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Hydrophobic Interaction Chromatography: Fundamentals and Applications in Biomedical Engineering

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1. Introduction

Hydrophobic interaction chromatography (HIC) a powerful technique used for separation and purification of biomolecules. It was described for the first time by Shepard & Tiselius (1949), using the term "salting-out chromatography". Later, Shaltiel & Er-el (1973) introduced the term "hydrophobic chromatography". Finally, Hjerten (1973) described this technique as "hydrophobic interaction chromatography", based on the retention of proteins on weakly hydrophobic matrices in presence of salt. Owing of its high versatility and efficiency, HIC is widely used for the separation and purification of proteins in their native state (Porath et al., 1973), as well as for isolating protein complexes (Chaturvedi et al., 2000) and studying protein folding and unfolding (Bai et al., 1997).

HIC has been applied in separating homologous proteins (Fausnaugh & Regnier, 1986), receptors (Zhang et al., 2008), antibodies (Kostareva et al., 2008), recombinant proteins (Lienqueo et al., 2003) and nucleic acids (Savard & Schneider, 2007). HIC shows similar capacity to ion exchange chromatography (IEC) and a high level of resolution. Since it exploits a different principle than IEC and other separation techniques it can be used as an orthogonal method to achieve the purification of complex protein mixtures (Haimer et al., 2007). In this chapter, the theoretical principles underlying macromolecule retention in HIC are reviewed and discussed in sight of their application for predicting macromolecule behavior in HIC. Besides, novel applications of HIC are discussed regarding their suitability on Biomedical Engineering.

2. Theoretical principles underlying macromolecule retention in Hydrophobic Interaction Chromatography

2.1 Thermodynamics fundamentals

Hydrophobicity can be defined, in general terms, as the repulsion between a non-polar molecule and a polar environment, such as that conferred by water, methanol, and other polar solvents. Two hydrophobic molecules (non-polar) located in a polar environment will show a trend to minimize the contact with the polar solvent. This is accomplished by coming in contact with each other thus minimizing the molecular surface exposed to the

solvent. This phenomenon is known as "hydrophobic interaction". Hydrophobic interaction is the most common macromolecular interaction in biological systems. It is also the driving force of several biological and physicochemical processes, such as protein folding, antigen-antibody recognition, stabilization of enzyme-substrate complexes, among others.

From a thermodynamic point of view, the interaction between hydrophobic molecules is an entropy-driven process, based on the second law of Thermodynamics and considering that temperature (T) and pressure (P) remain constant during the process, in this case, the hydrophobic interaction between two biological molecules. Considering equation (1), when a non-polar molecule enters in contact with a polar solvent (usually water), an increase in the degree of order of the solvent molecules that surround the hydrophobic molecule is observed, producing a decrease in entropy ($\Delta S < 0$). Given that enthalpy (ΔH) does not suffer a significant increase in this kind of processes (constant temperature) in comparison with $T\Delta S$, an overall positive change in the Gibbs energy ($\Delta G > 0$) is produced. Hence, the dissolution of a non-polar molecule in a polar solvent does not occur spontaneously, since it is thermodynamically unfavorable.

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

The thermodynamics situation changes when two or more non-polar molecules are located in a polar environment. In this case, the hydrophobic molecules spontaneously aggregate because of hydrophobic interaction, and in this way the hydrophobic surfaces of the macromolecules become hidden from the polar surrounding. Entropy increases ($\Delta S > 0$) owing to a displacement of the highly structured solvent molecules surrounding the exposed surface of the hydrophobic molecules towards the solvent bulk consisting of less structured molecules. As a consequence, the Gibbs energy decreases ($\Delta G < 0$), and therefore, hydrophobic interaction becomes a thermodynamically favorable process. In conclusion, the hydrophobic interaction between two or more non-polar molecules in a polar solvent solution is a spontaneous process governed by a change in entropy. Accordingly, hydrophobic interactions can be weakened by raising temperature or by modifying the solvent polarity through the addition of another solute.

2.2 Retention mechanisms in Hydrophobic Interaction Chromatography

Macromolecule retention in HIC occurs due to hydrophobic interactions between the hydrophobic ligands immobilized on a stationary phase and the hydrophobic moieties on the macromolecule surface (Queiroz et al., 2001). There is a variety of stationary phases used in HIC, corresponding to organic polymers or silica. Their main characteristics are being chemically modifiable, highly porous, and of high moisturizing power. Among them, the most commonly used are polyacrylamide (BiogelP™), cellulose (Cellulafine™), dextran (Sephadex™), agarose (Sepharose™), and others. These supports are further modified by linking hydrophobic ligands that become a sort of "active group" that allows hydrophobic interaction with the macromolecule to be separated from a solution. The ligand is linked to the support through a spacer arm (usually glycidyl ether), so that there is no steric impediment for macromolecule-ligand interaction, and avoiding hydrophobic interaction between the ligands. Figure 1 depicts the retention of a protein to a HIC stationary phase.

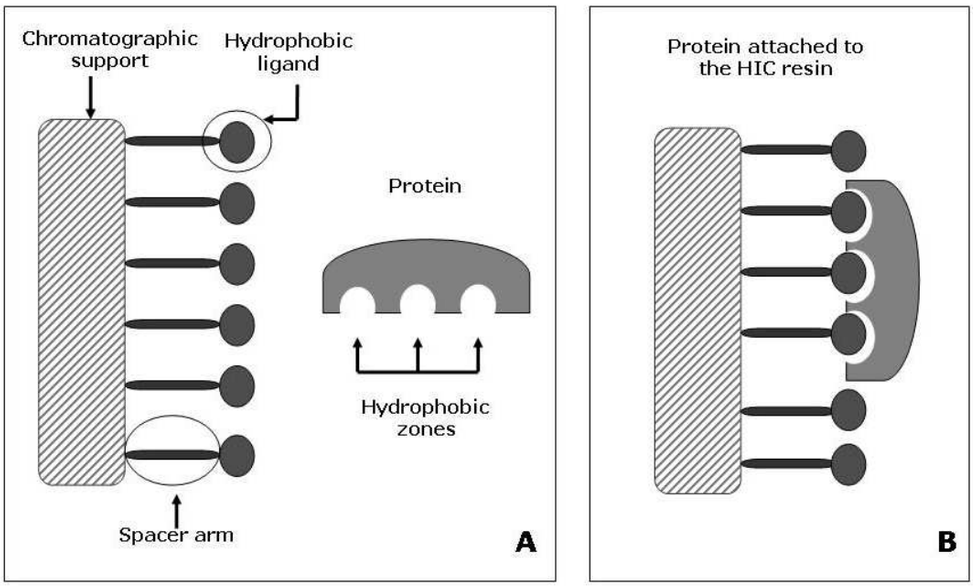


Fig. 1. Protein retention mechanism in HIC. (A) The basic structure of a HIC resin is depicted, and a protein is schematized highlighting the hydrophobic zones on the protein surface. (B) The protein gets in contact with the hydrophobic ligands of the resin, suffering a spatial reorientation. The hydrophobic ligands of the matrix interact with the exposed hydrophobic zones of the protein, and thus the protein is reversibly attached to the resin.

The most common hydrophobic ligands are alkyl or aryl groups of 4 to 10 carbons (Jennissen, 2000). The length of the carbon chain usually does not exceed 10 units in order to avoid self-folding. The nature of the hydrophobic ligand determines the performance of a HIC process. Figure 2 shows a scheme of stationary phases used in HIC and the chemical structure of the most commonly used alkyl and aryl groups, such as butyl (four carbons), octyl (eigth carbons) and phenyl (aromatic ring that promotes π-π interactions with the aromatic residues on a proteins surface). The hydrophobic interaction is directly proportional to the length of the alkyl chain. The most commonly used ligands in HIC resins are butyl, octyl and phenyl, in the following order in terms of relative interaction strength:

Phenyl > Octyl > Butyl

In the HIC process, retention is reinforced by the presence of a neutral salt. When a neutral salt is added to a solution consisting of a polar solvent, i.e. water, and a non-polar macromolecule, such as a protein, a competition for the water molecules that hydrate the macromolecule is observed, being more favorable to the salt. As a consequence, high salt concentration will reduce the number of solvent molecules that surround the macromolecules, thus favoring the hydrophobic interaction between them. Furthermore, if such solution comes in contact with a HIC resin, the interaction between the macromolecule and the hydrophobic ligand on the resin surface will be promoted, resulting in the adsorption of the macromolecule to the HIC stationary phase. From a process point of view, it is essential to choose the right type of salt and a concentration that minimizes macromolecule precipitation due to solubility decrease in the presence of high salt concentration ("salting-out").

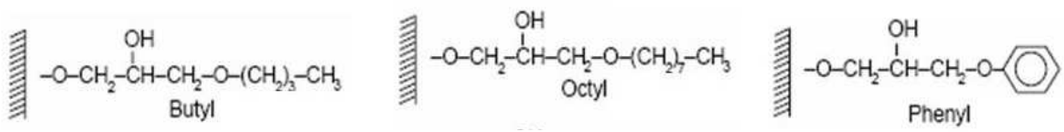
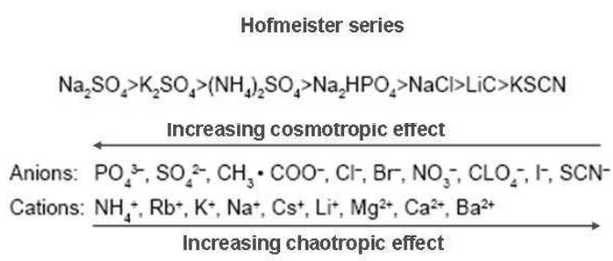


Fig. 2. Schematic representation of stationary phases used in HIC. Butyl is the shortest carbon chain used as HIC ligand and therefore the less hydrophobic one; octyl exhibits an intermediate hydrophobicity, and phenyl offers the strongest hydrophobic interaction.

