares represent the concentrations of first and the second component respectively. The full triangles represent the sum of the two components.
Figure 2.18: Breakthrough curve of of the polyclonal IgG mixture on Fractogel SE HiCap. The ionic strength is equal to 0.12 M and the feed concentration to 3.3 g/L. The open and the full squares represent the concentrations of first and the second component respectively. The full triangles represent the sum of the two components.
Figure 2.19: Breakthrough curve of the polyclonal IgG mixture on Fractogel SE HiCap. The ionic strength is equal to 0.17 M and the feed concentration to 2.85 g/L. The open and the full squares represent the concentrations of first and the second component respectively. The full triangles represent the sum of the two components.
At $I_m = 0.12$ (Figure 2.18), the behavior of the mixture is more complex. Keeping in mind that the mixture is made of a very large number of components and not only two pseudo-variants, as exemplified in this work, it can be noted from Figure 2.16 that the first pseudo-variant has a relatively small Henry value, while the second one a much larger one. As a result, the first pseudo-variant is breaking-through very early, possibly also due to the competition of the second variant. When this is eluting, the displacement of the first variant takes place, although this is less pronounced than in Figure 2.17. At high ionic strength ($I_m = 0.17$, Figure 2.19), the two components are both adsorbing very weakly. Accordingly, they are immediately eluting and almost no competition can be seen.

**Equilibrium capacity vs. ionic strength**

From the previous BTC experiments, it is possible to compute the equilibrium capacity of the two pseudo-variants as a function of the ionic strength in the eluent. This has been done by collecting the adsorbed antibody and by analyzing the relative quantities of the two pseudo-variants as described in the Experimental part. The result is plotted in Figure 2.20 and the corresponding values are summarized in Table 2.6. Note that the reported values of capacity are referring to the amount of solid volume. It can be seen that the equilibrium capacity of the two components is linearly decreasing with the ionic strength. Moreover, due to the lower affinity of the first pseudo-variant and the resulting displacement by the second one, the equilibrium capacity of the first pseudo-variant is very low. It is worth noting that in spite of the very early breakthrough at $I_m = 0.17$ M, the second pseudo-variant still has a reasonable equilibrium capacity, while the second one is practically non-adsorbing. The very early breakthrough can be probably explained with the strong diffusion limitations. In other words, the so-called dynamic capacity is small.

**Overloaded experiments**

The characterization of the PAb is finalized by carrying out some overloaded experiments. The results are shown in Figure 2.21 for a gradient of 20 min length (12 CV) and in Figure 2.22 for a gradient of 40 min length (24 CV). During the elution, differ-
2.4. Results and Discussion

<table>
<thead>
<tr>
<th>Ionic strength [M]</th>
<th>$c_1^{eq}[g/L]$</th>
<th>$c_2^{eq}[g/L]$</th>
<th>$q_1^{eq}[g/L]$</th>
<th>$q_2^{eq}[g/L]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>0.79</td>
<td>2.43</td>
<td>54.9</td>
<td>861.1</td>
</tr>
<tr>
<td>0.12</td>
<td>0.82</td>
<td>2.49</td>
<td>11.7</td>
<td>573</td>
</tr>
<tr>
<td>0.17</td>
<td>0.70</td>
<td>2.15</td>
<td>9.8</td>
<td>287</td>
</tr>
</tbody>
</table>

Table 2.6: Equilibrium capacities of the two pseudo-variants on Fractogel SE HiCap. The equilibrium capacities are given per mL of resin.

Figure 2.20: Equilibrium capacities (per solid volume) as a function of the ionic strength.
Figure 2.21: Overloaded linear gradient elution experiments of the polyclonal mixture on Fractogel SE HiCap. The polyclonal mixture (dashed curve and full triangles) and the first pseudo-variant (full curve and open squares) are shown. The gradient length is 20 minutes and increasing amounts are injected: 41, 89, 110 mg

Different fractions are taken and analyzed using the described analytical method. In both figures we can see that the peak front is shifted to the left as the injection volume is increased, while the peak back is always eluting at the same time. This is coherent with the theory for a (competitive) Langmuir isotherm. The shapes of the profiles are however deformed by the slow mass transport and the shock in the front is cancelled by the presence of dominant mass transport resistances. A similar displacement can be observed for the first pseudo-variant, although the broadness of the peak induced by the mass transport limitations and the actual presence of many variants is making impossible to conclude more about the behavior of this pseudo-variant.
Figure 2.22: Overloaded linear gradient elution experiments of the polyclonal mixture on Fractogel SE HiCap. The polyclonal mixture (dashed curve and full triangles) and the first pseudo-variant (full curve and open squares) are shown. The gradient length is 40 minutes. Increasing amounts are injected: 38, 79, 119 mg
2. Experimental Characterization of the Adsorption

2.5 Conclusions

The adsorption of a polyclonal IgG mixture on a preparative strong cation exchange column was studied in detail. First, the mixture was characterized using isoelectric focusing, size exclusion and cation exchange chromatography. The study evidenced the presence of many components, which are covering a broad and almost continuous spectrum of pI values. These components cannot be fully separated by preparative cation exchange. However, diluted linear gradient elution experiments are showing the presence of a shoulder in the elution profile of the mixture. This was then supposed to be comprised of two pseudo-variants only, corresponding to the shoulder and the main peak, respectively, and an analytical method to distinguish between the two was developed.

The behavior of the two pseudo-variants was studied in detail using short-cut methods. First, the column porosity was characterized by iSEC. The measurements showed a very limited pore accessibility for the two pseudo-variants. Mass transport parameters were then determined by Van Deemter experiments under non adsorbing conditions. It was shown that the main mass transport resistance is located in the particle pores. In particular, pore diffusion was found to be very hindered and its characteristic time comparable to the column residence time.

The linear part of adsorption isotherm was measured by diluted linear gradient elution experiments. The method of Yamamoto, applied separately for the two pseudo-components, indicated that the Henry coefficient strongly depends on ionic strength of the eluent. This dependence is stronger for the more retained pseudo-variant, due to the larger pI value. Since the two Henry functions are diverging, selectivity is expected to increase for smaller values of the ionic strength. The non linear part of the adsorption isotherm was investigated by frontal analysis. The breakthrough profiles of the two pseudo-variant were measured at three different values of the ionic strength. As expected, at low ionic strength, strong competition between the two variants was found. Most interestingly, the competition between the two pseudo-variants was found to continue also after that the column was fully saturated, i.e. a plateau value in the outlet total concentration was reached. From the three breakthrough curves, the equilibrium capacities for the two components were calculated and was found to linearly
2.5. Conclusions

decreasing with the ionic strength.

The characterization was finalized by overloaded linear gradient experiments. As expected, the overloaded peaks were moving towards shorter elution times for increasing injection masses. However, the shape of the profiles did not show any concentration shock in the peak front and, on the contrary, the peaks always remained rather symmetrical. This is due to the strong mass transport limitations.

The short-cut methods applied in this work allowed a simple and effective characterization of the behavior of the polyclonal antibody mixture. It is believed that most of the observations in this work can be readily applied to single monoclonal antibodies. In addition to this, it is worth to be noticed that in monoclonal antibodies produced by cell culture are very often present different variants, which are frequently inactive or even toxic and, therefore, have to be separated. Accordingly, the problems discussed in this work are closely resembling those typical of a monoclonal antibody purification. However, the complexity of this problem can be properly tackled only with the aim of a numerical model. This requires a more precise estimation of the mass transport and adsorption isotherm parameters, which the short-cut methods discussed in this work are not able to provide. This will be done in the following chapter, where all the parameters will be regressed using a suitable numerical model.
Chapter 3

Mathematical Modelling of the Adsorption

3.1 Introduction

The results of the previous chapter, have enlightened the complex behavior of the PAb mixture in conventional cation exchange supports. Short-cut methods can be hardly used to precisely estimate all the physicochemical parameters involved in the process. In fact, the nature of the adsorption isotherm and the severe mass transport limitations are making the system extremely sensitive to small errors in the parameters, so that their use in a numerical model would lead to largely inaccurate model forecasts. For this reason, it is often preferable to directly regress the parameters using the numerical model.

Two main approaches can be found in the literature to carry out the regression of the parameters needed to simulate the behavior of a chromatographic column: regression of batch uptake experiments or regression of elution profiles. In the first approach, the isotherm is determined directly from batch adsorption data and the mass transport is found byregressing of protein uptake experiments. This approach has been widely used for the adsorption of proteins on ion exchanger materials [26, 27, 29, 41, 46, 66, 117, 118, 119, 51]. Another option for the determination of the mass transport parameters is to use microscopic techniques to visualize the protein front moving to the center of the particles as the adsorption takes place. This can be done either by confocal microscopy
3. Mathematical Modelling of the Adsorption

[31, 32, 33] or by light microscopy [40]. However, these so-called "off-line" methods have the disadvantage of measuring the adsorption parameters in a different fluid dynamic environment than of the chromatographic column. This can be avoided when using the so-called "on-line" methods. Here the isotherm and the mass transport parameters are found by regression of elution profiles. This method has also been widely used for the adsorption of proteins on ion-exchange columns [17, 24, 120, 121, 122, 123].

For the simulation of the adsorption of large molecules like proteins, a very comprehensive model is needed. The most complete model present in literature is the so-called general rate model (GRM), where the concentration distribution of the different solutes in both the axial and the particle radial directions is accounted for [63]. Due to its complexity and the large number of differential equations involved, this model has gained importance in the last years due to the strong increase of computational power of the modern computers. It must be pointed out that the use of this model is mandatory in the presence of dominant mass transport resistances, as discussed by Kaczmarski et al. [88].

In this chapter, the parameter regression of the elution profiles is applied for a complete characterization (i.e. under diluted and overloaded conditions) of the adsorption of the polyclonal IgG on the preparative strong cation exchanger column. Note that the aim of this chapter is not only to estimate the relevant parameters to run the model simulation, but to use the model as a powerful tool for the understanding of the adsorption process. As it will be discussed in the following, through the model only it is possible to capture the full complexity of the system and the behaviors observed in the previous chapter.

As described in Chapter 2 of this Thesis, although the original polyclonal antibody mixture is made by a very large number of different antibodies, it can be approximated by considering only two so-called pseudo-variants, which in the following will be considered as single components. In order to simplify the regression procedure, the experiments were designed in such a way that as few parameter as possible are fitted together. The rationale behind the proposed regression procedure will be discussed in this work.
3.2 Model Development

The preparative separation of large molecules like proteins involve complex adsorption mechanism and slow mass transfer [63]. In order to achieve an accurate prediction of the elution profile a complete model is needed, which includes all contributions to the mass transport in the chromatographic column. The GRM is accounting for the concentration changes along the column axis and the particle radius. In this regard, the following assumptions are made:

- transport is taking place by convection and diffusion in the mobile phase, i.e. in the inter-particle voids; transport is purely by diffusion in the intra-particle voids, the so-called stagnant phase;
- packing is uniform. Therefore, all porosities are constant;
- there is no concentration gradient along the column radius;
- particles have spherical symmetry;
- transport inside the particle is due to diffusion in the liquid phase only. Solid diffusion is not accounted for;
- the adsorption process is always at equilibrium. Adsorption kinetics is neglected.

Considering the previous set of assumption, a model consisting of two sets of mass balance equations, for the mobile and stagnant liquid phase, respectively, can be written. The mass balance for the i-th component in the mobile phase is:

\[
\frac{\partial c_i}{\partial t} + u_{\text{int}} \frac{\partial c_i}{\partial z} + \varepsilon_{p,i} \left(1 - \varepsilon_b\right) \frac{k_{f,i}}{R_p} \left[c_i - c_{p,i}(r = R_p)\right] = D_{ax,i} \frac{\partial^2 c_i}{\partial z^2}
\]  

(3.1)

where \(\varepsilon_b\) is the column bed porosity, \(c_i\) the concentration of the i-th component, \(t\) the time, \(u_{\text{int}}\) the interstitial velocity of the mobile phase, \(\varepsilon_{p,i}\) the particle porosity accessible to the component \(i\), \(z\) the axial position, \(k_{f,i}\) the film mass transfer coefficient, \(R_p\) the radius of the particles and \(c_{p,i}(r = R_p)\) the concentration of the i-th component at the particle surface, \(r\) the radial position in the particle and \(D_{ax,i}\) the axial diffusion coefficient of the i-th component. The initial and boundary conditions for Equation 3.1 are:
3. Mathematical Modelling of the Adsorption

\[ t = 0 \quad c_i = c_i(0, z) \]

and [124]:

\[ z = 0 \quad \frac{\partial c_i}{\partial z} = \frac{u_{ax}}{D_{ax}}(c_i - c_{f,i}) \]

\[ z = L \quad \frac{\partial c_i}{\partial z} = 0 \]

where the feed concentration, \( c_{f,i} \), is a function of time and it is defined for a loading experiment as:

\[ z = 0 \quad c_{f,i}(t) = c_{f,i} \quad \text{for} \quad t > 0 \]

and for peak injection as:

\[ z = 0 \quad c_{f,i}(t) = c_{f,i} \quad \text{for} \quad 0 < t \leq t_{inj} \]

\[ c_{f,i}(t) = 0 \quad \text{for} \quad t > t_{inj} \]

where \( t_{inj} \) is the injection time.

The corresponding mass balance for the stagnant liquid in the particle pores can be written as:

\[
\frac{\partial c_{p,i}}{\partial t} + \left(1 - \varepsilon_{p,i}\right) \frac{\varepsilon_{b}}{\varepsilon_{p,i}} \frac{\partial q_i}{\partial t} = D_{p,i} \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial c_{p,i}}{\partial r}\right)
\]

(3.2)

where \( c_{p,i} \) is the pore concentration of the i-th component, \( q_i \) the corresponding concentration in the solid phase and \( D_{p,i} \) the pore diffusion coefficient. Equation 3.2 is subject to the following initial and boundary conditions:

\[ t = 0 \quad c_{p,i} = c_{p,i}(0, r) \]

and

\[ r = 0 \quad \frac{\partial c_{p,i}}{\partial r} = 0 \]

\[ r = R_p \quad \frac{\partial c_{p,i}}{\partial r} = \frac{k_{f,i}}{D_{p,i}}[c_i - c_{p,i}(r = R_p)] \]

Equations 3.1 and 3.2 can be rewritten in dimensionless form. The mass balance in the mobile phase is written as follows:

\[
\frac{\partial c_i}{\partial \tau} + \frac{\partial c_i}{\partial \eta} + \varepsilon_{p,i} \frac{1 - \varepsilon_b}{\varepsilon_b} S_{t_i} c_i - c_{p,i}(\rho = 1) = \frac{1}{P_{e_{ax,i}}} \frac{\partial^2 c_i}{\partial \eta^2}
\]

(3.3)
The one for the stagnant phase is written as:

$$\frac{\partial c_{p,i}}{\partial \tau} + \frac{(1 - \varepsilon_{p,i})}{\varepsilon_{p,i}} \frac{\partial q_i}{\partial \tau} = \frac{1}{P e_i} \frac{1}{\rho^2} \frac{\partial}{\partial \rho} \left( \rho^2 \frac{\partial c_{p,i}}{\partial \rho} \right)$$

(3.4)

where $\tau = (t u_{int})/L$ is the dimensionless time, $\eta = z/L$ the dimensionless axial position and $\rho = r/R_p$ the dimensionless radial position. Note that according to the previous definition, $\tau = 1$ corresponds to the retention time of a tracer totally excluded from the pores. The dimensionless numbers are defined as:

Axial Peclet number:

$$P e_{ax,i} = \frac{u_{int}/L}{D_{ax,i}/L^2} = \frac{t_{ax}}{t_{conv}}$$

(3.5)

Peclet number:

$$P e_i = \frac{u_{int}/L}{D_{p,i}/R_p^2} = \frac{t_{pore}}{t_{conv}}$$

(3.6)

Sherwood number:

$$Sh_i = \frac{k_{f,i}/R_p}{D_{p,i}/R_p^2} = \frac{t_{pore}}{t_{film}}$$

(3.7)

Stanton number:

$$St_i = 3 \frac{Sh_i}{Pe_i} = 3 \frac{k_{f,i}/R_p}{u_{int}/L} = \frac{t_{conv}}{t_{film}}$$

(3.8)

where $t_{ax}$, $t_{conv}$, $t_{pore}$ and $t_{film}$ are the characteristic times for axial diffusion, convection, pore diffusion and film mass transfer, respectively. The initial and boundary conditions for the mass balance equations in the mobile (Eq. 3.3) and in the stagnant (Eq. 3.4) phases can be then rewritten as:

$$\tau = 0 \quad c_i = c_i(0, \eta)$$

and

$$\eta = 0 \quad \frac{\partial c_i}{\partial \eta} = P e_{ax,i} (c_i - c_{f,i})$$

$$\eta = 1 \quad \frac{\partial c_i}{\partial \eta} = 0$$

For Equation 3.4 the initial and boundary conditions become:

$$\tau = 0 \quad c_{p,i} = c_{p,i}(0, \rho)$$
Due to its complexity, the general rate model has no analytical solution. Numerical methods have to be applied. In this work the finite difference method is used to solve the original system of partial differential equations. This method consist in transforming the space derivatives in difference equations over a small discretization interval [125, 126]. The interval is achieved discretizing the space (radial and axial) coordinate. In this work, 9 grid points along the particle radius and 99 along the column axis are used. The final system of equations (ODEs) then consists of 9x99 ordinary differential equations per solute. The numerical code has been written in Fortran-95 and the system of ODEs solved using the LSODI package [127]. The simulations were run on a Pentium 4 Dual Core 3.2 MHz computer (average CPU time is about 1 to 5 sec). More details on the numerical method used can be found in Chapter 4.

