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## Hydrogel Network in a Porous Resin Matrix:

## a study for chromatographic applications

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# Abstract

In this work the possibility of filling porous poly(styrene-divinylbenzene) resin particles with hydrogel has been investigated, and the viability of such a composite material for chromatographic purposes has been assessed. The main focus was put on the loading capacity of the material with respect to proteins, but ion capacity and swelling properties were measured as well. Both resin and hydrogel were expressly produced for this study: porosity and particle size distribution of the resin were accurately controlled. Moreover, several compositions of the hydrogel were tested, changing both nature and quantities of the various components. Different processes for combining the two materials were developed, in order to achieve optimal chromatographic properties of the final composite. All materials produced were packed into a chromatography column and tested inside a suitable high performance liquid chromatography cycle and with respect to an appropriate protein, so as to verify their viability for biochromatography. The obtained results proved that a few improvements are required in order to make the material suitable for industrial applications.


Key words: Porous poly(styrene-divinylbenzene) resin particles, Hydrogel, Protein loading capacity, Biochromatography, Industrial applications.

# Sommario

In questo lavoro è stata esplorata la possibilità di riempire particelle porose di poli(stirene-divinilbenzene) con un idrogel, e l'attitudine di un simile materiale composito a scopi cromatografici è stata valutata. L'attenzione è stata rivolta principalmente alla capacità di carico del materiale verso le proteine, ma anche le proprietà di swelling e di capacità ionica sono state misurate. sia la resina che l'idrogel sono stati appositamente prodotti per questo studio: la porosità e la distribuzione della dimensione delle particelle sono state attentamente controllate. Inoltre, varie composizioni sono state sperimentate per l'idrogel, cambiando sia la natura che le quantità dei diversi componenti. Diversi processi atti a combinare i due materiali sono stati sviluppati, in modo da ottenere proprietà cromatografiche ottimali del composito finale. I materiali prodotti sono stati impaccati in colonne cromatografiche e testati in un opportuno ciclo di cromatografia liquida ad alta resa e con una proteina adatta, così da verificarne le potenzialità per applicazioni in biocromatografia. I risultati ottenuti hanno dimostrato che con gli opportuni miglioramenti il materiale potrebbe prestarsi ad applicazioni su scala industriale.

Parole chiave: Particelle di resina di poli(stirene-divinilbenzene), Idrogel, Capacità di carico verso le proteine, Biocromatografia, Applicazioni industriali.

# Contents

# List of abbreviations

AA……………………………………………………… Acrylamide

AIBN………………………………………………….. Azobisisobutyronitrile

AMPS…………………………………………………. 2-Acrylamido-2-methylpropane sulfonic acid

APS…………………………………………………. Ammonium persulfate

AETAC……………………………………………… [2-(Acryloyloxy)ethyl] trimethylammonium chloride

AU……………………………………………………… Absorbance units

DVB…………………………………………………. Divinylbenzene

EC……………………………………………………. Electrical conductivity

EtOH………………………………………………….. Ethanol

h…………………………………………………………… hour(s)

$H_2SO_4$…………………………………………………. Sulphoric acid

HCl…………………………………………………….. Hydrochloric acid

HETP………………………………………………….. Height equivalent to a theoretical plate

$HNO_3$………………………………………………… Nitric acid

HPLC…………………………………………………….. High-Performance Liquid Chromatography

$KNO_3$…………………………………………………….. Potassium nitride

KPS…………………………………………………….. Potassium persulfate

mBAM…………………………………………………N,N'-methylenebisacrylamide

$MgCl_2$…………………………………………………….Magnesium chloride

$N_2$………………………………………………. Nitrogen (gas)

$Na_2HPO_4$………………………………………… Sodium phosphate dibasic

$NaCl$……………………………………………… Sodium chloride

$NaH_2PO_4$………………………………………. Sodium phosphate monobasic

$NaHCO_3$………………………………………… Sodium carbonate

$NaOH$…………………………………………… Sodium hydroxide

PEEK……………………………………………….. Polyether ether keton

PS-DVB…………………………………………… Poly(styrene-divinylbenzene)

SDS………………………………………………… Sodium dodecyl solphate

SEM………………………………………………… Scanning electron microscopy

SPAN 20………………………………………….. Sorbitan monolaurate

SPAN 40………………………………………….. Sorbitan monopalmitate

Sty…………………………………………………… Styrene

TEMED……………………………………………Tetramethylethylenediamine

UV-Vis……………………………………………Ultraviolet-visible spectrophotometry

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# 1 Introduction

In this chapter both the concept and the history of column chromatography are presented; then, the reasons for which this research was carried out are explained, after a brief presentation of the various materials that have already been developed for the same purpose.

# 1.1 Excerpt on column chromatography

The word "chromatography" comes from Greek (*chroma* "color" and *graphein* "to write"), and was first used by Mikhail Tsvet in 1900 to describe the process he invented to reveal, separate and study pigments found in plants [1]. All these natural pigments have a particular colour, different from those of the other ones, so Tsvet decided to name the process chromatography, because the technique revealed each pigment by its colour, as can be seen in Figure 1-1 below.
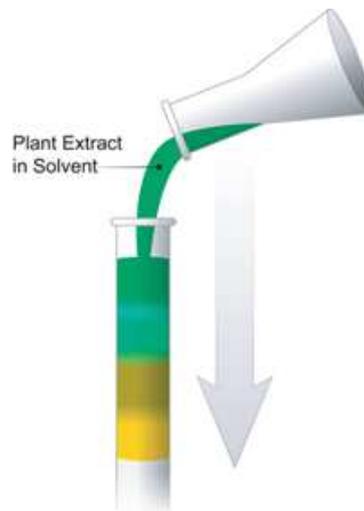


**Figure 1-1: separation of vegetal pigments by means of chromatography, as intended by Mikhail Tsvet [2]**

The technique was later further developed and applied to other purposes, but the name has so far been kept, even though the color of the substances subjected to this process is not of any importance anymore.

The term chromatography indicates a series of different techniques the purpose of which is to separate the components of a homogenous mixture. The basic idea is to have the homogenous mixture (generally a fluid phase with dissolved substances in it, usually referred to as the "mobile phase") flowing in contact with a second phase (in most cases a solid, called "stationary phase"); due to thermodynamic imbalance between the two

phases, there is a material transport of species from the mobile phase to the surface of the stationary phase, where adsorption is taking place. The extent of this adsorption is different for the different species, depending upon their affinity for the solid surface. After establishing equilibrium conditions, i.e. long enough contact between the two phases, a properly selected solvent, capable of displacing the adsorbed molecules, is let through the stationary phase and collected, so that only the previously adsorbed molecules will be found in it. Such solvent is called eluent.

The effectiveness of the process is determined by the characteristics of all the chemical species involved, which means mobile phase, stationary phase and eluent. This implies that both stationary phase and eluent must be properly selected according to the kind of substance that is going to be extracted from a solution.

Chromatography can be used both for preparation and for analysis purposes. The purpose of preparative chromatography is to separate the different substances present in a solution so that they can later be used for further applications, and is therefore a form of purification; analytical chromatography is done normally with significantly smaller amounts of material and is applied for measuring the relative proportions of chemical species present in a mixture. It is important to note that none of the two exclude the other: it is possible to cover both aspects of the process with the same procedure. The distinction is mainly about the amount of the mixture subjected to the process.

The most widespread chromatographic technique for preparation purposes is the so-called column chromatography, in which the adsorbent is filling a cylinder that is usually made of glass, polymer or stainless steel and is named "column". In the specific case of ion exchange chromatography  for separation of bio-macromolecules (such as proteins), which is the main focus of this work, the procedure of interest is the one called High-Performance Liquid Chromatography (HPLC), which is known for its high value of separation efficiency. Modern HPLC relies on complex systems such as that shown in Figure 1-2: these apparatuses consist of: a high pressure pump capable of generating and measuring a specified flow rate of eluent (solvent delivery system); an injector that introduces the sample in the continuously flowing mobile phase (sample manager); a column, which contains the stationary phase (chromatographic packing material) that is responsible for effectively carrying out the separation; a detector, which is used to

observe the separated compounds coming out of the column; a computer data station, that collects the detected data and plots them on a diagram called "chromatogram".
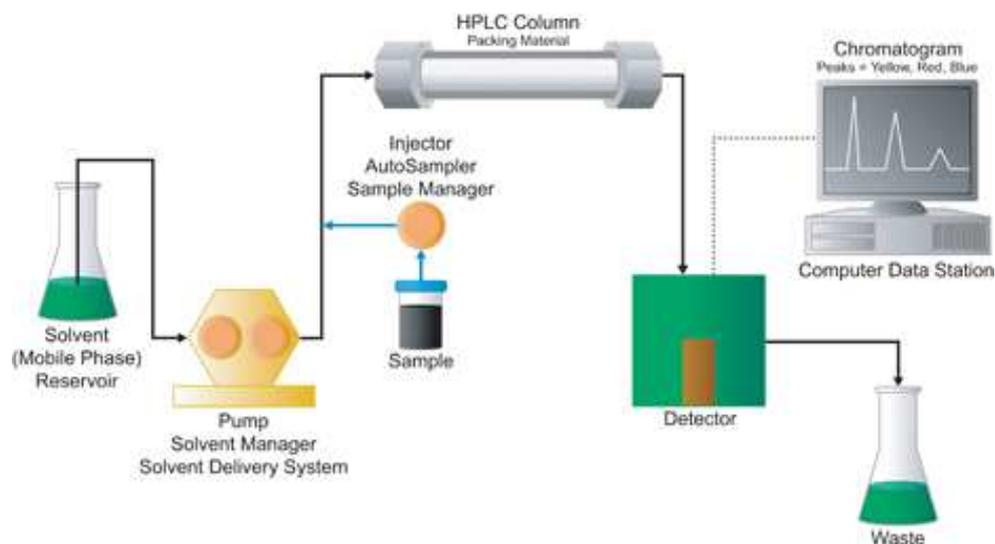


**Figure 1-2: components of a typical HPLC system [2]**

The improved performance of HPLC is due to the particular structure of the stationary phase, that consists of small particles tightly packed together thus making the mass transport through the particles quicker, which allows to optimize process times. Furthermore, being that the substance to be separated from the mobile phase is adsorbed on the surface of the stationary phase, a greater surface to volume ratio increases the amount of such substance that can be adsorbed and separated by a column. This quantity can be measured (by static binding capacity measurements, for example) and is actually one of the most important characteristics in a column for HPLC: it is referred to as the capacity of the column.

Adsorption is due to binding forces, which can be very different in nature and in strength. This also means that the adsorption equilibria vary a lot in strength. It is possible to distinguish two main forms of interaction: physisorption, which happens through weak interactions (van der Waals forces), and chemisorption, that involves

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much stronger interactions (valence forces or chemical bonds) [3]. For ion exchange chromatography, the subject of the present study, valence forces are the ones involved in the binding of proteins to the stationary phase: therefore, in order to grant total reversibility of the adsorption, a proper desorption step must be designed.

The capacity of a column is particularly important for preparative chromatography: in this case, using columns with a greater capacity increases the amount of solution that can be processed in one cycle. In an industrial process, this means minimizing process times (as long as the flow rate can be kept constant), which ultimately increases the income for that process. It is then rather obvious that maximizing column capacity is a problem of great relevance for pharmaceutical industries, as well as for all those industries that use HPLC in their productive chain. Monoclonal antibodies, which are of critical importance for pharmaceutical industries nowadays, accounting for roughly 35% of all biologics in clinical development, make a good example for portraying the relevance of this topic: the downstream processing is responsible for up to 80% of the total production costs of such antibodies [4]. Therefore, a significant reduction in the costs for purification of the product will cause a consistent drop in the cost of the good.

## 1.2 History of HPLC materials

When HPLC was first introduced, many different materials meant to be used as stationary phases of the columns were developed to optimize the process. After the first decade, there was a commonly accepted standard that dominated the market from 1975 until 2000: a stainless steel tube with an internal diameter of 4.6 mm, packed with particles having a 5 to 10 μm diameter [5]. These particles were macroporous: since they are packed as tightly as possible in the column, the mobile phase has to go through the network of pores, so that adsorptive substances can be adsorbed; moreover, this adsorption is usually enhanced by proper functionalization of the surfaces of the pores.

This standard remained unaltered for about 25 years, except for a progressive reduction in the length of the column, which went from 300 to 150 mm. Although finer particles were produced and tested by several research groups, they did not make it to the

market [5]. This was due to the higher pressure requirements that would have noticeably increased the capital expense, and to a significant issue with local temperature maxima due to friction around the particles that would cause changes in viscosity and, consequently, peak broadening (a process called viscous fingering).

The first alternative to these stationary phases was commercialized by Merck in 1999: it was called Chromolith, and consisted of a large (100 mm x 4.6 mm) silica monolith instead of packed microscopic particles. While the monolith was basically a single block of material, it consisted of two regions characterized by different pore sizes, distinguishable in macropores (2 μm) and mesopores (10-15 nm).

A common approach for comparing chromatographic performances of different stationary phases is to plot their normalized efficiency (i.e. HETP, cfr. section 2.5) as a function of the flow rate of the mobile phase [6]. This method is quick and useful in a lot of cases, but it does have the notable limitation of not taking into account kinetic parameters such as analysis times or pressure restrictions. To evaluate performances of a column material without neglecting relevant variable, an alternative term is required: one such parameter is called "impedance", which represents the resistance encountered by the mobile phase as it moves down the column. This parameter is defined as:

$$E = \Delta P\, t \big/ \eta\, N^2 \tag{1}$$

Where $\Delta P$ is the pressure drop, $t$ is the dead time of the chromatographic system, $\eta$ is the dynamic viscosity of the mobile phase and $N$ is the number of theoretical plates [6]. This separation impedance can be seen as the difficulty to achieve a certain performance and should be minimized to optimize a column material.

Another useful parameter for comparing performances of column materials is the so-called "resolution", which is the ability to separate two different signals, as the general meaning of the word would suggest; in the specific case of column chromatography, resolution is defined as the ratio between the time interval between maxima of two peaks and their average width at base:

$$R_s = 2\Delta t \big/ (w_{b1} + w_{b2}) \tag{2}$$

# 1 INTRODUCTION

This term should be maximized in order to optimize column performance. Several models that relate resolution to adjustable column parameters (such as efficiency and retention times) exist, each based on more or less restrictive hypotheses; on the other hand, all these model agree that column resolution grows linearly with the square root of N (which is once again the number of theoretical plates in the column) [6]:

$$R_s \propto \sqrt{N} \qquad (3)$$

The first silica monoliths that were developed made it possible to prepare columns with impedance around 50% of what could be obtained at the time with 5 µm particles. On the other hand, this new technology had the drawback of inducing largely turbulent flows, which lead to band broadening [5]. This meant that process times were reduced, but the quality of the analysis or separation was decreased as well.

The introduction of silica-based monolithic stationary phases renewed the interest around column technology: the threat of competition posed by this new monolithic stationary phases incited a rapid development of the previously existing technology of macroporous particles. In a few years fully porous particles were reduced from 5 to 1.5 µm in diameter, as columns became shorter (from 150 to 50 mm in length) while still maintaining their efficiency: this means that capital expense is reduced because less material is needed to pack the column.

The impedance of columns packed with particles having such a small diameter was only slightly larger than that of monolithic columns [5]. These columns have to operate at high pressure to overcome the high pressure drops associated with the reduced particle size (1000 bar or more), but they have the advantage of having a resolution more than 4 times higher than their monolithic counterpart [5]. This is due to the low efficiency of monolithic silica rods, given by radial heterogeneity of the monoliths and poorly designed sample distributors [5]; a second generation of monolithic stationary phases free from such issues is to be hoped for.

In parallel to these finer particles, new brands of core-shell particles, with a slightly larger diameter (2-3 µm) were also developed.