The effect of different types of salt on macromolecule retention in HIC follows the Hofmeister (or lyotropic) series according to their positive influence in increasing the molal surface tension of water (Melander & Horvath, 1977). Besides, anions and cations exhibit cosmotropic or chaotropic properties. The salts at the beginning of the series are known as "cosmotropic" or "antichaotropic", since they promote hydrophobic interactions (as well as protein precipitation due to the "salting-out" effect) because of their water structuring ability. On the other hand, the salts at the end of the series, called "chaotropic", tend to randomize the structure of water and therefore they disfavor hydrophobic interactions. The salts ammonium sulfate and sodium chloride are most preferred in HIC.



Once the macromolecule of interest is attached to the stationary phase, it is necessary to detach it in order to recover it as a bio-product. Desorption is most commonly accomplished by reducing the ionic strength in the mobile phase, by building a decreasing gradient of salt concentration (Fausnaugh et al., 1984). In this stage, the hydrophobic interaction between the macromolecule and the ligand is weakened as salt concentration diminishes in the mobile phase. As a consequence, the macromolecule is desorbed when a specific salt concentration is reached. This salt concentration, or ionic strength, depends on the physicochemical properties of the macromolecule. In this way, HIC can be used to selectively detach different macromolecules in a solution, thus becoming a powerful separation process.

Protein retention in HIC has been interpreted in the light of the underlying thermodynamic phenomena, by considering the effect of salt. Melander et al. (1989) proposed a thermodynamic model that describes protein retention in terms of electrostatic and hydrophobic interactions. This model describes protein retention due to only electrostatic interactions (case of ion Exchange Chromatography), only hydrophobic interactions (case of HIC), and both types of interactions (case of a weakly hydrophobic support or a chromatographic support bearing both hydrophobic and charged ligands). Simplifications of this model have been used to develop methodologies to predict protein retention in HIC. This model is described below.

2.3 Thermodynamic model for protein retention in HIC

The thermodynamic model proposed by Melander et al. (1989) to describe the effect of salt concentration on macromolecule retention in chromatography (IEC and HIC) can be applied to any stationary phase consisting of a highly hydrated surface modified with charged ligands (in the case of IEC), weakly hydrophobic moieties (in the case of HIC), or both. Electrostatic and hydrophobic interactions between the macromolecule and the stationary phase are treated separately. Electrostatic interaction is modeled based on the Manning's counter ion condensation theory (Manning, 1978), whereas hydrophobic interaction is treated by considering an adaptation of the Sinanoglu's solvophobic (Sinanoglu, 1982) theory that relates the salting out of proteins with their retention in HIC (Melander & Horvath, 1977). Figure 3 depicts protein retention due to hydrophobic interactions, electrostatic interactions, and both hydrophobic and electrostatic interactions.

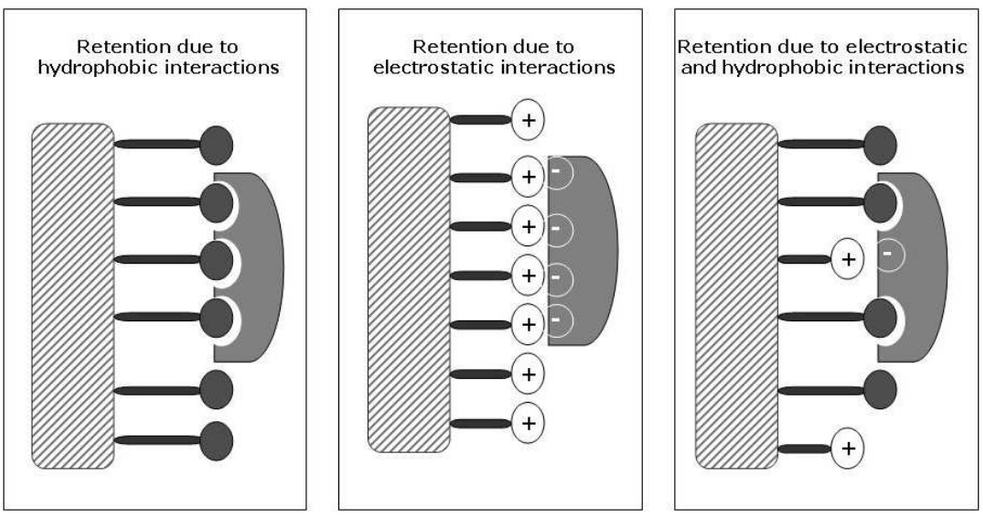


Fig. 3. Protein retention due to hydrophobic, electrostatic, and both interactions. Electrostatic interactions are long-range interactions, and then moieties with opposite charges do not need to be in physical contact. Hydrophobic interactions are short-range, and then interacting hydrophobic moieties must be in contact. As a consequence, when hydrophobic and charged moieties are present, both types of interactions may occur.

The main assumptions considered in the model are listed below:

i. The dimensions of the pores of the support are large with respect to the macromolecule size, and their shape is approximately a cylinder of infinite radius and the size-exclusion effects are negligible.
ii. The immobilized charges or hydrophobic moieties are uniformly spaced and equally accessible at the pore wall.
iii. The macromolecule is spherical and presents uniformly distributed and equally accessible fixed charges and hydrophobic patches on its surface.
iv. The macromolecule does not suffer conformational changes during the adsorption-desorption process.
v. Only a small fraction of the binding moieties on the stationary phase are occupied by the macromolecule.
vi. There are no specific interactions between the salt and the macromolecule.

2.3.1 Electrostatic interaction

The Gibbs energy of binding (ΔG^0_{es}) of the macromolecule to a stationary phase in presence of salt (that acts as a counter ion) is given by equation (2). Here m_s is the molal salt concentration, N_{Av} the Avogadro's number, "e" the base of the natural logarithm, "b" the average spacing of fixed charges on the surface, δ_p the thickness of the condensation layer over the surface of the stationary phase where each fixed charge occupies an area of b^2, δ_s the layer thickness of salt counter ion, Z_p the characteristic charge of the protein, Z_s the valence of the salt counter ion, and ξ a dimensionless structural parameter that characterizes the charged surface. R is the universal constant of gases and T the absolute temperature.

$$\frac{-\Delta G^0_{es}}{2.3 \cdot R \cdot T} = \log\left(\frac{N_{Av} \cdot b^2 \cdot \delta_p}{1000 \cdot e}\right) + \frac{Z_p}{Z_s}\log\left(\frac{1000 \cdot e}{\left(N_{Av} \cdot b^2 \cdot \delta_s \cdot m_s\right) \cdot \left(1 - Z_s \cdot \xi\right)}\right) \tag{2}$$

2.3.2 Hydrophobic interaction

The contact between the hydrophobic patches on the macromolecule surface that are exposed to the solvent and the hydrophobic ligands on the stationary phase, trigger the retention due to hydrophobic interaction. The Gibbs energy of hydrophobic interaction (ΔG_{hp}) is expressed in terms of the molal surface tension increment of the salt (σ_s), as shown in equation (3), which is valid only in the absence of specific salt effects. In Equation (3) m_s is the salt molality, ΔG^0_{aq} represents the reduction in Gibbs energy due to other effects different form hydrophobic interactions, $\Delta A'$ is the difference between the molecular surface area of the unbound macromolecule (A_M) and the molecular surface area of the macromolecule attached to the stationary phase (A_s). $\Delta A'$ corresponds to the surface contact area between the bound protein and the hydrophobic site of the matrix.

$$\Delta G^0_{hf} = \Delta G^0_{aq} - \Delta A' \cdot \sigma_s \cdot m_s \tag{3}$$

2.3.3 Combined electrostatic and hydrophobic interaction

The retention factor (k'), given in equations (4) and (5), is represented in terms of salt molality when both electrostatic and hydrophobic interactions are present. This is accomplished by combining equations (2) and (3) to give equation (6). In equation (5), K is the equilibrium constant and ϕ is the phase ratio (stationary phase mass / mobile phase mass). In equation (6) α is the phase volume ratio (stationary phase/mobile phase). Equation (6) can be written in a simplified form, as given by equation (7), where A is a constant determined by all the system characteristics, B the electrostatic interaction parameter and C the hydrophobic interaction parameter. In equation (7), the term C accounts for the hydrophobic surface contact area between the macromolecule and the stationary phase, and is given by equation (8).

$$\log K = \left(\frac{-\Delta G^0_{es}}{2.3 \cdot R \cdot T}\right) - \left(\frac{-\Delta G^0_{hf}}{2.3 \cdot R \cdot T}\right) \tag{4}$$

$$k' = \phi \cdot K \tag{5}$$

$$\log k' = \log\left(\frac{N_{Av}\cdot b^2\cdot\delta_p}{1000\cdot e}\right) + \frac{Z_p}{Z_s}\log\left(\frac{1000\cdot e}{\left(N_{Av}\cdot b^2\cdot\delta_s\cdot m_s\right)\cdot\left(1-Z_s\cdot\xi\right)}\right) + \frac{\Delta G^0_{aq}}{2.3\cdot R\cdot T} + \frac{\Delta A'\cdot\sigma_s\cdot m_s}{2.3\cdot R\cdot T} + \log\alpha \tag{6}$$

$$\log k' = A - B\cdot\log m_s + C\cdot m_s \tag{7}$$

$$C = \frac{\Delta A'\cdot\sigma_s}{2.3\cdot R\cdot T} \tag{8}$$

Equation (7) corresponds to the Simplified Thermodynamic Model for Electrostatic and Hydrophobic Interactions. This model is of practical usefulness, since its parameters can be obtained from experimental runs in a relatively simple manner, depending on the salt concentration present in the macromolecule solution. At low salt concentration, up to 0.5 molal, hydrophobic interactions can be neglected, and therefore the parameters A and B in equation (7) can be estimated by means of a linear regression between isocratic retention factors obtained at different salt molalilies. At high salt concentration, electrostatic interactions are negligible, and hence the parameters A and C can be obtained in a similar way, considering the isocratic retention factors. The hydrophobic contact area (ΔA′ in equation (8)) can easily be obtained from the slope of the limiting plot of log k′ versus molal salt concentration.

The simplified thermodynamic model has been used to investigate the effect of surface hydrophobicity distribution of proteins on retention in HIC (Mahn et al., 2004). The applicability of the model to predict protein retention time in HIC was demonstrated, and for the first time it was experimentally proven that surface hydrophobicity distribution has an important effect on protein retention in HIC. Furthermore, it was shown that the parameter ΔA′ that comes from equations (7) and (8) was able to represent the protein retention in HIC with salt gradient elution. However, the methodology proposed by Mahn et al. (2004) requires the generation of a considerable amount of experimental data, thus limiting its application.