### 3.2.1 Mass balance of the salt

Due to its large diffusion coefficient (with respect to the proteins) it is assumed that the transport of the salt inside the particle is infinitely fast and, therefore, the salt concentration in the pores is everywhere the same as in the mobile phase. The mass balance for the salt can be then simplified as in the following:

\[
\frac{\partial c_s}{\partial \eta} + \frac{\partial c_s}{\partial \tau} + \frac{(1 - \varepsilon_b)}{\varepsilon_b} \left( \varepsilon_{p,s} \frac{\partial c_s}{\partial \tau} + (1 - \varepsilon_{p,s}) \frac{\partial q_s}{\partial \tau} \right) = \frac{1}{Pe_{ax,s} \varepsilon_{p,s}} \frac{\partial^2 c_s}{\partial \eta^2} \tag{3.9}
\]

\(c_s\) and \(q_s\) are the salt concentration in the liquid and in the solid respectively and \(\varepsilon_{p,s}\) is the particle porosity for the salt.

### 3.2.2 Adsorption isotherm

For the salt and the proteins, Langmuir-type adsorption isotherms are assumed. In addition to this, due to the large excess of salt with respect to protein, it is assumed that
there is no competition between salt and antibodies [70]. As a result, the adsorption isotherm of the salt reduces to a single component Langmuir, which can be written as:

\[ q_{eq}^s(c_s) = \frac{H_s c_{eq}^s}{1 + \frac{H_s}{c_{eq}^s} c_s} \] (3.10)

where \( H_s \) is the Henry constant of the salt and \( q_{eq}^\infty \) the saturation capacity. In other words, the adsorption behavior of the salt is independent from the antibody concentration. Similarly, the adsorption isotherm of the different antibodies reduces to a competitive multi-component Langmuir isotherm, as in the following:

\[ q_{eq}^i(c_{p,i}, c_s) = \frac{H_i(c_s) c_{eq}^{p,i}}{1 + \sum_{j=1}^{n} \frac{H_j(c_s)}{c_{eq}^{p,j}} c_{eq}^{p,j}} \] (3.11)

where \( c_{p,i} \) is the pore concentration of the \( i \)-th pseudo-variants, \( H_i \) the Henry constant and \( q_{eq}^\infty \) the saturation capacity of \( i \)-th component respectively. In this expression, it can be noted that there is an explicit competition among the antibodies, but no explicit competition with the salt. The role of the salt is implicit and it is embedded in the definitions of the Henry coefficient and of the saturation capacity. For the former, the expression derived by Yamamoto [70] is used:

\[ H_i = \alpha_i I_m^{-\beta_i} \] (3.12)

where \( \alpha_i \) and \( \beta_i \) are two constants. No expression can be found in the literature for the dependence of the saturation capacity. Following the experimental results obtained in the previous chapter, a simple linear dependence on the salt concentration is suggested:

\[ q_{eq}^\infty = \gamma_i - \delta_i I_m \] (3.13)

### 3.3 Parameter Determination

The mass transport parameters and the adsorption isotherm for all components have to be determined. This will be done by a proper combination of literature correlations and parameter regression. For the regression of the parameters, the experiments presented in Chapter 2 will be used. It is important to notice that, in the following, it is assumed that all the IgG variants have identical size and, therefore, mass transport coefficients. Moreover, it is assumed that the presence of dimers in the original mixture is negligible (refer to Chapter 2.4.2).
3. Mathematical Modelling of the Adsorption

### 3.3.1 Parameters from literature correlations

The axial diffusivity ($\text{Pe}_{\text{ax}}$) and the film mass transfer coefficient ($\text{St}$) can be calculated from literature correlations. The film mass transport coefficient is calculated from the equation of Wilson and Geankoplis [100]:

$$k_f = \frac{D_m}{2R_p} \frac{1.09}{\varepsilon_b} \left( \frac{u_s 2R_p}{D_m} \right)^{1/3}$$

where $u_s$ is the superficial velocity and $D_m$ the molecular diffusion coefficient. This can be calculated from the equation by Young et al [101]:

$$D_m = 8.31 \times 10^{-8} \frac{T}{\eta_b M^{1/3}}$$

$T$ is the absolute temperature, $\eta_b$ the solvent viscosity and $M$ the molecular weight of IgG. The axial diffusion can be calculated assuming that hindered molecular diffusion and eddy diffusion are additive [80]:

$$D_{\text{ax},i} = 0.7D_{m,i} + u_{\text{int}} R_p$$

Considering that the molecular diffusion of a molecule in water is in the order of $10^{-5}\text{cm}^2/\text{s}$, the first term in Equation 3.16 is approximately equal to $7 \cdot 10^{-6}\text{cm}^2/\text{s}$. The stationary phase used in this work has a particle diameter of $65 \mu\text{m}$ and the interstitial velocity is $0.11 \text{cm/s}$. The second term is, therefore, equal to $3 \cdot 10^{-4}\text{cm}^2/\text{s}$ and, thus, dominant over the first one, which will be neglected.

### 3.3.2 Parameter regression

As already mentioned, the determination of the effective pore diffusivity and the adsorption isotherm is carried out by regression of the experimental elution profiles, i.e. by minimizing the error between the simulated and the experimental profiles. In this work, an adaptive simulating annealing (ASA) optimization routine [128] is used to find the global minimum of the regression function. The determination of the confidence interval of the regressed parameter is a very important issue. However, for the sake of space, it will not be discussed in this work.
3.4 Results and Discussion

The parameter regression has been divided into separate regression steps, in order to isolate different sets of parameters and to reduce the complexity of the overall procedure. The strategy adopted for this work is discussed in the following.

3.4.1 Column characterization

The column porosity has been characterized by inverse size exclusion chromatography (iSEC): tracers with different dimension were injected under non adsorption conditions and the average retention volume is measured. [94]. The measured porosities are summarized in Table 3.1. It is worth recalling that, assuming that the IgG has a diameter of 11 nm [112], only 28% of the total particle volume is accessible for this molecule.

3.4.2 Polyclonal IgG mixture characterization

The detailed characterization of the polyclonal mixture can be found in Chapter 2.4.2. It was shown that for the system studied the polyclonal IgG is comprising of a large number of monoclonals, whose pI value is ranging between 6.5 and 10. In spite of this, the mixture could be approximated by two pseudo components only. An analytical procedure was developed at this regard.

<table>
<thead>
<tr>
<th>Porosity value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon_b$</td>
<td>0.39</td>
</tr>
<tr>
<td>$\varepsilon_t$</td>
<td>0.83</td>
</tr>
<tr>
<td>$\varepsilon_{t,IgG}$</td>
<td>0.56</td>
</tr>
<tr>
<td>$\varepsilon_{p,IgG}$</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 3.1: Bed porosity, column total porosity and extrapolated total and particle porosities for IgG at $I_m = 0.52 M$. 
3.4.3 Mass transport parameters

As it can be observed from the mass balance equations reported above (Equations 3.3 and 3.4), in the absence of adsorption the number of parameters reduces to the porosity values ($\varepsilon_b$ and $\varepsilon_p$) and to the non-dimensional mass transport parameters ($Pe_{ax}$, $Pe$ and $St$). The bed and the total porosity have been evaluated above by iSEC. The axial Peclet and the Stanton number can be estimated using literature parameters only (see Equations 3.5 and 3.8): $Pe_{ax} = 1342$ and $St = 61$. As a result, only the pore Peclet number ($Pe$) and the pore accessibility of IgG ($\varepsilon_p$), must be estimated. These are determined by regression of isocratic experiments under non-adsorbing conditions, run at different interstitial velocities (from 0.02 to 0.31 cm/s). The fitted and the experimental profiles are shown in Figure 3.1. It can be observed that the skewness of the peak increases as the interstitial velocity increases, as effect of the transport limitations in the pores. The fit is very good and the following values of the particle porosity, $\varepsilon_p = 0.29$, and effective pore diffusivity, $D_{eff}^p = 2.98 \times 10^{-8}$ cm$^2$/s, were regressed. Note that the regressed specific IgG accessible porosity is well matching with the value extrapolated from the iSEC curve, assuming a hydrodynamic diameter of 11 nm for IgG [112] and reported in Table 3.1. It is also worth noticing that, for the regression of the pore effective diffusivity, it was assumed that the characteristic diffusion length inside the particle is equal to the entire particle radius. At $u_{int} = 0.11$ cm/s, the fitted effective pore diffusivity, corresponds to a Peclet number of 3.41. This means that the mass transport into the pores is very hindered, being the characteristic time for diffusion inside the particles more than three times larger than the characteristic time for convection. The regressed effective pore diffusivity value is very close to the value found from the Van Deemter’s plot shown in Chapter 2.4.3 ($D_{eff}^p = 2.31 \times 10^{-8}$ cm$^2$/s).

3.4.4 Isotherm determination

The salt isotherm is determined by the perturbation method (not shown). For the experimental conditions used in this work, a linear adsorption isotherm with $H_s = 0.25$ is found. In the following, it is described the procedure to regress the adsorption isotherm parameters for the two pseudo-variants as a function of the ionic strength (see Equations 3.12 and 3.13).
Figure 3.1: Fitted isocratic elution profiles of the polyclonal IgG mixture under non-adsorption conditions and different interstitial velocities changing from 0.02 to 0.31 cm/s. The arrow shows the direction of increasing velocity.

Diluted linear gradient elution experiments

As discussed in Chapter 2.4.4, the determination of the adsorption isotherm using isocratic experiments is unpractical, due to the strong dependence of the isotherm parameters on the salt concentration, the strong mass transport limitations and the presence of many components into the original IgG mixture. For this reason, linear gradient elution (LGE) experiments will be used in the following. From LGE experiments under diluted conditions, the linear part of the isotherm is determined. Since no competition effects are presence under linear adsorption conditions, the $\alpha$ and $\beta$ constants of the Henry coefficient (Equation 3.12) can be regressed independently for two pseudo-variants. This procedure also avoids that large errors in the regression of the less concentrated variant are compensated by small errors in the more concentrated, being the initial concentration of the two pseudo-variants in the feed very different. It is worth pointing out that the Peclet number will also be regressed together with the parameters $\alpha$ and $\beta$. In fact, it should be recalled that the two pseudo-variants are actually comprising of many variants, each of them with an individual adsorption behavior. All these variants have been then lumped into two macro-components.
Therefore, the Peclet number is used to compensate for the additional peak broadening due to the presence of many different components.

The first pseudo-variant has been obtained by fractionating the original PAb mixture by applying the analytical method described in Chapter 2.4.2. A small amount of the first pseudo-variant (0.004 mg in 10 µL) was injected in the column. After injection, the ionic strength was kept constant at 0.07 M for 20 min (corresponding to about 10 column volumes) to elute impurities and compensate for salt disturbances due to the injection. The ionic strength was then increased linearly to 1.02 M in 120 (75 CV) to 240 minutes (150 CV). The same experiments were performed with the polyclonal mixture injecting by 0.0165 mg in 10 µL. The elution profile of the second pseudo component is found by subtracting the profile of the first component from that of the mixture. The regression of the elution profiles of the first and the second components are shown in Figure 3.2 and Figure 3.3 respectively. In the case of the first pseudo-variant (Figure 3.2), a very good regression of the experimental data was obtained. In this case, a value of β equal to 5.17 was regressed. In the case of the
Figure 3.3: Fitted linear gradient elution profiles for the second pseudo-variant (obtained by subtracting the first component profile from the mixture profile). The gradient length is varied from 120 to 240 min (from left to right). The interstitial velocity is equal to 0.11 cm/s. The curves and the points represent the regression and the experimental profiles, respectively.
second pseudo component (Figure 3.3), the matching between experiments and model is not as good. The mean retention time of the peak (first moment) is predicted with good approximation. However, the experimental elution profiles show very long tails and asymmetric peaks, which cannot be predicted by the model. This is due to the presence of a small portion of antibody molecules with very large affinity to the stationary phase, whose presence is not accounted for. The regressed value of $\beta$ is 5.76. All the regressed parameters ($\alpha$, $\beta$ and Pe) are summarized in Table 3.2. In Figure 3.4 the Henry constants of the two variants (calculated by Equation 3.12) are plotted as a function of the ionic strength. Both functions are very strongly dependent on the ionic strength. The fitted functions can be compared to those found with the method of Yamamoto in Chapter 2.4.4. The values calculated with the method of Yamamoto in the correspondence of the ionic strengths used for the breakthrough experiments (discussed in the following) are shown in Figure 3.4 (points). The agreement with the two methods is good especially for the pseudo-variant two. On the other hand, the method of Yamamoto predicts a smoother dependence for the first pseudo-variant Henry constant on salt. The difference is particularly important at large salt concentrations, i.e. at low retention times. Note that at $I_m = 0.17$ M, the first pseudo-variant is close to non-adsorption conditions. In such conditions, the mistake introduced by the method of Yamamoto in the definition of the Henry constant (see Equation 2.14 in Chapter 2.3.3) is probably not negligible. It is also worth noting that the regressed values of $\alpha$ and $\beta$ are very correlated to each other. In particular, if the values of $\alpha$ and $\beta$ in the proximity of the solution are plotted against each other, they are almost falling on a line. However, the effect of this strong correlation on the behavior of the Henry coefficient as a function of salt is negligible. More details on this can be found in Chapter 4.

Table 3.2: $\alpha$, $\beta$ and Pe for the two pseudo-variants, obtained from the regression of diluted linear gradient elution experiments.

<table>
<thead>
<tr>
<th>Pseudo-variant 1</th>
<th>$\alpha$ [-]</th>
<th>$\beta$ [-]</th>
<th>Pe [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.76 10^{-3}</td>
<td>5.17</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>Pseudo-variant 2</td>
<td>9.82 10^{-3}</td>
<td>5.76</td>
<td>1.98</td>
</tr>
</tbody>
</table>
Figure 3.4: Henry constants of the two pseudo-variants plotted as a function of the ionic strength. The curves are the function found from the regression of the linear gradient elution experiments. The points are calculated by applying the equations found with the method of Yamamoto in Part I of this work in correspondence to the ionic strengths of the breakthrough curves (discussed in the following). The dashed curve and open symbols represent the first pseudo-variant; the full curve and symbols the second.
An additional comment is due on the regressed values of the pore Peclet number in Figures 3.2 and 3.3. Unexpectedly, the following values have been obtained for the two pseudo-variants: \( P_{e_1} = 1.16 \) and \( P_{e_2} = 1.98 \). These Peclet numbers are actually smaller than the ones regressed from the isocratic experiments under non-adsorption conditions (Chapter 3.4.3). This contradictory result can be explained by supposing that some unspecific adsorption mechanism (e.g. hydrophobic interaction) is operating in the absence of ion exchange even at the large salt concentrations used in Figure 3.1. The presence of some (small) adsorption can then lead to peak broadening and, thus, to an underestimation of the pore effective diffusion rate. It is worth noting that a very similar value of the Peclet number under non-adsorption conditions have been obtained here by peak regression and in the first part of this work from the van Deemter’s plot. In other words, this result is independent of the method used to estimate pore diffusivity. On the other hand, it is legitimate to assume that such kind of non-specific adsorption is not influencing the determination of the IgG isotherm parameters, since the cation exchange mechanism is most probably dominant at low salt concentrations.