Core-shell particles such as that of Figure 1-3 consist of a highly cross-linked, rigid full core, and a lightly cross-linked, soft porous shell. This means that only the shell volume

is accessible to the mobile phase, which limits the overall surface area of the material; on the other hand, the rigid core is capable of bearing relatively high compression stresses, showing elastic behavior up to 60 MPa [7], which makes the material suitable for HPLC applications. Furthermore, since the mobile phase is not able to penetrate the core of the particles, diffusion distances are reduced and there can be no band broadening due to stagnant mobile phase mass transfer [8], which improves efficiency and resolution of columns packed with this kind of particles.
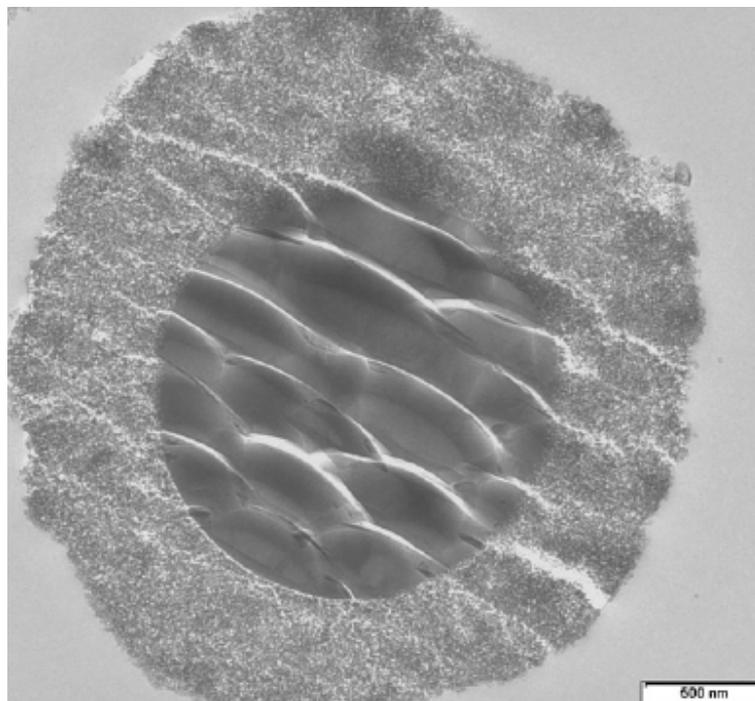


**Figure 1-3: TEM photograph of a 2.7 µm core-shell Halo particle by Advanced Materials Technology (picture reproduced from [9])**

First, Advanced Materials Technology patented Halo particles in 2006, then Kinetex was commercialized in 2009 and Poroshell 120 followed in 2010: columns packed with these materials have an impedance around 1000, which is one third of what the finest particles can have, and they also have an improved resolution (up to 50% higher) [5]. Meanwhile, EMD Millipore introduced Fractogel, which consists of much larger particles (20-150 µm in diameter) with very large porosity (60-70% of the particle volume)

functionalized with polymeric tendrils occupying part of the volume of the pores, as can be seen in Figure 1-4.

These tendrils are made of a copolymer with repeated units having a sulfonic group, they can adsorb molecules such as proteins and they can rearrange so that efficiency of adsorption is maximized. This can be considered the current state of the art of stationary phase materials for HPLC.
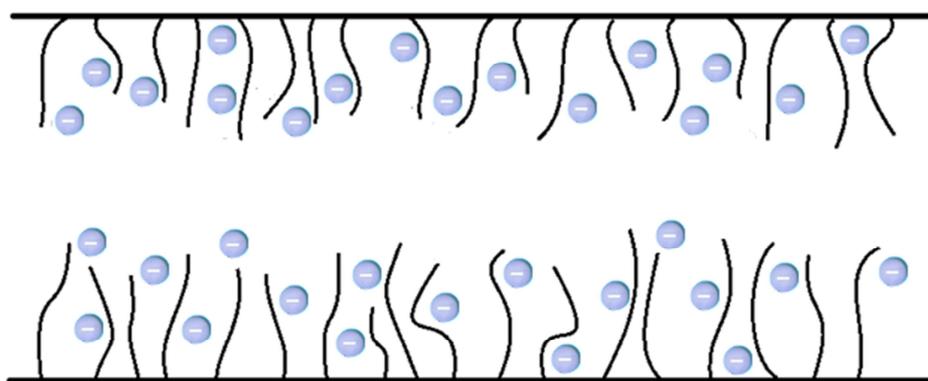


**Figure 1-4: representation of a pore in a particle of Fractogel resin**

# 1.3 Purpose of the research

This project aims at developing an industrially feasible process for producing a new composite material for HPLC application with good commercial viability. The novelty of the material consists in an increased extent of functionalization in core-shell PS-DVB resins that have been proposed as solid stationary phase for column chromatography [5], [9]: such feature will be achieved by filling the pores with a hydrogel network in which polymer chains have a high number of charged groups. To allow the reader a better understanding of this idea, a schematic representation of the target material is given in Figure 1-5; the picture is purposefully similar to Figure 1-4 to make the comparison between the two materials easier. Indeed, it can be easily noticed that the polymer chains in the pores of the target material are filling the entire pore volume, as opposed to the

chains in Fractogel that fill the pores only partially. Also, the chains are highly cross-linked, resulting in a continuous hydrogel.
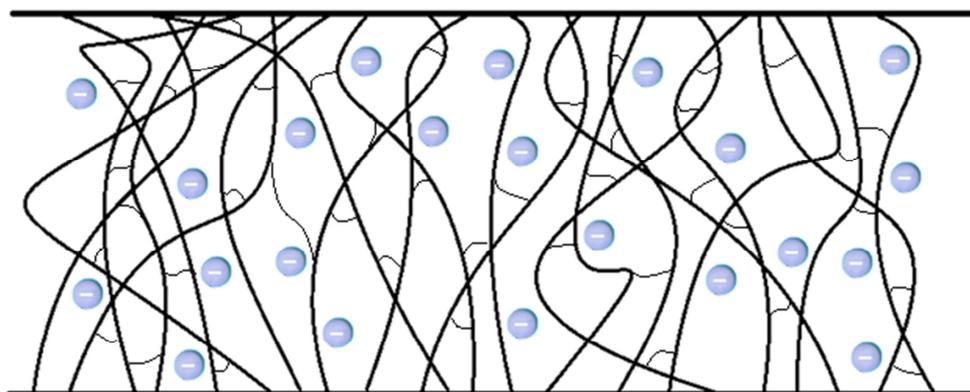


**Figure 1-5: representation of a pore in a resin particle of the target material**

The reason for filling the pores with a hydrogel is that gels have a peculiar structure, which grants that some proprieties typical of solid state materials, and some proprieties typical of the liquid state. Specifically, hydrogels consist of a complex network made of cross-linked polymer chains in which the monomer unit is a hydrophilic molecule [10]: this makes absorption of water very favorable, so that in practice hydrogels left in contact with a watery phase become water constrained by a polymer network [11]. In the particular case in which the monomer unit is ionic, the hydrogel will resemble a volume filled with immobile charges suspended in water [12]: this is an ideal medium for separation of charged species, because it can have a high density of charges while still allowing fluid-state diffusion [13]. Of course, diffusion through a hydrogel is not as fast as diffusion through pure water because the hydrogel network hinders diffusion of solutes, but it is definitely faster than diffusion in solid matter [13].

Given these premises, it is expected that hydrogels could be used to further increase the capacity of HPLC columns having a stationary phase consisting of resin particles. Although a hydrogel network may also suppress convective flow inside the pore volume, access to the functionalized volume should still be granted by means of diffusion; furthermore, since diffusion is a phenomenon driven by concentration gradient, as

expressed by Fick's first law:

$$\mathbf{J} = -D\,\nabla C_i \tag{4}$$

diffusion from a fluid phase into a hydrogel is expected to be rather quick [14], meaning that no major reduction of flow rate is required. It will be necessary to develop a procedure capable of producing hydrogel inside the volume of the pores of the resin particles without filling the interstitial spaces between particles, because that would prevent flow of the mobile phase through the column in which the material is packed.

# 2 Theory

In this chapter, all the knowledge needed beforehand for understanding the procedure that was followed to produce the desired material is given. Since this material is a composite, based on PS-DVB resin as the matrix for a hydrogel, both these classes of materials are presented in the chapter. A few elements of column kinetics are given to the reader, to allow for a better understanding of the issues that are being addressed. Finally, an introduction to titration is given to explain its relevance to this study.

# 2.1 Poly(styrene-divinylbenzene) resins

In recent times, PS-DVB resins have been widely used in chemical industries for many different purposes, including chromatography, because of their high chemical and mechanical stability in most environments and their ability to be made into several different conformations including gel-type, macroporous and core-shell resin particles [15], [9]. In addition to this, the productive process for these particles is relatively cheap and simple to carry out [15], making PS-DVB resins a good choice as basic study material for chromatographic applications. The polymerization reaction depicted in Figure 2-1 is usually carried out in well-stirred reactors, at 70 °C for 24 hours, using AIBN as initiator: such a reaction will follow the free-radical mechanism.
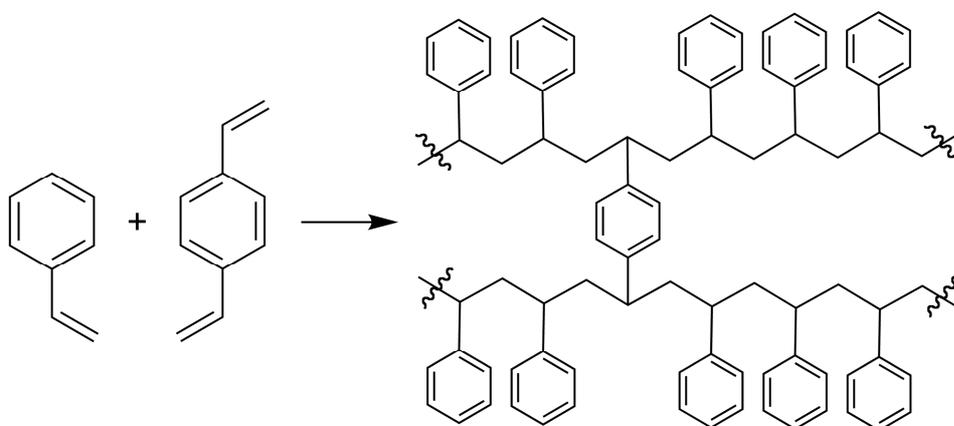


**Figure 2-1: reaction of DVB and Sty to form a PS-DVB copolymer**

Due to the presence of a difunctional monomer (DVB), the final polymer is cross-linked, thus exhibiting the attractive properties mentioned above. In order for PS-DVB resin particles to be useful for chromatography, they must be made so that their volume is at least partially accessible to a liquid phase (either for functionalization of the material or to increase the contact area for the mobile phase). This effect can be achieved in two ways:

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- By swelling the resin through a thermodynamically good solvent: such a swollen resin will be soft, easily compressible and rather fragile in shear. This type of materials are produced keeping the concentration of DVB in the copolymer low (0,5-2,0% mol), so that the obtained resin is only lightly cross-linked [15].
- By employing a proper porogen (usually an organic solvent non compatible with the polymer) during the polymerization: this generates a complex network of permanent pores that run across the volume of each particle. A higher concentration of DVB is needed for these particles to be made, resulting in harder and more rigid material. Pores found in such particles are easier to access for thermodynamically poor solvents [15].

# 2.2 Titration method

Titration is an effective technique to assess the amount of charged groups present on the surface of a material. In order to do this as precisely as possible, the method described by Stone and Carta [16] was selected as a suitable option for this study: the main focus of this research is to maximize protein capacity of a stationary phase for column chromatography; in the case of ion affinity chromatography, this can be simply evaluated as the number of charged groups present and accessible on the surface of the stationary phase. By means of titration, one can find the exact number of charged groups present in a column, which can be a useful indicator; nevertheless, the effective capacity can only be measured by an actual chromatography run with proteins on a HPLC machine. As a matter of fact, what is being measured by titration is actually the ionic capacity, which can differ from the protein capacity.

The titration method proposed by Stone and Carta describes a 3 steps preparation:

1. Bathe the material in a large volume of concentrated (1M) HCl solution: this will protonate all negatively charged groups. When titrating sulfonic groups, the reaction is:

$$RSO_3^- + HCl \rightarrow R\ SO_3^-H^+ + Cl^-$$
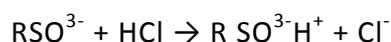
2. Wash with a large volume of pure water: this will clean the residual acid left among the resin particles during step 1, without desorbing any proton, so that electroneutrality is preserved.

3. Bathe in 25 mL of a 0.5M/0.05M NaCl/NaOH aqueous solution: this will substitute the protons with Na, consuming hydroxides to form water, as per the reaction:

$$R\ SO_3^-H^+ + NaOH \rightarrow R\ SO_3^-Na^+ + H_2O$$
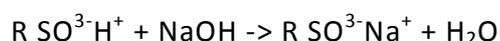
4. Collect and titrate 15 mL of the solution described in step 3 with an appropriate volume of HCl.

What is actually being titrated with this method is the NaOH molecules that did not react with the material during step 3; by subtracting such amount from the number of molecules of NaOH that were used to bathe the sample, the amount of reacted molecules is evaluated and, given the experimental conditions, it is safe to assume that all charged groups reacted with NaOH. Indeed, the high concentration of NaCl in the solution is meant to grant that all protons that reacted with sulfonic groups are replaced by $Na^+$ ions; then, those $Na^+$ coming from dissociation of NaCl will react with the less stable NaOH, regenerating the salt and releasing $OH^-$, which should then react with the protons released in the solution with the first reaction, giving water. Therefore, the overall reaction is:

$$R\ SO_3^-H^+ + NaOH \rightarrow R\ SO_3^-Na^+ + H_2O$$
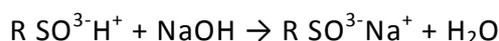
This way, it becomes possible to reckon the number of accessible charged groups on the surface of the resin. If the number of moles of generic negatively charged groups $X^-$ is $N_1$, the number of moles of NaOH revealed by titration is $N_2$ and the total number of moles of NaOH that went through the column as per step 3 is $N_3$, then it can be written:

$$N_1 = N_3 - N_2 \tag{5}$$

The resulting value for $N_1$ must then be normalized by the volume of the column.

This method is supposed to provide a very precise evaluation for the ionic capacity of a material. Since this characteristic is usually expressed in µmol/mL of column volume, the

total amount of charged groups measured by titration has to be divided by the total volume of the column.

## 2.3 Polymer hydrogels

Polymer hydrogels are polymer networks that, due to cross-linking of the polymer chains, remain insoluble in water, while still being able to adsorb large quantities of water thanks to the physical and chemical nature of polymers [10]: to be a good candidate for preparing hydrogels, a monomer must possess a hydrophilic group, such as the carboxylic acid group. Molecules having this characteristic need to be stabilized by coordination with a proper counterion [17]. Neglecting the effect of this counterion, the unbalanced charges in the chain would repel each other, leading to uncoiling of the polymer chains, as shown in Figure 2-2.
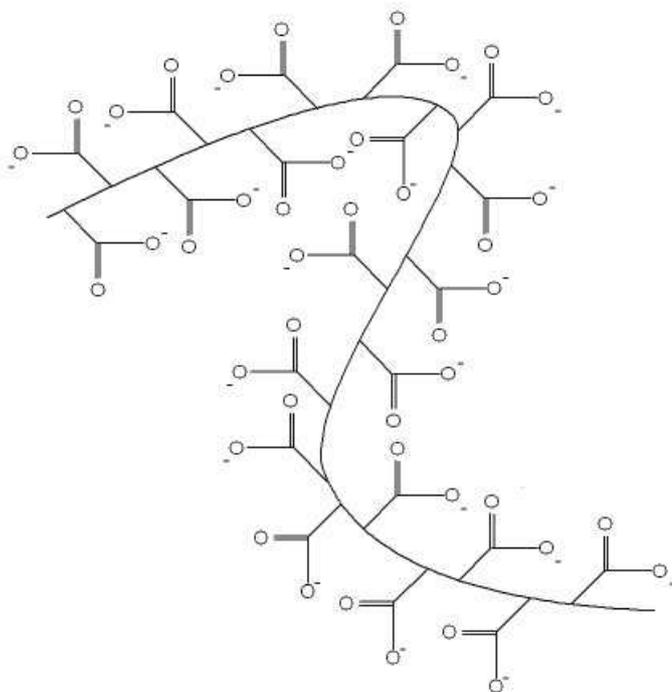


**Figure 2-2: uncoiling of a polymer chain due to repulsion of unbalanced charges**

However, polar molecules (such as water) will be attracted by these unbalanced charges, forming hydrogen bonds with them when possible, as depicted in Figure 2-3: in this state, hydrogels can adsorb hundreds of times their own weight in water [18], [19]. This phenomenon, called swelling, is what qualifies hydrogels [19] and will be discussed in section 2.4.
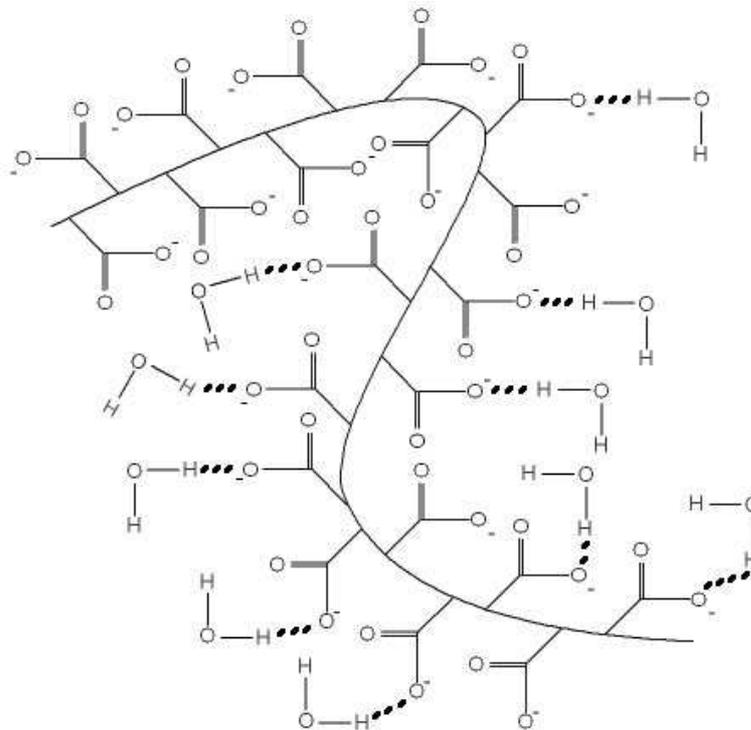


**Figure 2-3: water adsorbing on a hydrogel network**

Hydrogels can be produced either by free radical polymerization of monomer with an appropriate bifunctional cross-linker or by polymerization of large bifunctional macromers [11]. In this work, the first approach was followed, as discussed more specifically in the following.