3. Hydrophobic Interaction Chromatography process

The HIC process consists of injecting a macromolecule solution in a column packed with a stationary phase specifically designed to promote hydrophobic interaction with macromolecules such as proteins (solute). Usually retention is accomplished under high salt concentration conditions. Elution is achieved by decreasing the ionic strength in the mobile phase, building a decreasing salt gradient. At a microscopic level, the macromolecule enters in contact with the hydrophobic ligands at the pores surface of the resin, suffering a spatial reorientation. The hydrophobic ligands of the stationary phase interact with the hydrophobic zones of the macromolecule exposed to the solvent (usually aqueous solution), and thus the protein is reversibly attached to the resin.

Figure 4 shows a schematic representation of a HIC process. Here, A and B represent the vessels that contain the buffers used to manage the chemical environment in order to promote adsorption and desorption of the macromolecules present in the sample. The

solution in A corresponds to a buffer with a low concentration of a neutral salt (usually 0.1 M), aiming to stabilize the macromolecular three-dimensional structure. The solution in B corresponds to buffer "A" added with a high salt concentration (usually higher than 1 M). Adsorption is promoted by using buffer "B", while desorption is induced by mixing both A and B forming a decreasing gradient salt concentration.

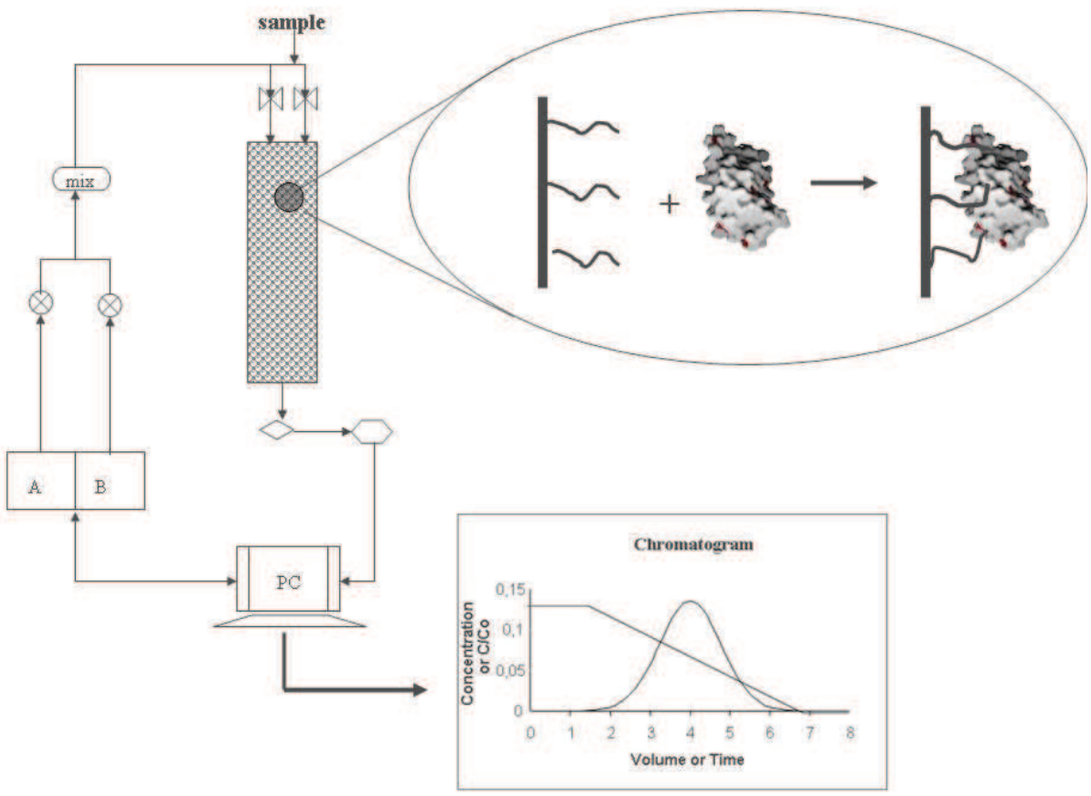


Fig. 4. Schematic representation of the HIC process. The HIC process consists of injecting a protein sample in a hydrophobic column under high salt concentration conditions such that hydrophobic interaction between the protein and the resin is promoted. Elution is achieved by decreasing the ionic strength in the mobile phase, building a decreasing salt gradient. In a microscopic level, the hydrophobic patches on the protein surface interact with the hydrophobic ligands of the resin, being reversibly attached to it. The protein concentration in the outlet is recorded as a function of time, and then a chromatogram is obtained.

The macromolecule concentration in the outlet solution is continuously determined through absorbance at 280 nm, and finally the elution curve or "chromatogram" is obtained. The chromatographic behavior in HIC can be characterized by several parameters, including the elution curve (most commonly by using the theoretical plate theory), the retention time or volume, or other parameters based on the preceding ones. To predict the behavior of proteins in HIC, the preferred parameter is the "Dimensionless Retention Time" (DRT), given by equation (9), where t_R is the time corresponding to the peak maximum, t_0 is the time at the beginning of the elution gradient, and t_f the time at the end of the gradient. In

HIC, the exploited property is hydrophobicity (Eriksson, 1998), and accordingly retention time is highly influenced by this property. Therefore, knowing macromolecule hydrophobicity allows predicting its behavior in HIC. Currently there is no universally agreed definition of protein hydrophobicity, but there is consensus in that it is determined by the hydrophobic contribution of the amino acids that compose the protein (Tanford, 1962).

$$DRT = \frac{t_R - t_0}{t_f - t_0} \tag{9}$$

On the other hand, protein retention in HIC is significantly affected by the operating conditions, which influence the resolution and selectivity of purification processes that include a HIC step (Ladiwala et al., 2006). From a process point of view, it is essential to count on methodologies and mathematical models to describe and to predict a protein behavior in HIC, ideally under varying operating conditions. Many efforts have been carried out to develop theories to explain this behavior based on protein properties, mainly protein hydrophobicity. At this point, controversial approaches have been proposed to theoretically estimate or experimentally determine protein hydrophobicity. These approaches include different amino acid hydrophobicity scales as well as diverse methodologies to perform calculations that use some scale to describe and predict protein retention time in HIC.

3.1 Protein hydrophobicity

3.1.1 Amino acid hydrophobicity scales

As stated above, protein hydrophobicity is determined by the hydrophobicity of the amino acids that compose it. Hence, it becomes necessary to quantify in any way the hydrophobic contribution of each amino acid. For this purpose, different approaches have been proposed to assign a hydrophobicity value to each one of the standard amino acids (Biswas et al., 2003; Kovacs et al., 2006). These methods are based on theoretical calculations and/or experimental determinations. Besides, the amino acid hydrophobicity scales differ in the hydrophobicity value assigned to each amino acid as well as in the relative position occupied by each one. These scales have been classified into several categories by different authors (Lienqueo et al., 2002; Mahn et al., 2009), based on their underlying principles.

Despite the differences between the hydrophobicity assigned to each residue by the different scales; it is clear a global tendency. Isoleucine shows the highest hydrophobicity in most scales, followed by Tryptophan. Glycine usually has an intermediate hydrophobicity level, i.e. neutral hydrophobicity, and the lowest level is mostly assigned to Aspartic acid (Lienqueo et al., 2007), i.e., this is the most hydrophilic amino acid. The suitability of the hydrophobicity scale depends on the use that will be given to the estimation of the protein or peptide hydrophobicity, as well as on the way to estimate this property. The scales proposed by Miyazawa & Jernigan (1996) and by Cowan & Whittaker (1990) are the most adequate to estimate protein hydrophobicity based on its three-dimensional structure (Lienqueo et al., 2007), regarding its behavior in HIC. Additionally, Salgado et al. (2005) proposed that the scale developed by Wertz & Scheraga (1978) is the most adequate to estimate protein hydrophobicity based on the amino acid composition of that protein.

	Cowan & Whittaker (1990)		Miyazawa & Jernigan (1996)		Wertz & Scheraga (1978)	
	Original	*Normalized*	*Original*	*Normalized*	*Original*	*Normalized*
ALA	0.420	0.660	5.330	0.391	0.520	0.375
ARG	-1.560	0.176	4.180	0.202	0.490	0.321
ASN	-1.030	0.306	3.710	0.125	0.420	0.196
ASP	-0.510	0.433	3.590	0.105	0.370	0.107
CYS	0.840	0.763	7.930	0.819	0.830	0.929
GLN	-0.960	0.323	3.870	0.151	0.350	0.071
GLU	-0.370	0.467	3.650	0.115	0.380	0.125
GLY	0.000	0.557	4.480	0.252	0.410	0.179
HIS	-2.280	0.000	5.100	0.354	0.700	0.696
ILE	1.810	1.000	8.830	0.967	0.790	0.857
LEU	1.800	0.998	8.470	0.908	0.770	0.821
LYS	-2.030	0.061	2.950	0.000	0.310	0.000
MET	1.180	0.846	8.950	0.987	0.760	0.804
PHE	1.740	0.983	9.030	1.000	0.870	1.000
PRO	0.860	0.768	3.870	0.151	0.350	0.071
SER	-0.640	0.401	4.090	0.188	0.490	0.321
THR	-0.260	0.494	4.490	0.253	0.380	0.125
TRP	1.460	0.914	7.660	0.775	0.860	0.982
TYR	0.510	0.682	5.890	0.484	0.640	0.589
VAL	1.340	0.885	7.630	0.770	0.720	0.732

Table 1. Amino acid hydrophobicity scales useful in HIC.