As previously discussed, in the gradient experiments the original complex mixture was approximated by two pseudo-variants only. This would also explain the different Pe values found for the two components. The effective pore diffusivities calculated from the Peclet numbers for the two pseudo components are equal to: \( D_{p,1}^{\text{eff}} = 8.81 \times 10^{-8} \) cm\(^2\)/s and \( D_{p,2}^{\text{eff}} = 5.16 \times 10^{-8} \) cm\(^2\)/s. Even if these diffusion rate constants are not those of a single IgG, as discussed above, they are still typical for an antibody on this type of resin. It is also worth noting that these values of Peclet are not correlated to the values of \( \alpha \) and \( \beta \) found above. This result can be explained by recalling that, under diluted conditions, the average retention time of the solute is a function of the adsorption isotherm only.

**Overloaded linear gradient elution experiments**

The non-linear region of the adsorption isotherm, that is the saturation capacity, is regressed by carrying out overloaded linear gradient elution experiments. In this regression, the values of the Henry as function of the ionic strength functions and the Peclet numbers found from the regression of the diluted linear gradients are kept con-
3.4. Results and Discussion

The values of $\gamma$ and $\delta$ (see Equation ??) are regressed using the data from two different gradient lengths and different injection volumes. It is important to notice that, in order to obtain reliable values for the saturation capacity, very large feed amounts had to be used. The separate evaluation of the saturation capacity for the two pseudo-variants, although preferable from many points of view, was not feasible. In fact, this would require a very large consumption of the PAb mixture for the separation of the two pseudo-variants. Accordingly, the regression has been carried out by injecting the original IgG mixture and, then, by analyzing the fraction collected at the column outlet. This procedure has also the advantage of introducing the competition for adsorption between the two pseudo-variants, which must be properly predicted by the model. In addition to this, considering that the two components are made of very similar molecules (IgG clones), it is reasonable to assume that they have the same (or, at least, similar) saturation capacities. The estimation of the saturation capacity is then reduced to the regression of two parameters only, $\gamma$ and $\delta$, which are kept the same for the two components.

Large amounts of the polyclonal mixture (2 to 110 mg) are injected into the cation exchanger column. The experimental procedure is similar to the diluted gradients. The mixture is loaded to the column via multiple injection at very low ionic strength (0.07M). Due to the extremely large Henry constants found at low ionic strength, it reasonable to assume that the components are immediately adsorbing and are not moving during the intervals between successive injections. After the last injection, the ionic strength is kept constant to 0.07 M for 5 minutes (3 CV). The ionic strength is successively linearly increased to 1.02 M in 20 (12 CV) and 40 minutes (24 CV), respectively. The elution profiles are fractionated and their composition analyzed. The comparison between the experimental data and the model simulations using the regressed values of $\gamma$ and $\delta$ are shown in Figure 3.5 and 3.6 for the 20 and the 40 minutes gradients, respectively. The regression is generally good especially for the shallower gradient. Note that, in the presence of negligible mass transport resistances, it would be expected that: (i) the peak front is showing a shock, which is moving to smaller elution volumes at larger injection volumes; (ii) the peak rear does not move and elutes at the same elution volume as in linear conditions; (iii) that the components with lower
Figure 3.5: Regressed overloaded linear gradient elution profiles for the first pseudo-variant (dashed curve and open symbols) and the second (full curve and symbols). The gradient length is equal to 20 minutes and the interstitial velocity to 0.11 cm/s. The injected mass of the mixture is 41 (squares), 89 (triangles) and 110 (circles) mg. The curves and the points represent the regression and the experimental profiles, respectively.
3.4. Results and Discussion

Figure 3.6: Regressed overloaded linear gradient elution profiles for first pseudo-variant (dashed curve and open symbols) and the second (full curve and symbols). The gradient length is equal to 40 minutes and the interstitial velocity to 0.11 cm/s. The injected mass of the mixture is 38 (squares), 79 (triangles), 119 (circles) mg. The curves and the points represent the regression and the experimental profiles, respectively.
affinity are displaced towards lower elution volumes. From Figure 3.5 and 3.6, it can be noticed that indeed the peak front is moving to the left for larger injection volumes, whereas the rear is not. This, as in the case of diluted LGE, exhibits a more pronounced tailing than what predicted by the model. On the other hand, the overall peak do not bend and, on the contrary, remain rather symmetrical. This is due to the large diffusion resistances. This feature is well predicted by the model, which is also well predicting the overall peak width without any adjustment of the Peclet number. Accordingly, it is possible to suppose that diffusion limitations are similar in both diluted and overloaded LGE experiments. When analyzing the elution of the first pseudo-variant, it can be noticed that the entire peak is displaced to lower elution volumes, while remaining sufficiently symmetrical. However, despite the evident data scattering, it appears that the peak rear of the first pseudo-variant is not displaced, as instead predicted by the model. In general, the satisfactory agreement between the model and the experiments and the capability of the former to correctly predict the elution time of both pseudo-variants and the peak width, is indicating a good description of both the mass transport resistances and of the adsorption isotherm.

Finally, it should be noticed that the best regression was obtained with the following parameters: $\gamma = 1902$ mg/mL and $\delta = 49.5$ M.mg/mL. The regression result seems to indicate that the confidence on these two parameters is large and that there is little correlation between the two. It can be concluded that the saturation capacity is almost constant (it is now ranging from 1898 to 1893 mg/mL, at an ionic strength of 0.07 and 0.17 M, respectively). This observation is coherent with a view of the saturation capacity which is only representative of the maximal surface coverage and it seems contradicting the different experiments reported in literature showing a decreasing saturation capacity for increasing salt concentrations [35, 40, 107, 108, 109, 110].

**Breakthrough experiments**

Using all the isotherms and mass transport parameters regressed so far, i.e. without any adjustable parameter, the breakthrough curves at three ionic strengths are predicted using the model. These are shown in Figure 3.7, 3.8 and 3.9. The qualitative shape of the breakthrough, as well as the breakthrough position is well predicted by the
3.4. Results and Discussion

Figure 3.7: Frontal analysis experiment at $I_m = 0.07$ M and $u_{int} = 0.11$ cm/s. The empty black circles and dashed curve represent the first pseudo-variant, the full black circles and curve the second pseudo-variant. The gray triangles and curve represent the mixture. The curves and the points represent the regression and the experimental profiles, respectively.
Figure 3.8: Frontal analysis experiment at $I_m = 0.12$ M and $u_{int} = 0.11$ cm/s. The empty black circles and dashed curve represent the first pseudo-variant, the full black circles and curve the second pseudo-variant. The gray triangles and curve represent the mixture. The curves and the points represent the regression and the experimental profiles, respectively.
3.4. Results and Discussion

Figure 3.9: Frontal analysis experiment at $I_m = 0.17$ M and $u_{int} = 0.11$ cm/s. The empty black circles and dashed curve represent the first pseudo-variant, the full black circles and curve the second pseudo-variant. The gray triangles and curve represent the mixture. The curves and the points represent the regression and the experimental profiles, respectively.
simulation. This is indicating that the saturation capacity was correctly predicted by the overloaded LGE experiments. The experimental data are, however, slightly steeper than the simulated curves. The mass transport seems to be enhanced in these experiments, with respect to the overloaded gradients. The most probable reason for such behavior is the progressive obstruction of the particle pores by the adsorbed IgG (refer to Chapter 5). This would decrease the diffusion path in the particles pores and thus decrease the pore Peclet number, i.e. increase the effective mass transport. It is important to notice that the competition between the two pseudo components is well reproduced at the smallest ionic strength, thus indicating that the selectivity of the two components is well predicted even at low values of the ionic strength. The experimental profile of the first component in the breakthrough curve at $I_m = 0.12 \, \text{M}$ (Figure 3.8) shows instead a shoulder, which is not reproduced by the model. In such an intermediate situation, at the boundary between no adsorption and adsorption for many IgG variants, the simplification of the original mixture to only two single pseudo-variants is showing its limit. The first shoulder is probably caused by a fraction of the variants belonging to the first pseudo-variant, which are practically non adsorbing at this ionic strength. The peak maximum in the elution profile of the first pseudo-variant is instead due to the competition between the remaining portion of the first pseudo-variant and the second pseudo-variant. In spite of this, the agreement is qualitatively still good and both the breakthrough time and the competition are well predicted. In Figure 3.9, it can be seen that the affinity of both pseudo-variants has been probably slightly overestimated at high salt concentrations. In fact, the model is predicting a slightly later breakthrough. However, in this case, the affinity of the two pseudo-variants is very small, i.e. the experiment was operated very close to non-adsorption conditions.

**3.4.5 Analysis of the column behavior under loading conditions**

The pore model is exploited to visualize the concentration profiles along the column axis and the particle radius. This is shown in the case of the BTC at $I_m = 0.07\, \text{M}$ in Figures 3.10, 3.11 and 3.12.
3.4. Results and Discussion

Figure 3.10: Liquid concentration of the first (a) and the second (b) pseudo-variant, plotted as a function of the nondimensional axial position and the nondimensional time for the BTC at $I_m = 0.07M$. 
In Figures 3.10, the axial profiles of the first and second pseudo-variants are plotted as a function of the nondimensional time $\tau$. For $\eta = 0$ the concentration of the two components is equal to the inlet concentration, with the exception of $\tau = 0$ where it is zero. As the loading proceeds, the concentration front of the two components moves towards the outlet of the column. In these figures, it can be appreciated the fact that the front of the first pseudo-component is travelling faster and, at the same time, it is progressively displaced by the second one. As a consequence, the concentration of the first pseudo-component builds up, thus creating the typical overshoot effect, evident at $\tau = 100$. At about $\tau = 210$ the adsorption equilibrium is established and the feed concentrations are reached at the outlet of the column.

The radial profiles of the two components, at the entrance and at the outlet of the column, are shown in Figure 3.11 and 3.12, respectively. Let us start discussing the radial concentration profiles at the entrance of the column ($\eta = 0$). It is interesting to notice how the same displacement mechanism acting along the column axis in the mobile phase, and shown in Figures 3.10, is replicated at the particle level. The same discussion as for the axial profiles is valid. The concentration profile of the first pseudo-variant moves towards the particle center faster than that of the second pseudo-variant and, as a result, at $\tau > 50$ it starts to be displaced. As a consequence, the concentration overshoot is pushed towards the center of the particle, thus creating a large concentration gradient which is favoring the diffusion of the excess of the first pseudo-variant out of the particle. At $\tau = 100$ the particle is completely equilibrated. Note that $\tau = 100$ is roughly the time at which the concentration overshoot starts building up in the mobile phase and the two pseudo-variant are breaking through the column. The radial profiles at the outlet of the column (at $\eta = 1$) are shown in Figure 3.12. The same kind of mechanism already observed for the particles at the column inlet (Figure 3.11) is observed here. However, the effect is stronger in this case. In fact, differently from the column inlet, where the concentration of the two components at the particle surface is constant and equal to the feed concentration, at the column outlet the particles are also observing the build up of the first pseudo component at the particle surface ($\rho = 1$). As a result, the build-up of the first component in the particle center is more pronounced.
Figure 3.11: Liquid concentration of the first (a) and the second (b) pseudo-variant, plotted as a function of the nondimensional time and the nondimensional radial position at the entrance of the column ($\eta = 0$). The BTC at $I_m = 0.07M$ is simulated.
Figure 3.12: Liquid concentration of the first (a) and the second (b) pseudo-variant, plotted as a function of the dimensionless time and the dimensionless radial position at the outlet of the column ($\eta = 1$). The BTC at $I_m = 0.07$M is simulated.
3.5 Conclusions

Previous figures are evidencing the consequences of the limited mass transport inside the particle, which is at the origin of the difference between the so-called dynamic binding capacity and the column static capacity. It is also showing that there is a need of increasing the pore diffusivity of large proteins in order to increase the performance of this materials. For higher pore diffusivity, the elution profiles are steeper and therefore the dynamic binding capacity higher. Higher pore diffusivity can be reached for example by increasing the pore radius.

3.5 Conclusions

The adsorption of a polyclonal IgG mixture was studied in detail with the aid of a mathematical model. In particular, a suitable model was developed for the simulation of the adsorption process and a parameter regression strategy has been proposed.

As discussed in the previous chapter, the polyclonal mixture was simplified by assuming that two pseudo-variants are present only. The mass transport parameters were calculated by literature correlations, with the exception of the effective pore diffusivity, which was regressed on isocratic experiments under non adsorption conditions. Very low values of diffusivity were found, in agreement with the value found from the Van Deemter’s experiments of Chapter 2.

The adsorption isotherms were estimated by regression of diluted and overloaded linear gradient elution experiments. In order to compensate for the errors produced by the mixture simplification, the Pe number was kept as an adjustable parameter in the regression of the diluted linear gradient experiments. A very good agreement between experiments and model results was generally found. More specifically, diluted gradients were used to separately estimate the Henry coefficients for the two pseudo-variants as a function of ionic strength. These values were then used to regress the dependence of the saturation capacity. Surprisingly, an almost constant saturation capacity as a function of the ionic strength was found. It is also worth noticing that the Pe values regressed from the linear gradient elution experiments were smaller than the one obtained from regression of the experiments under non adsorption conditions. This is believed to indicate that non-specific adsorption mechanisms, such as hydrophobic interaction, are
operating at large salt concentrations.

The model was finally used in a completely predictive way to simulate three breakthrough curves at different ionic strengths. The results indicate that the model with the parameter values regressed by gradient elution experiments is able to predict the elution profile of the breakthroughs with good approximation. The breakthrough time and the competition between the two pseudo-variants could be as well reproduced by the model.

The model was also exploited to visualize the concentration profiles of the two pseudo-variants along the column axis and the particle radius. The overshoot of in the concentration of the first pseudo-variant typical of competing systems could be observed both along the column axis and the particle radius. The shape of the profiles was found to be strongly affected by the small effective pore diffusivity of the two pseudo-variants.

The model has proven to be well suited for the modelling of the adsorption process of PAbs on cation exchange resins. Moreover the parameter regression strategy proposed was proven to be robust, to require low solute consumption and to be readily applicable to the adsorption of any protein on ion exchange resins. However, if a precise determination of the elution profiles under different operative conditions is required, the polyclonal mixture cannot be simplified to two components only. In fact, the presence of long tailing and shoulders in the elution peaks can be predicted only if a finer description of the mixture is carried out. This work will be discussed in the following chapter.
Chapter 4

Multi Component Mathematical Modelling of the Adsorption

4.1 Introduction

In the previous two chapters of this Thesis, an investigation of the behavior of a polyclonal antibody (PAb) mixture on a strong cation exchanger (sCIEX) has been carried out. In particular, in Chapter 2, the general behavior of the mixture has been determined with short cut methods, whereas, in Chapter 3, the investigation of both the mass transport and the adsorption isotherm parameters has been repeated by peak regression using a suitable numerical model. From these two analyses, it became immediately evident that it is impossible to consider the PAb mixture as a single monoclonal antibody. The different antibody variants in the mixture are characterized by a broad, almost continuous, spectrum of pI values. As a consequence, the different components of the mixture have very different adsorption behaviors once injected into a sCIEX column. On the other hand, in these two chapters it has been shown that the initial complexity of the PAb mixture can be reduced to two so-called pseudo-variants only, where the characteristics of a large number of different antibodies are lumped into two single components. The advantages of these procedure lies in the simplicity of the analytical procedure and of the corresponding numerical model, which must account for the competition of two components only.