The overall polymerization process in the case of free radical polymerization involves four different reaction steps:

1.  Initiation
2.  Propagation
3.  Chain transfer
4.  Termination

The initiation reaction produces free radicals: this can happen by thermal degradation of the monomer, which can be induced by temperature (but may cause unwanted reactions on rare occasions), or by addition of a suitable initiator. It is common practice to use this second method to avoid undesired reactions that could reduce the purity of the final product.   Radical initiators are characterized by a bond that can be cleaved homolytically, splitting the molecule in two, often identical, radicals. For most initiators, this reaction can be started by either an increase in temperature or UV irradiation, depending on the nature of the initiator.

Thermally activated initiators are generally either peroxides (such as APS or KPS) or diazocompounds (such as AIBN): the molecular structure of the initiators used in this work is shown in Figure 2-4
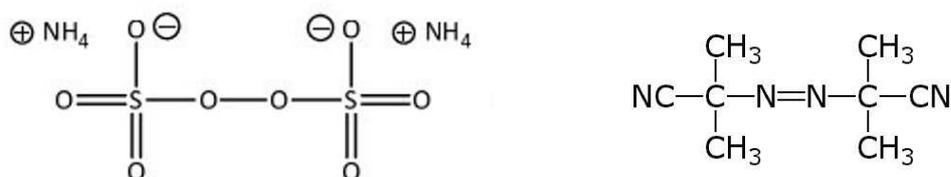


**Figure 2-4: initiators used in this work: APS (left) and AIBN (right)**

For an initiator to be considered suitable for a particular polymerization reaction, two conditions must be met:

1.  The dissociation temperature of the initiator must be lower than that at which the monomer degrades.

2. The free radicals released by dissociation of the initiator must react with the desired monomer.

3. The characteristic time of initiator dissociation has to be much longer than the reaction duration, in order to ensure a quite constant radical production all along the reaction.

As a relevant example, the decomposition of APS is portrayed in Figure 2-5 below
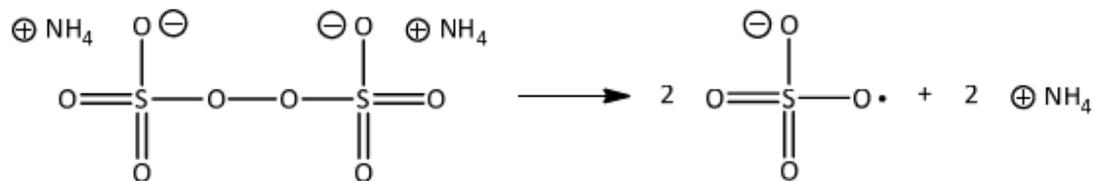


**Figure 2-5: dissociation reaction of APS**

This reaction can happen either at 60 °C over a few hours or in less than one minute at room temperature in presence of a promoter such as TEMED [20].

In presence of free radicals, the propagation step can happen, leading to formation of polymer chains. Most free radical polymerizations are polyaddition reactions, usually characterized by fast chain kinetics [21]. When the polymerizing monomer is a vinylic one, such as acrylamide, partial constitutive control is granted by steric hindrance; however, in absence of external factors, configurational control can only be very limited, because reactivity of a growing molecule with an active centre is very high [21]: polymers produced by these reactions will then usually be atactic. As an example, the propagation reaction of a polyacrylamide chain is portrayed in Figure 2-6
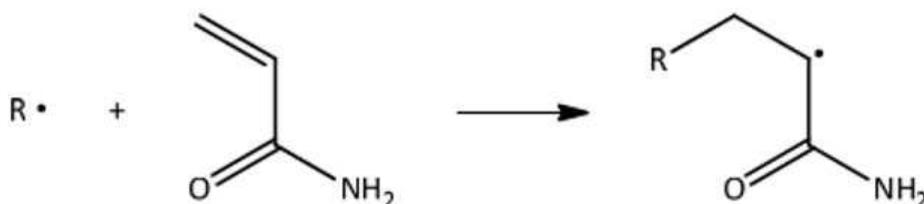
**Figure 2-6: propagation of a polyacrylamide chain**

When a bifunctional unit is present in solution with the monofunctional monomer, a growing chain will also react with such cross-linker molecule, leading to the incorporation of a pendant double bond along the chain backbone. Such double bond will react with another active chain, thus forming a bridge between the two chains, i.e. a cross-linking. By repetition of this event, highly branched chains and eventually networked macromolecules are formed and these highly interconnected chains are called cross-linked polymer. When the reactivity of bifunctional and monofunctional monomer is similar, the spacing between each crosslinking point (i.e. the number of monomer units between two bifunctional units) will approximately be equal to the molar ratio between monomer and cross-linker [21]; this approximation is more accurate when the concentration of cross-linker relative to the monomer content is low, resulting in a high cross-linking efficiency (cfr. section 2.4).

In chain transfer reactions the active centre of a growing polymer chain is moved to a different molecule. As a result, the original macroradical stops growing, but a new radical species is formed, possibly resulting in a new kinetic chain. From a chemical point of view, chain transfer reactions consist of an hydrogen atom being transferred to the macroradical from a different molecule, which may be solvent, initiator, monomer or already formed polymer, as shown in Figure 2-7 which depicts a chain transfer reaction that characterizes polymerization of LDPE.
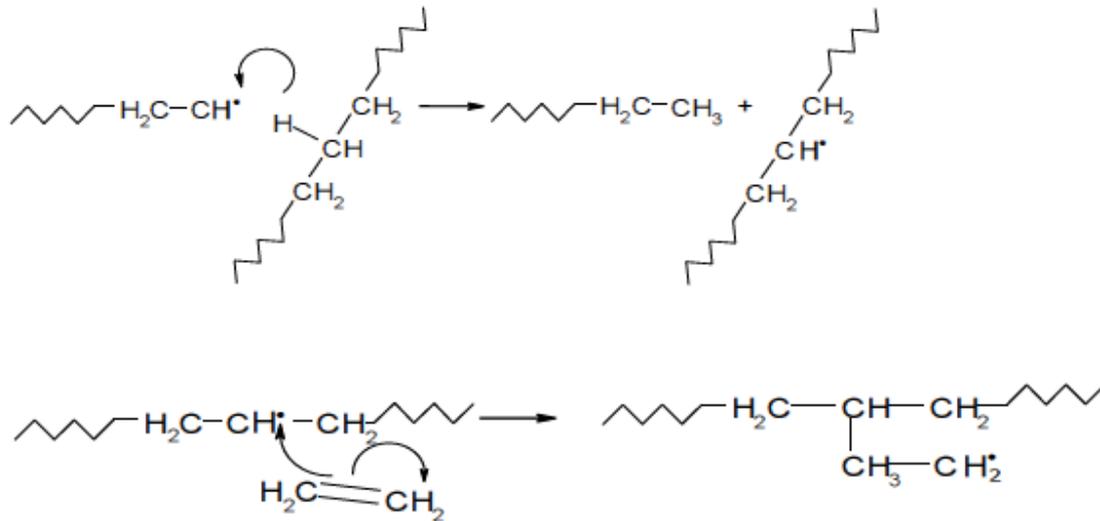
**Figure 2-7: chain transfer reaction during the free-radical polymerization of LDPE**

The main effect of chain transfer reactions is a reduction of the molecular weight of the obtained polymer; depending on the reactivity of the radical produced by the reaction, there might also be a change in the kinetics of the overall polymerization process [22].

In processes such as emulsion polymerization, very high molecular weights are easily obtained; this may make mechanical processing of the polymer difficult or impossible, because viscosity increases exponentially with the molecular weight, as given by Mark-Houwink equation [23]

$$[\eta] = \mathbf{KM}^a \tag{6}$$

in which K and $a$ are Mark-Houwink parameters that depend on the particular polymer-solvent system, and $[\eta]$ and M are the intrinsic viscosity and the molecular weight of the polymer, respectively. For most flexible polymers $a$ ranges from 0,5 and 0,8: a value of 0,5 is usual for theta solutions, while 0,8 indicates a thermodynamically good solvent [18]. In order to reduce the final molecular weight in these processes, it is possible to add a transfer agent; thiols and mercaptans are commonly used for this purpose in

industrial processes [24]. The reaction induced by these compounds is generically represented by Figure 2-8:
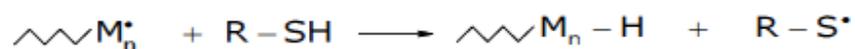


**Figure 2-8: chain transfer reaction induced by a generic transfer agent, R-SH**

Finally, growth of a polymeric chain ends by a bimolecular termination reaction. Such a reaction can occur according to two mechanisms: combination, represented in Figure 2-9(top) or disproportionation, described by the lower part of Figure 2-9.
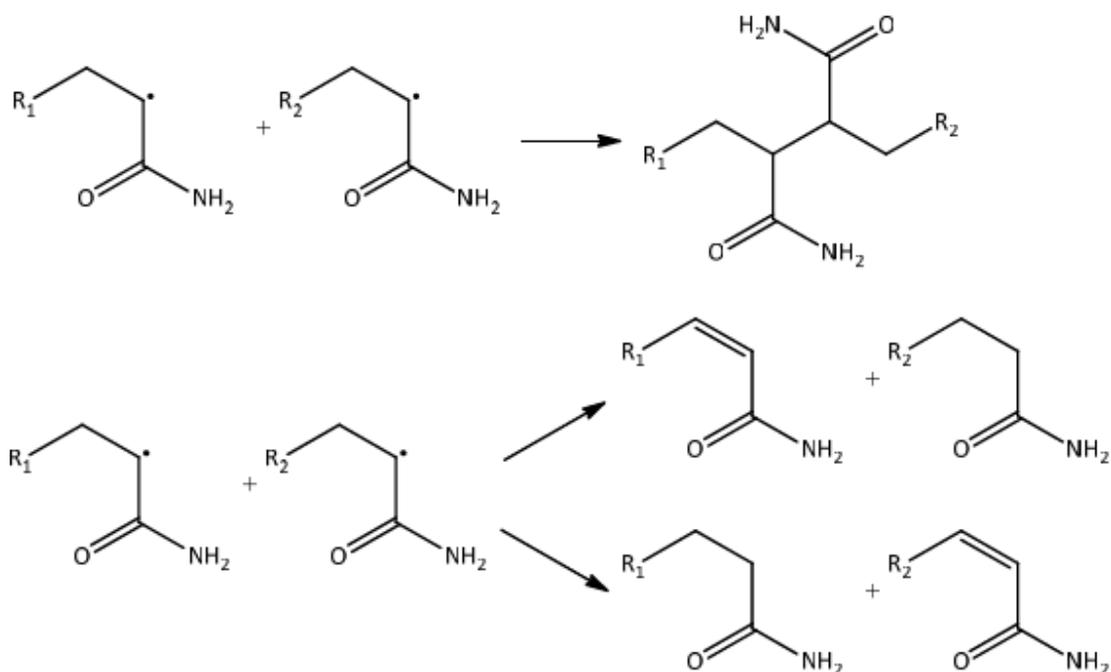


**Figure 2-9: bimolecular termination reactions in polymerization of a polyacrylamide chain: combination (above) and disproportionation (below)**

The two mechanisms are usually coexisting and competitive in a polymerization process, depending on the process variables [23]. In a combination reaction two unpaired electrons from two different macroradicals combine to form a single macromolecule having a molecular weight equal to the sum of the two macroradicals. In a disproportionation reaction a hydrogen atom is transferred from a macroradical to another: a terminal double bond forms on the macroradical that lost the hydrogen and the chain lengths of the two molecules remain unaltered.

# 2.4 Hydrogel swelling

A hydrogel in contact with an aqueous solution will absorb water, thus increasing its own volume: this phenomenon is called swelling. The swelling behavior of a hydrogel can be quantified by the mass swelling ratio ($Q_m$), which equals the ratio between the mass of the swollen hydrogel ($m_s$) and the mass of the dry hydrogel ($m_d$), or by the volume swelling ratio ($Q_V$), defined as the ratio between the volume of the swollen hydrogel ($V_s$) and that of the dry hydrogel ($V_d$) [18]. The mass swelling ratio is also commonly referred to as the swelling ratio ($Q$).

$$Q \equiv Q_m = \frac{m_s}{m_d} \tag{7}$$

$$Q_V = \frac{V_s}{V_d} \tag{8}$$

In thermodynamic theories describing the swelling behavior of polymer gels at equilibrium, the polymer volume fraction ($\varphi$) is typically used, which is the reciprocal of the volume swelling ratio $Q_V$ [18].

$$\varphi = \frac{1}{Q_V} \tag{9}$$

Since the mass swelling ratio is generally more convenient to measure, this same approach was followed in this work to evaluate the swelling behavior of the produced hydrogels.

If the absorption of water into the hydrogel network is thermodynamically favored, as discussed in the previous chapter, it must cause an increase of the entropy of the system; at the same time, there will be an elastic contribution to the chemical potential caused by the resistance of the polymer chains to uncoiling, which will reduce the entropy of the system: accordingly, the change in chemical potential of the solvent $\Delta\mu$ is given by the difference between the chemical potential of water absorbed into the hydrogel, $\mu^{gel}$, and that of water in the external solvent, $\mu^{solvent}$. More specifically, such difference of chemical potential is expressed as the sum of mixing ($\Delta\mu_{mix}$) and elastic contribution ($\Delta\mu_{elastic}$) [23] [19], as expressed by the following equation:

$$\Delta\mu = \mu^{gel} - \mu^{solvent} = \Delta\mu_{mix} + \Delta\mu_{elastic} \qquad (10)$$

According to this equation, an equilibrium swelling ratio will be established when the elastic contribution is balancing the mixing contribution, thus inhibiting further water absorption.

The swelling behavior of non-ionic polymer networks is fully described by the Flory-Rehner theory [25]; for ionic polymer networks, such as those of hydrogels under examination in this work, more complex models exist [26] [27]. These models require assessment of parameters that need expensive and time consuming experiments to be measured. On the other hand, they provide useful information such as the cross-linking efficiency, which is the ratio of molecules of cross-linker actually forming bridges among macromolecules over the total number of cross-linker molecules reacted in the system [25]. If this ratio is low, the network has a high number of narrow twists, due to the fact that many polymer chains are linked to themselves [19]: this renders part of the charges found on their monomer unit inaccessible to large molecules such as proteins, making the material less interesting for chromatographic purposes.

In the frame of this research, swelling tests were carried out in order to determine the equilibrium swelling ratio: these tests consist of letting a hydrogel swell in an aqueous medium until it reaches an equilibrium volume, which can be measured; furthermore, they can give qualitative information about the mechanical properties of a hydrogel left in contact with an aqueous medium for extended periods of time. These data are of critical importance for the present study, since significant changes in the volume of the gel can induce severe stresses; depending on the mechanical behavior of both resin and

gel, this can lead to formation of fragments that could block an HPLC column during service.

# 2.5 Column kinetics

Mass transfer kinetics is a determining factor for the performance of columns used in gas, liquid and supercritical chromatography [14]. Most currently accepted mathematical models describing mass transfer kinetics inside a HPLC column rely on the concept of height equivalent to a theoretical plate (HETP). This empirical concept represents the length of a hypothetical zone in which a phase featuring a nonzero chemical potential gradient reaches equilibrium with its surroundings: this leads to models consisting of "equilibrium stages", or layers, characterized by a certain height. The HETP can then be defined as the thickness of the layer in which a solution is equilibrated with the mean concentration of solute contained in the stationary phase throughout the layer [28].

In the particular case of linear chromatography, a popular equation has been developed by van Deemter to evaluate the corresponding HETP:

$$h = A + {B}/{v} + Cv \tag{11}$$

where $h$ is the reduced plate height (given by the ratio between HETP and diameter of the particles constituting the stationary phase), $v$ is the reduced velocity (given by velocity of the mobile phase times the ratio between diameter of the particles constituting the stationary phase and the diffusion coefficient of the solute in the mobile phase) and $A$, $B$ and $C$ are numerical parameters characteristic of the column: in particular, $A$ is a parameter related to turbulent flow and channeling through a non-ideal packing (eddy current parameter), $B$ is the diffusion coefficient of the eluting molecules in the longitudinal direction (resulting in dispersion) and $C$ is the coefficient of resistance to mass transfer of the analyte between mobile and stationary phase.

The resolution of a HPLC column is defined as the ability to separate two species into two separate chromatographic peaks and is given by the equation

$$R = \frac{(t_{r2} - t_{r1})}{1/2(w_1 + w_2)} \tag{12}$$

where $t_{r1}$ and $t_{r2}$ and $w_1$ and $w_2$ are elution times and widths, respectively, of the two peaks. Resolution is related to HETP, because each plate adds to the precision of the measurement, so that the number of plates in a column is directly proportional to the second power of the resolution of a column [29], as expressed by:

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_B}{k_B + 1}\right) \tag{13}$$

where $N$ is the number of plates (given by the ratio between the length of the column and HETP), $k_B$ is the retention factor (given by the ratio of the time spent by the analyte and the time spent by a non-adsorbing species inside the column) and $\alpha$ is the selectivity factor (given by the ratio $k_B/k_A$).