The Miyazawa & Jernigan (1996) scale is based on the three-dimensional structure of proteins, and it represents the contact energy between adjacent amino acids in folded protein. The Wertz & Scheraga (1978) scale is also based on knowledge of the folded protein structure, and it estimates the amino acid hydrophobicity as the ratio between the number of buried residues and the number of residues exposed to the solvent, for each type of standard amino acid. Both scales are based on clusters composed by a significant number of proteins whose three-dimensional structure had been elucidated through experimental methods. Both scales have been classified as indirect scales (Mahn et al., 2009). On the other hand, the Cowan & Whittaker (1990) scale, which has been considered a direct scale, assigned a hydrophobicity value to each standard amino acid based on the retention time of z-derivatives of each amino acid in HPLC. The scales mentioned above are presented in Table 1.

3.1.2 Estimation of protein hydrophobicity

There are different approaches to estimate protein hydrophobicity, which are based on different principles. The classical approach consists of estimating the "average surface hydrophobicity" ($\phi_{surface}$) based on the three-dimensional structure of the macromolecule in its native conformation (Lienqueo et al., 2002; Berggren et al., 2002). This approach considers only the amino acid residues that are accessible to the solvent at the protein surface, by using three-dimensional structural data. This method considers that each amino acid on the protein surface has a hydrophobic contribution proportional to its solvent accessible area, and the hydrophobicity of each residue is given by the amino acid hydrophobicity scale

developed by Miyazawa & Jernigan (1996) or Cowan & Whittaker (1990), in their normalized form (see Table 1), as shown by equation (10).

$$\phi_{surface} = \frac{\sum (s_{aai} \cdot \phi_{aai})}{s_p} \tag{10}$$

Here, $\phi_{surface}$ is the calculated value of the surface hydrophobicity for a given protein, i (i =1, . . ., 20; different i-values indicate different standard amino acids), s_{aai} is the solvent accessible area occupied by the amino acid i, ϕ_{aai} is the hydrophobicity value assigned to amino acid i by the hydrophobicity scale, and s_p is the total solvent accessible area of the entire protein. It has to be noted that for proteins with a prosthetic group s_p is bigger than the sum of the solvent accessible area occupied by the amino acids; and for proteins without prosthetic group, these values are equal. Table 2 shows the average surface hydrophobicity for a group of proteins using the amino acid hydrophobicity scales given in Table 1, and calculated by equation (10). This method for estimating protein hydrophobicity has proven to be valid in several cases (Lienqueo et al., 2002; Lienqueo et al., 2003; Lienqueo et al., 2007); however, this methodology is not valid for proteins that exhibit a highly heterogeneous distribution of the hydrophobic patches on their surfaces (Mahn et al., 2004).

Protein	Cowan & Whittaker	Miyazawa & Jernigan	Wetz & Scheraga
α-amylase	0.447	0.282	0.319
Citochrome c	0.362	0.185	0.171
Conalbumin	0.421	0.233	0.242
Concanavalin A	0.448	0.273	0.308
α-lactalbumin	0.491	0.318	0.304
β-lactoglobulin	0.468	0.279	0.284
Lysozyme	0.425	0.274	0.307
Myoglobin	0.392	0.214	0.220
Ovalbumin	0.457	0.257	0.270
Chymotrypsin	0.474	0.306	0.313
Chymotrypsinogen	0.468	0.298	0.305
Ribonuclease A	0.406	0.230	0.255
Thaumatin	0.464	0.269	0.279

Table 2. Surface hydrophobicity of proteins estimated by equation (9).

Genetic engineering is often used to improve the performance of separation and purification methods. Specifically in HIC, its performance has been improved by the fusion of short hydrophobic peptide tags such as T3, (TP)3, T3P2, T4, (TP)4, T6, T6P2, T8, (WP)2, (WP)4 to a protein of interest (Brandmann et al., 2000; Rodenbrock et al., 2000; Fexby & Bülow, 2004), thus increasing its original hydrophobicity. This genetic engineering strategy has the advantage that the structure/function changes are minimized in relation to the original properties of the native protein. Furthermore, the use of hydrophobic polypeptide tags allows investigating simple and less expensive stationary phases (in comparison with affinity chromatography supports), such as those used in HIC.

As a consequence, methods to calculate the surface hydrophobicity of tagged proteins have been proposed. One of those methods is the one proposed by Simeonidis et al. (2005) that allows computing the "tagged surface hydrophobicity" (ϕ_{tagged}), by equation (11). The surface hydrophobicity of the tagged protein is estimated as the average surface hydrophobicity of the original protein (without the tag) plus the hydrophobicity of the peptide tag. In this case, a fully exposed surface of the amino acids in the tag is assumed. In equation (11), n_k is the number of amino acids of "k" type (usually hydrophobic amino acids, such as tryptophan, leucine and isoleucine) in the tag, and s_{tag_aak} is the fully exposed surface of amino acid "k" in the tag.

$$\phi_{tagged} = \frac{\sum\left(s_{aai} \cdot \phi_{aai}\right)}{s_p} + \sum\left(\frac{\left(s_{tag_aak} \cdot n_k\right)}{s_p + \sum\left(s_{tag_aak} \cdot n_k\right)} \cdot \phi_{aak}\right) \tag{11}$$

Despite the remarkable results reached by the methods described above to estimate protein hydrophobicity, the need of knowing the three-dimensional structure appears as a serious disadvantage. This is especially clear from the ratio between the number of proteins of known three-dimensional structure available in the PDB database (Bermann et al., 2000) and the number of proteins sequenced in the UniProtKB/Swiss-Prot database (Bairoch et al., 2005). Currently (January 2011) this number is closer to 0.13 (70695/534420). This situation points out the need of a procedure based on low level information, such as the amino acidic composition. Salgado et al. (2005) developed a mathematical model to predict the average surface hydrophobicity of a protein based only on its amino acidic composition and, therefore, avoiding the use of its three-dimensional structure.

Equation (12) shows the basic structure of the model. In this equation, ASH represents the average surface hydrophobicity, n_i is the number of amino acids of class i in the protein, ^l is the normalized length of the protein sequence, and c_i correspond to adjustable parameters. The function f accounts for a correction of the amino acid composition of the protein according to different assumptions about the amino acids trend to be exposed to the solvent. The simplest form of f considers all the amino acids completely exposed. Parameters for building the function f were determined in a large set of non-redundant proteins by Salgado et al. (2005).

$$ASH = c_0 + \sum_{i=1}^{20} c_i \cdot f\left(n_i\right) + c_{21}\hat{l} \tag{12}$$

3.2 Methods for predicting retention time in HIC

The approaches discussed above to calculate protein hydrophobicity have been used to predict protein retention time by different methods. The simplest methodology uses straightforward quadratic models, whose parameters depend on the chromatographic conditions used in the HIC run (Lienqueo et al., 2007), and whose variables are DRT and the average surface hydrophobicity of the protein to be separated ($\phi_{surface}$). The most appropriate hydrophobicity scale was found to be that proposed by Miyazawa & Jernigan (1996), in its normalized form. The general model is given by equation (13), where A′, B′ and C′ are the model parameters that depend on the chromatographic conditions, such as type and concentration of salt and type of stationary phase. These parameters have been obtained

from adjusting experimental data to the quadratic model. Table 3 shows the values of A′, B′ and C′ obtained for different operating conditions. The model given by equation (13) is useful for predicting retention times of structurally stable proteins that have a relatively homogeneous distribution of the surface hydrophobicity, such as ribonuclease A.

$$DRT = A' \cdot \phi_{surface}^2 + B' \cdot \phi_{surface} + C' \tag{13}$$

Figure 5 shows a scheme of the methodology to predict DRT based on protein hydrophobicity. The procedure begins with the calculation of the protein surface accessible to the solvent, and the fraction of that surface occupied by each kind of amino acid. To calculate this, it is necessary to count on a PDB file, i.e. to know the spatial coordinates of each atom composing the macromolecule, preferably determined experimentally through X-ray crystallography or nuclear magnetic resonance (NMR). Experimentally determined structures can be obtained in The Protein Data Bank (PDB; www.rcsb.org/pdb) database (Bermann et al., 2000). Additionally, three-dimensional models can be found in other databases such as ModBase (http://modbase.compbio.ucsf.edu/modbase-cgi/search_form.cgi) (Pieper et al., 2009). Also it is required using a computational program or suit to perform the calculation, such as the software GRASP (Nicholls et al., 1991). With this information, the average surface hydrophobicity is calculated by means of equation (10) and using the Miyazawa & Jernigan hydrophobicity scale, in its normalized form. Finally, through a quadratic model like equation (13) the retention time of the protein can be estimated as DRT.

Resin	Salt	Initial Salt molarity	A′	B′	C′
Phenyl Sepharose	Ammonium sulfate	1	11.79	-0.29	0.35
Phenyl Sepharose	Ammonium sulfate	2	-12.14	12.7	-1.14
Phenyl Sepharose	Sodium chloride	2	-77.10	42.33	-5.13
Phenyl Sepharose	Sodium chloride	4	-65.01	37.55	-4.71
Butyl Sepharose	Ammonium sulfate	1	36.76	-16.07	1.73
Butyl Sepharose	Ammonium sulfate	2	10.02	0.45	-0.38
Butyl Sepharose	Sodium chloride	2	-12.05	6.51	-0.80
Butyl Sepharose	Sodium chloride	4	-1.74	5.55	-1.01

Table 3. Parameters of equation (12) for different operating conditions.

The surface hydrophobicity of tagged proteins (ϕ_{tagged}) has been used by Lienqueo et al. (2007) for predicting the DRT of cutinases tagged with hydrophobic peptides in different matrices for HIC, by means of equation (13) and the methodology represented in Figure 3. The coefficients of the linear model are constants for each set of operating conditions. This approach has proven to be effective in predicting the behavior of tagged proteins in HIC, since it showed a low deviation between predicted and experimental DRT (in the order of 2%), for the tagged cutinases that were studied. Finally, the ASH obtained from equation

(11) based on amino acidic composition was used to predict chromatographic behavior in HIC, resulting in a performance 5% better than that observed in the model based on the three-dimensional structure of proteins (equation (10)) (Salgado et al., 2008).