Besides the intrinsic difficulties connected to the investigation of the behavior of
proteins on CIEX columns, previous analysis soon showed all its limits. First, in many experiments it was impossible to correctly describe the complex behavior of the mixture and the presence of shoulders or long tailings. Second, in this analysis the mass transport parameters were used as adjustable parameters to compensate for the broadening introduced by the different adsorption behavior of the different components in the two pseudo-variants. As a result, the main advantage of using a sophisticated numerical model as the general rate model (GRM) [75, 76, 77, 78] were partly lost. In fact, as for other lumped models, such as the lumped pore model or the kinetic rate model, the regressed mass transport parameters were actually lumped parameters, which depend on the mass transport characteristics of the system and on their adsorption behavior.

As previously discussed in this Thesis and by other authors, as Melter et.al. [15], analytical weak cation exchange columns have an outstanding resolution power with respect to protein having different pI values. In fact, the small differences in the net protein charge lead to very different adsorption behaviors. This has been also suggested by Yamamoto et.al. [70], who proposed the following expression of the Henry coefficient on ion exchange columns:

$$H = \alpha \cdot c_s^{-\beta}$$

where $C_s$ is the salt concentration and $\alpha$ and $\beta$ constant parameters. This expression has been derived from the mass action law [65] and it can be demonstrated that the constant $\beta$ is directly correlated to the protein net charge. Clearly, being the expression of the Henry coefficient in Equation 4.1 a power function of $C_s$, small changes in the value of $\beta$ are leading to very different adsorption affinities for small variations in the salt concentration. In addition to this, non-porous analytical columns are avoiding the problems connected to the limited mass transport of the proteins in macro-porous particles and, thus, the peak spreading remains very limited.

In this chapter, the resolution power of non-porous analytical cation exchange columns is exploited. In particular, a simple method to rapidly identify a finite number of pseudo-variants is presented. It is worth pointing out that the model regression of all the mass transport and adsorption isotherm parameters is then exponentially increasing with the number of pseudo-variants identified in the mixture. Accordingly,
a compromise must be found between the need of precisely reproduce the complex behavior of the mixture components and the time needed to estimate all the parameters, so to have a numerical model able to predict (and not only simulate) the column output under different operative conditions.

In the following on this chapter, the numerical model and the analytical procedure are first discussed. Then, the regression procedure of all the involved parameters is analyzed. A discussion on the optimal number of pseudo-variants is not present in this work. On the contrary, it has been arbitrarily decided to simulate the PAb mixture using six pseudo-variants only. It should be noticed that, as discussed by Kaczmarski et.al. [78, 88], in the case of slow mass transport inside the particles, a numerical model explicitly accounting for the solute concentration profile along the particle radius, as the GRM, is mandatory. This is a rather computationally expensive model, especially when the competition for adsorption among a large number of solutes in the presence of very non-linear adsorption isotherms must be simulated. All the work to reduce the computational time of such model to few seconds, so to carry out an extensive parameter regression, is also not discussed.

4.2 Materials and Methods

4.2.1 Stationary phase and columns

The column discussed in Chapter 2.2.1 is used in the following. Moreover, an analytical weak cation exchanger column (250x4 mm, ProPac WCX-10) from Dionex is used for the analysis of the polyclonal IgG mixture.

4.2.2 Mobile phase and chemicals

Experiments under no adsorption conditions are run using 50 mM phosphate buffer at pH = 7, whereas adsorption experiments are run using a 20 mM sodium acetate buffer at pH=5. The buffer preparation is equivalent to the one discussed in Chapter 2.2. Inverse size exclusion experiments are carried out using dextran standards (Sigma-Aldrich, Switzerland). For the experiments a polyclonal IgG mixture (Gammanorm, Octapharma, Switzerland) is used.
4.2.3 Chromatography equipment

The same chromatographic equipment as in the previous chapters is used in the following.

4.3 Model Description

4.3.1 Main Assumptions

In this work, the so-called general rate model (GRM) is used to simulate the behavior of the different solutes inside a chromatographic column [75, 76, 77, 78]. In this model, it is assumed that the mobile phase percolates through the interstitial spaces left by the macro-porous particles (i.e. the beads). In this region of space (the mobile phase), the transport is almost purely convective. From this stream, diffusion takes place into the liquid filled pores of the particles (the stagnant phase). Here, transport takes place exclusively by diffusion and in every point of the particle the liquid phase is thermodynamically in equilibrium with the pore surface (the solid phase), where the adsorption of the solutes takes place.

The following additional assumptions are made in writing the GRM:

- The concentration profiles along the column radius are flat and wall-effects are ignored. Spherical symmetry is assumed for the macro-porous particles.

- The so-called bed porosity, that is the voids in between the particles, is constant along the column axis. The particle pore size distribution is also constant along the particle radius.

- The adsorption mechanism is described by using a competitive Langmuir adsorption isotherms. Kinetic limitations to adsorption are ignored.

- Due to very large salt concentration of the salt with respect to the IgG molecules, the competition for adsorption between salt and antibodies is neglected. Accordingly, the adsorption isotherm of the salt is reduced to a single component isotherm, whereas a competitive Langmuir is describing the adsorption behavior.
of the antibody mixture. In the latter, the role of the salt on adsorption is hidden in the definition of the Langmuir coefficients.

- For sake of simplicity, the concentration of the solutes in the solid phase is computed as a volumetric concentration, i.e. the amount of solute adsorbed per unit volume of solid.

- All antibodies are assumed to have the same hydrodynamic size and, thus, to access the same pore volume. Therefore, no size exclusion is present among the antibodies and all the corresponding mass transport parameters are equal. However, it should be noted that the salt has a different pore accessibility than the antibodies and, in particular, it is assumed to access the entire particle porosity.

- The transport of the solute in the intra-particle voids takes place by diffusion in the liquid phase only. Additional transport mechanisms, as solid-diffusion, are ignored. The transport of the salt in the particle pores is assumed to be much faster than the transport of the solutes and, therefore, it is supposed to take place instantaneously. Accordingly, the salt concentration is constant everywhere in the liquid phase in each column section.

Note that this assumptions are the same as used in Chapter 3.

### 4.3.2 Mass Balance Equations

According to previous model assumptions, it is possible to write the mass balances for the solutes in each phase. First, the mass balance on the $i$-th solute in the mobile phase can be written as follows:

$$\frac{\partial c_i}{\partial t} + u \frac{\partial c_i}{\partial z} + \varepsilon_p \nu_b \frac{3}{R_p} J_i = D_{ax} \frac{\partial^2 c_i}{\partial z^2}$$

(4.2)

where $c_i$ is the concentration in the mobile phase of the $i$-th solute, $u$ the interstitial velocity, $J_i$ the mass flux of the solute from the mobile to the stagnant phase. Note that it has been assumed that only a fraction $\varepsilon_p$ of the total particle surface is available for the diffusion process. The initial and boundary conditions (Danckwerts’ conditions)
corresponding to the previous equations are:

\[
c_i = 0; \quad t = 0; \quad 0 \leq z \leq L
\]

\[
-D_{ax} \frac{\partial c_i}{\partial z} = u (c_{f,i}(t) - c_i); \quad t > 0; \quad z = 0
\]

\[
\frac{\partial c_i}{\partial z} = 0; \quad t > 0; \quad z = L
\]

(4.3)

where \(c_{f,i}\) is the time dependent inlet concentration of the \(i\)-th solute. Previous mass balance must be coupled to the mass balance on each solute in the macroporous particles:

\[
\frac{\partial c_{p,i}}{\partial t} + \nu_p \sum_{j=1}^{N} \frac{\partial q_i}{\partial c_{p,j}} \frac{\partial c_{p,i}}{\partial c_{p,j}} + \nu_p \frac{\partial q_i}{\partial c_m} \frac{\partial c_m}{\partial t} = \frac{D_p}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial c_{p,i}}{\partial r} \right)
\]

(4.4)

where \(q_i\) is the solute equilibrium concentration in the solid phase and is a function of the solutes and modifier concentrations. The following initial and boundary conditions are valid in this case:

\[
c_{p,i} = 0; \quad t = 0; \quad 0 \leq r \leq R_p
\]

\[
D_p \frac{\partial c_{p,i}}{\partial r} = J_i = k_f (c_i - c_{p,i}); \quad t > 0; \quad r = R_p
\]

(4.5)

\[
\frac{\partial c_{p,i}}{\partial r} = 0; \quad t > 0; \quad r = 0
\]

The following non-dimensional independent variables can be defined:

\[
\tau = \frac{t}{u}, \quad \eta = \frac{z}{L}, \quad \rho = \frac{r}{R_p}
\]

(4.6)

and substituted in the previous mass balance equations and boundary conditions (Equations 4.2-4.5). The new set of mass balance equations using non-dimensional variables is reported in the following, for the mobile phase:

\[
\frac{\partial c_i}{\partial \tau} + \frac{\partial c_i}{\partial \eta} + \varepsilon_p \nu_b St (c - c_p) = \frac{1}{Pe_{ax}} \frac{\partial^2 c_i}{\partial \eta^2}
\]

(4.7)

\[
c_i = 0; \quad \tau = 0; \quad 0 \leq \eta \leq 1
\]

\[
-\frac{1}{Pe_{ax}} \frac{\partial c_i}{\partial \eta} = c_{f,i} - c; \quad \tau > 0; \quad \eta = 0
\]

(4.8)

\[
\frac{\partial c_i}{\partial \eta} = 0; \quad \tau > 0; \quad \eta = 1
\]
4.3. Model Description

and for the macroporous particles:

\[
\frac{\partial c_{p,i}}{\partial \tau} + \nu_p \sum_{j=1}^{N} \frac{\partial q_i}{\partial c_{p,j}} \frac{\partial c_{p,j}}{\partial \tau} + \nu_p \frac{\partial q_i}{\partial c_{m}} \frac{\partial c_{m}}{\partial \tau} = \frac{1}{P e_p \rho^2} \frac{\partial}{\partial \rho} \left( \rho^2 \frac{\partial c_{p,i}}{\partial \rho} \right) \tag{4.9}
\]

\[
c_{p,i} = 0; \ \tau = 0; \ 0 \leq \rho \leq 1
\]

\[
\frac{\partial c_{p,i}}{\partial \rho} = Sh (c_i - c_{p,i}); \ \tau > 0; \ \rho = 1
\]

\[
\frac{\partial c_{p,i}}{\partial \rho} = 0; \ \tau > 0; \ \rho = 0
\]

In previous equations (Equations 4.7-4.10), the following non-dimensional groups can be identified:

\[
St = \frac{3 L/u}{R_p/k_f} = 3 \frac{t_{\text{conv}}}{t_{\text{film}}}
\]

\[
P_{e_{ax}} = \frac{L^2/D_{ax}}{L/u} = \frac{t_{\text{diff,ax}}}{t_{\text{conv}}}
\]

\[
P_{e_p} = \frac{R_p^2/D_p}{L/u} = \frac{t_{\text{diff,p}}}{t_{\text{conv}}}
\]

\[
Sh = \frac{R_p^2/D_p}{R_p/k_f} = \frac{P e_p St}{3} = \frac{t_{\text{diff,p}}}{t_{\text{film}}}
\]

where \( t_{\text{conv}}, t_{\text{film}}, t_{\text{diff,ax}}, t_{\text{diff,p}} \) are the characteristic times of convection, film mass transport, axial diffusion and particle pore diffusion, respectively.

4.3.3 Adsorption Isotherms

As mentioned above, due to the large excess of salt with respect to protein, it is assumed that there is no competition between salt and antibodies [70]. As a result, the adsorption isotherm of the salt reduces to a single component Langmuir, which can be written as:

\[
q_{eq}^{s}(c_s) = \frac{H_sc_s^{eq}}{1 + H_sc_s^{eq}/q_s^{\infty}} \tag{4.11}
\]

where \( H_s \) is the Henry constant of the salt and \( q_s^{\infty} \) the saturation capacity. In other words, the adsorption behavior of the salt is independent from the antibody concentration. Similarly, the adsorption isotherm of the different antibodies reduces to a competitive multi-component Langmuir isotherm, as in the following:

\[
q_{eq}^{i}(c_{p,i}, c_s) = \frac{H_i(c_s)c_{p,i}^{eq}}{1 + \sum_{j=1}^{n} H_j(c_s)c_{p,j}^{eq}/q_j^{\infty}(c_s)} \tag{4.12}
\]
where $H_i$ is the Henry constant and $q_i^\infty$ the saturation capacity of i-th component, respectively. In this expression, it can be noted that there is an explicit competition among the antibodies, but no explicit competition with the salt. The role of the salt is implicit and it is hidden in the definitions of the Henry coefficient and of the saturation capacity. For the former, the expression derived by Yamamoto [70] and reported in Equation 4.1 is used. No expression can be found in the literature for the dependence of the saturation capacity. Differently as in Chapter 3, here the following S-shape dependence on the salt concentration is used:

$$q_i^\infty = \frac{\gamma}{1 + e^{\delta c_s - \epsilon}}$$

(4.13)

Previous choice can be justified by the need of an expression which is asymptotically going to zero for infinite salt concentration (i.e. under no-adsorption conditions) and to $\gamma$ for zero salt. This last value should represent the true saturation capacity due to full coverage of the available surface. In between the two asymptotes, the function is almost linear: the slope and the position of the inflection point are determined by $\delta$ and $\epsilon$, respectively.

### 4.3.4 Numerical Solution of the Mass Balance Equations

The original mass balance equations consist of a set of partial differential equations. This system has been reduced to a system of ordinary differential equations (ODEs) using the finite difference method. The first and second order space derivates were reduced using the so-called central scheme. A forward and a backward difference scheme has been used for the axial coordinate at the column inlet and outlet, respectively. A forward scheme was used for the space derivatives in the particle center, whereas a second order polynomial expansion has been used on the particle surface ($\rho = 1$):

$$\frac{\partial c(\rho = 1)}{\partial \rho} \sim \frac{c_{N-1} - 4c_N + 3c_{N+1}}{2\Delta \rho}$$

(4.14)

where $c_i$ is the estimated concentration at $\rho = i/(N+1)$ and $N$ is the total number of grid points along the radial coordinate. Both axial and radial directions have been discretized using two equispaced grids. Typically, 99 grid points have been used along the column axis and 15 along the particle radius.
The resulting system of ODEs has been solved using the LSODI routine [129]. This routine is solving the system in the form:

\[ A(t, y) \cdot \dot{y} = g(t, y) \]  

(4.15)

where \( y \) is the vector of the unknown functions, \( \dot{y} \) its time derivatives, and \( A \) is a square matrix of the coefficients. It can be shown that, by a proper selection of the order of the differential equation, the matrix of the coefficients \( A \) can be reduced to a banded matrix with a width equal to the number of grid points along the radial coordinate, thus minimizing the computational effort.

4.3.5 Parameter Regression

Parameter regression has been carried out by using the least square method [131], where the difference between the observed outlet concentration values and the values predicted by the model is minimized. The *nlinfit* package from Matlab was used to find the different local minima of the least square function [130]. In this procedure, the starting point for the minimum search was randomly generated and a procedure was repeated until a sufficiently large number of minima were found, which were different by 2% maximum. These local minima were then used for the correlation analysis.

4.3.6 Fraction Analysis

The fraction collected from all the experiments discussed in the following are analyzed on the analytical column (see Materials and Methods). The elution chromatogram of the original Gammanorm mixture is shown in Figure 4.1, where the UV signal as a function of the elution time is shown. After the injection the ionic strength is kept constant at 0.07 M for 5 min. Then a gradient is started, which reaches a final ionic strength of 52M in 30 minutes at a flow rate of 0.5 ml/min. Note that, in this figure, the dead time was already subtracted and a base line connecting the UV signal at 10 and 35 minutes was arbitrarily applied. In the same figure, the algorithm to estimate the variant composition is show. The chromatogram has been approximated by triangular
Figure 4.1: UV signal (280 nm) as a function of the elution time for the original Gammanorm mixture on the analytical column Propack.
4.4. Results and Discussion

Table 4.1: Composition of the original Gammanorm mixture, as defined with the procedure represented in Figure 4.1 and described in text.