According to van Deemter equation, an optimal fluid velocity exists at which HETP is minimized. By differentiating the equation for the velocity and setting the result to zero, the optimal velocity is found to be:

$$u = \sqrt{B/C} \tag{14}$$

This optimal velocity is generally rather low, so it is impractical to run a chromatography cycle at that rate due to low productivities or long analysis times.

# 3 Experimental

In this chapter, after a list of all chemicals and instruments involved, a detailed description of all the experiments carried out in this work is given. Each of the techniques that were devised and tested is fully illustrated in its own paragraph.

## 3.1  Materials

## 3.1.1  Chemicals

Ammonium persulfate (APS), N,N'-methylenebisacrylamide (MBAA), 2-Acrylamido-2-methylpropane sulfonic acid (AMPS), N,N,N',N'-tetramethylethylenediamine (TEMED), [2-(Acryloyloxy)ethyl] trimethylammonium chloride (ATAC, 80 wt. % in H2O, with 600 ppm of monomethyl ether hydroquinone as inhibitor), Acrylamide (AA), Divinylbenzene (DVB), Potassium persulfate (KPS), Styrene (Sty), Sulfuric acid ($H_2SO_4$), Sodium hydroxide (NaOH), Sodium chloride (NaCl), Magnesium chloride ($MgCl_2$), Hydrochloric acid (HCl), Safranin O, Ethanol (EtOH), Sorbitan monolaurate (SPAN 20), Sorbitan monopalmitate (SPAN 40), Sodium phosfate monobasic ($NaH_2PO_4$), Sodium phosfate dibasic ($Na_2HPO_4$), Sodium dodecyl sulphate (SDS), Sodium bicarbonate ($NaHCO_3$), Nitric acid ($HNO_3$), and Potassium nitrate ($KNO_3$) were purchased from Sigma-Aldrich; Silicone oil 350 from SILITECH SA; EXXSOL D100S from EXXONMobil Chemical; Titrisol 0.1 M hydrocloric acid (titrisol HCl), and Titrisol 0.1 M Sodium hydroxide (titrisol NaOH) from Merck.

All water used in any process was deionized, unless otherwise specified. Water used for polymerization reaction was also stripped of oxygen with nitrogen jet for 30 minutes.

## 3.1.2  Equipment

- Mettler Toledo LabMax 4 liters jacket laboratory automatic reactor

- Mettler Toledo AT250 balance

- Mettler Toledo PM4000 balance

- Metrohm 665 Dosimat (titrator)

- Vacuum drying oven Heraeus Vacutherm serie VT 6060 M

- Microfluidics Corporation HC5000 Homogenizer (microchannel)

- Bischoff HPLC compact pump (offline pump)

- Agilent 1100 Series HPLC equipped with G1322A Degasser, G1311A Quat Pump, G1313A Autosampler, G1316A Thremostatted Column Compartment and G1314A Variable Wavelength Detector (HPLC)

- Varian Cary 4000 UV-Vis spectrophotometer

- GE Healthcare Tricorn HPLC columns 50 x 5.0 mm, glass

- GE Healthcare Tricorn HPLC columns 100 x 5.0 mm, glass

- Metrohm Metrosep RP Trap 1 HPLC columns 50 x 4.0 mm, PEEK

## 3.1.3    Column Geometry

All columns used in this research were made out of PEEK or of glass. PEEK columns were used whenever the packed material needed to stay at high temperature for a long period of time; otherwise glass columns were preferred because they allowed for visual inspection of the packed material.

An example of a Tricorn glass column is shown in Figure 3-1. Along with the column itself, the necessary parts for its use are displayed as well. Tricorn columns are available in many sizes, although they all have an internal diameter of 5 mm; in this thesis, only columns measuring either 50 or 100 mm in height were used. However, it is important to note that Tricorn columns have a very long end cap: this can be used to reduce the actual height of the column when needed, by screwing the filter holder in for up to 15 mm.

**Figure 3-1: 50 x 5 mm Tricorn glass column, complete with all its parts (from the left: end cap, glass tube, 2 o-rings, adapter unit and filter holder) [30]**

The Metrohm PEEK column used for this work, shown in Figure 3-2, does not offer such a possibility. Its internal diameter measures 4 mm, and its height is 50 mm: therefore, its internal volume equals 628.3 µL.

**Figure 3-2: 50 x 4 mm Metrohm Metrosep PEEK column, with end caps and stoppers at both ends [31]**

As an alternative to the external stoppers shown in Figure 3-2, it is possible to use internal PEEK plugs to seal the column: this is useful when the column needs to be closed while staying at high temperatures for a long period of time, because the external stoppers are not made of PEEK and cannot bear such conditions.

## 3.2 Preparation and preliminary experiments

## 3.2.1  Resin preparation

600mL of PS-DVB resin were produced with a 5 steps process:

1. Core particle production: 1250 mL of 0.2% weight solution of sodium dodecyl sulfate were charged in the Labmax reactor, trying to avoid foaming as much as possible. The solution was then heated up to 70 °C, after which 10 mL of a 2% KPS aqueous solution were added. After this, the feed started: through the Labmax membrane pump, 625 mL of a 80% styrene in divinylbenzene concentrated solution mixed with 550 mL of a 0.2% sodium dodecyl sulfate aqueous solution were fed to the reactor, while 100 mL of a 2% KPS aqueous solution were fed through a VitFit syringe pump over 14 hours.

2. Shell production: the "core particle" latex solution was diluted in 1336 mL of water and then charged into the Labmax reactor. Again, the solution was heated up to 70 °C, then 50 mL of a 6.5% KPS solution were added. Afterwards, 277.4 g of a 99% concentrated solution of styrene in divinylbenzene were fed through the Labmax membrane pump over 11 hours, while 100 mL of a 3.5% KPS aqueous solution were added through the VitFit syringe pump at 10 mL/h.

3. Swelling: 2 L of the latex were then collected in a suitable container and left under gentle stirring (45 rpm) while 60 mL of a solution consisting of 48 mL of styrene, 12 mL of divinylbenzene and 0.6 g AIBN were dripping into the latex solution.

4. Aggregation: the latex was then aggregated with a high-shear microchannel device operating at an overpressure of 168 bar. The latex was first diluted with 4135 mL of a 1.3 mM $MgCl_2$ solution and then carefully dripped at the inlet of said pump, so that the channel would not run empty at any given time, and the aggregated resin was collected as a slurry at the other end of the pump. The process lasted only a few minutes.

5. Post-polymerization: finally the slurry was left under gentle stirring in a heated tank at 70 °C for 5 hours. More than 13 L of such mixture were collected; the final volume was reduced to approximately 3.6 L of resin at 18.6% of dry content by decantation.

All particles with a diameter larger than 200 μm or smaller than 45 μm were removed by multiple sieving cycles. Light scattering analysis and mercury intrusion were run on the resin to measure particle size distribution and porosity of the final material. Also, a few

pictures of resin particles were taken by Scanning Electron Microscopy (SEM).

Part of the resin was separated into 3 fractions with different particle diameters: this was done by a manifold sieving procedure. To obtain 3 fractions each characterized by a different range of possible diameters of the resin particles, 4 stainless steel sieves with different mesh size were used. First, particles with diameter above 125 µm were removed; then, particles with diameter less than 100 µm were separated from the rest, obtaining the first fraction containing particles with diameters comprised between 100 and 125 µm; then, from the remaining resin, particles with diameter less than 45 µm were sifted out, and the remaining solid was further divided into two more fractions with another sieving step: the second fraction included particles with diameters between 63 and 100 µm, while the third one had the particles with diameters in the range 45 to 63 µm. During each step, the resin to be separated was poured on the sieve, and then sprayed with a few mL of ethanol. Then water was poured onto the resin, while a 45 µm mesh size sieve was placed under the one that was currently holding the resin, so that it was be possible to keep what would be passing through during the step. When water had drained from the resin, the particles left on the 45 µm mesh sieve would be collected and stored and the process would be repeated until no particles could be found on the 45 µm sieve. Then, the resin left on the sieve with the larger mesh was sprayed with ethanol, this time in large quantity (100-200 mL). The resin that got through the mesh and onto the 45 µm mesh was collected, and all particles left on the other sieve were considered to have a diameter larger than the mesh size of the sieve.

## 3.2.2    Bulk hydrogel composition

A few compositions were tested. First, monomer solutions with TEMED as a promoter for the dissociation of APS were studied.

40 mL of a 10% concentrated monomer solution were prepared, with 4% in weight (with respect to the monomer) concentration for the cross-linker:

- 3.84 g AMPS

- 0.16 g MBAm

- 0.04 g APS

- 34.00 g $H_2O$

- 0.04 g TEMED

The solution was stirred for 30 minutes to ensure complete dissolution and homogenization.

A set of 20 mL solutions with different composition, summarized in Table 3-1, was produced; based on the results of the previous experiment (discussed in section 4.1.2) NaOH was added to the original recipe.

Table 3-1: compositions of AMPS bulk hydrogels produced with TEMED

|        | HG1 (5%) | HG2 (10%) | HG3 (15%) | HG4 (20%) | HG5 (25%) |
|--------|----------|-----------|-----------|-----------|-----------|
| AMPS   | 0.98 g   | 1.97 g    | 2.96 g    | 3.940 g   | 4.93 g    |
| MBAm   | 0.04 g   | 0.08 g    | 0.12 g    | 0.160 g   | 0.20 g    |
| TEMED  | 0.01 g   | 0.02 g    | 0.03 g    | 0.040 g   | 0.05 g    |
| $H_2O$ | 19.00 g  | 18.00 g   | 17.00 g   | 16.00 g   | 15.00 g   |
| APS    | 0.01 g   | 0.02 g    | 0.03 g    | 0.040 g   | 0.05 g    |
| NaOH   | 0.16 g   | 0.38 g    | 0.59 g    | 0.790 g   | 1.30 g    |

Experiments on monomer solutions without TEMED were run too. Initially, 40 mL of an AMPS solution 10% concentrated in weight, 4% cross-linked were prepared with the following composition:

- 3.84 g AMPS

- 0.16 g MBAm

- 0.04 g APS

- 34.00 g $H_2O$

The solution was stirred for 15 minutes, then heated to 60 °C and kept at that temperature for 5 hours. While in the oven, the glass vials containing the solution were kept closed, and their lids were sealed with *parafilm* to prevent reactions with oxygen from the atmosphere. This procedure was followed every time a monomer solution kept inside a vial needed to polymerize in an oven.

A set of solutions with different monomer concentrations was produced once again. The composition of each of those 20 mL solutions is listed in Table 3-2:

**Table 3-2: composition of AMPS bulk hydrogels produced without TEMED**

|          | HG1 (5%) | HG2 (10%) | HG3 (15%) | HG4 (20%) | HG5 (25%) |
|----------|----------|-----------|-----------|-----------|-----------|
| **AMPS** | 0.98 g   | 1.97 g    | 2.96 g    | 3.940 g   | 4.93 g    |
| **MBAm** | 0.04 g   | 0.08 g    | 0.12 g    | 0.160 g   | 0.20 g    |
| **$H_2O$** | 19.00 g | 18.00 g   | 17.00 g   | 16.00 g   | 15.00 g   |
| **APS**  | 0.01 g   | 0.02 g    | 0.03 g    | 0.04 g    | 0.05 g    |

The production of different kinds of hydrogel was also attempted. A positively charged hydrogel, made of AETAC monomer cross-linked with MBAA was sought. As a first experiment, 20 mL of an AETAC monomer solution 10% concentrated in weight and 4% cross-linked were prepared with the following composition:

- 2.50 g AETAC (80%)

- 0.08 g MBAA

- 0.02 g APS

- 17.50 g $H_2O$

The solution was stirred in a 30 mL glass vial for 30 minutes and stored in an oven at 60 °C for 24 hours.

The experiment was repeated for a monomer solution 20% concentrated in weight, with the following composition:

- 4.92 g AETAC (80%)

- 0.08 g MBAA

- 0.02 g APS

- 15.11 g $H_2O$

As in the aforementioned case, the solution was stored in a 30 mL sealed glass vial, stirred for 30 minutes and put into an oven at 60 °C for 24 hours.

The first AETAC experiment was repeated, this time with salt present in solution along with the other chemicals, leading to the following mixture:

- 2.50 g AETAC (80%)

- 0.079 g MBAA

- 0.02 g APS

- 17.51 g $H_2O$

- 0.175 g NaCl

After 30 minutes of stirring in a 30 mL glass vial, the solution was put in an oven for 5 hours at 60 °C.

The experiment was repeated for similar solutions with lower monomer concentration. Their exact compositions are listed in Table 3-3:

**Table 3-3: composition of AETAC bulk hydrogels produced with salt**

|  | HG1 (5%) | HG2 (10%) | HG3 (15%) |
|---|---|---|---|
| AETAC | 1.24 g | 2.48 g | 3.73 g |
| MBAm | 0.04 g | 0.08 g | 0.12 g |
| TEMED | 0.01 g | 0.02 g | 0.03 g |
| $H_2O$ | 18.75 g | 17.52 g | 16.21 g |
| APS | 0.01 g | 0.02 g | 0.03 g |
| NaCl | 0.167 g | 0.158 g | 0.149 g |

A third kind of hydrogel was also prepared. In this case, an AA/AETAC solution 20% concentrated in weight and 4% cross-linked was produced with the following specifics:

- 1.50 g AA

- 0.625 g AETAC (80%)

- 0.080 g MBAA

- 0.02 g APS

- 17.92 g $H_2O$

The solution was stirred in a 30 mL glass vial for 30 minutes and put in an oven at 60 °C for 5 hours.

A set of AA/AETAC solutions with different monomer concentrations was produced. Their exact compositions are listed in Table 3-4:

**Table 3-4: compositions of AA/AETAC bulk hydrogels**

|  | HG1 (5%) | HG2 (15%) | HG3 (20%) |
|---|---|---|---|
| AETAC | 0.259 g | 0.942 g | 1.289 g |
| AA | 0.75 g | 2.26 g | 2.99 g |
| MBAm | 0.04 g | 0.12 g | 0.16 g |
| TEMED | 0.01 g | 0.03 g | 0.04 g |
| $H_2O$ | 18.97 g | 16.89 g | 15.91 g |
| APS | 0.01 g | 0.03 g | 0.04 g |

# 3.2.3   Titration of the resin

A four-steps procedure was followed in order to titrate the PS-DVB resin.

1. Flush the column with 60 mL of a 1 M HCl solution over 1 hour.

2. Flush the column with 60 mL of distilled water over 1 hour.

3. Circulate 25 mL of 0.5 M NaCl/0.05 M NaOH aqueous solution through the column for 2 hours. The solution was circulated thoroughly through the column to ensure that all sulphonic groups reacted.

4. Flush the column with 25 mL of distilled water over 25 minutes, and collect the liquid that comes out (this includes the solution that was previously circulating through the column).

The solution obtained this way was then titrated by a 0.1 M HCl solution. A Dosimat 665 titrator by Metrohm was used with the cell under nitrogen atmosphere maintained by continuous flow of water-saturated nitrogen.

The titrator consists of a precise pump (able to control injected volume down to 1 µL)

connected to a computer: the control software allows the operator to input number of injections, their volume and time for equilibration between injections as well; the same computer is also connected to a pH-meter and an Electro Conductivity-meter, so that both pH and electrical conductivity are measured and recorded on a text file at the end of each equilibration step and before each new injection. The titrator is completed by a magnetic stirrer and a set of glass tanks, each having different volumetric capacities, but all characterized by a truncated cone geometry; the magnetic stirrer has a steel rod protruding upwards from its back, to which is bound a plastic disc with five round holes, so that the disc is free to move vertically, up and down along the rod; the disc has also a metallic jaw below it that can open up to a 75° angle.

The solution to be titrated goes into the tank with the appropriate volume; these tanks are made so that a ring with two protrusions is fixed at their top, in a way that they can be fixed to the bored disc thanks to the jaw that closes on it and is locked in position by the protrusions on the tank. The disc has to be lowered down as far as it can without touching the surface of the stirrer. The magnet may be inserted in the tank by letting it through one of the holes, then the stirrer can be turned on; the central hole in the disc is for the inlet of the pump, a tube that lets drop of the titrating solution in the tank: this tube is connected to a bottle of the appropriate solution through the pump of the titrator, which regulates the rate at which the solution must be added in the tank, according to the data that were previously chosen and written on the program by the operator. The electrodes of both pH-meter and EC meter are fitted in the holes so that they can touch the solution to be titrated, but not the walls of the tank. Finally, a tube connected to an external source of nitrogen must be connected to a glass coupling (which ends in a shape that fits the holes perfectly); a similar coupling that lets the gas out is fit in the last hole. When everything is set up, the operator may give the necessary input for the computer to start the procedure.

When the procedure is over, the data recorded on the computer can be interpreted, and possibly plotted on a diagram (either pH or electrical conductivity vs injected volume). In this work, the diagram was merely drawn to check if there were significant deviations from the expected result, meaning discontinuities along the curve; other than that, the only meaningful information for the purpose of this research, is the injected volume at which neutrality is achieved in the solution (which is a single point in such a diagram). This volume, given that the concentration of the titrating solution injected is extremely

precise, can directly be converted in an amount of moles of charged groups in the solution to be titrated; therefore, for neutrality to be achieved at that point, the titrated solution must contain the same molar amount of ions.