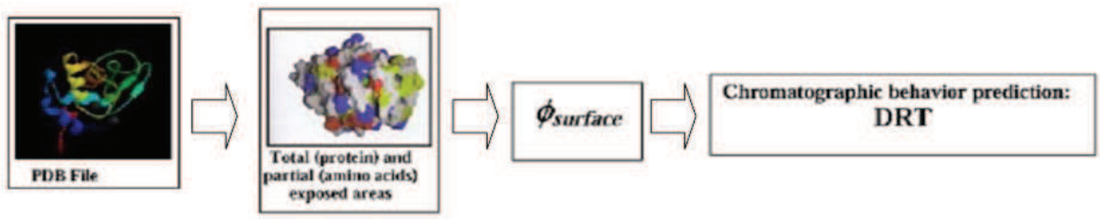


Fig. 5. Methodology for predicting protein retention time in HIC based on surface hydrophobicity. Using a PDB file as input to the program GRASP, the total and partial accessible areas of the exposed amino acids is determined. Using an amino acid hydrophobicity scale and equation (12), the average surface hydrophobicity can be obtained. Then, through simple mathematical correlations the DRTof the protein can be estimated.

4. Applications in biomedical engineering

4.1 General applications

Currently, many proteins of pharmacological and industrial interest are obtained through highly optimized purification processes, typically consisting of two or three chromatographic separation stages. Usually these processes involve one or two IEC steps followed by a HIC step (Asenjo & Andrews, 2004). In addition, most recombinant proteins can be obtained at therapeutic grade of purity, by processes of the same structure (Asenjo & Andrews, 2008). Then, HIC often forms part of processes to yield a purified macromolecule of biomedical interest, such as therapeutic proteins (Seely & Richey, 2001), DNA vaccines (Diogo et al., 2000), and enzymes (Teng et al., 2010), among others. Besides, the use of HIC to purify protein complexes (McCue et al., 2008), as well as to study protein folding from a thermodynamic point of view (Geng & Wang, 2007), have been reported. Some applications of HIC for purifying enzymes and protein complexes, and to studying protein folding are described below.

4.1.1 Purification of proteins and enzymes by HIC

Recently, many strategies that involve a HIC step to purify proteins and enzymes of industrial and/or biomedical interest have been reported. For instance, Liu et al. (2010) developed a purification process to isolate and characterize an antifungal protein from *Bacillus subtilis,* which can be used as a bio-control agent. The process consisted of a preliminary precipitation with ammonium sulfate at 30-70% saturation, followed by HIC (using Phenyl Sepharose as stationary phase) and finally an IEC step. The process gave an overall recovery of 1.2% of total protein in the cell extract. The antifungal protein showed ribonuclease, protease and hemagglutinating activities.

On the other hand, Teng et al. (2010) purified and characterized an endo-β-1,4-glucanase from the giant snail (*Achatina fulica frussac*) by means of a process consisting of three chromatographic steps: size exclusion chromatography (SEC), anion exchange chromatography (AEC), and finally hydrophobic interaction chromatography. A 29-fold purity increase was achieved, and an overall recovery of 14.7% was reached. In addition, this

novel enzyme has a particularly high stability at a broad pH range, acidic pH optimum, and a very high thermostability, and therefore it would have a great potential use in industry.
Lavery et al. (2010) reported the purification of a peroxidase from horseradish roots (*Armoracia rusticana*) by means of a three-step strategy, consisting of ultrasonication, ammonium sulfate precipitation, and HIC (using Phenyl Sepharose). In this strategy, the only high-resolution purification step corresponded to HIC. An overall yield of 71% and a 291-fold purification were achieved, thus demonstrating the high efficiency of this technique. The purified peroxidase was extremely stable in different media, and therefore its commercialization seems promising. Bhuvanesh et al. (2010) used a single-step method to purify a filarial protein (expressed heterologously in *E. coli*) with great potential as a vaccine for preventing human lymphatic filariasis. The purification method consisted of a HIC step. An overall recovery of 60% and 100% purity were achieved.

4.1.2 Purification of protein aggregates by HIC

The use of HIC to separate product-related impurities in the biopharmaceutical industry is well documented (Queiroz et al., 2001). This method is also used to separate multimers from monomeric forms of proteins of biomedical interest, since these conformations often differ in surface hydrophobicity. This difference owes to the fact that the stabilization of quaternary structures occurs due to hydrophobic interaction between the monomers, resulting in protein aggregation. In this way, the hydrophobic patches of a multimer are somewhat hidden, and therefore less accessible to the hydrophobic ligands of a HIC support, unlike the monomer whose hydrophobic patches are exposed to the solvent and, accordingly, accessible to the HIC ligands. The adsorption mechanism of protein aggregates in HIC is complex and not fully understood so far.
Mc Cue et al. (2010) developed a chromatography model to predict the separation of monomer and aggregate species. Equation (14) shows the Langmuir isotherm that describes equilibrium between the protein adsorbed to the resin and the protein that remains in solution. Here, C is the protein concentration in the mobile phase, q is the protein concentration in the stationary phase, q_m is the resin maximum capacity and k is the equilibrium constant. Equation (15) shows the mass balance used to describe the protein concentration profiles. Mass conservation was assumed and the intra-particle mass transfer was considered to be driven by homogeneous diffusion. In equation (15), D_{eff} is the effective diffusivity of the protein from the mobile phase bulk to the inner of the porous resin bead, t is time and r is the radial coordinate. The validity of the model was assessed by experimental determinations. A fraction of the aggregate proteins bound irreversibly to the HIC resin, becoming the major factor governing the process. This phenomenon was adequately described by the model.

$$C = \frac{q}{k \cdot (q_m - q)} \tag{14}$$

$$\frac{\partial q}{\partial t} = D_{eff} \cdot \left(\frac{\partial^2 q}{\partial r^2} + 2 \cdot \frac{\partial q}{r \cdot \partial r} \right) \tag{15}$$

4.1.3 Protein folding in HIC

Protein folding is relevant from a process point of view, since most recombinant proteins produced in bacteria such as *E. coli* are accumulated as inclusion bodies, and therefore

protein refolding constitutes an additional stage in the production and purification process in order to yield a "functional" product (especially in the case of enzymes). Hydrophobic interaction chromatography has been used to study thermodynamics aspects of protein folding. For instance, Geng et al. (2005) performed calorimetric determinations on the enthalpy change ($\Delta H_{folding}$) of denatured lysozyme during its adsorption to a hydrophobic surface, with the simultaneous protein refolding. The surface consisted of PEG-600 made of a silica base HP-HIC (High Performance- Hydrophobic Interaction Chromatography) packing. At 25°C, $\Delta H_{folding}$ was found to be - 34 439 KJ/mol, involving adsorption, dehydration and molecular conformation enthalpies changes.

Later, Geng & Wang (2007) used the concept of "Protein Folding Liquid Chromatography" (PFLC), to describe a chromatographic process aiming to either raise the efficiency, or shortening the time of protein folding. Besides, an optimal PFLC should be able to simultaneously remove denaturant substances, separate contaminant proteins, promote refolding of the target protein, and ease denaturant recovery. Any type of chromatography can be used in PFLC, mainly Size Exclusion Chromatography, Ion Exchange Chromatography, Affinity Chromatography, and Hydrophobic Interaction Chromatography.

In HIC, the process is governed by thermodynamic equilibrium and so does the protein folding. PFLC provides the chemical equilibrium that favors the conversion from aggregate to desorbed protein, resulting in a higher refolding efficiency and shorter refolding time. The unfolded proteins, at a high ionic strength, are driven by hydrophobic interactions from the mobile phase to the HIC stationary phase, and the hydrophobic patches on the proteins surface get attached to the hydrophobic ligands, while the hydrophilic zones of the unfolded molecules remain in contact with the solvent. As a consequence, unfolded molecules are not able to aggregate. The unfolded molecules desorb from the HIC support as ionic strength in the mobile phase decreases. Protein molecules with incorrectly folded domains would be corrected by the spontaneous disappearance of the domains in the mobile phase due to their thermodynamic instability. After many HIC runs, the incorrectly folded domains will decrease, while the correctly folded molecules will predominate, resulting in protein refolding at high efficiency.

4.2 Applications in biomedical engineering

Biomedical applications of HIC are broad, since this technique offers some advantages over other chromatographic techniques, such as Affinity Chromatography (AC) and Reverse-Phase Chromatography (RPC). The use of AC depends on the availability of a specific ligand for the protein or group of proteins to be separated, thus limiting their applicability and raising its cost. The main disadvantage of RPC relies on the nature of the solvent in which the purified protein is recovered, usually an organic solvent not suitable for human or animal use. Then, HIC constitutes a purification tool suitable for biomedical applications, such as vaccines, therapeutic proteins, plasmids and mainly antibodies. In addition, the use of chromatography in high-throughput studies, such as proteomics and protein interactions, is increasing. Some of these Biomedical Engineering applications of HIC are discussed below.

4.2.1 Antibodies purification

At the beginning of the antibody industry, purification was performed through AC. For instance, protein A - AC was used for purifying monoclonal antibodies (MAbs), due to the

extremely low MAb concentration in the initial solution (fermentation broth), and the high amount of contaminant proteins. Therefore, affinity chromatography was the most suitable technique, given its high selectivity and resolution. Unfortunately, this purification technique has a serious disadvantage given by the high affinity of the MAb for the ligand (such as protein A), making it difficult to release the MAb from the ligand, with the consequent economical detriment. Moreover, MAbs are highly hydrophobic macromolecules, and then the use of HIC has been suggested (Asenjo & Andrews, 2008). At the present time molecular biology advances have enabled reaching high concentrations of MAbs in the fermentation broth, making it possible to use less selective but cheaper purification techniques, such as HIC. Figure 6 depicts a monoclonal antibody (A) and the antibody attached to a HIC stationary phase (B).