<table>
<thead>
<tr>
<th>Pseudo-Variant No.</th>
<th>Fractional Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.091</td>
</tr>
<tr>
<td>2</td>
<td>0.177</td>
</tr>
<tr>
<td>3</td>
<td>0.338</td>
</tr>
<tr>
<td>4</td>
<td>0.254</td>
</tr>
<tr>
<td>5</td>
<td>0.100</td>
</tr>
<tr>
<td>6</td>
<td>0.040</td>
</tr>
</tbody>
</table>

functions, $v_i$, on an equispaced grid, $t_i$:

$$v_i(t) = \begin{cases} 
\frac{t - t_{i-1}}{t_i - t_{i-1}} & (t_{i-1} < t \leq t_i) \\
\frac{t - t_{i+1}}{t_i - t_{i+1}} & (t_i < t \leq t_{i+1}) \\
0 & (t \leq t_{i-1}, t > t_{i+1})
\end{cases} \quad (4.16)$$

where $i$ is the grid point, which is ranging from 0 to $N + 1$. As shown in figure 4.1, six grid points were used ($N = 6$) on a grid ranging from $t_0 = 12$ to $t_{N+1} = 26$ minutes. The area of each triangle functions was assumed to represent a so-called pseudo-variant. Accordingly, it is possible to measure the composition of the original Gammanorm mixture, as reported in Table 4.1. An identical procedure was adopted to determine the pseudo-variant composition of the fractions collected from the experiments discussed in this work.

4.4 Results and Discussion

4.4.1 Experiments under Non-Adsorption Conditions

As discussed in Chapter 3, it is suspected that the preparative cation exchange column used in this work has little non-specific adsorption, probably hydrophobic interaction, when working at large ionic strength values, i.e. under non-adsorption conditions for the ion exchange mechanism. For this reason, so-called van Deemter experiments, where
4. Multi Component Mathematical Modelling of the Adsorption

<table>
<thead>
<tr>
<th>Porosity</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total porosity</td>
<td>$\varepsilon_{\text{tot}}$ 0.729</td>
</tr>
<tr>
<td>Total IgG porosity</td>
<td>$\varepsilon_{\text{tot},\text{IgG}}$ 0.514</td>
</tr>
<tr>
<td>Bed porosity</td>
<td>$\varepsilon_b$ 0.373</td>
</tr>
<tr>
<td>Total particle porosity</td>
<td>$\varepsilon_{\text{p,tot}}$ 0.567</td>
</tr>
<tr>
<td>IgG particle porosity</td>
<td>$\varepsilon_{\text{p,IgG}}$ 0.225</td>
</tr>
<tr>
<td>Fractional IgG particle porosity</td>
<td>$K_{d,IgG}$ 0.396</td>
</tr>
</tbody>
</table>

Table 4.2: Porosity data computed by the first order moment analysis of the elution peaks under non-adsorbing conditions. The total porosity is that corresponding to the salt (NaCl). The bed porosity has been calculated from the mid value of the elution front of a highly concentrated injection of dextran 2M. This is supposed to be fully excluded from the pores.

the liquid velocity is changed in order to estimate the mass transport resistances, are not used in this chapter. On the contrary, the estimation of the mass transport resistances is carried under adsorbing conditions in the next section.

In spite of this, several experiments at different velocities of the mobile phase were carried out in order to estimate the column porosity (not shown). In fact, this is independent of the peak shape, when the average retention time is correctly computed from the peak first order moment. The porosity data are reported in Table 4.2. It can be seen that the IgG molecules have a small pore accessibility to the stationary phase. Note that it was assumed that all the components of the Gammanorm mixture have identical size and, thus, pore accessibility.

4.4.2 Diluted Linear Gradient Experiments

The strong dependence of the adsorption isotherm on the ionic strength, the strong mass transport limitations and the presence in the original antibody mixture of a large number of components makes impossible to use isocratic experiments to estimate the Langmuir parameters. On the other hand, the use of linear gradient experiments (LGEs) is particularly convenient. In fact, experiments in both diluted (linear ad-
4.4. Results and Discussion

Figure 4.2: UV signal (280 nm) as a function of the elution time for different linear gradient elution experiments using the original Gammanorm under diluted conditions. The times are referring to the gradient duration. Initial ionic strength = 0.07 M; final ionic strength = 1.02 M; gradient start = 5 min.

Figure 4.2: UV signal (280 nm) as a function of the elution time for different linear gradient elution experiments using the original Gammanorm under diluted conditions. The times are referring to the gradient duration. Initial ionic strength = 0.07 M; final ionic strength = 1.02 M; gradient start = 5 min.

sorption conditions) and overloaded conditions can be carried out and the influence of disturbances in the ionic strength are minimized.

LGEs were first carried out under diluted conditions (0.165 mg in 10 µm are injected) to estimate the affinity towards the stationary phase of the different pseudo-variants composing the original mixture. Five different experiments were carried out using the original Gammanorm mixture, using different salt gradients. These are shown in Figure 4.2, where the outlet UV signal is plotted versus the elution experimental time. In the figure, it can be noticed the sharpening effect due to the increase in the steepness of the gradient. At the same time, it can be noticed that the typical shoulder on the front of the peaks is more and more pronounced for longer gradients.

The gradients of Figure 4.2 have been fractionated and the composition of each
fraction measured on the analytical column, with the procedure described before. The \( \alpha \) and \( \beta \) constants of the Henry coefficient (see Equation 4.1) have been regressed individually. In fact, under linear adsorption conditions, the elution behavior of each pseudo-variant is independent of the others. This procedure also avoids that large errors in the estimation of the elution behavior of the less concentrated pseudo-variants are compensated by small errors in the evaluation of the behavior of the more concentrated ones.

The composition of each pseudo-variant in the different diluted LGE experiments can be observed in Figures 4.3, where the UV signal is plotted as a function of the non-dimensional time. The dashed curve is representing the experimental composition, as computed from the analytical column for each gradient. It can be observed that for the more concentrated pseudo-variants (variants 2 to 4 in Figures 4.3(b) to (d), respectively), the computed composition is has a regular behavior with a clear maximum, which is moving to shorter elution times for the shortest gradients. In the case of the other pseudo-variant, the computed composition is less regular, especially in the case of pseudo-variants 5 and 6 (Figures 4.3(e) and (f), respectively). This is due to the fact that these components have small concentrations and are located at the extremes of the peak. Accordingly, the determination of their concentration is largely affected by the peak rumor. Note that, in order to remain under linear adsorption conditions, the injected mass of the Gammanorm mixture had to remain small, making the analysis of the fractions very difficult.

Previous problems are reflected in the regression of the adsorption isotherm parameters. It can be noticed that, for the main pseudo-variants (Figures 4.3(b) to (d)) a good regression of the main peaks was possible. The regression of the other variances is still acceptable, although due to the large scattering of the experimental data, the regression residuals are larger. In all cases, it can be concluded that the model is able to correctly regress the average elution time of the peaks for the different gradient slopes. It is worth pointing out that, together with the \( \alpha \) and \( \beta \) constants of the Henry coefficient, for each pseudo-variant, the Peclet and the Stanton numbers were regressed, in order to determine the mass transport parameters of each variant. The feed concentration was not a regression parameter. In all the cases, it was used the feed concentration of
Figure 4.3: UV signal (280 nm) as a function of the elution time for pseudo-variants 1 to 6 in figures (a) to (f), respectively. In each subplot, it is plotted the concentration corresponding to the different gradient slopes shown in Figure 4.2. Solid curves: model regression; dashed curves: experimental data.
the pseudo-variant computed from the feed concentration of the Gammanorm mixture and from the composition computed from Figure 4.1. Accordingly, the good matching between the experimental and the model peak areas is confirming the effectiveness of the analytical method.

In Figure 4.4, the correlation plots are shown for the fourth pseudo-variant. All the points shown in the figures correspond to a local minimum found by the regression routine. Minima whose value of the objective function was within 2% of the global minimum are plotted only. In the different regression plots between all possible couples of parameters, it can be observed that a clear correlation can be observed between the values of $\alpha$ and $\beta$ and between the Peclet and the Stanton numbers. In other words, it can be said that the mass transport and the adsorption isotherm parameters are not correlated with each other. This result can be explained by recalling that the average retention time of the peaks under linear adsorption conditions is a function of the isotherm only. Similar plots were obtained for the remaining variants (not shown).

It is now interesting to analyze the correlation plots between $\alpha$ and $\beta$ for the different pseudo-variants. This is shown in Figure 4.5, where it is shown, for the different pseudo-varinats, the points corresponding to a local minimum whose value of the objective function was within 0.25% of the global minimum (a stricter requirement than that used for Figure 4.4). It can be observed that, moving from the first to the last pseudo-variant, the value of $\beta$, which represent the steepness of the Henry function as a function

<table>
<thead>
<tr>
<th>Pseudo-variant</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$Pe$</th>
<th>$St$</th>
<th>$\alpha$-$\beta$ Correlation</th>
</tr>
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<tr>
<td>1</td>
<td>2.9700</td>
<td>1.6740</td>
<td>$6.4129 \cdot 10^{-3}$</td>
<td>41.094</td>
<td>$\beta \simeq -0.18134 \alpha + 2.2113$</td>
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<tr>
<td>2</td>
<td>1.4465</td>
<td>2.2958</td>
<td>$1.2467 \cdot 10^{-3}$</td>
<td>39.065</td>
<td>$\beta \simeq -0.40936 \alpha + 2.8880$</td>
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<tr>
<td>3</td>
<td>1.1958</td>
<td>2.6505</td>
<td>$6.8535 \cdot 10^{-7}$</td>
<td>41.635</td>
<td>$\beta \simeq -0.52807 \alpha + 3.2824$</td>
</tr>
<tr>
<td>4</td>
<td>1.1452</td>
<td>2.8966</td>
<td>$2.5342 \cdot 10^{-5}$</td>
<td>34.286</td>
<td>$\beta \simeq -0.60041 \alpha + 3.5849$</td>
</tr>
<tr>
<td>5</td>
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<td>3.5056</td>
<td>$2.6398 \cdot 10^{-1}$</td>
<td>40.286</td>
<td>$\beta \simeq -1.1222 \alpha + 4.2786$</td>
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<tr>
<td>6</td>
<td>0.40981</td>
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<td>$9.0435 \cdot 10^{-1}$</td>
<td>947.75</td>
<td>$\beta \simeq -2.1000 \alpha + 4.8250$</td>
</tr>
</tbody>
</table>

Table 4.3: Values of the parameters corresponding to the best regression of the diluted LGE experiments (cf. Figure 4.3). The last column represents the best linear fit of the $\alpha$-$\beta$ correlation plots shown in Figure 4.5.
Figure 4.4: Correlation plots for pseudo-variant 4. Each point corresponds to a local minimum. The corresponding value of the objective function is within 2% of the global minimum.
Figure 4.5: Correlation plots between the values of $\alpha$ and $\beta$ for the different pseudo-variants and obtained in the diluted LGE experiments (cf. Figure 4.2). Each point corresponds to a local minimum. The corresponding value of the objective function is within 0.25% of the global minimum. The dashed lines represent the regression best linear fit. PV = pseudo-variant.

of the salt concentration, is increasing. As discussed by Yamamoto et.al [70], the value of $\beta$ is directly correlated to the net protein charge. In fact, pseudo-variant 6 is the last eluting variant on the preparative cation exchange column and, thus, the one with the largest net positive charge. At the same time, the regressed value of $\alpha$ is decreasing. If a linear fit of these points is observed (dashed lines of Figure 4.5), it is interesting to notice that the slope of the fit line is increasing from pseudo-variant 1 to 6. In other terms, in the case of the first pseudo-variant, to a large confidence interval on the parameter $\alpha$ corresponds a small interval on $\beta$, whereas the opposite situation is found for the last pseudo-variant.

It is interesting to observe the affect of the regression on $\alpha$ and $\beta$ has on the corre-
4.4. Results and Discussion

The respondent behavior of the Henry coefficient as a function of the salt concentration (see Equation 3.12). This is shown in Figure 4.6(a), where the best regression values used in Figure 4.3 have been used to define the values of $\alpha$ and $\beta$ of the Henry function for the different pseudo-variants. All the Henry coefficients are strong functions of the salt concentration. However, it can be noticed that the differences in the value of $\beta$ observed in Figure 4.5 are such that at low salt concentration the affinity of the different pseudo-variants towards the stationary phase are very different. On the contrary, all the Henry coefficient of the various components seem to cross at a salt concentration between 0.4 and 0.5 M, where the affinity is anyway low. More interesting is to observe that influence of the confidence interval of $\alpha$ and $\beta$ on the behavior of the Henry coefficient. This is shown in Figure 4.6(b) for the pseudo-variants 3 and 4. Note that the fit lines of Figure 4.5 for these two pseudo-variants are never crossing and, on the contrary, they are roughly parallel. In spite of this, the effect on the Henry coefficients of choosing different values of $\alpha$ and $\beta$ along the fit line is very strong and the selectivity (i.e. the ratio between the Henry coefficients) at very low salt concentrations is changing between about 1.2 and 9. It can be concluded that another type of experiments is needed to better regress the Henry coefficient at low salt concentrations.

Finally, it is instructive to discuss the correlation between the pore Peclet and the Stanton numbers. As shown in Equations 4.11, these two non-dimensional groups are defining the importance of the mass transport limitations with respect to convection, in the pores and in the laminar film around the particles, respectively. The correlation plots for all the variants are shown in Figure 4.7. It can be noticed that the correlation between the two transport parameters obtained independently for the different pseudo-variants are very similar. This is confirming that the pseudo-variants can be actually considered as single components, in spite of the fact that they are composed by many IgG molecules. In fact, as discussed in the previous chapter, the presence of different variances could artificially make the peak broader, due to the different adsorption behaviors of the components of the pseudo-variant. This in turn could lead to an overestimation of the mass transport resistances. The Peclet number and the Stanton number are much smaller and much larger than one, respectively, indicating that the characteristic time for mass transport is smaller than that for convection. Clearly, as
Figure 4.6: Henry coefficient as a function of the salt concentration. (a) Henry function for the different pseudo-variants found using the values of $\alpha$ and $\beta$ corresponding to the best regression of Figure 4.3. (b) Henry function of the pseudo-variants 3 and 4 found by moving along the fit line of Figure 4.5.
4.4. Results and Discussion

Figure 4.7: Correlation plots between the values of the Peclet and Stanton numbers for the different pseudo-variants and obtained in the diluted LGE experiments (cf. Figure 4.2). Each point corresponds to a local minimum. The corresponding value of the objective function is within 1% of the global minimum. PV = pseudo-variant.

shown in Figure 4.7, when the pore resistances are overestimated (large values of $Pe$), the film transfer resistances must be underestimated to compensate (large values of $St$). In general, for Stanton number in between 30 and 40, the regression becomes independent of the Peclet number, if a value smaller than 0.01 is used.

4.4.3 Overloaded Linear Gradient Experiments

Overloaded LGE experiments can be used to fix the dependence of the saturation capacity on the ionic strength, once the dependence of the Henry coefficients is established by the diluted LGE experiments. In Figure 4.8, the outlet concentration as a function of the elution time is shown for three overloaded experiments (the corre-
Figure 4.8: Outlet concentration as a function of the elution time for different linear gradient elution experiments using the original Gammanorm under overloaded conditions. The masses are referring to the injected mass of Gammanorm. Initial ionic strength = 0.07 M; final ionic strength = 1.02 M; gradient start = 5 min; gradient duration = 30 min.

It can be observed that, as the injection mass is increased, the peak front is moving towards shorter elution times, as a consequence of the saturation of the stationary phase. The peak rear is not moving, coherently with the theory for Langmuir isotherms. On the other hand, the typical concentration shock on the peak front predicted by the theory is absent and, on the contrary, the peak remains rather symmetrical. This effect is due to the dominant mass transport limitations.

The regression of the overloaded experiments proceeded as follows. First, the coefficients $\gamma$, $\delta$ and $\epsilon$ of the saturation capacity expression (see Equation 4.13) were assumed to be equal for all the pseudo-variants. In fact, it is here assumed that the saturation
4.4. Results and Discussion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pseudo-variant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
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<td>2.1462</td>
</tr>
<tr>
<td>$\beta$</td>
<td>1.8660</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>7468.2</td>
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<tr>
<td>$\delta$</td>
<td>18.027</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>-29.427</td>
</tr>
</tbody>
</table>

Table 4.4: Values of the parameters corresponding to the best regression of the overloaded LGE experiments (see Figure 4.9).