The aforementioned Dosimat 665 was set up so that 20 mL were injected in 400 steps of 50 μL each. The accuracy of the measurements given by the titrator was increased implementing a custom LabView program; under its supervision, pH and conductivity of the solution were measured until the values of pH within a given time interval did not exceed a given range of values. For the purpose of this thesis, the program was set to keep the titrator measuring until the pH values did not vary more than 0.02 for 2 seconds. As no major mass transfer resistances exist in the protocols applied in this work, this procedure was considered accurate enough for all titrations carried out in this work.

## 3.2.4    Functionalization of the resin

300 mL of the plain (unfunctionalized) resin suspension were dispersed in 500 mL of $H_2SO_4$ (concentrated); after 24 hours of stirring at 80 °C the suspension was poured into a 2 L volumetric cylinder and diluted with 1200 mL of distilled water. After some hours the resin was resting on the bottom of the volumetric column, so it was possible to decant most of the supernatant and repeat the dilution process until the pH of the supernatant was neutral. About 400 mL of resin were collected.

A Stone-Carta titration was run on a column filled with sulfonated resin. The exact same procedure that was described for the titration of the standard PS-DVB resin in section 3.2.3 was followed.

## 3.2.5    Swelling Tests

Nine solutions were prepared in order to produce the gels to be tested. Their exact

composition is listed in Table 3-5 and Table 3-6:

Table 3-5: compositions of hydrogels with increasing monomer content

|  | HG1 (5%) | HG2 (10%) | HG3 (15%) | HG4 (20%) | HG5 (25%) |
|---|---|---|---|---|---|
| AMPS | 0.98 g | 1.97 g | 2.96 g | 3.940 g | 4.93 g |
| MBAm | 0.04 g | 0.08 g | 0.12 g | 0.160 g | 0.20 g |
| TEMED | 0.01 g | 0.02 g | 0.03 g | 0.040 g | 0.05 g |
| $H_2O$ | 19.00 g | 18.00 g | 17.00 g | 16.00 g | 15.00 g |
| $NaHCO_3$ | 0.0798 g | 0.0756 g | 0.0714 g | 0.069 g | 0.064 g |
| APS | 0.01 g | 0.02 g | 0.03 g | 0.040 g | 0.05 g |
| NaOH | 0.16 g | 0.38 g | 0.59 g | 0.790 g | 1.30 g |

Table 3-6: compositions of hydrogels with increasing cross-linker content

|  | HG6 (2%) | HG2 (4%) | HG7 (6%) | HG8 (8%) | HG9 (10%) |
|---|---|---|---|---|---|
| AMPS | 1.97 g | 1.97 g | 1.97 g | 1.97 g | 1.97 g |
| MBAm | 0.04 g | 0.04 g | 0.12 g | 0.16 g | 0.20 g |
| TEMED | 0.02 g | 0.02 g | 0.02 g | 0.02 g | 0.02 g |
| $H_2O$ | 18.00 g | 18.00 g | 18.00 g | 18.00 g | 18.00 g |
| $NaHCO_3$ | 0.0756 g | 0.0756 g | 0.0756 g | 0.0756 g | 0.0756 g |
| APS | 0.02 g | 0.02 g | 0.02 g | 0.02 g | 0.02 g |
| NaOH | 0.38 g | 0.38 g | 0.38 g | 0.38 g | 0.38 g |

NaOH was added in order to achieve a pH value suitable for the gelation. The required

amount of it was estimated by first calculating the number of moles of monomer present in the solution, and then calculating the weight of the same number of moles of NaOH plus a 15% of excess. Once the solutions were prepared and the pH was known to be in the desired range, all the nine vials containing each solution were left for 10 minutes to rest at room temperature.

All the gels obtained had very similar initial volumes. All of them were removed from their respective vials and put in 50 mL beakers containing a phosphate buffer (15.5 mM $NaH_2PO_4$, 9.5 mM $Na_2HPO_4$). They were left in the beakers, sealed with *Parafilm,* and periodically their weight was measured, to assess whether they had achieved equilibrium volume or not. Whenever one sample appeared to be about to fill a beaker, it would be moved to a larger container, in order to allow further expansion.

# 3.3 Solution soaking

10 mL of resin suspension were poured in a monomer solution with the following composition:

- 3.94 g AMPS

- 0.16 g MBAA

- 16.00 g $H_2O$

- 0.04 g APS

The resin was then sieved with two different filters: one was a standard paper filter, the other one was a 20 μm polymer mesh.

The paper filter was placed upon a glass funnel leading to a 100 mL jar, in which the excess solution could be collected. Half of the wet resin was poured onto it and let filter through. After 30 minutes, the final, wet slurry was removed from the filter with a stainless steel spatula and placed in a 100 mL glass jar.

As for the polymer mesh, it was placed upon a Buchner ceramic funnel, which was set on

a 500 mL waste bottle connected to a vacuum pump. The mesh was wetted with ethanol and then the vacuum pump was set on 500 mbar. Finally, the remaining resin was poured into the funnel, spreading all over the polymer mesh, so that an homogenous layer as thin as possible (roughly 2 mm) could be obtained. After a few seconds the dry resin was collected and put into a 100 mL glass jar.

Both jars were sealed and put in an oven at 60 °C for 5 hours. After that, the resin was dispersed in a solution of water and ethanol: this was done by spraying ethanol on the dry resin, and then pouring water into the jar.

Light scattering analysis was run on the samples. Large aggregates were sieved out with a 125 μm opening stainless steel sieve, then the samples were once more subjected to light scattering analysis. This was repeated until the particle size distribution obtained by light scattering confirmed that no more aggregates were present in the samples. Afterwards, the resin was packed in a column. In order to avoid formation of air bubbles it was necessary to wash the column with ethanol first.

For the packing, a 20% ethanol solution was let through the column with an increasing flow rate, to avoid abrupt increases in stress that could cause critical failure in the material:

1. 15 minutes at 1 mL/min

2. 10 minutes at 2 mL/min

3. 10 minutes at 3.5 mL/min

4. 5 minutes at 5 mL/min

The column was then titrated, following the procedure described in section 3.2.3.

A similar procedure was also followed with sulfonated PS-DVB resin. 10 mL of functionalized resin were soaked in a 20% weight concentrated AETAC solution (with 15% weight concentration of cross-linker with respect to the monomer) with the following composition:

- 4.25 g AETAC

- 0.60 g MBAA

- 0.02 g APS

- 15.74 g $H_2O$

- 0.175 g NaCl

Again, about half of the resin was poured and sifted on a paper filter placed upon a glass funnel leading into a 100 mL glass jar, while the rest was sifted on a 20 µm opening polymer mesh that was previously wetted with ethanol and placed upon a Buchner ceramic funnel, which was set on a 500 mL waste bottle connected to a vacuum pump.

Once properly dried, both samples were collected and stored into glass jars that were put in an oven at 60 °C for 5 hours.

Afterwards, each sample was dispersed in 100 mL of 20% ethanol solution. Both samples were analyzed by static light scattering to verify that no aggregation of resin particles took place during polymerization.

The samples were then packed into two 0.5 mL glass HPLC columns, which were previously washed with ethanol. The packings of both columns were titrated to evaluate the R-$NMe_3^+$ groups. The titration procedure described in section 3.2.3 had to be modified as follows to make it suitable for detecting positively charged groups:

1.  Flush the column with 60 mL of a 1 M NaOH solution over 1 hour.

2.  Flush the column with 60 mL of distilled water over 1 hour.

3.  Circulate 25 mL of 0.5 M NaCl/0.05 M HCl aqueous solution through the column for 2 hours.

4.  Flush the column with 25 mL of distilled water over 25 minutes, and collect the liquid that comes out (this includes the solution that was previously circulating through the column).

Afterwards, both columns were cleaned with a 1 mL/min flow of 20% ethanol solution for 20 minutes and stored.

# 3.4 Oil dispersion

The procedure for this technique starts off in a way very similar to that of the solution soaking technique. First, a monomer solution with the following composition was prepared.

- 39.40 g AMPS

- 1.60 g MBAA

- 160.00 g $H_2O$

- 0.20 g APS

Once all chemicals were fully dissolved, 50 mL of plain PS-DVB resin suspension were poured into the mixture while it was still being stirred.

After 15 minutes, the excess solution could be sifted out. A 10 μm polymer mesh was placed upon a Buchner ceramic funnel, which was set on a 500 mL waste bottle connected to a vacuum pump. The mesh was wetted with ethanol, and then the vacuum pump was set to 200 mbar.

The remaining resin was poured into the funnel, spreading all over the polymer mesh, so that a homogenous layer as thin as possible (about 2 mm thick) could be obtained. In a few seconds the resin became dry, so it was collected and put in a 100 mL glass jar.

The sample was then dispersed in a 250 mL beaker, filled with 160 mL of silicone oil: the resin formed large aggregates of irregular shape. The beaker was placed inside a second larger beaker (500 mL) containing 100 mL of silicon oil; this second beaker was in turn placed on a heating plate, and equipped with an overhead stirrer. The head of the stirrer was then lowered so that it could submerge in the suspension and the rotor was set at 65 rpm. As soon as the resin seemed reasonably dispersed (no aggregate larger than 2 mm in diameter could be spotted), the heating plate was set to 60 °C, and a thermometer was put in the silicone oil inside the larger beaker, so that the actual temperature of the system could be checked during the process. After the desired temperature was reached  the sample was kept stirring for 5 hours.

Afterwards, the suspension was sieved on a polymer mesh (25 μm mesh size) placed upon a ceramic funnel sitting on top of a 500 mL glass bottle connected to a vacuum pump. An underpressure of up to 300 mbar was used to accelerate the leaching of the oil. To remove remaining traces of oil left on the resin, 1.6 L of 10% SPAN 20 in water were used to wash it. This was done by carefully pouring said surfactant solution over the resin, while it was still on the polymer mesh, with a weaker vacuum pressure (roughly 150 mbar), taking care not to let the resin run dry.

Light scattering analysis was run on the sample. The material was put in a centrifuging bottle (using water and ethanol to remove it from the surfaces it was sticking on) and then subjected to 3 subsequent centrifuging cycles:

- Add 10% SPAN 20 so that the bottle contains around 60% of SPAN 20 solution (the rest being resin, water and ethanol) and disperse on a shaking plate at 100 rpm for 1 hour.

- Centrifuge at 4000 rpm for 20 minutes.

- Let the bottle rest for at least 3 hours.

- Decant the supernatant, keeping all sedimented resin.

After centrifugation, 3 phases could be easily distinguished: resin resting on the bottom, SPAN 20 dissolved in water above it and silicone oil on top of everything else, thus making decantation an easy task. After that, more SPAN solution has to be added, and then the mixture was put on a shaking plate for a while so that the resin could be dispersed in it.

After the third centrifuging cycle, a second PSD analysis was run. Some minor differences with the original PSD could be found, but they were thought to be negligible, and possibly due to the mechanical stresses to which the material had been subjected during the extended processing described above. In fact, the difference was only apparent in a noticeable "shoulder" in the size distribution diagram towards the fine diameters (25-50 μm). Therefore, the sample was considered ready for column packing and titration.

The experiment was then repeated using EXXSOL D100S instead of silicone oil for

dispersing the resin during polymerization of the gel. After the first cleaning, the change in PSD was dramatic: both very large aggregates (over 150 μm in diameter) and fine particles (below 25 μm in diameter) were detected by light scattering. This lead to the conclusion that the use of carbon-based oils causes plasticization of the resin, that in turn makes it unsuitable for column packing.

# 3.5 Liquid penetrant

A solution was prepared according to the following recipe:

- 39.40 g AMPS

- 1.60 g MBAA

- 0.20 g TEMED

- 95.24 g $H_2O$

- 0.40 g $NaHCO_3$

All ingredients were put in a 250 mL bottle, along with a magnet, so that the solution could be mixed on a magnetic stirring plate.

After the complete solubilization of all ingredients, 50 mL of PS-DVB resin (suspended in water) were poured into the bottle as well. The mixture was put on a shaking plate for 1.5 hours at medium speed (around 80 rpm), so that the monomer solution had time to permeate the pore network inside the resin particles.

Then, the content of the bottle was poured onto a 25 μm polymer mesh, which was lying on the inside of a ceramic funnel leading into a 500 mL waste bottle, connected to a vacuum pump. A small underpressure was applied through the pump, but only very little was needed in order to sift out the excess solution (about 150 mbar). After a few seconds the vacuum pump was shut down and the partially dried resin was collected and stored in a sealed 250 mL bottle at room temperature.

A second solution was then prepared in a 100 mL beaker by mixing:

- 95.24 g $H_2O$

- 0.20 g $NaHCO_3$

- 0.1 g APS

Then the beaker was emptied into the bottle containing the resin that was prepared for the experiment and said bottle was placed on the magnetic stirrer set at 350 rpm.

Finally, NaOH was added until the pH of the solution became higher than 8.5. The stirring speed was then lowered to 150 rpm and maintained in this condition for 15 minutes. The mixture was poured onto a new 25 μm polymer mesh, set up as always on a ceramic funnel leading into a 500 mL waste bottle connected to a vacuum pump, which was set to produce a pressure of 350 mbar, in order to remove all excess solution. After only a few seconds, it was possible to spot several rather large cracks (up to 5 cm in length and up to 300 μm wide) along with a few smaller ones. The dry resin was then collected by scraping it off the filter with a spatula and putting it in a 30 mL vial, along with a magnet. The collected resin was then re-dispersed in 10 mL of water and 2 mL of ethanol, and the vial was placed on the magnetic stirrer, so that the resin particles could disperse well. After 10 minutes, the resin was ready for column packing.

Once again, both the filling volume and the actual column were washed with a few milliliters of ethanol, to improve the quality of the packing. As for the pump, the usual increasing flow rate was used.

To titrate the final material an alternative method was developed. The functionalized resin was dried: first, the column was unpacked and emptied on a 20 μm polymer mesh, placed inside a ceramic funnel lying on top of a 500 mL waste bottle, connected to a vacuum pump. To get all the resin out of the column, some ethanol was also used to wash its volume. The pump was then set to 300 mbar; after 1 minute, the pressure was increased to 400 mbar, and after another minute to 500 mbar. After 2 more minutes of sieving, the resin was removed from the polymer mesh and put on a weighing plate (which had been weighed earlier), and then heated in an oven. After 2 hours at 40 °C the plate was removed from the oven, and the resin was weighed.

Being that the resin could not be packed into a column due to strong backpressure rising during the packing procedure (even at flow rates as low as 0.7 mL/min), the sample was titrated in a manner more similar to the standard Stone-Carta method [16] summarized in section 2.2. The resin was poured in a 30 mL vial, and to it 25 mL of 1M HCl were added. The mixture was stirred with a magnet for 1 hour and then the content was poured onto a 10 μm polymer mesh lying on a ceramic funnel connected to the vacuum pump through a 500 mL waste bottle. The pump was set to 150 mbar, to make the drying of the resin more rapid. After about 30 seconds of drying, the resin was collected and put back in the vial, along with 25 more mL of 1M HCl. Again, the mixture was stirred for 1 hour and then the resin was dried in the same exact manner as before. The soaking in 1 M HCl was repeated a third time. After the last drying step, the resin was left on the polymer mesh. Distilled water was carefully poured onto the resin, so that the resin could not dry. The pH of the runoff was periodically checked and addition of water stopped when it reached a neutral value. The pressure was then set to 300 mbar and the material dried for one minute. The weight of the filtered material was compared to the weight that was measured after it was thermally dried to ensure it was dried as much as possible in this step; if not, the drying step was continued until the difference between the two measured weights would be less than 0.1% of the measured values.

Finally, 25 mL of 0.05 M NaOH/0.5 M NaCl solution were added to the resin. This amount of solution was taken as precisely as possible with a volumetric pipette. The mixture was stirred for 2 hours with a magnet. A known amount of supernatant was collected by filtration through a 20 μm polymer mesh and the still moist resin was stored in a closed vial. The collected supernatant was titrated with 25 mL of 0.01 M HCl solution, in 500 steps of 50 μL each, with a wait time of 10 seconds between each step.

## 3.6 Solution flow-through

About 10 mL of resin (63-100 μm in diameter fraction) were packed into a PEEK column, with an increasing flow rate:

- 5 minutes at 1 mL/min

- 5 minutes at 2 mL/min

- 5 minutes at 3.5 mL/min

- 10 minutes at 5 mL/min

Then, 10 mL of monomer solution with the following composition were injected in the column:

- 0.160 g MBAA

- 3.946 g AMPS

- 16.000 g $H_2O$

- 0.02 g APS

To ensure a steady flow inside the column, a syringe pump was used for the injection: a capillary tube was connected with the tip of a 20 mL syringe, and the monomer solution was taken up with it. The syringe was then placed on the pump, the speed of which was set to a low value (around 0.8 mL/min). A custom head with proper screw thread was placed at the end of the capillary tube, which allowed for it to be safely connected to the PEEK column containing the packed resin; this connection was made so that the monomer solution would be fed into the column from the same direction from which water had been flowing during packing of the resin, to avoid rearrangement of the resin particles. The other end of the column was placed upon a 30 mL vial, so that the eluted monomer solution could be stored for analysis, if necessary.