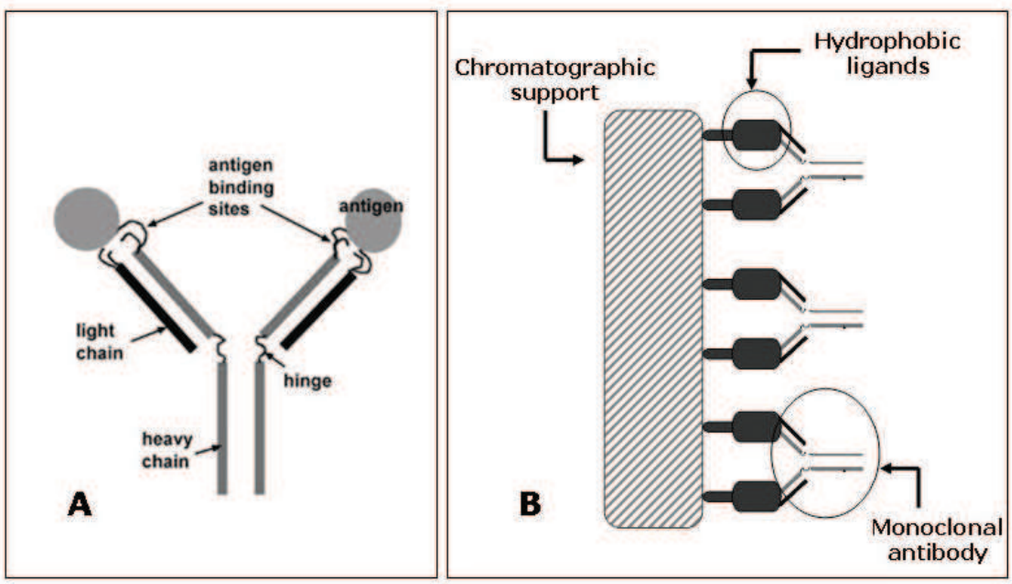


Fig. 6. (A) Schematic representation of a MAb. The antigen binding sites of the MAb are highlighted. Since this zone is characterized by an extremely high hydrophobicity, MAbs exhibit a high attraction for the hydrophobic ligands used in HIC resins. (B) Schematic representation of MAbs attached to a HIC resin. The antigen binding site interacts directly with the hydrophobic ligands of the HIC resin.

HIC is used as a polishing step in the purification processes of immunoglobulin-related products, since it has the ability to remove aggregated forms of the antibody (Rinderknecht & Zapata, 2006). Despite the high resolution offered by HIC, there are some drawbacks for its use in MAbs purification, given by the relatively low binding capacity of HIC supports and the consequent low yield in MAb recovery, compared to AC. Besides, MAb elution is usually achieved at a relatively high salt concentration, which implies that the solution containing the purified MAb also contains a high amount of salt that hinders sample manipulation and transitions during large-scale production.

This has encouraged research on HIC optimization, mainly regarding chromatographic supports. Recently, Chen et al., (2008) showed that the optimization of pore size of a HIC support significantly improved Immunoglobulin G binding capacity and also increased HIC

efficiency, maintaining the MAb stability. Optimizing pore size facilitates mass transfer from mobile phase bulk towards the hydrophobic ligand. Kostareva et al. (2008) purified a heteropolymer (a kind of MAb consisting of a dual antibody conjugate) by HIC. They found that using a Propyl-HIC resin the heteropolymer was efficiently separated from free MAbs, thus confirming the ability of HIC for separating aggregates from monomers, and also its suitability for purifying MAbs.

4.2.2 Proteomics

Proteomics can be defined as the study of all the proteins codified by a genome, in a given tissue of a given organism at a given time. It involves studying how the concentration or "relative abundance" of the proteins change under a certain stimulus, protein conformational changes, protein – protein interactions (or "interactomics"), among others, as well as the use and development of experimental and bioinformatics technologies necessary to perform these studies. In this regard, protein separation techniques are essential. The fundamental separation methods used in proteomics are Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) and/or Two-Dimensional Gel Electrophoresis (2DGE) and mass spectrometry (MS); the latter is used as separation but also as identification tool. Figure 7 depicts a classical proteomics experiment, starting from a biological sample, followed by preliminary fractionation by liquid chromatography and after that separation by 2DGE, and finally identification of protein spots by MS.

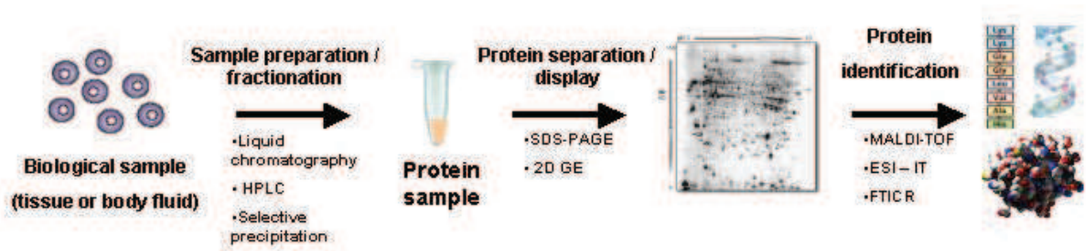


Fig. 7. Simplified representation of a gel-based proteomics experiment. Starting from a biological sample, a protein extract is obtained using different biochemical techniques to fractionate the sample. These fractionation steps allow the enrichment of protein fractions in low abundance proteins and to reduce the complexity of the sample. The protein fractions are then resolved by SDS-PAGE or 2D GE, and finally protein spots are excised form the gel and then analyzed by mass spectrometry in order to determine their identity and structural properties.

From a Biomedical point of view, proteomics is an important field in the task of discovering new biomarkers that reflect the health/disease status of living organisms. The use of proteomics with this purpose has been somewhat limited due to technical hurdles related to the high complexity of the biological samples to be analyzed, usually blood serum or plasma, but also cerebrospinal fluid, urine and tears. These samples show a wide dynamic range of protein concentration, exceeding 10^{10}. This means that the most abundant protein in plasma (albumin), for example, has a concentration 10^{10} times higher than that of the less abundant protein (such as transcription factors).

Two-dimensional gel electrophoresis can resolve a concentration range of up to 10^4, and therefore 2DGE images or "maps" of blood plasma are dominated by the highly abundant

proteins, namely albumin, immunoglobulin, fibrinogen, among others, thus preventing the detection of low abundance proteins (Hoffmann et al., 2007). Mass spectrometry can resolve a range of 10^3 in a single spectrum, but combined with separation steps it can resolve a range of up to 10^6 (Jacobs et al., 2005). This range is still wide, and thus many proteins cannot be detected. Then, chromatographic separation steps should be used before 2DGE in order to reduce the dynamic range of proteins concentration, and consequently increase resolution.

The most abundant proteins in blood plasma are albumin, immunoglobulin, transferrin, haptoglobin, fibrinogen and α-1-antitrypsin, which amount to 90% of total protein mass. Then, total or partial depletion of these proteins allows detecting low abundance proteins. Different methods can be used to deplete these proteins, being liquid chromatography the most popular one (Nakamura et al., 2008). Different chromatographic strategies are available for this purpose, including affinity dye-based chromatography for albumin depletion, affinity to protein A and G for immunoglobulin depletion, specific antibody-affinity columns (Linke et al., 2007), and affinity columns containing lectins, peptides or inorganic ligands (Salih, 2005). Liquid chromatography has the advantage of being easy to use and to scale-up, but are relatively expensive, especially those involving affinity columns. Another drawback of affinity chromatography is the non-specific interactions that lead to the loss of some proteins, with the consequent loss of information (Altintas & Denizli, 2006).

In order to overcome the disadvantages of affinity chromatography for its use in blood plasma proteomics, several complementary strategies have been examined, such as sequential anion and cation exchange chromatography followed by 2DGE; and strong cation exchange chromatography followed by liquid-phase isoelectric focusing (Ottens et al., 2005; Barnea et al., 2005). Since these approaches considerably improve the capacity to detect low abundance proteins, it was suggested that the optimization of combinatorial processes by coupling immuno-affinity depletion with other conventional separation methods such as hydrophobic interaction chromatography will probably lead to significant advances in proteomics (Mahn et al., 2010). Despite the research conducted in this area, there is still a lack of optimized processes that ensure detection of the complete proteome of a tissue or cell.

4.2.2.1 Plasma fractionation by HIC

The applicability of HIC as a plasma fractionation method has been recently proposed. Geng et al. (2009) developed a two-dimensional liquid chromatography resin having two types of ligands, and hence that functions in two retention modes: cation exchange and hydrophobic interaction. This method could be applied to the fast fractionation of intact proteins before mass spectrometry analysis. The results obtained by HIC were similar to those obtained by ion exchange chromatography. On the other hand, a HIC matrix consisting of highly acetylated agarose has been used for the isolation of immunoglobulin from porcine serum, with a relative success (Ramos-Clamont et al., 2006).

Recently, Mahn et al. (2010) investigated if the performance of 2DGE could be improved by fractionating blood plasma through a HIC step, thus reducing the relative concentration of some highly abundant proteins in plasma. First, the hydrophobicity of the main 56 proteins present in blood plasma was determined. To do this, the amino acidic composition of the proteins was considered, and hydrophobicity was calculated by equation (16) based on the methodology proposed by Salgado et al. (2005). In equation (16), ϕ_{aai} is given by the Cowan–Whittaker hydrophobicity scale in its normalized form (see Table 1), n_i is the number of amino acids of type i in the protein, $s_{i,max}$ is the maximum solvent accessible area that an

amino acid X can have when forming part of the G–X–G tripeptide in extended conformation (Miller et al., 1987).

$$ASH = \sum_{i=1}^{20} \left(\phi_{aai} \cdot \frac{n_i \cdot s_{i,\max}}{\sum_{j \in A} n_j \cdot s_{j,\max}} \right) \tag{16}$$

After that, a cluster analysis was performed in order to classify them as low, medium or high hydrophobicity proteins. This analysis showed that the highly abundant proteins, i.e. albumin, immunoglobulins, fibrinogen and haptoglobin, exhibited a medium hydrophobicity, and thus they fell in the same cluster. With this information, a HIC step was designed to deplete highly abundant proteins from rat plasma samples. The HIC step consisted of stepwise elution to separate the three groups of proteins (low, medium and high hydrophobicity) using a maximum concentration of 2 M ammonium sulfate, and concentration for elution of 0.6 M (to desorb low hydrophobicity proteins), 0.5 M (to desorb medium hydrophobicity proteins), and 0.0 M (to desorb the highly hydrophobic proteins).