The overall quality of the fit is good. In particular, it can be noticed that the fit is
Figure 4.9: Outlet concentration as a function of the elution time for the different LGE experiments. The first five subplots are referring to the regression of the diluted LGE experiments of Figure 4.2; the remaining three subplots to the regression of the overloaded LGE experiments of Figure 4.8. Solid curves: model regression; dashed curves: experimental data.
particularly good in the case of the overloaded experiments, where a very good matching between experiments and model is obtained for both the overall concentration (dashed curves) and the concentrations of the single pseudo-variants (solid curves). It can be observed that the peak displacement at large overloading conditions is very well predicted. To this regard, it must be pointed out that the quality of the overloaded experiments was particularly good, due to the fact that the large outlet concentration in each fraction made easy the following composition analysis on the analytical IEX column. The regular behavior of the experiments, as compared to the scattering of the ones in the diluted gradients, clearly helped the regression. This is particularly true for the less concentrated pseudo-variants at the peak extremes. This fact can be noted by comparing the regressed values of the parameters for the diluted (Table 4.3) and for the overloaded experiments (Table 4.4). It can be observed that small changes have been introduced in the values of $\alpha$ and $\beta$. This is especially the case of the central (more concentrated) pseudo-variants, whereas larger changes are seen for the first and the last pseudo-variants. In this case, the good quality of the overloaded gradients allowed to adjust the uncertainty in the Henry parameters due to the large scattering of the diluted gradients. A better view upon the values of $\alpha$ and $\beta$ is found in Figure 4.10, where the regressed values of these parameters for all the variants is shown for the diluted (circles) and the overloaded (diamonds) LGE experiments. As for Figure 4.5, only the points corresponding to a local minimum whose value of the objective function was within 0.25% of the global minimum were plotted. It can be noted that for pseudo-variants 2 to 5 the old regression points are overlapping to the new ones. This is not the case for pseudo-variants 1 and 6, although the differences remain limited.

Finally, it is interesting to analyze the correlation between the remaining parameters. There is little correlation among the values of $\beta$ of the different variants (not shown). The objective function for the regression is defined as the quadratic error between the experimental concentration and that predicted by the model for each pseudo-variant. As a consequence, the parameter correlation was minimized. On the other hand, some degree of correlation can be observed for the parameters defining the saturation capacity, $\gamma$, $\delta$ and $\epsilon$. This can be observed in Figure 4.11. No correlation can be observed between $\gamma$ and the other two parameters. However, it is clear that some
Figure 4.10: Correlation plots between the values of $\alpha$ and $\beta$ for the different pseudo-variants and obtained in the diluted (circles) and the overloaded (diamonds) LGE experiments (cf. Figure 4.2). Each point corresponds to a local minimum. The corresponding value of the objective function is within 0.25% of the global minimum. The dashed lines represent the regression best linear fit. PV = pseudo-variant.
correlation exists between $\delta$ and $\epsilon$. The reason of this correlation can be found by analyzing the computed behavior of the saturation capacity as a function of the ionic strength. By inserting any of the value of $\delta$ and $\epsilon$ in the definition of the saturation capacity (Equation 4.13), it can be seen that for ionic strength much smaller than one the argument of the exponent is always much smaller than zero, i.e. the exponent is tending to zero. Accordingly, under these conditions the saturation capacity is constant with the ionic strength and equal to $\gamma$. As already noticed in Chapter 3, this result seems to indicate that the saturation capacity is really representing the maximum surface occupation of the stationary phase, coherently with the definition of the Langmuir isotherm. This in spite of the fact that many works indicated that also the saturation capacity is a function of the ionic strength (i.e. emptying the parameter of its physical meaning) [35, 40, 107, 108, 109, 110].

### 4.4.4 Prediction of Breakthrough Experiments

Previous values of the mass transport and isotherm parameters are used here to predict the elution profiles of two breakthrough experiments carried out at different salt concentrations. This is shown in Figures 4.12(a) and (b), where the outlet concentration of the single pseudo-variants is shown as a function of time for the breakthrough experiment using an ionic strength of 0.07 and 0.12M, respectively. The general agreement is satisfactory. This is particularly true for the breakthrough curve at the smaller ionic strength (Figure 4.12(a)). Here, it can be observed that the slope of the total concentration, as well as the breakthrough time, are well predicted. The same can be said for the competition between the pseudo-variants, with the exception of the first one, which is eluting too early. In the case of the second breakthrough (Figure 4.12(b)), the agreement is not as satisfactory. The breakthrough time is well predicted. However, the overall concentration profile is not exhibiting the shoulder observed in the experimental data. Moreover, the competition between the pseudo-variants is almost absent, while it is very pronounced in the experiments.

The parameters of the Henry coefficients have been regressed again, together with all the LGE experiments, in order to improve the description of the previous breakthrough experiments. The parameters were left to change within 20% of the value
Figure 4.11: Correlation plots between the saturation capacity parameters (cf. Equation 4.13) regressed from the overloaded LGE experiments (cf. Figure 4.2). Each point corresponds to a local minimum. The corresponding value of the objective function is within 0.25% of the global minimum.
Figure 4.12: Outlet concentration as a function of time for two breakthrough experiments, using an ionic strength of (a) 0.07 and (b) 0.12M. The values of the parameters found in the LGE experiments are used. Solid curves: pseudo-variant concentration; dashed curves: total concentration; black curves: model prediction; gray curves: experimental data.
regressed in the overloaded LGE experiments (see Table 4.3). The same saturation capacity as in the overloaded LGE experiments has been used, still keeping this value equal for all the pseudo-variants. The final result of the regression is shown in Figures 4.13(a) and (b), where the outlet concentration of the single pseudo-variants is shown as a function of time for the breakthrough experiment using an ionic strength of 0.07 and 0.12M, respectively. The fit on all the LGE experiments is shown in Figure 4.14. In Figures 4.13, it can be observed that the regression of the first breakthrough experiment is further improved. The overall concentration is almost perfectly matching the experimental profile. This result has been mainly obtained by adjusting the Henry coefficients of the first pseudo-variant, which was early eluting in Figure 4.12(a). Also the overall profile of the second breakthrough is improved (Figures 4.13(b)). However, the competition among the pseudo-variants is still not predicted correctly. Negligible variations can be observed in the prediction of the remaining LGE experiments shown in Figure 4.14.

4.5 Conclusions

In this Chapter, a new procedure for the identification of a pre-defined number of pseudo-variants from a single analysis on a non-porous analytical weak cation exchange column is presented. The polyclonal antibody mixture analyzed in this study is made of a very large number of different antibodies and the analytical identification of all this components can be very difficult. On the other hand, the description of the adsorption behavior of the mixture can be carried out by identifying a limited number of pseudo-variants, which are covering the entire range of components of the original mixture.

A regression procedure for the determination of the mass transport and the adsorption isotherm parameters is discussed. Simple isocratic experiments under non-adsorption conditions were carried out to determine the column porosity. The mass transport parameters and the linear part of the adsorption isotherm have been instead estimated by diluted linear gradient elution experiments. The regression has been done separately for each variant. The analysis of the regression result evidenced that the transport parameters are strongly correlated to each other, as well as the parameters of
Figure 4.13: Outlet concentration as a function of time for two breakthrough experiments, using an ionic strength of (a) 0.07 and (b) 0.12M. The values of the parameters have been regressed together with the LGE experiments. Solid curves: pseudo-variant concentration; dashed curves: total concentration; black curves: model prediction; gray curves: experimental data.
4.5. Conclusions

Diluted LGE - 10 min

Diluted LGE - 20 min

Diluted LGE - 30 min

Diluted LGE - 45 min
Figure 4.14: Comparison between the experimental data (gray curves) and the model regression (black curves) for the LGE experiments shown in Figure 4.9. The regressed parameter values used for Figure 4.13 are used in this figure.
the Henry coefficient. On the other hand, no important correlation was found between the two groups of parameters.

A further refinement of the adsorption parameters has been done using the overloaded linear gradient elution experiments. With the exception of the least concentrated pseudo-variant, for which a large data scattering was present in the diluted gradients, negligible changes have been introduced in the regressed values of the Henry parameters. On the other hand, it has been possible to estimate the saturation capacity of the column. As in the case of Chapter 3, it has been confirmed that this can be assumed roughly independent of the ionic strength and equal for all the pseudo-variants. In fact, a very good description of the overall elution profile, as well as of the displacements effects among the components, has been obtained.

Unfortunately, the very good results in the description of the gradients experiments did not lead to equally good results in the description of the breakthrough experiments. Although there is a general acceptable agreement between the experiments and the model prediction (i.e. without any parameter adjustment), the increase in model complexity did not bring substantial improvements in the prediction capabilities of the model. The same result has been obtained after an additional regression of all the experiments. This is particularly true for the breakthrough experiment at larger ionic strength.

Many reasons can be found for this result. First, the very large sensitivity of the adsorption isotherm to the operating conditions (ionic strength and pH) makes this experiments very difficult to be carried out. This is particularly true for the largest ionic strengths, for which the adsorption isotherms are no more rectangular. On the other hand, when using very small salt concentrations, the isotherm becomes soon rectangular and, being the saturation capacity almost constant, a smaller sensitivity can be expected. In fact, under this conditions it is possible to well reproduce the experimental profiles. Another cause for the unsatisfactory regression can be found is possible changes of the pH value due to salt displacement. This would affect the adsorption isotherm is a way that is not accounted for in this work. Finally, a large influence could be played by the changes in porosity due to adsorption, as reported by many authors [15, 18, 27, 41, 42]. This could dramatically change the competition
behavior inside the particles. This argument is the object of investigation of the next chapter.
Chapter 5

Porosity Investigation

5.1 Introduction

High pressure liquid chromatography (HPLC) is the method of choice for the downstreaming process of various active pharmaceutical ingredients (APIs) in the pharmaceutical industry [132]. Although relatively expensive, liquid chromatography has the big advantage of allowing good separations using very mild conditions. This is very important especially for very sensible biomolecules like peptides and proteins. Packing materials with highly porous beads are typically used for this scope. Among the different beads available on the market, those based on polymeric supports are particularly important in biomolecule purifications, due to the possibility of sanitize them [133]. These materials can be functionalized with different groups, such as charged groups (ion-exchange chromatography), groups with an hydrophobic character (hydrophobic interaction chromatography) or groups which can interact specifically with other molecules (affinity chromatography). Ion exchange chromatography (IEX) is widely used for the purification of proteins, since the different proteins can be effectively separated according to their charge distribution [116]. Moreover, the entire purification process can be done under physiological conditions and therefore not affecting the bioactivity of the protein [70].

In preparative IEX chromatography, 40 to 100 $\mu$m highly porous particles, with pore diameters ranging from 100 to 1000 Å, are generally used [134]. The formation of the particle porosity must meet two conflicting needs. On one hand, that of forming very
small pores, so to increase the total surface area available for adsorption. On the other, to have pores large enough to allow for the transport of the solutes. This dichotomy becomes especially important in the case of large biomolecules. Large proteins can have in fact hydrodynamic diameters as large as 100 Å, that is very similar to the average diameter of most of the available stationary phases, and therefore pore accessibility of only 20 to 60% of the total available pore volume [94, 134, 135]. This is not only detrimental to the total static capacity of the support, but it has major consequences on the protein mass transport inside the beads. Under such conditions in fact hindrance becomes very pronounced and, thus, the effective pore diffusion rate of these molecules, already small due to their size, becomes even smaller. As a consequence, elution peaks become broader, thus decreasing peak resolution, and the dynamic capacity drops fast with increasing flow rates [134].

Several factors can have a large influence in determining pore accessibility of biomolecules. First, hindrance can clearly worsen under adsorption conditions, since the presence of adsorbed molecules further reduces the available pore diameter for transport. Several authors have investigated the change in the effective pore diffusion rate with increasing loading, reporting a sensible decrease [18, 41, 42]. The same effect can be expected in those materials where the ionic ligand is carried by the so-called tentacles [136], that is a polymeric chain attached to the polymer surface. Tentacles made of some hundreds of monomer units can have dimensions similar to those of the pores and, thus, a large influence on the transport of large molecules. In particular, it is interesting to investigate the behavior of such tentacles under different operating conditions, i.e. salt concentrations. Charge shielding and salting-out effects can in fact change the tentacle conformation and, then, its hindrance effect on the transport of large molecules.

The knowledge of the pore size distribution is especially important in the numerical modelling of chromatographic processes. Peak shape and elution time are a function of the accessible pore volume, the overall retention factor, $k'$, and the overall rate of mass transport [63]. All these parameters are direct function of the pore size distribution. The retention factor can in fact be regarded as given by the product of two coefficients: the specific retention factor of the surface, which is quantifying the affinity of the
solute towards the support, and the total accessible surface area. At the same time, the rate determining step in the mass transport of large biomolecules is pore diffusion. Accordingly, the knowledge of how the pore size distribution changes for different operating conditions is fundamental for the prediction of the column behavior, and small errors can lead to highly inaccurate model predictions [15].

Several works exist which are measuring the pore size distribution of stationary phases under non-adsorbing conditions [94, 134, 135, 137]. Few reports can be found on the change of the pore size distribution under adsorbing conditions, i.e. low salt concentrations [66]. At the same time, several works can be found where the pore diffusivity of biomolecules is measured directly with off-line experiments [138], or indirectly by regressing it with both off- [41, 66] and on-line [116, 139, 140] experiments. However, to our best knowledge, no investigation is available on the pore size distribution under true working conditions, i.e. low salt concentrations and high loadings. In this chapter, we focus on the direct measurement of porosity on ion-exchange resins with tentacles, namely Fractogel EMD COO- (S) and Fractogel LLD (M). The former is a commercial material, which is particularly suited for the purification of large bio-molecules. The latter has been specifically synthesized for this work and is characterized by a lower tentacle density on the surface. As it will be discussed, the measure of the pore size distribution under typical working conditions was possible thanks to the careful choice of a polymeric tracer, which does not interact neither with the support nor with the adsorbed proteins.

5.2 Materials and Methods

Stationary phase and columns

Two ion exchange materials have been selected for this work: Fractogel EMD COO- (S) and Fractogel LLD (M). Fractogel EMD COO- (S) is a commercial stationary phase and was purchased from Merck (Germany). Fractogel LLD (M) was synthesized for this work by Merck (Germany). The properties of the two materials are summarized in Table 5.1. The chemistry of the base matrix is the same for both materials and both are functionalized using the tentacles technology [141]. Fractogel EMD COO-
(S) is a weak cation exchanger with carboxy ethyl functional terminal groups attached to the tentacles; Fractogel LLD (M) is a strong cation exchanger with sulfoisobuthyl terminal groups. The two materials have different tentacle density. Fractogel LLD (M) has a low ligand density (144 $\mu$mol/g) compared to Fractogel EMD COO-(S) (about 300 $\mu$mol/g). Packing has been done following the indications of the manufacturer, into two 50x5 mm Tricorn columns (GE Healthcare, UK) at 2 ml/min. The quality of the packing has been tested with acetone by measuring the corresponding HETP. Note that the use of such small columns is justified by the need to limit the consumption of proteins during the loading experiments.

<table>
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<th>Fractogel LLD (M)</th>
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<td>crosslinked PMA</td>
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<td>saturation capacity** for HSA</td>
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Table 5.1: Properties of the ion exchange resins used in this work.*: before derivatization, **: measured in 20 mM acetate buffer at pH=5 and 0.05 M NaCl

Mobile phase and chemicals

All the experiments have been performed at pH=5 using 20 mM sodium acetate buffer. The buffer was made mixing sodium acetate (Merck, Germany) and acetic acid (Carlo Erba reagents, Italy). NaCl (J.T. Baker, USA) was used as modifier. Each component of the buffer was exactly weighted using a precision balance (METTLER AT250, Mettler-Toledo, Switzerland). The protein used in the loading experiments were Immunoglobulin G (IgG, Gammanorm, Octapharma GmbH, Germany) and human serum albumin (HSA, Sigma, Switzerland). The concentration of these proteins in solution was determined using the analytical stationary phase ProPac WCX-10 (Dionex, USA).
Chromatography equipment

The same chromatographic equipment as in the previous chapters has been used.