After collecting 10 mL of monomer solution leaving the column, the pump could be stopped.

The capillary tube was detached from the column and from the syringe. The syringe itself was removed from the pump and disposed of. The tube was then attached to 500 mbar of nitrogen pressure, to flush out most of the monomer solution left in the capillary. Then, the pressure was set to 200 mbar. The column was fixed with a clasp above a clean beaker of known weight. Without turning the pump off (or changing the pressure that was set up) the capillary was reconnected with the column. The nitrogen flow was let through the column for 5 minutes; meanwhile, the ejected solution was

collected. Afterwards, the pump was turned off and the collected solution was weighed.

This experiment was repeated three more times, each time changing the pressure of the nitrogen flow. All columns were flushed for 5 minutes. The 4 specimens obtained were:

- Column 1 flushed with 200 mbar $N_2$ flow

- Column 2 flushed with 400 mbar $N_2$ flow

- Column 3 flushed with 600 mbar $N_2$ flow

- Column 4 flushed with 800 mbar $N_2$ flow

Each column was sealed with full PEEK plugs, secured in place by the column's end caps at both ends. A graphical representation of this precaution is given in Figure 3-3
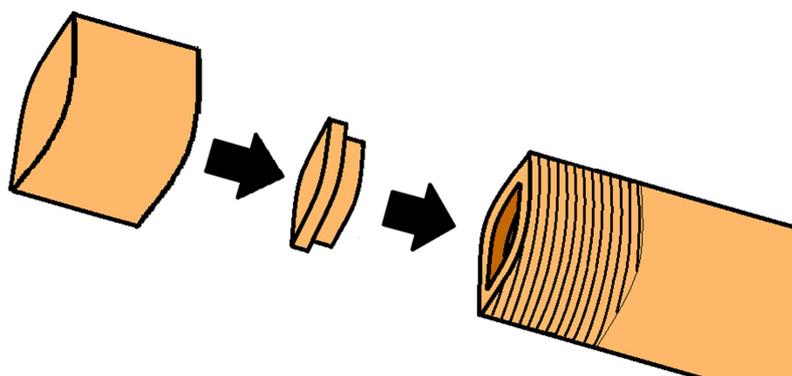


Figure 3-3: temperature resistant sealing of a PEEK column

All columns were placed in an oven at 60 °C for 5 hours to let polymerization of the gel happen. Afterwards, all full plugs were removed, all frits were replaced with new ones and the punched plugs were put back in place. Finally, all columns were titrated.

Based on the results of these experiments (which will be discussed in section 4.5) a second set of tests were conducted to better identify the optimal pressure for the nitrogen flow used to flush out the excess monomer solution left amongst the resin particles packed into a column.

The pressures which were investigated this time were 450, 400, 350, 300 and 250 mbar.

Another set of experiments was run to test the effect of the hydrogel's chemical composition on HPLC viability for columns produced with this technique. For this purpose, four different monomer solutions were prepared to reproduce the experiment. Their exact compositions are summarized in Table 3-7.

**Table 3-7: monomer solution compositions for solution flow-through technique**

|          | HG 1   | HG 2   | HG 3   | HG 4   |
|----------|--------|--------|--------|--------|
| **AMPS** | 1.00   | 0.75   | 0.50   | 0.25   |
| **MBAm** | 0.04   | 0.03   | 0.02   | 0.01   |
| **APS**  | 0.0050 | 0.0038 | 0.0025 | 0.0013 |
| **$H_2O$** | 4.00 | 4.25   | 4.50   | 4.75   |

The solution flow-through technique was also tested for producing an anion exchange stationary phase. Two PEEK columns were packed with sulfonated resin (functionalized as described in section 3.2.4), with an increasing flow rate of pure water:

- 5 minutes at 1 mL/min

- 2 minutes at 2 mL/min

- 2 minutes at 3 mL/min

- 2 minutes at 4 mL/min

- 1 minute at 5 mL/min

Two monomer solutions were prepared. Their compositions are listed in Table 3-8.

**Table 3-8: composition of AETAC monomer solutions used for flow through technique**

|  | HG 1 | HG 2 |
|---|---|---|
| AETAC | 0.49 | 2.02 |
| AA | 1.51 | 0 |
| MBAm | 0.08 | 0.30 |
| APS | 0.02 | 0.02 |
| $H_2O$ | 7.97 | 17.53 |
| NaCl | 0.06 | 0.18 |

Both solutions were injected into one of the two PEEK columns packed with sulfonated resin. To do this a syringe pump set to 0.8 mL/min was used, with an analogue setup as that which was used for the injection of the AMPS monomer solution, described earlier in this chapter.

A 400 mbar nitrogen jet was used to eject the excess solutions left inside the columns: these excess solutions were collected in two beakers and weighted for future reference.

The columns were sealed and left in an oven at 60 °C for 5 hours. Afterwards, contents of both columns were titrated, each with 25 mL of a 0.1 M NaOH solution. In order to do this, the titration method described in section 0 had to be modified to make it capable of titrating positively charged chemical groups. The modified four-step procedure that was used can be summed up as follows:

1.  Flush the column with 60 mL of a 1 M NaOH solution over 1 hour.

2.  Flush the column with 60 mL of distilled water over 1 hour.

3.  Circulate 25 mL of 0.5 M NaCl/0.1 M HCl aqueous solution through the column for 2 hours.

4. Flush the column with 25 mL of distilled water over 25 minutes, and collect the liquid that comes out (this includes the solution that was previously circulating through the column).

Once again, what is actually titrated is the supernatant collected at the fourth step of the procedure depicted just above.

Sample HG 2 was also subjected to ultraviolet-visible spectrophotometry. Namely, 200 mL of a 25 mM phosphate buffer were prepared with the following composition:

- 200.00 g $H_2O$

- 0.3719 g NaH2PO4

- 0.2697 g $Na_2HPO_4$

This buffer was poured in two 150 mL bottles, so that one contained precisely 100 g of it: in this bottle 150 mg of albumin were dissolved. By mixing these two phosphate buffers in different proportions, 3 reference samples were obtained. Their exact compositions are listed in Table 3-9.

**Table 3-9: composition of reference samples for UV-Vis analysis**

|  | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| **Pure buffer** | 10.013 g | 4.982 g | 0 |
| **Albumin buffer** | 4.995 g | 10.006 g | 14.989 g |

The adsorption spectra of these samples were measured with a Varian spectrophotometer.

Sample HG 2 was flushed with 50 mL of the pure phosphate buffer.

A fourth albumin solution was produced by dissolving 0.050 g of albumin in 25.00 g of pure phosphate buffer.

A 50 mL bottle was weighted and then the albumin solution was poured into it. This solution was then circulated through sample HG 2 for 2 hours. The remaining pure buffer was used to flush the column, and all of the eluted solution was collected in the same bottle that was used to let the albumin solution circuit through the column. The bottle was weighted once more and then a 2 mL sample of it was taken in order to run UV-Vis spectrometry analysis.

# 4 Results

In this chapter all results of the experiments described in chapter 3 are listed. An explanation is given where possible. A brief clarification about the ideas that inspired the four procedures that were followed is given as well.

# 4.1 Preliminary tests

## 4.1.1    Resin preparation

A mercury intrusion test of the PS-DVB resin revealed a total porosity of 83.11% and an average pore diameter of 4.29 μm. These values are rather high: having a high porosity can be good for the sake of this study, because it increases the volume accessible to the mobile phase (therefore allowing for a higher capacity of the stationary phase), but the high average diameter can be an issue for multiple reasons; in the particular case of this study, the main problem would be that a large pore diameter strongly reduces capillary forces, which have been used to retain the monomer solution inside the pores before polymerization, so that hydrogel could form only within them. In fact, the Brooks-Corey correlation, one of the most widely accepted models for the capillary pressure encountered by a fluid in a porous medium states:

$$p_c = cS_w^{-1/\lambda} \qquad (15)$$

Where $p_c$ is the capillary pressure of the medium, $c$ is the entry capillary pressure, $S_w$ is the normalized water saturation and $\lambda$ is the pore size distribution index (an empirical parameter that can have any positive value). Under the assumption of cylindrical pores $c$ can be calculated with Laplace-Young equation [32]:

$$c = \frac{2\gamma cos\theta}{r} \qquad (16)$$

Where $\gamma$ is the interfacial tension, $r$ is the radius of the interface (i.e. the pore radius for the present study) and $\theta$ is the contact angle between fluid and solid phases. As for $S_w$ and $\lambda$ in Equation 15, they are both to be measured experimentally [32]: such measurements require significant time and effort, and the exact value of the capillary force in the porous resin media used in the experiments carried out over the course of the present study was not judged worthy; it was thought sufficient to know that it scales linearly with $1/r$ and exponentially with a parameter that depends on the pore size distribution of the resin (and ranges from 0.2 to 1 for most media [33], decreasing as the

distribution broadens). Note that $S_w$ is a material property independent of experimental conditions, and that it strictly ranges from 0 to 1.

Under the assumption of cylindrical pores, the entry capillary pressure can be estimated from Equation 16: using $\gamma$ = 34 $10^{-3}$ J/m$^2$ and $\theta$ = 87.4° (literature values for the system polystyrene-water [34], [35]), and an average pore radius of 2.15 $10^{-6}$ m (the value measured for the resin used in this work by mercury intrusion), such pressure is equal to 1435 $Pa$ ($=$ 14.35 $mbar$). This means that, neglecting the effects of the pore size distribution, an external pressure less than 14 mbar should be enough to remove the fluid retained inside the pores of such resin. This value is rather low: while it is desirable to be able to functionalize the material with a low pressure (thus reducing the costs of the procedure), a too low capillary pressure would make it difficult to retain any liquid inside the pores, even before applying any external pressure. In order to increase the capillary force inside the pores, it would be advisable to reduce the average pore size of the resin particles, even by one order of magnitude if possible. It may also be considered that the value obtained from Equation (16) neglects the influence of the pore size distribution: in a real material (with a non-razor-sharp pore size distribution) the actual capillary pressure found inside the pores will be significantly higher, as suggested by Equation (15).

Light scattering showed that the resin produced for the study had a rather broad particle size distribution, with significant shoulders both in the short and in the long diameter regions. This is shown in Figure 4-1.
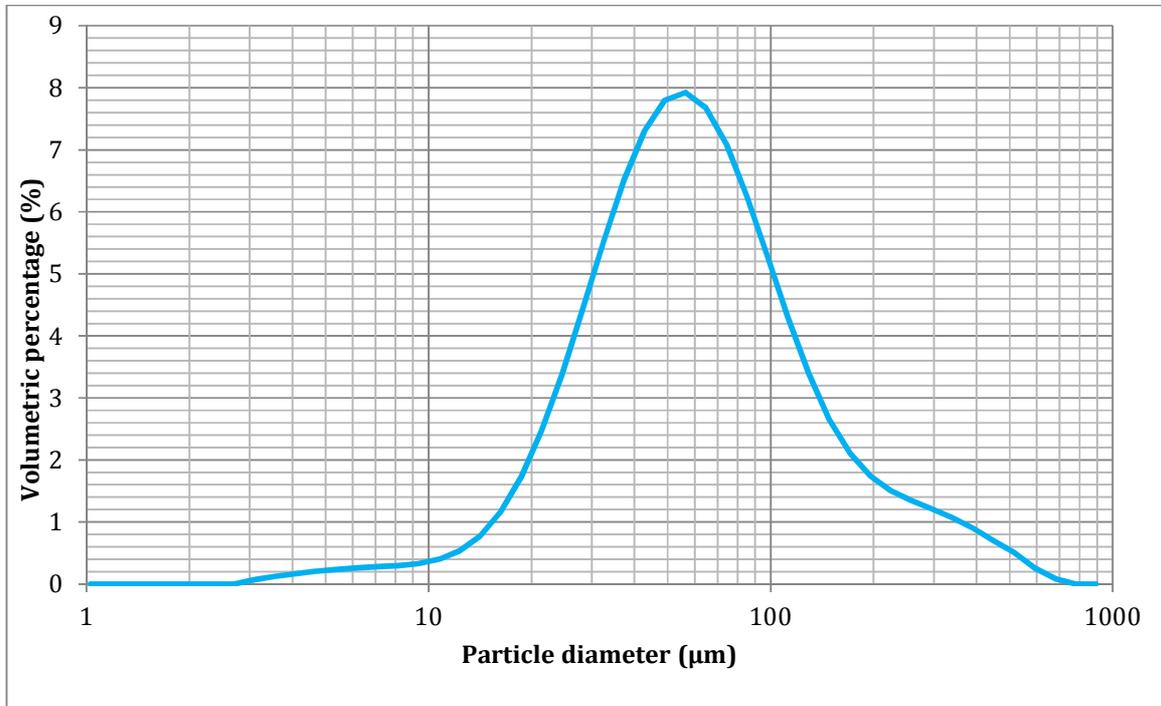
**Figure 4-1: Particle size distribution of the original PS-DVB resin**

Remember that this result was obtained after a thorough sieving of the resin aimed at removing particles with diameter less than 45 µm or greater than 200 µm: as it can be seen, a significant amount of particles was left both in the small and in the large regions. Also notice that the measurement is volumetric, so the shoulder in the smaller diameter region indicates a very high number of fine particles in the resin. A broad PSD can have deleterious effects: some of the techniques that were experimented are very susceptible to the pore size distribution, which strongly depends on the PSD of the resin; a broader PSD can generally cause problems during packing of the resin into a column; but most importantly, a broad PSD increases randomness of all results that are affected by the diameter of the pores or by that of the particles, and it consequently reduces the experimental reproducibility. Therefore, it was decided to narrow the PSD by fractionation. Three fractions were produced:

1. Particles with diameter between 45 µm and 63 µm

2. Particles with diameter between 63 μm and 100 μm
3. Particles with diameter between 100 μm and 125 μm

In addition, a fourth batch of resin was produced by combining the first and the third fraction in a 1:1 ratio. This was done in order to obtain a batch of resin with particles having all the diameters present in the original resin, but with a narrower PSD.

All fractions had their PSD analyzed by light scattering analysis. The results are reported in Figure 4-2 along with the PSD of the original resin for comparison.
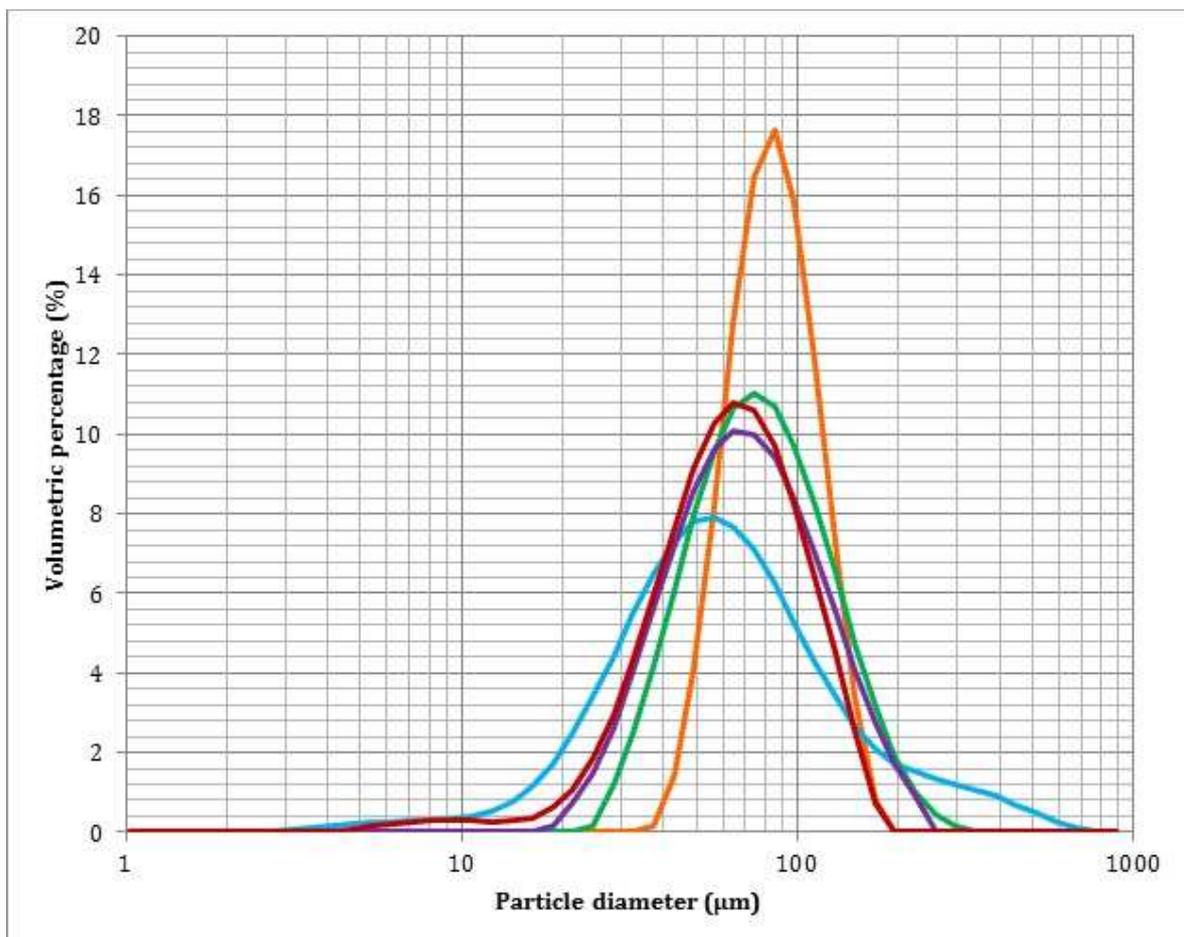


**Figure 4-2: particle size distributions of the original PS-DVB resin (light blue), the 45-63 μm fraction (purple), the 63-100 μm fraction (orange), the 100-125 μm fraction (red) and the mixed fraction (green)**

It is easily noticeable how all fractions have a narrower PSD than that of the original resin, except for the second fraction (the one containing particles with diameters between 63 μm and 100 μm), which is significantly narrower than the others. Also, it is interesting to note that the fraction containing the smaller particles has a significant shoulder in the larger diameter region, while the fraction containing the larger particles has a noticeable shoulder in the small diameter region. The first effect could be caused by aggregation of fine particles, meaning that small particles tend to form aggregates that are seen as large particles by light scattering; the second effect could be caused either by large particles collapsing and shattering into a large number of finer particles, due to the high stirring rate used during the analysis (3000 rpm), or by fine particles getting trapped among the large ones during sieving: the former explanation is thought to be less likely, given the high mechanical resistance of PS-DVB particles.