Finally, the depleted samples were analyzed by 2DGE and the performance of the HIC pre-fractionation step was compared with that exhibited by a commercial immuno-affinity column. The reproducibility of 2DGE was similar to that obtained from immuno-affinity depleted plasma. However, HIC was more successful in depleting albumin and α-1-antitrypsin. Besides, HIC resulted in a much lower increment of immunoglobulin and haptoglobin abundances than the immuno-affinity column. Then, HIC depletion allowed detecting twice the number of protein spots than immuno-affinity depletion did. Therefore, HIC could be used as a depletion method complementary to affinity columns. The operating conditions in HIC could be optimized in order to maintain the high number of spots that are detected if HIC is used as the sole depletion method. Finally, given the relatively low cost of HIC supports and HIC operation, its use could be proposed as a convenient choice for depleting highly abundant proteins in plasma samples prior to 2DGE-based proteomics.

4.2.2.2 Analysis of protein interaction networks by HIC

Protein–protein interactions are essential in biological processes. All the interactions in a cellular system are known as protein interaction network or 'interactome'. In Biomedicine there is great interest in recognizing these interactions, aiming to establish the role they play in certain diseases. The traditional approaches to study protein-protein interactions are the antibody pull-down method (APD) and the yeast two-hybrid method (YTH). Despite their popularity, these methods have some disadvantages. It is very likely that a protein forms part of different complexes; then, in an APD experiment, antibodies targeting such a protein will pull down together all the complexes where the protein participates, making them appear to be part of a single large complex, confusing the biological interpretation of the results. The YTH is an "*in vivo*" method that allows detecting only binary interactions. It tends to give false positives and is limited to binary interactions. Therefore it is not useful in studying the dynamics of complex formation triggered by different stimuli (Corvey et al., 2005).

Liu et al. (2008) investigated the potential of chromatography to allow the simultaneous examination of multiple protein complexes along with comparing and validating results from the traditional methods. Since protein complexes remain intact during mild forms of

elution in AC, a similar behavior should be expected in other chromatographic supports, such as IEC and HIC. They studied the extent to which protein interaction partners from yeast (*S. cerevisiae*) lysate remain associated during IEC, SEC and HIC. Most protein complexes remained intact, and all the proteins forming part of the complex migrated as a single unit. Protein complexes exhibited a chromatographic behavior different from that shown by the individual proteins that compose the complex. Accordingly, studying protein complexes could be easily performed by multidimensional chromatographic methods when at least one of the fractionation dimensions included SEC of native proteins. This method enables the study and recognition of several protein complexes simultaneously, avoiding the use of genetic engineering.

5. Conclusion

HIC is a powerful tool for purifying macromolecules of biomedical interest whose potential has been relatively under-exploited so far. Its applications are diverse, including industrial processes as well as analytical methods. The performance of HIC can be improved by optimizing the supports and the operation mode considering the hydrophobicity of the macromolecule to be separated. Research on optimization of HIC for biomedical applications should be encouraged, since this method allows reducing production cost of biopharmaceuticals such as antibodies and therapeutic proteins.

6. Acknowledgement

Fondecyt Programme.

7. References

Altintas, E.B., Denizli, A. (2006). Efficient removal of albumin from human serum by monosize dye-affinity beads. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 832, 2, 216-223.

Asenjo, J.A., Andrews, B.A. (2008). Challenges and trends in bioseparations. *Journal of Chemical Technology and Biotechnology*, 83, 117-120.

Asenjo, J.A., Andrews, B.A. (2004). Is there a rational method to purify proteins? From expert systems to proteomics. *Journal of Molecular Recognition*, 17, 236-247.

Bai, Q., Wei, Y.M., Geng, M.H., Geng X.D. (1997). High performance hydrophobic interaction chromatography - A new approach to separate intermediates of protein folding .1. Separation of intermediates of urea-unfolded alpha-amylase. *Chinese Chemical Letters*, 8, 67-70.

Bairoch, A., Apweiler, R., Wu, C.H., Barker, W.C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M.J., Natale, D.A., O'Donovan, C., Redaschi, N., Yeh, L.S. (2005). The universal protein resource (UniProt). *Nucleic Acids Research*, 33, D154-D159.

Bandmann, N., Collet, E., Leijen, J., Uhlen, M., Veide, A., Nygren, P.A. (2000). Genetic engineering of the Fusarium solani pisi lipase cutinase for enhanced partitioning in PEG-phosphate aqueous two-phase systems. *Journal of Biotechnology*, 79, 161-172.

Barnea, E., Sorkin, R., Ziv, T., Beer, I., Admon, A. (2005). Evaluation of prefractionation methods as a preparatory step for multidimensional based chromatography of serum proteins. *Proteomics*, 5, 3367-3375.

Berggren, K., Wolf, A., Asenjo, J.A., Andrews, B.A., Tjerneld, F. (2002). The surface exposed amino acid residues of monomeric proteins determine the partitioning in aqueous two-phase systems. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology*, 1596, 253-268.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E. (2000). The Protein Data Bank. *Nucleic Acids Research*, 28, 235-242.

Bhuvanesh, S., Arunkumar, C., Kaliraj, P., Ramalingam, S. (2010). Production and single-step purification of Brugia malayi abundant larval transcript (ALT-2) using hydrophobic interaction chromatography. *Journal of Industrial Microbiology & Biotechnology*, 37, 1053-1059.

Biswas, K.M., DeVido, D.R., Dorsey, I.G. (2003). Evaluation of methods for measuring amino acid hydrophobicities and interactions. *Journal of Chromatography A*, 1000, 637-655.

Chaturvedi, R., Bhakuni, V., Tuli, R. (2000). The delta-endotoxin proteins accumulate in Escherichia coli as a protein-DNA complex that can be dissociated by hydrophobic interaction chromatography. *Protein Expression and Purification*, 20, 21-26.

Chen, J., Tetrault, J., Ley, A. (2008). Comparison of standard and new generation hydrophobic interaction chromatography resins in the monoclonal antibody purification process. *Journal of Chromatography A*, 1177, 272-281.

Corvey, C., Koetter, P., Beckhaus, T., Hack, J., Hofmann, S., Hampel, M., Stein, T., Karas, M., Entian, K.-D. (2005). Carbon source-dependent assembly of the Snf1p kinase complex in Candida albicans. *Journal of Biological Chemistry*, 280, 25323-25330.

Cowan, R., Whittaker, R.G. (1990). Hydrophobicity indices for amino acid residues as determined by high-performance liquid chromatography. *Peptide Research*, 3, 75-80.

Diogo, M.M., Queiroz, J.A., Prazeres, D.M. (2001). Studies on the retention of plasmid DNA and Escherichia coli nucleic acids by hydrophobic interaction chromatography. *Bioseparation*, 10, 211-220.

Diogo, M.M., Queiroz, J.A., Monteiro, G.A., Martins, S.A., Ferreira, G.N., Prazeres, D.M. (2000). Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography. *Biotechnology and Bioengineering*, 68, 576-583.

Eriksson, K., in: J.-C. Janson, L. Ryden (Eds.), Protein Purification: Principles, High-resolution Methods and Applications, 2nd ed., Wiley-Liss, New York, 1998, p. 151.

Fausnaugh, J.L., Regnier, F.E. (1986). Solute and mobile phase contributions to retention in hydrophobic interaction chromatography of proteins. *Journal of Chromatography*, 359, 131-146.

Fausnaugh, J.L., Kennedy, L.A., Regnier, F.E. (1984). Comparison of hydrophobic-interaction and reversed-phase chromatography of proteins. *Journal of Chromatography*, 31, 141-155.

Fexby, S., Bülow, L. (2004). Hydrophobic peptide tags as tools in bioseparation. *Trends in Biotechnology*, 22, 511-516.

Geng, X., Ke, C. , Chen, G., Liu, P., Wang, F., Zhang, H., Sun, X. (2009). On-line separation of native proteins by two-dimensional liquid chromatography using a single column. *Journal of Chromatography A*, 1216, 3553-3562.

Geng, X., Wang, C. (2007). Protein folding liquid chromatography and its recent developments. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 849, 69-80.

Haimer, E., Tscheliessnig, A., Hahn, R., Jungbauer, A. (2007). Hydrophobic interaction chromatography of proteins IV - Kinetics of protein spreading. *Journal of Chromatography A*, 1139, 84-94.

Hjerten, S. (1973). Some general aspects of hydrophobic interaction chromatography. *Journal of Chromatography*, 87, 325-331.

Hoffman, S.A., Joo, W.A., Echan, L.A., Speicher, D.W. (2007). Higher dimensional (Hi-D) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 849, 43-52.

Jacobs, J.M., Adkins, J.N., Qian, W.J., Liu, T., Shen, Y., Camp 2nd, D.G., Smith, R.D. (2005). Utilizing human blood plasma for proteomic biomarker discovery. *Journal of Proteome Research*, 4, 1073-1085.

Jennissen, H.P. (2000). Hydrophobic interaction chromatography. *International Journal of Bio-Chromatography*, 5, 131-138.

Kostareva, I., Hung, Campbell, F.C. (2008). Purification of antibody heteropolymers using hydrophobic interaction chromatography. *Journal of Chromatography A*, 1177, 254-264.

Kovacs, J.M., Mant, C.T., Hodges, R.S. (2006). Determination of intrinsic hydrophilicity/hydrophobicity of amino acid side chains in peptides in the absence of nearest-neighbor or conformational effects. *Biopolymers*, 84, 283-297.

Ladiwala, A., Xia, F., Luo, Q., Breneman, C.M., Cramer, S.M. (2006). Investigation of protein retention and selectivity in HIC systems using quantitative structure retention relationship models. *Biotechnology and Bioengineering*, 93, 836-859.

Lavery, C. B., MacInnis, M.C., MacDonald, M.J., Williams, J.B., Spencer, C.A., Burke, A.A., Irwin, D.J., D'Cunha, G.B. (2010). Purification of Peroxidase from Horseradish (Armoracia rusticana) Roots. *Journal of Agricultural and Food Chemistry*, 58, 8471-8476.

Lienqueo, M.E., Mahn, A. V., Asenjo, J.A. (2002). Mathematical correlations for predicting protein retention times in hydrophobic interaction chromatography. *Journal of Chromatography A*, 978, 71- 79.