Selection of the porosity tracers

Polymeric tracers for porosity measurements must fulfill the following characteristics:

- negligible adsorption under the experimental conditions
- narrow molecular weight distribution
- well defined molecular size (or hydrodynamic radius)
- negligible change in size at the investigated operating conditions
- good UV and RI visibility

A polymer fulfilling most of the above requirements is polyvinylpyrrolidone (PVP). However, PVP is only commercially available with broad molecular weight distributions. Three PVP products were selected: PVP K12 (avg MW 3 500 g/mol, Acros organics, USA), PVP K30 (avg MW 40 000 g/mol AppliChem, Germany) and PVP K90 (avg MW 360 000 g/mol, Acros organics, USA). In order to obtain narrow distributed polymer fractions, PVPs were fractionated using two size exclusion chromatography (SEC) columns in series: a 300x7.5 mm TSKgel G4000PWXL column (Tosoh bioscience, Japan) followed by a 300x10 mm Supedex75 10/300 GL column (GE Healthcare, UK). The exclusion limits for the two columns are (in dextran equivalents): 1 000 to 700 000 g/mol and 500 to 40 000 g/mol, respectively. Pure water was used as solvent in the fractionation. Successively, the collected fractions were dried in an oven in order to obtain pure polymers in solid state. The SEC traces of the selected fractions as measured on the TSKgel G4000PWXL SEC column are shown in Figure 5.1, where the traces of the original polymers are also shown. The same column, previously calibrated using polyethyleneglycol (PEG) tracers with known size (from 200 to 35 000 g/mol) [143], was used to evaluate the average molecular weight of the PVP fractions. PVP 90 fraction 1 is excluded from the pores of the SEC column and its hydrodynamic diameter was measured using dynamic light scattering (DLS). The characteristics of
the selected tracers are summarized in Table 5.2. Dextran 2000 (avg MW 2 000 000 g/mol, Pharmacia, Sweden) was used to measure the bed porosity. The hydrodynamic radii of IgG and HSA have been taken from literature [18, 139, 144].

Figure 5.1: SEC traces of the selected PVP fractions (full curves) as well as the original polymers (dashed curves). The PVP fraction are corresponding to (from right to left): PVP 12 fraction 11, PVP 12 fraction 10, PVP 12 fraction 6, PVP 30 fraction 10, PVP 30 fraction 9, PVP 30 fraction 8, PVP 30 fraction 7, PVP 30 fraction 5, PVP 90 fraction 6 and PVP 90 fraction 1.

The diameter of one PVP fraction (PVP 90 fraction 6, see Table 5.2) was measured by DLS at different salt concentrations, ranging from 0 to 1.0 mol/l. The observed change in diameter was found to be within the experimental error of the method (± 2nm). Accordingly, it is assumed that the change in size due to changes in the eluent salt concentration is negligible and, therefore, the PVP fractions fulfill all the requirements for a tracer for porosity determination reported above.
5.2. Materials and Methods

<table>
<thead>
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</tr>
<tr>
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<td>1</td>
<td>83.70*</td>
</tr>
</tbody>
</table>

Table 5.2: Hydrodynamic diameter of the PVP fractions measured with size exclusion chromatography (*: measured with DLS).

**Porosity measurements**

The porosity measurements have been carried out using different salt concentrations and different amounts of protein adsorbed on the material. In the first type of measurements, five buffers with 20 mM sodium acetate at pH=5 and different NaCl concentrations were prepared. In order to minimize signal disturbances, the solid PVP fractions were solved in the same buffers as in the experiments. The column was equilibrated with 10 column volumes (CV) of buffer before injection. Tracers were detected by the UV detector at 220 nm.

In the second type of measurements, the column was first equilibrated with a 20 mM sodium acetate buffer at pH=5 containing 0.05 M NaCl, after which the protein solution (initially with a concentration of 4 g/l in 0.05 M NaCl buffer) was pumped in a loop (at 0.5 ml/min) through the column until the outlet signal was constant (the minimal equilibration time was 3 hours). The protein concentration was determined before and after adsorption. From the difference between the initial amount of protein in the liquid phase and the amount left after equilibration, the amount adsorbed per volume of column was determined. At this point, the PVP fractions were dissolved in
the equilibrium protein solution (from the loop) and injected into the column. Their retention time was measured by the RI detector after the reference cell of the detector was flushed with the mobile phase. After all the tracers were measured, a given amount of buffer containing 0.5M NaCl was added to the protein pool to decrease the protein amount adsorbed on the column. After waiting for the new steady state, the procedure above was repeated.

The total liquid volume accessed by a molecule, $V_{t,i}$, with respect to the total column volume, $V_c$, is referred to as total porosity [95]:

$$
\varepsilon_{t,i} = \frac{V_{t,i}}{V_c} \tag{5.1}
$$

$V_{t,i}$ is calculated from the peak maximum retention time. Even if it represents an approximation, the choice of this method is justified by the non ideal shape of the polymers elution peaks (see Figure 5.1). The particle volume accessed, $V_{p,i}$, with respect to the particle volume is referred to as particle porosity:

$$
\varepsilon_{p,i} = \frac{V_{p,i}}{V_p} = \frac{V_{p,i}}{(1 - \varepsilon_b)V_c} \tag{5.2}
$$

where $\varepsilon_b$ is the bed porosity, that is the total porosity accessed by a molecule entirely excluded from the particle pores. Therefore, the total and the particle porosity are correlated by the following equation:

$$
\varepsilon_{t,i} = \varepsilon_{p,i} \cdot (1 - \varepsilon_b) + \varepsilon_b \tag{5.3}
$$

Using tracers with different dimension, the so called inverse size exclusion chromatography (iSEC) curve is constructed, where the total porosity is plotted against the logarithm of the hydrodynamic diameter of the different tracers [94] (see e.g. Figure 2.1. Two limits can be identified in the iSEC curve: the column free volume, $\varepsilon_t$, that is the volume accessed by a tracer so small to enter every pore; and the bed volume, $\varepsilon_b$, that is the volume accessed by those tracers entirely excluded by all particle pores. Note that in this work, the particle (and not the total) porosity is plotted against the hydrodynamic radius in the iSEC curves, for sake of clarity.
5.3 Results and discussion

5.3.1 Porosity as a function of salt concentration

Different PVP fractions were injected in the columns pre-equilibrated with four salt concentrations: 0.05, 0.1, 0.5 and 1.0 M NaCl, respectively. IgG and HSA were injected at 1.0 M, since they are not retained at such large salt concentration. The change in the measured particle porosity for the different PVP fractions is shown in Figures 5.2 and 5.3 for Fractogel EMD COO- (S), and in Figures 5.4 and 5.5 for Fractogel LLD (M).

Figure 5.2: Change in particle porosity for PVP fractions as a function of the salt concentration on Fractogel COO- (S). ♦: PVP 12 fraction 11, ▲: PVP 12 fraction 10, ■: PVP 12 fraction 6, ●: PVP 30 fraction 9, ◆: PVP 30 fraction 7, △: PVP 30 fraction 6, ◇: PVP 90 fraction 6

Let us discuss the results for Fractogel EMD COO- (S) first. Dextran 2000 and PVP
90 fraction 1 have the same pore accessibility. Due to their size, they are believed to be both fully excluded from the pores and, therefore, are used for the determination of the bed volume of the column. The total porosity of these tracers is not changing with the salt concentration (not shown). This means that the dimension of the particles in the column remains constant at the different salt concentrations and, therefore, only the pore dimension is affected by changes in the salt concentration. On the other hand, by observing Figure 5.2, it can be seen that there is a small increase in particle porosity for increasing salt concentrations for the PVP fractions not fully excluded from the pores, as also observed by Stone and Carta [66]. The observed size exclusion can be due to both steric and electrostatic effects. As the salt concentration is increased, the charge of the ionic groups on the tentacles is shielded, thus minimizing electrostatic effects. At the same time, salting-out effects can take place [94], making the tentacles more hydrophobic and causing their collapse on the support surface. This effect would reduce the steric exclusion (except for the steric exclusion due to the pore structure of the support).

In Figure 5.3, the inverse size exclusion (iSEC) graph is plotted for the same set of experimental data. In this figure, the small role played by NaCl in affecting the particle porosity is more evident, even though a small increase in particle porosity can be observed for increasing salt concentration for the largest tracers. In the same figure, the particle porosities of IgG and HSA (at 1M NaCl) are shown (circles). These are very similar to those of the corresponding PVP tracers of the same size, which are not charged. In fact, at such large salt concentration, all the protein charges are shielded and exclusion is due to steric effects only. The residual differences in porosity can be caused by small non-specific interactions between the proteins and the base matrix at 1M NaCl (most probably hydrophobic interactions), or by small errors in the evaluation of the hydrodynamic radii of both the polymer tracers and the proteins. In spite of this, the agreement is sufficiently good to confirm the reliability of PVP as porosity tracer.

The same analysis as above has been repeated for Fractogel LLD (M). The particle porosities for the different polymer tracers at different salt concentrations are shown in Figures 5.4 and 5.5. In this material, dextran 2000 exhibits a smaller total porosity than
Figure 5.3: iSEC curve of Fractogel COO- (S) measured with the PVP fractions as a function of the salt concentration (♦: 1.00M NaCl, ▲: 0.50M NaCl, △: 0.10M NaCl, ◆: 0.05M NaCl). ◇: HSA and IgG particle porosity measured at 1M NaCl.
Figure 5.4: Change in particle porosity for PVP fractions as a function of the salt concentration on Fractogel LLD (M). ♦: PVP 12 fraction 11, ▲: PVP 12 fraction 10, ■: PVP 12 fraction 6, ●: PVP 30 fraction 9, ◊: PVP 30 fraction 7, △: PVP 30 fraction 6 □: PVP 30 fraction 5, ○: PVP 90 fraction 1

the largest PVP standard (not shown). Accordingly, dextran 2000 is used to evaluate the bed porosity, while about 10% porosity seems to be accessible to PVP 90 fraction 1. It cannot be excluded that this last result is caused by some small adsorption of dextran 2000. Two additional observations can be made from the comparison with Fractogel EMD COO- (S): (i) the iSEC curves are steeper for Fractogel LLD (M) (see Figures 5.3 and 5.5), thus indicating a narrower pore size distribution; and (ii) this material has a larger sensitivity to variations in the salt concentration. When considering the two smallest tracers only (PVP 12 fraction 11 and PVP 12 fraction 10), a large variation in the porosity and values larger than one can be observed in Fractogel LLD (M). This material has a smaller ligand density than Fractogel EMD COO- (S) and, thus, the underivatized surface is larger. For this reason, the two small tracers mentioned
5.3. Results and discussion

Figure 5.5: iSEC curve of Fractogel LLD (M) measured with the PVP fractions as a function of the salt concentration (◊: 1.00M NaCl, ▲: 0.50M NaCl, △: 0.10M NaCl, ♦: 0.05M NaCl). ○: HSA and IgG particle porosity measured at 1M NaCl.
above can probably access part of this free surface and adsorb on it by hydrophobic interaction. This effect is probably increased at larger salt concentrations by salting-out effects. On the other hand, larger tracers should not access the surface between the functional groups and, therefore, adsorb on the material. This is confirmed by the similarity of the porosity data between the polymer tracers and the two proteins, IgG and HSA at 1M NaCl (see Figure 5.5). However, as for the largest tracer, little interactions cannot be excluded. Tracers between 2 and 9 nm have a larger pore accessibility than in the case of Fractogel EMD COO- (S). However, the porosity of the largest tracers is very similar to that measured on Fractogel EMD COO- (S).

It is possible that, for such large molecules, the accessibility to the pores is mostly determined by the pore size distribution of the unfunctionalized support (which is similar for the two materials). In spite of this, the sensitivity of the accessible porosity to changes of salt concentration appears to be more pronounced than in the case of Fractogel EMD COO- (S) (see Figure 5.2), especially for large tracers. In case of dominant electrostatic effects, all the tracers should be similarly affected, being their chemical composition identical. The fact that this effect is noticeable only for the largest tracers should suggest that collapsing of the tentacles on the pore surface is the most probable mechanism behind the increased porosity for the largest tracers.

It should be noted that the two materials are produced starting with the same base material, but with two different derivatization techniques. This, combined with the smaller ligand density, is probably the cause of the bigger pore size distributions measured for the Fractogel LLD material. The different chemistry of the functional groups, i.e. carboxy ethyl-groups and sulfoisobutyl-groups, could also be the reason behind the different salt sensitivity of the two materials.

From the iSEC curves the pore size distribution and thus the average pore size of the material can be calculated. The average pore size at 1.0 M NaCl is around 30 nm for Fractogel COO- (S), and around 50 nm Fractogel LLD. This confirms the higher porosity of Fractogel LLD. Moreover it is important to note that the average pore diameters are only 3 and 5 times larger than IgG, respectively.

By extrapolation of the previous data from PVP tracers, it is possible to calculate the pore accessibility of IgG and HSA as a function of salt concentration. This is
5.3. Results and discussion

![Graph showing predicted change in particle porosity for HSA (♦) and IgG (▲) as a function of the salt concentration on Fractogel COO- (S).]

Figure 5.6: Predicted change in particle porosity for HSA (♦) and IgG (▲) as a function of the salt concentration on Fractogel COO- (S).

shown in Figures 5.6 and 5.7 for Fractogel EMD COO- (S) and Fractogel LLD (M), respectively. For both proteins, the change in porosity is modest and it is confirmed that it is larger in the case of Fractogel LLD (M). As for the larger tracers, the porosity accessed by IgG is very similar for both materials, with the exception of the smallest salt concentration. On the other hand, the two materials behave very differently in the case of HSA (and smaller molecules). In particular, the accessibility is larger for Fractogel LLD (M). Note that pore accessibility for proteins is generally supposed to be constant and independent of the operating conditions, especially when measuring the solute affinity to the stationary phases. From Figures 5.6 and 5.7, it is seen that changes in the pore accessibility are not negligible and are generally lumped into the estimated adsorption isotherm. The independent knowledge of the pore accessibility allows avoiding this mistake.
In the previous discussion, no mention is given to the fact that the two materials have different particle sizes. It is believed that the effect of the particle size on the porosity is secondary. In fact the different characteristic times for diffusion in the two materials due to the different particle size have no effect on previous measurements. The average retention time is independent of mass transport limitations.

From the analysis above we can conclude that, as expected, there is an influence of salt concentration on the porosity accessible to a given molecule. This effect is modest and limited to the largest polymer tracers, i.e. those similar in size to the two proteins used in this work. The two materials show different behaviors with respect to the increase on salt. The difference can be explained by the different chemical nature of the functional group, the different derivatization technique or the different tentacles density.
5.3.2 Porosity as a function of the amount of protein loading

The second set of measurements is aimed at measuring the change in the accessible porosity for increasing amounts of protein adsorbed on the material. Let us first analyze Fractogel EMD COO- (S). The porosity of the PVP fractions for this material is plotted as a function of the amount of adsorbed IgG and HSA in Figures 5.8 and 5.9, respectively. Note that the amount of adsorbed proteins in equilibrium with the liquid phase is expressed as a concentration, that is $\text{mg}_{\text{protein}}/\text{mL}_{\text{column}}$.

![Graph showing change in particle porosity for PVP fractions on Fractogel COO- (S) as a function of adsorbed IgG concentration]

Figure 5.8: Change in particle porosity for PVP fractions on Fractogel COO- (S) as a function of the adsorbed amount of IgG. The porosity values at $q_{\text{eq, IgG}} = 0$ correspond to the particle porosities measured at 0.5 M NaCl, without protein. ♦: PVP 12 fraction 6, ▲: PVP 30 fraction 10, ■: PVP 30 fraction 9, ♦: PVP 30 fraction 8, △: PVP 30 fraction 7 □: PVP 90 fraction 1.