In Figure 4-3 it is possible to see an actual picture taken by SEM of a particle of the resin used in this work.
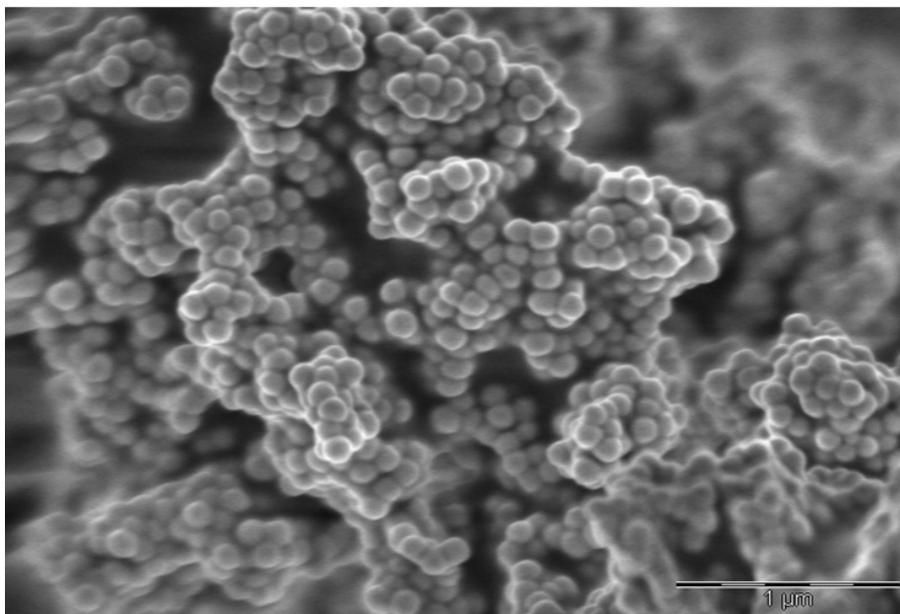


**Figure 4-3: SEM picture of a resin particle produced for this study**

It is easy to notice that the resin particles are actually made of a large number of aggregated styrene nanoparticles; also, the pores are quite irregular in shape and size, and the same should be true for the particles too.

A simple way to portray the final particles used in this work is shown in Figure 4-4:



**Figure 4-4: schematic representation of a core-shell PS-DVB resin particle**

The aggregated nanoparticles constitute the continuous, solid matrix, while the void spaces (clearly visible in the previous SEM picture) are the large pores. This final morphology is quite well-suited for  accommodating large molecules as well as the hydrogels, as discussed later.

# 4.1.2 Bulk hydrogel composition

The experiments carried out to produce hydrogels with different compositions in bulk were aimed at verifying which reactions would actually produce a suitable hydrogel. Initially, only the effect of monomer concentration was studied, and not that of the cross-linker, the concentration of which was kept at 4% in weight with respect to the monomer.

First, reactions using high temperature to induce fast spontaneous dissociation of APS were tested: solutions having a monomer concentration higher than 5% in weight always turned into a solid product, although the gel was softer than expected according to previous experiments on acrylamide. To increase the gel stiffness, a 20% weight concentration of monomer was used in most monomer solutions made for later experiments: this is a rather high value for some systems, but it did help significantly in producing stiffer hydrogels and increasing the amount of charge groups per volume. Therefore, this monomer mixture concentration was used as a standard in most experiments carried out in this thesis.

The reaction with TEMED turned out to be favored only at slightly basic pH. No reaction happened at pH = 1, nor at pH = 14. Between pH 8 and 10 it worked well. This particular condition was achieved by substituting water with a buffer solution (50 mM $H_2CO_3$) and adding NaOH until the pH reached the desired value.

There is no apparent difference in the gel produced by TEMED induced reaction and the one by the temperature induced reaction. The main difference between the two reaction lies in their kinetics: while the temperature induced reaction takes 5 hours, the TEMED induced one reaches full conversion in just a few seconds. In fact, by using TEMED the technique defined "liquid penetrant technique" (discussed in section 3.5) became feasible, since it needs to be carried out in a time frame much shorter than that of the reaction without TEMED. Still, the temperature induced reaction was preferred because introducing salts in the solution was thought to be a possible cause for a reduction in capacity, taking into account the electrostatic nature of the separation mechanism employed by these hydrogels.

Similar compositions were tested for AETAC hydrogels as well, even though some of the concentrations that worked well for AMPS gels did not work for AETAC. A 20% concentrated AETAC solution would not gel, even after 24 hours at 60 °C. It was thought that the problem could be the excessive charge density preventing an extent of cross-linking high enough to achieve gelation of the polymer. To address this issue, two possible solutions were devised and tested: the first was to add salt in the monomer solutions, so as to screen the repulsive interactions between charged groups in the monomer; the second was to add a second, uncharged monomer, so that polymer chains would actually be made of two different monomer units, plus the occasional cross-linker unit.

In order to have a statistical copolymer, a second monomer unit with a similar chemical activity was needed: therefore acrylamide was chosen for the first test and, since it gave good results, it was used in all following experiments. In fact, monomer solutions using a mixture of AA and AETAC in a 3:1 proportion gelled at concentrations ranging from 10% to 25%.

When adding salt, solutions with 20% monomer in weight would gel, but would always exhibit a few milliliters of unreacted liquid phase; these solutions also took significantly longer time to gel, compared to all the other monomer solutions containing TEMED that were tested. Solutions with 5% or 10% monomer content had no issue in polymerizing, while solutions with 15% monomer concentration in weight would polymerize quite oddly: despite having a rather tough bulk compared to less concentrated hydrogels, their free surface was very soft and possibly not completely polymerized.

## 4.1.3    Titration of the resin

During the reaction between styrene and divinylbenzene to form a PS-DVB resin (Figure 2-1), the other chemicals present in the mixture may induce parasitic reactions that alter the proprieties of the material. In particular, the presence of KPS may introduce charged groups on the surface of the resin particles: such charged groups will provide some ionic capacity to the material, which will be referred to as "surface capacity" of

the support from now on.

When measuring the ionic capacity of the composite material that is the objective of this study, both the capacity of the hydrogel and the capacity of the resin are measured: in order to assess if and how much gel was introduced into the pores of the resin by a particular functionalization process, it is necessary to distinguish between the two. Therefore, it was decided to titrate the resin used as a matrix for the hydrogel: once the capacity of the resin would be measured, it could be possible to subtract it from the value obtained for the final material to reckon the actual increase in capacity introduced by the procedure.

In this research, a few slight changes were applied to the original Stone-Carta method, in order to make it possible for packed columns to be titrated with an offline pump, making the sample preparation the multiple steps procedure which is described in section 3.2.3: this titration technique was first tested on a HPLC column packed with PS-DVB resin (which was made with the process described in section 3.2.1). This was a necessary step, to make sure that the method was suitable to measure the capacity of a column filled with the resin that was produced for this purpose, which was meant to exclude any relevant influence of the untreated material on the final measure.

The method used in this work still goes through the steps described by Stone and Carta, but with an additional final step at the end, in which water is circulated through the column containing the material to be titrated, meaning that a known amount of water is let through the column multiple times: this is done to make sure that none of the solution that went through the column in the previous step is left inside the pump or in the various capillary tubes used in the process.

The pH measurements are plotted in Figure 4-5. The measurement closest to the equivalence point is highlighted with a red diamond, and its coordinates on the diagram are written next to it.
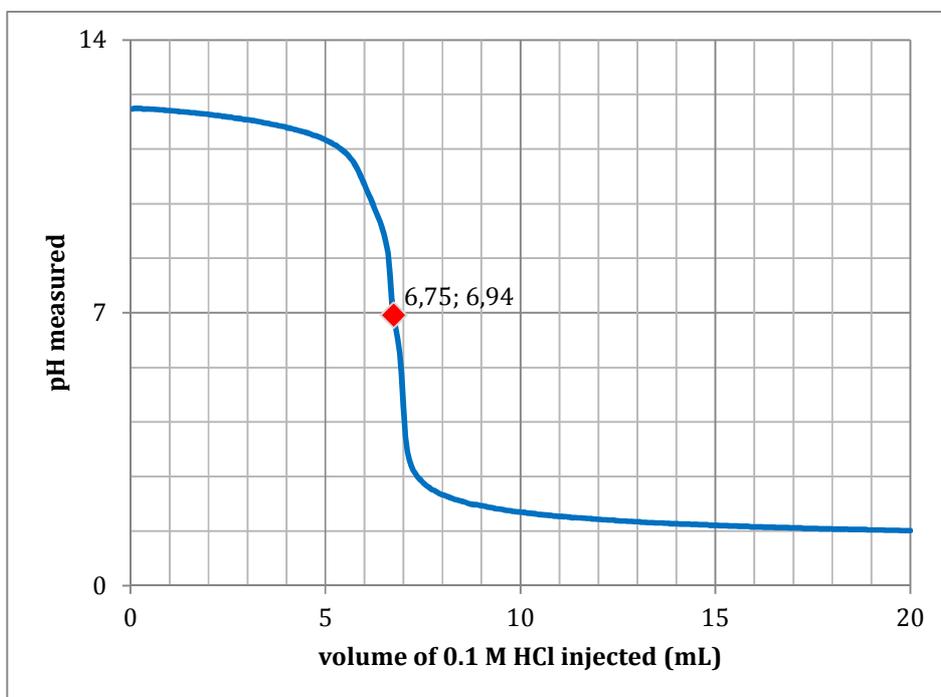
**Figure 4-5: pH measurements obtained by titration of plain PS-DVB sample**

Recalling Equation (5), the difference between the number of moles of NaOH circulated through the column in step 3 (0.675 mmol in this case) and the amount of moles of NaOH left in the titrated solution is equal to the amount of sulphonic groups on the surface of the material. Since 6.75 mL of 0.1 M HCl were required to reach pH 6.94, and the dependence of pH on the volume of injected HCl is quite linear, 0.6692 mmol of NaOH must have been present in the solution, from which the number of moles of charged groups on the resin surface was estimated as 5.8 µmol. Note that this value is low compared to the measured number of moles (it is smaller by 2 orders of magnitude): therefore, even a small error in the measurements would significantly affect this result.

Since the column volume was 0.628 mL, an ionic capacity of 9.24 µmol/mL column volume was measured for the blank resin. The potential for error is large with this method, especially when only small amounts of ion exchange groups are present: therefore, not much emphasis should be put onto the specific value but rather on the

fact that only very few groups are present.

# 4.1.4    Functionalization of the resin

Being that PS-DVB resin has very low polarity, it is expected to be hydrophobic. Indeed, during the experiments that were carried out in this study, this hydrophobic behavior was apparent in several instances: especially while sieving, particles would hardly disperse in water, and would rather stick to the metallic surface of the sieve, making the procedure much harder. Furthermore, when the resin was stored in glass bottles without filling them completely, resin particles would adhere to the glass walls that were left untouched by the water and stick to those walls until ethanol was sprayed on them.

This could have been a major issue, for the functionalization of the stationary phase was supposed to happen through a reaction in liquid phase at the surface of the resin: if the resin could not be wetted by an aqueous solution, the reaction could hardly happen.

To address this possible issue, part of the resin that was formerly produced was sulfonated in order to make it more hydrophilic. A poly-styrene resin treated in such fashion is a lot less hydrophobic, and the reaction to modify a standard PS-DVB resin is rather simple [36]: basically, the desired amount of resin is suspended in sulfuric acid at 80 °C for 24 hours while gently stirred (at 25 rpm). Since this was done in the early stage of this work, only a fraction of the poly-styrene resin was modified, just to be prepared in case the non-sulfonated resin would demonstrate itself unsuitable for the purpose of this study.

After sulfonation, a very slight beige coloring could be detected as the only apparent difference with plain PS-DVB resin (which looks pearl white); furthermore, from the behavior of the sulfonated resin during the production and stocking steps, it seemed to be much less hydrophobic than its plain counterpart.

The sulfonation process introduces new sulfonic groups in a PS-DVB resin. This increases the ionic capacity of the material. To measure the extent of this change, a sample of

functionalized resin was titrated: based on the collected data (reported in Figure 4-6), an ionic capacity of 77.4 µmol/mL column volume was measured.
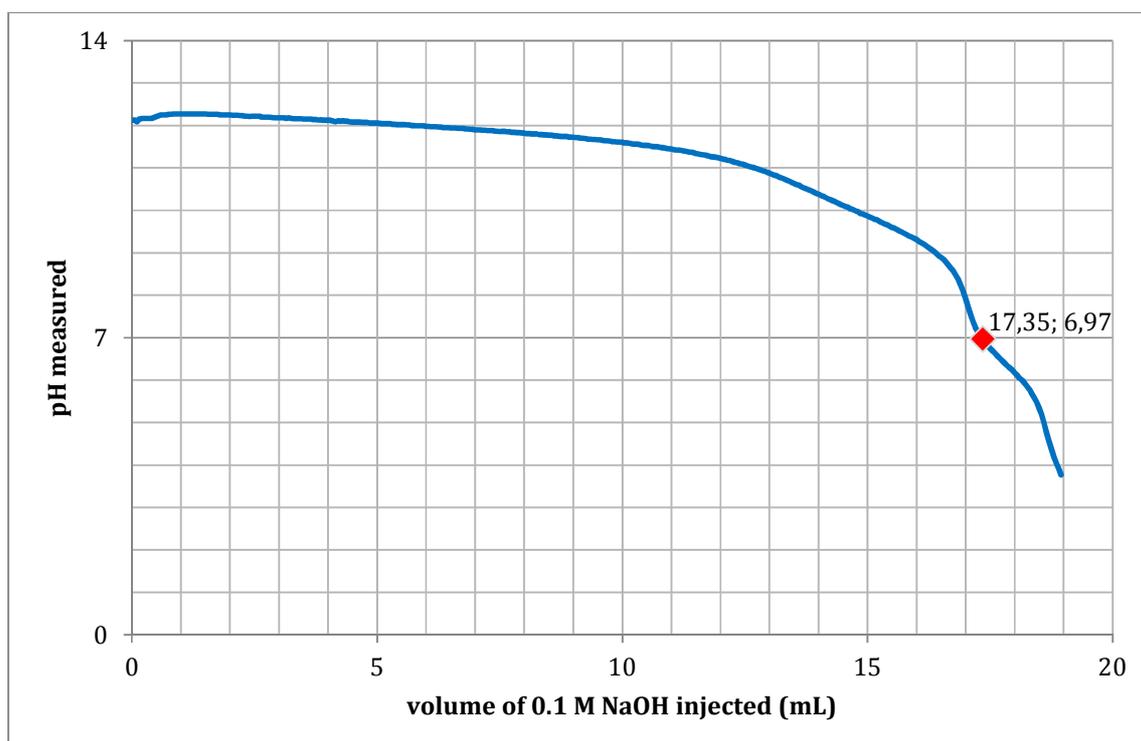


**Figure 4-6: pH measurements obtained by titration of a sulfonated PS-DVB sample**

This means that sulfonation of blank PS-DVB resin significantly increases the "support surface capacity" of a stationary phase. This is what causes the decrease in hydrophobicity of the material; on the other hand, being that the charges introduced on the surface of the resin particles by sulfonation are negative (sulfonic groups), such a resin will not be an ideal matrix for a cation exchange hydrogel (such as an AMPS-based one). However, it will be a better recipient for an anion exchange hydrogel (such as an AETAC-based one).

# 4.1.5    Swelling tests

During the preparation of the hydrogel solutions, solution number 5 needed addition of a large amount of NaOH in order to reach an acceptable pH value. The cause for this remains unclear.

Another issue that was encountered during the preparation of hydrogel solutions was that solution number 1 did not gel. This was not entirely unexpected, given the results discussed in section 4.1.2: it was concluded that 5% is below the minimum monomer concentration in weight needed for an AMPS solution to polymerize into a gel.

As for the measurements of equilibrium volumes of all produced specimens, the results are reported in Table 4-1 below.