Lienqueo, M.E., Mahn, A., Vasquez, L., Asenjo, J.A. (2003). Methodology for predicting the separation of proteins by hydrophobic interaction chromatography and its application to a cell extract. *Journal of Chromatography A*, 1009, 189-196.

Lienqueo, M.E., Salazar, O., Henriquez, K., Calado, C.R.C., Fonseca, L.P., Cabral, J.M. (2007). Prediction of retention time of cutinases tagged with hydrophobic peptides in hydrophobic interaction chromatography. *Journal of Chromatograohy A*, 1154, 460-463.

Linke, T, Doraiswamy, S., Harrison, E.H. (2007). Rat plasma proteomics: Effects of abundant protein depletion on proteomic analysis. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 849, 273-281.

Liu, B., Huang, L. Buchenauer, H., Kang, Z. (2010). Isolation and partial characterization of an antifungal protein from the endophytic Bacillus subtilis strain EDR4. *Pesticide Biochemistry and Physiology*, 98, 305-311.

Liu, X., Yang, W., Gao, Q., Regnier, F. (2008). Toward chromatographic analysis of interacting protein networks. *Journal of Chromatography A*, 1178, 24-32.

Mahn, A. V., Lienqueo, M.E., Asenjo, J.A. (2004). Effect of surface hydrophobicity distribution on retention of ribonucleases in hydrophobic interaction chromatography. *Journal of Chromatography A*, 1043, 47-55.

Mahn, A., Lienqueo, M.E., Salgado, J.C. (2009). Methods of calculating protein hydrophobicity and their application in developing correlations to predict hydrophobic interaction chromatography retention. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 1216, 1838-1844.

Mahn, A., Reyes, A., Zamorano, M., Cifuentes, W., Ismail. M. (2010). Depletion of highly abundant proteins in blood plasma by hydrophobic interaction chromatography for proteomic analysis. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 878, 1038-1044.

McCue, J.T., Engel, P., Ng, A., Macniven, R., Thömmes, J. (2008). Modeling of protein monomer/aggregate purification and separation using hydrophobic interaction chromatography. *Bioprocess and Biosystems Engineering*, 31, 261-275.

Melander, W.R., el Rassi, S., Horvath, Cs. (1989). Interplay of Hydrophobic and Electrostatic Interactions in Bio-polymer Chromatography - Effect of Salts on the Retention of Proteins. *Journal of Chromatography*, 469, 3-27.

Miller, S., Janin, J., Lesk, A.M., Chothia, C. (1987). Interior and surface of monomeric proteins. *Journal of Molecular Biology,* 196, 641-656.

Miyazawa, S., Jernigan, R. (1996). Residue-residue potentials with a favorable contact pair term and an unfavorable high packing density term, for simulation and threading. *Journal of Molecular Biology*, 256, 623-644.

Nakamura, T., Kuromitsu, J., Oda, Y. (2008). Evaluation of comprehensive multidimensional separations using reversed-phase, reversed-phase liquid chromatography/mass spectrometry for shotgun proteomics. *Journal of Proteome Research*, 7, 1007-1011.

Nicholls, A., Sharp, K.A., Honig, B. (1991). Protein Folding and Association - Insights from the Interfacial and Thermodynamic Properties of Hydrocarbons. *Proteins-Structure Function and Genetics*, 11, 281-291.

Ottens, A.K., Kobeissy, F.H., Wolper, R.A., Haskins, W.E., Hayes, R.L., Denslow, N.D., Wang, K.K. (2005). A multidimensional differential proteomic platform using dual-phase ion-exchange chromatography-polyacrylamide gel

electrophoresis/reversed-phase liquid chromatography tandem mass spectrometry. *Analytical Chemistry*, 77, 4836-4845.

Pieper, U., Eswar, N., Webb, B.M., Eramian, D., Kelly, L., Barkan, D.T., Carter, H., Mankoo, P., Karchin, R., Marti-Renom, M.A., Davis, F.P., Sali, A. (2009). MODBASE, a database of annotated comparative protein structure models and associated resources. *Nucleic Acids Research*, 37, D347-D354.

Porath, J., Sundberg, L., Fornstedt, N., Olsson I. (1973). Salting-out in amphiphilic gels as a new approach to hydrophobic adsorption. *Nature*, 245, 465-466.

Queiroz J.A., Tomaz, C.T., Cabral, J.M.S. (2001). Hydrophobic interaction chromatography of proteins. *Journal of Biotechnology*, 87, 143-159.

Ramos-Clamont, G., Candia-Plata, M.C., Guzman, R., Vazquez-Moreno, L. (2006). Novel hydrophobic interaction chromatography matrix for specific isolation and simple elution of immunoglobulins (A, G, and M) from porcine serum. *Journal of Chromatography A,* 1122, 28-34.

Rinderknecht, E.H., Zapata, G.A. (2006). US Patent 7,038,017.

Rodenbrock, A., Selber, K., Egmond, M.R., Kula, M.R. (2000). Extraction of peptide tagged cutinase in detergent-based aqueous two-phase systems. *Bioseparation,* 9, 269-276.

Salgado, J.C., Andrews, B.A., Ortúzar, M.F., Asenjo, J.A. (2008). Prediction of the partitioning behaviour of proteins in aqueous two-phase systems using only their amino acid composition. *Journal of Chromatography A*, 1178, 134-144.

Salgado, J.C., Rapaport, I., Asenjo, J.A. (2005). Prediction of retention times of proteins in hydrophobic interaction chromatography using only their amino acid composition. *Journal of Chromatography A*, 1098, 44-54.

Salih, E. (2005). Phosphoproteomics by mass spectrometry and classical protein chemistry approaches. *Mass Spectrometry Reviews*, 24, 828-846.

Savard, J.M., Schneider, J.W. (2007). Sequence-specific purification of DNA oligomers in hydrophobic interaction chromatography using peptide nucleic acid amphiphiles: Extended dynamic range. *Biotechnology and Bioengineering*, 97, 367-376.

Seely, J.E., Richey, C.W. (2001). Use of ion-exchange chromatography and hydrophobic interaction chromatography in the preparation and recovery of polyethylene glycol-linked proteins. *Journal of Chromatography A*, 908, 235-241.

Shaltiel, S., Er-el, Z. (1973). Hydrophobic chromatography: use for purification of glycogen synthetase. *Proceedings of the National Academy of Sciences U.S.A.*, 70, 778-781.

Shepard, C.C., Tiselius, A. (1949). In "Chromatographic Analysis" p. 275. *Discussions of the Faraday Society*, 7. Hazell, Watson and Winey. London.

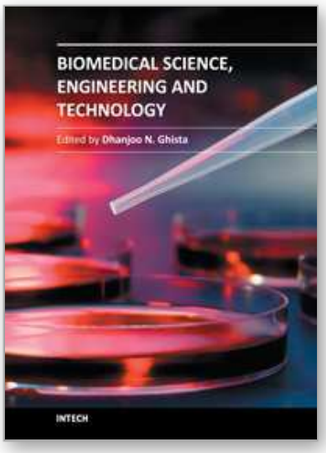
Simeonidis, E., Pinto, J.M., Lienqueo, M.E., Tsoka, S., Papageorgiou, L.G. (2005). MINLP models for the synthesis of optimal peptide tags and downstream protein processing. *Biotechnology Progress*, 21, 875-884.

Tanford, C. (1962). Contribution of hydrophobic interactions to the stability of globular conformation of proteins. *Journal of the American Chemical Society*, 84, 4240-4247.

Teng, Y., Yin, Q., Ding, M., Zhao, F. (2010). Purification and characterization of a novel endo-beta-1,4-glucanase, AfEG22, from the giant snail, Achatina fulica frussac. *Acta Biochimica et Biophysica Sinica*, 42, 729-734.

Wertz, D.H., Scheraga, H.A. (1978). Influence of water on protein structure. An analysis of the preferences of amino acid residues for the inside or outside and for specific conformations in a protein molecule. *Macromolecules*, 11, 9-15.

Zhang, Y., Martínez, T., Woodruff, B., Goetze, A., Bailey, R., Pettit, D., Balland, A. (2008). Hydrophobic interaction chromatography of soluble Interleukin 1 receptor type II to reveal chemical degradations resulting in loss of potency. *Analytical Chemistry*, 80, 7022-7028.



Biomedical Science, Engineering and Technology

Edited by Prof. Dhanjoo N. Ghista

ISBN 978-953-307-471-9

Hard cover, 902 pages

Publisher InTech

Published online 20, January, 2012

Published in print edition January, 2012

This innovative book integrates the disciplines of biomedical science, biomedical engineering, biotechnology, physiological engineering, and hospital management technology. Herein, Biomedical science covers topics on disease pathways, models and treatment mechanisms, and the roles of red palm oil and phytomedicinal plants in reducing HIV and diabetes complications by enhancing antioxidant activity. Biomedical engineering coves topics of biomaterials (biodegradable polymers and magnetic nanomaterials), coronary stents, contact lenses, modelling of flows through tubes of varying cross-section, heart rate variability analysis of diabetic neuropathy, and EEG analysis in brain function assessment. Biotechnology covers the topics of hydrophobic interaction chromatography, protein scaffolds engineering, liposomes for construction of vaccines, induced pluripotent stem cells to fix genetic diseases by regenerative approaches, polymeric drug conjugates for improving the efficacy of anticancer drugs, and genetic modification of animals for agricultural use. Physiological engineering deals with mathematical modelling of physiological (cardiac, lung ventilation, glucose regulation) systems and formulation of indices for medical assessment (such as cardiac contractility, lung disease status, and diabetes risk). Finally, Hospital management science and technology involves the application of both biomedical engineering and industrial engineering for cost-effective operation of a hospital.

How to reference

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Andrea Mahn (2012). Hydrophobic Interaction Chromatography: Fundamentals and Applications in Biomedical Engineering, Biomedical Science, Engineering and Technology, Prof. Dhanjoo N. Ghista (Ed.), ISBN: 978-953-307-471-9, InTech, Available from: http://www.intechopen.com/books/biomedical-science-engineering-and-technology/hydrophobic-interaction-chromatography-fundamentals-and-applications-in-biomedical-engineering

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