In both figures we can observe a very strong decrease of the PVP accessible porosity for increasing amounts of adsorbed protein. Accordingly, adsorbed proteins hinder sig-
Figure 5.9: Change in particle porosity for PVP fractions on Fractogel COO- (S) as a function of the adsorbed amount of HSA. The porosity values at $q_{eq,HSA} = 0$ correspond to the particle porosities measured at 0.5 M NaCl, without protein. ♦: PVP 12 fraction 6, ▲: PVP 30 fraction 10, ■: PVP 30 fraction 9, ◇: PVP 30 fraction 8, △: PVP 30 fraction 7 □: PVP 90 fraction 1

nificantly pore accessibility and this is more pronounced when the bulky protein IgG is used (Figure 5.8). For instance, PVP 30 fraction 7 (empty triangles), which has an hydrodynamic diameter of 8.7 nm and it is similar in size to IgG, has a particle porosity of 35% when no IgG is adsorbed, which reduces to only 5% when the column is loaded with about 100 mg/mL of IgG. When HSA is adsorbed, the porosity decrease is about 25%. A similar effect is observed for all the tracers, with the exception of those entirely excluded from the pores, where as shown by the open squares the particle porosity is always zero. For both IgG and HSA adsorption, the porosity decreases initially fast, to reach an asymptotic value at large amount of protein adsorbed. This behavior demonstrates that even small amounts of adsorbed proteins are able to significantly reduce
pore accessibility. Note in fact that such materials are very irregular and characterized by limited pore connectivity [145]. Therefore, each time the adsorption of a large protein is blocking a small pore, it is reasonable to suppose that this is preventing the access to a large number pores (maybe larger in size). The fact that a plateau value in porosity is always reached for large loadings indicates that there is always a fraction of pores that remains accessible in spite of protein adsorption. This fraction is clearly larger the smaller the adsorbed molecule and the smaller the tracer.

To get some insight on the pore exclusion process happening at high protein loading, some characteristic iSEC chromatograms are shown. Figure 5.10 shows the iSEC chromatograms of PVP 12 fraction 6, PVP 30 fraction 10 and PVP 30 fraction 7 measured at the highest and lowest loading of IgG on Fractogel COO- (S). The shift in retention time due to the reduced pore accessibility can clearly be seen. The peak shape is not macroscopically changing as a function of the loaded protein amount. The influence of increased pore hindrance on the peak shape and, thus, on the effective pore diffusivity has, however, to be studied in more detail.

The porosities accessible to IgG and HSA are calculated by interpolating the porosity data of the PVP fractions. The corresponding results are shown in Figure 5.11. The full triangles represent the accessible porosity of IgG, whereas the empty diamonds that of HSA. The dashed curves have been measured after adsorption of IgG, the full curves after HSA adsorption. From this figure, it is confirmed that the effect of the adsorbed protein on porosity is extremely strong. The particle porosity accessible to IgG, for example, goes to almost zero for a fully loaded column. This result clearly demonstrates that such changes cannot be ignored in modeling the chromatographic behavior of these columns.

The same experiments were repeated on the Fractogel LLD (M) material. The results are shows in Figure 5.12 and 5.13 for IgG and HSA adsorption, respectively. Also on this material the porosities are decreasing significantly for increasing amount of protein loaded. The decrease is however smaller than what observed for Fractogel EMD COO- (S), as demonstrated by the porosity change predicted for IgG and HSA as a function of protein loading, shown in Figure 5.14. Differently from Fractogel EMD COO- (S), where the IgG accessibility went to almost zero for fully loaded materials,
5. Porosity investigation

Figure 5.10: Traces of PVP 12 fr 6 (a), PVP 30 fr 10 (b) and PVP 30 fr 7 (c) on the Fractogel COO- (S) column loaded with 98 (solid curve) and 9 (dashed curve) mg/ml IgG.
5.3. Results and discussion

Figure 5.11: Predicted change in particle porosity for HSA (♦) and IgG (▲) as a function of the adsorbed amount of HSA (black line) and IgG (dashed line) on Fractogel COO- (S).

when dealing with Fractogel LLD (M) about 20% porosity remain accessible even at very large loadings.

It can be concluded that, differently from the effect of salt, the effect of the adsorbed amount of protein on pore accessibility is very strong and may sensibly influence the static (total surface) and the dynamic (mass transport) behavior of a column. In particular, the material with the higher starting porosity (Fractogel LLD) has a considerable volume of pores which remains always accessible, even for large loading values. This is clearly beneficial for the protein mass transport inside the particle pores. In spite of this, by comparing Figure 5.11 and Figure 5.14, it can noticed that the material with smaller ligand density has a smaller capacity (the larger capacity value was been obtained starting from the same protein concentration in the feed). In fact, total capacity must be proportional to the tentacle density. Increasing the ligand density is,
however decreasing the porosity. This is suggesting that an optimum must be found between the amount of protein that can be loaded on the support and the accessibility at large loadings, that is between static and dynamic binding capacity.

![Figure 5.12: Change in particle porosity for PVP fractions on Fractogel LLD (M) as a function of the adsorbed amount of IgG. The porosity values at $q_{eq, IgG} = 0$ correspond to the particle porosities measured at 0.5 M NaCl, without protein. ♦: PVP 12 fraction 6, ▲: PVP 30 fraction 10, ■: PVP 30 fraction 9, ◊: PVP 30 fraction 7, △: PVP 30 fraction 5, □: PVP 90 fraction 1]

5.4 Conclusions

The change in porosity of two ion exchange stationary phases with different functional groups has been investigated. In particular, the change in porosity due to changes in the salt concentration and in the amount of protein adsorbed was measured. This has been possible due to the careful choice of a set of polymeric tracers which are
5.4. Conclusions

Figure 5.13: Change in particle porosity for PVP fractions on Fractogel LLD (M) as a function of the adsorbed amount of HSA. The porosity values at $q_{eq,HSA} = 0$ correspond to the particle porosities measured at 0.5 M NaCl, without protein. ♦: PVP 12 fraction 6, ▲: PVP 30 fraction 10, ■: PVP 30 fraction 9, ◊: PVP 30 fraction 7, Δ: PVP 30 fraction 5 □: PVP 90 fraction 1

not interacting with the stationary phases and the adsorbed proteins. Notably, the analyzed conditions are closely representing typical working conditions in biomolecule purification. More specifically, under such conditions the analyzed proteins strongly bind to the stationary phase and therefore its pore accessibility cannot be measured directly.

The influence of the salt concentration on the accessible porosity was found to be modest. Moreover, mostly the largest tracers are influenced by a change in salt concentration, which suggest that the change in porosity is mostly due to the tentacle collapse on the support surface. However, this hypothesis must be further verified.

A much stronger influence has been observed when loading the column with different
5. Porosity investigation

Figure 5.14: Predicted change in particle porosity for HSA (♦) and IgG (▲) as a function of the adsorbed amount of HSA (black line) and IgG (dashed line) on Fractogel LLD (M).

The available porosity fast decreases with increasing loading values and, in the case of IgG loading, almost all the pores become hindered to large tracers when the material is saturated. A smaller drop in porosity was found for the material with higher porosity.

It is worth noting that the accessible porosity is typically assumed constant in numerical simulations of the behavior of chromatographic processes. The results reported in this chapter urge the development of models accounting for a non-constant pore accessibility. In particular, we believe that the knowledge of the entire pore size distribution of the support at different operating conditions is the key-point for estimating other quantities needed in numerical simulations, namely the void fraction, the surface available for adsorption and the effective diffusion rate in the particle pores. Due to the
large adsorption capacities and the slow diffusion rates typical of such materials when dealing with large biomolecules, the knowledge of the previous quantities is strongly necessary to have a reliable simulation tool.
Chapter 6

Conclusions

A detailed study of the adsorption of a polyclonal IgG on a preparative strong cation exchange column has been presented in this Thesis. The aim of this work was twofold: first, to understand the underlying phenomena operating in protein chromatography; second, to develop a numerical model aimed to better understand and study these phenomena and to design protein chromatographic purifications. Different levels of complexity have been introduced in this study. First, a purely experimental approach was used to characterize the process, based on well known short-cut methods. Then, a model based approach was introduced, in which the polyclonal IgG mixture was extremely simplified and reduce to two "macro-components" only. Finally, the model was used to account for the presence of a larger number of different components. From this analysis, it was possible to identify two main problems: (i) the very hindered transport of the antibodies inside the macro-porous pores. This is largely affecting the performance of the studied stationary phase. (ii) The large sensitivity of the adsorption isotherm, which, together with the limited mass transport, made the experimental analysis particularly complex and which makes the process very sensitive to little variations in the operating parameters. The first problem was already addressed in this Thesis, and a detailed analysis of the influence of the operating conditions on the pore size distribution of the stationary phase (and, thus, on the pore mass transport limitation) was carried out.

Although very satisfactory results have been already obtained in this work, a large amount of work has still to be done in order to properly integrate previous observations.
into a fully comprehensive numerical model. First, the correct influence of the pore size distribution on the mass transport inside the particles must be implemented. Porosity is largely affecting the column performance and it can sensibly change during the process, as a result of e.g. adsorption. The corresponding reduction of the accessible pore volume and, most probably, the change in the diffusion length inside the pores must be properly characterized in the future. Clearly, this can be done by either developing short-cut methods or by fully integrating the pore structure into the model.

At the same time, the influence of the ionic strength only on the adsorption isotherm was considered in this work. However, a similar sensitivity can be observed in response to pH changes. Moreover, it is to be expected that different molecules are reacting in very different ways to simultaneous changes in ionic strength and pH, due to the complexity of the charge distribution and type inside the proteins. Accordingly, this kind of changes must be considered in the future not as a undesired perturbation in the system in response to simple changes in the operating conditions, but as a tool to be exploited in order to further enhance the resolution power of these stationary phases.
List of Symbols

A  column cross-sectional area [cm$^2$]
$c_{eq}^i$  equilibrium liquid phase concentration of the i-th component [g/l]
$c_{eq}^s$  equilibrium salt concentration in the liquid phase [mg/ml]
$c_i$  concentration of the i-th component in the mobile phase [mg/ml]
$c_{f,i}$  feed concentration of the i-th component [g/l]
$c_{p,i}$  concentration of the i-th component in the stagnant liquid phase in the particle pores [mg/ml]
$c_s$  concentration of salt in the mobile phase [mg/ml]
CV  column volume [-]
$D_{ax}$  axial diffusion coefficient [cm$^2$/s]
$D_m$  molecular diffusion coefficient [cm$^2$/s]
d$_p$  particle diameter [cm]
$D_p$  pore diffusion coefficient [cm$^2$/s]
$D_{eff}^p$  effective pore diffusion coefficient (= $\varepsilon_p D_p$) [cm$^2$/s]
g  gradient slope [M/ml]
GH  gradient slope normalized with respect to the column stationary phase volume (= g(V$_t$ − V$_0$)) [M]
f  flow rate [ml/s]
F  phase ratio (= $\varepsilon_b/(1 - \varepsilon_b)$) [-]
$H_i$  Henry constant of the i-th component [-]
$H_s$  Henry constant of salt [-]
HETP  height equivalent to a theoretical plate [-]
$I_m$  ionic strength [M]
$I_R$  conductivity at peak maximum [M]
$J_i$  mass flux of the i-th component [mg/(s cm$^2$)]
$K_p$  hindrance factor [-]
$K_i$  distribution coefficient of the i-th component [-]
$K'$  distribution coefficient of salt [-]
k$_f$  film mass transport coefficient [cm/s]
List of Symbols

\( L \) \quad \text{column length [cm]}
\( M \) \quad \text{molecular weight [g/mol]}
\( m_{\text{ads},i} \) \quad \text{adsorbed mass of the i-th component [mg]}
\( N \) \quad \text{total number of grid points [-]}
\( \text{Pe}_{\text{ax},i} \) \quad \text{axial Peclet number of the i-th component (} = \frac{u_{\text{int}}/L}{D_{\text{ax},i}/L^2} \text{) [-]}
\( \text{Pe}_{\text{ax},s} \) \quad \text{axial Peclet number of the salt (} = \frac{u_{\text{int}}/L}{D_{\text{p},i}/R_p^2} \text{) [-]}
\( \text{Pe}_i \) \quad \text{Peclet number of the i-th component (} = \frac{u_{\text{int}}/L}{D_{p,i}/R_p^2} \text{) [-]}
\( q^e_{i} \) \quad \text{equilibrium solid phase concentration of the i-th component [g/l]}
\( q^e_{s} \) \quad \text{equilibrium solid phase concentration of salt [mg/ml]}
\( q^\infty_{i} \) \quad \text{saturation capacity of the i-th component [g/l]}
\( q^\infty_{s} \) \quad \text{saturation capacity of salt [mg/ml]}
\( q_i \) \quad \text{solid phase concentration of the i-th component [mg/ml]}
\( q_s \) \quad \text{solid phase concentration of salt [mg/ml]}
\( r \) \quad \text{radial position in the particle [cm]}
\( R_p \) \quad \text{particle radius [cm]}
\( \text{Sh}_i \) \quad \text{Sherwood number of the i-th component (} = \frac{k_{f,i}/R_p}{D_{p,i}/R_p^2} \text{) [-]}
\( \text{St}_i \) \quad \text{Stanton number of the i-th component (} = 3 \frac{\text{Sh}_i}{\text{Pe}_i} = 3 \frac{k_{f,i}/R_p}{u_{\text{int}}/L} \text{) [-]}
\( T \) \quad \text{absolute temperature [K]}
\( t \) \quad \text{time [s]}
\( t_{\text{ax}} \) \quad \text{characteristic time for axial diffusion [s]}
\( t_{\text{conv}} \) \quad \text{characteristic time for convection [s]}
\( t_{\text{film}} \) \quad \text{characteristic time for film mass transport [s]}
\( t_{\text{inj}} \) \quad \text{injection time [s]}
\( t_{\text{pore}} \) \quad \text{characteristic time for pore diffusion [s]}
\( u_i \) \quad \text{triangular function for the components determination}
\( u_{\text{int}} \) \quad \text{interstitial velocity (} = f/(A\varepsilon_b) \text{) [cm/s]}
\( u_s \) \quad \text{superficial velocity (} = f/A \text{) [cm/s]}
\( V_0 \) \quad \text{column void volume [ml]}
\( V_c \) \quad \text{total column volume [ml]}
\( V_d \) \quad \text{dead volume [ml]}
\( V_{p,i} \) \quad \text{particle pore volume accessed by the i-th component [ml]}
\( V_p \) \quad \text{total particle volume [ml]}
$V_{t,i}$  total liquid volume accessed by the i-th component [ml]
$z$  axial position [cm]

**Greek letters**

$\alpha_i$  $\alpha$ parameter of the Henry vs ionic strength function for the i-th component [1/M$^{-\beta}$]
$\beta_i$  $\beta$ parameter of the Henry vs ionic strength function for the i-th component [-]
$\delta_i$  $\delta$ parameter of the saturation capacity vs ionic strength function for the i-th component [mg/(ml M)]
$\varepsilon$  $\varepsilon$ parameter of the saturation capacity vs ionic strength function for the i-th component [mg/(ml M)]
$\varepsilon_b$  bed porosity [-]
$\varepsilon_{p,i}$  particle porosity of the i-th component [-]
$\varepsilon_{p,s}$  particle porosity of salt [-]
$\varepsilon_{t,i}$  total porosity of the i-th component [-]
$\varepsilon_{\text{tot}}$  total porosity of the column [-]
$\gamma_i$  $\gamma$ parameter of the saturation capacity vs ionic strength function for the i-th component [mg/ml]
$\nu_b$  phase ration (= (1 - $\varepsilon_b$)/$\varepsilon_b$) [-]
$\mu_1$  first moment [min]
$\mu_2$  second centered moment [min$^2$]
$\eta$  dimensionless axial position (= $z$/$L$) [-]
$\eta_b$  solvent viscosity [Cp]
$\rho$  dimensionless radial position (= $r$/$R_p$) [-]
$\tau$  dimensionless time (= (t $u_{\text{int}}$)/$L$) [-]
$\tau_{\text{inj}}$  dimensionless injection time [-]
$\tau$  tortuosity (Chapter 2) [-]
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[142] Personal communication


Curriculum Vitae

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Publications

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