Table 4-1: equilibrium volumes (mL) of AMPS hydrogels of various compositions

| | | Days since gelation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 21 | 30 | 41 | 50 | 62 | 75 | 90 | 98 |
| Specimen | HG 2 | 20 | 144.08 | 220.92 | 243.40 | 275.45 | 293.36 | 307.76 | 320.96 | 323.22 |
| | HG 3 | 20 | 96.32 | 163.53 | 170.27 | 170.45 | | | | |
| | HG 4 | 20 | 136.87 | 185.48 | 193.43 | 194.60 | | | | |
| | HG 5 | 20 | 158.96 | 167.95 | 169.09 | 169.41 | | | | |
| | HG 6 | 20 | 156.13 | 315.48 | 356.68 | 392.41 | 422.86 | 429.67 | 434.88 | 435.54 |
| | HG 7 | 20 | 94.91 | 126.21 | 127.43 | 127.48 | | | | |
| | HG 8 | 20 | 82.05 | 86.30 | 87.69 | 87.98 | | | | |
| | HG 9 | 20 | 60.73 | 73.81 | 75.99 | 76.61 | | | | |

Most samples reached equilibrium in less than two months, but quite noticeably, two out of eight samples swelled for a much longer time (more than three months), reaching a much larger final volume long after the other samples had already stopped their own

swelling.

Obtained data were subdivided into two series: one for the hydrogels produced at different monomer concentration and one for those at different cross-linker concentration, to highlight the relative importance of the two components. The corresponding diagrams are shown in Figure 4-7 and Figure 4-8 below:
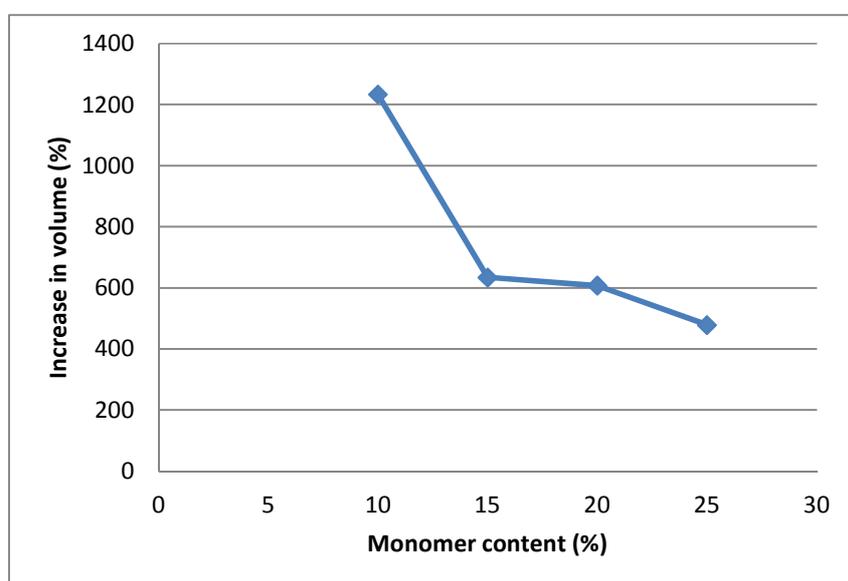


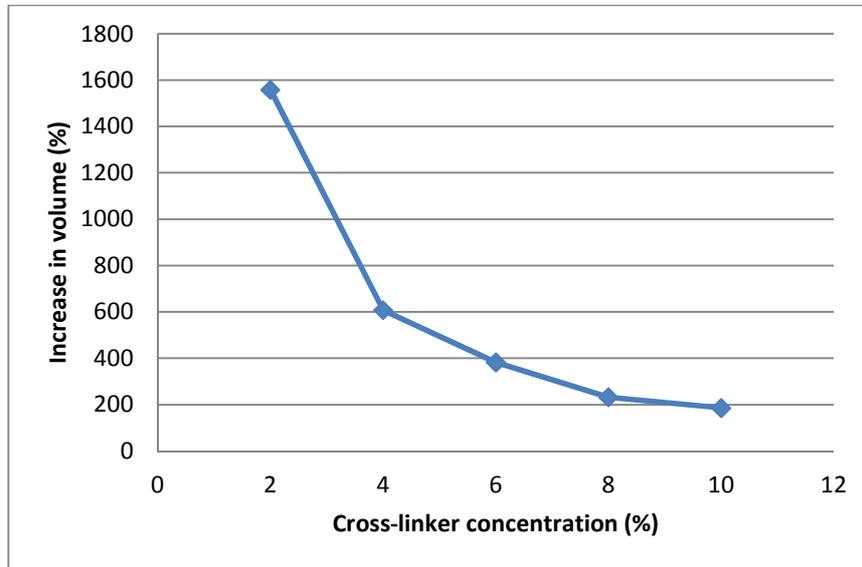**Figure 4-7: effect of monomer concentration on swelling behaviour**

**Figure 4-8: effect of cross-linker concentration on swelling behaviour**

The effect of the monomer seems to be very strong at low concentrations, whereas it becomes much weaker above 15% of monomer content. As for the cross-linker, the effect on swelling behaviour seems to vary more continuously with increasing concentration.

From the results above, it can be hypothesized that in order to maximize swelling one could just aim for the composition with the least monomer and cross-linker concentration at which gelation still happens; to achieve the minimum possible swelling on the other hand, one should try to aim for the highest cross-linker concentration possible, which will be limited by the solubility of MBAm in water (at 20 °C it is about 20 mg/mL), and then add monomer until a concentration of at least 15% is reached: after that point, the effect of adding more monomer will not be as strong. In general, this behavior is fully consistent with that typical of crosslinked polymers: increasing monomer mixture concentration as well as cross-linker content results in higher degree of chain reticulation, thus reducing the swelling capacity of the material.

# 4.2 Solution soaking

The solution soaking technique was the first one to be devised. It is clearly a very simple idea, and does not provide any control over where gelation of the monomer solution can or cannot occur: nothing prevents the formation of hydrogel outside of the pores. The procedure only consists of three steps, portrayed in Figure 4-9:
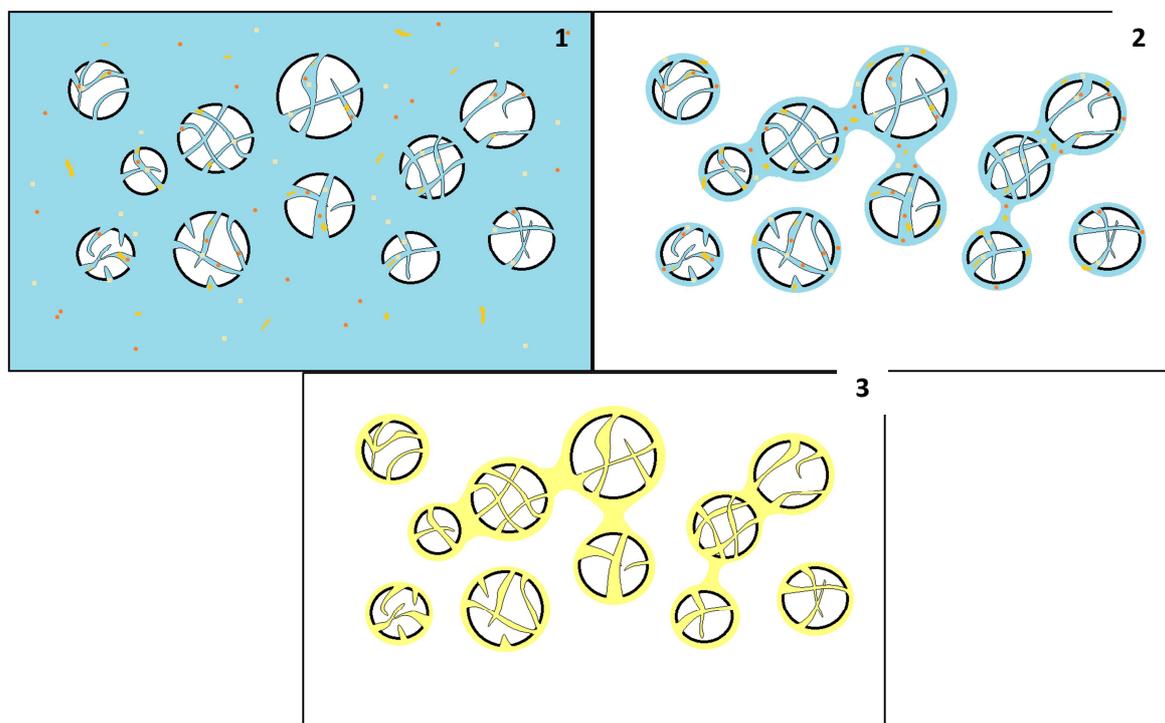


Figure 4-9: graphic interpretation of the 3 steps involved in the solution soaking technique: soaking in monomer solution (1), removal of excess solution (2) and heating at 60 °C for 5 hours (3)

The most evident weakness of this technique lies in step 2: there is no way to tell if the solution is staying in the pores and measuring the amount of solution retained by the resin is rather complicated. The easiest method to do it would be to completely dry the resin before the soaking step, weigh it, then proceed with the first two steps of the

procedure and weight the resin again: the difference between the two measured weights would then be the amount of solution retained. However, it is not possible to establish whether this solution is inside the pores of the resin particles as it should be, or if it is actually trapped in the interstitial spaces among the particles.

These possible issues were foreseen before the technique was experimented: however, since the procedure as described in section 3.3 is very simple to carry out, it was thought to provide important information about how to handle the materials and the most relevant issues that could arise during their processing using different techniques.

In the case of AMPS gel grown on PS-DVB resin, sieving on a polymer mesh made the material extremely hard to be re-dispersed in water or aqueous solutions; re-dispersion in solutions at various concentrations of NaOH (0.05 to 5 M) could not be obtained either. When the sieving is carried out on paper filter, leaving the resin noticeably more moist during the drying step, re-dispersion is much easier and the resin can actually be functionalized: this lead to the belief that partial drying is preferable for this procedure.

Based on the data shown in Figure 4-10, an ion capacity of up to 357.2 μmol/mL column volume could be achieved with this technique.
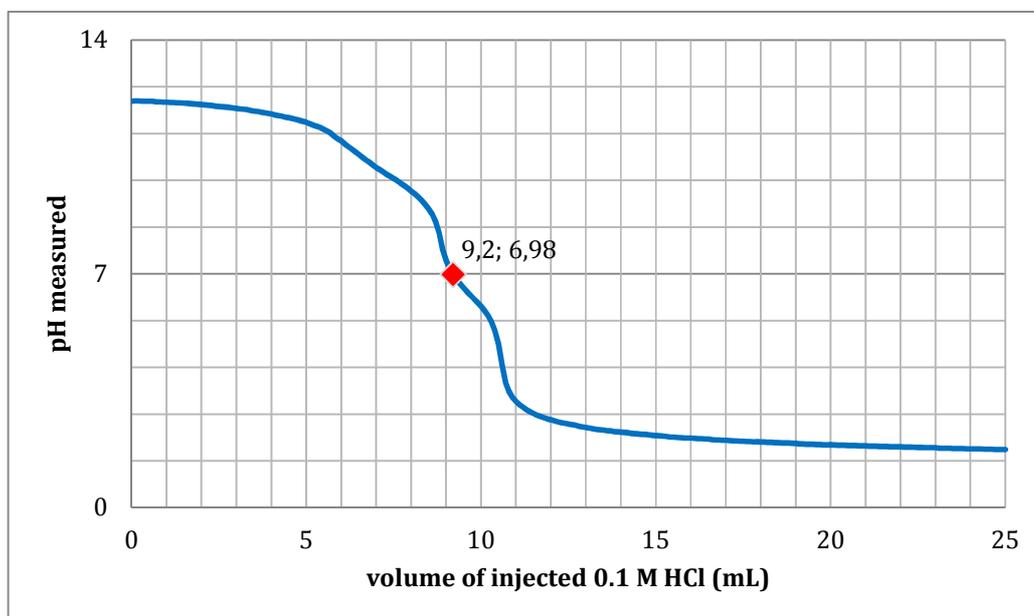
**Figure 4-10: pH measurements obtained by titration of a resin sample subjected to solution soaking technique with AMPS gel**

Still, there is no way to predict where the gel will form with respect to the resin particles: the irreversible clogging that was observed seems to point out that gel formed on the outside of the particles; the titration procedure that was used required an extended exposition to a great amount of aqueous solution, that could cause swelling of the gel, resulting in the filling of the column empty volume.

In the case of the AETAC gel grown on sulfonated PS-DVB resin, sieving did not seem to affect the quality of the final product. There was no major issue with immiscibility in any of the solutions involved. Still, titration of the columns revealed very low capacities in both cases, 26.02 μmol/mL column volume for the resin dried on paper filter and 12.86 μmol/mL column volume for the other one (cf. Figure 4-11 and Figure 4-12).
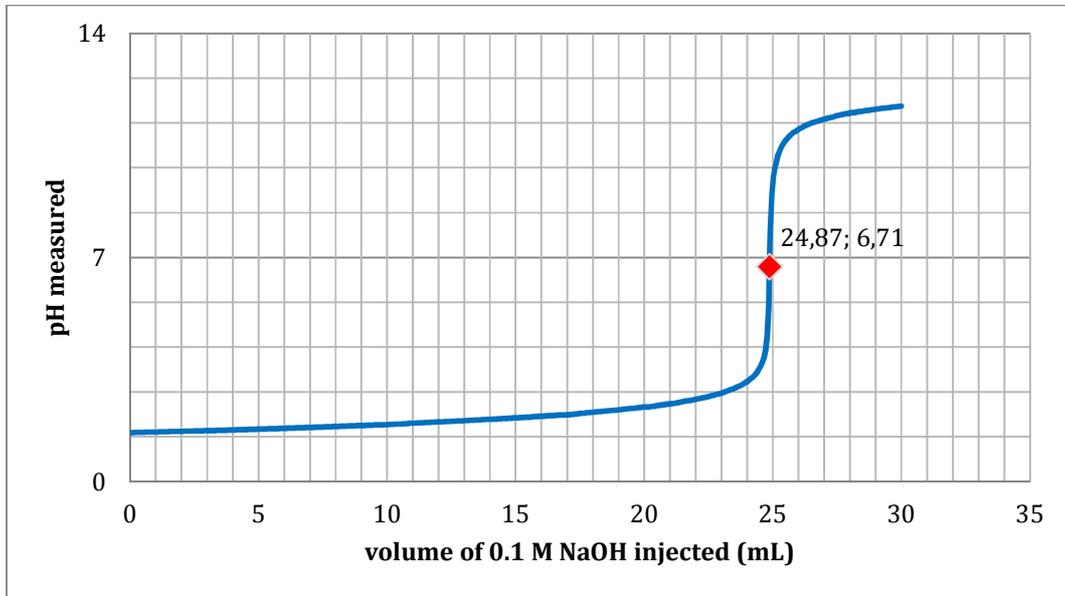
**Figure 4-11: pH measurements obtained by titration of a resin sample subjected to solution soaking technique with AETAC gel (dried on paper filter)**
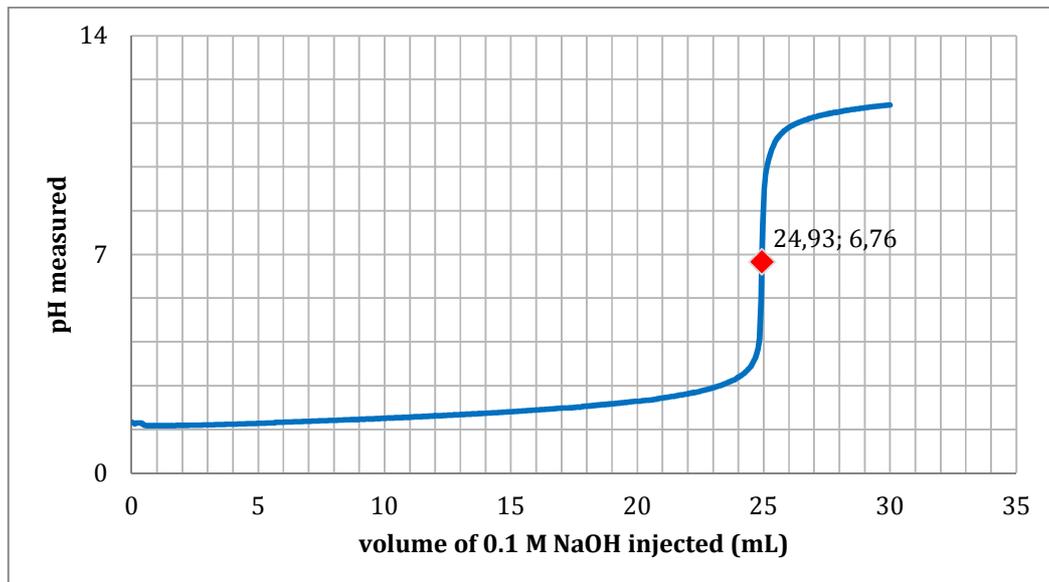


**Figure 4-12: pH measurements obtained by titration of a resin sample subjected to solution soaking technique with AETAC gel (dried on polymer mesh)**

It is to be noted that both these values are well below the support surface capacity of the sulfonated resin, which was reported to be 77.41 µmol/mL column volume in section 4.1.4. This could be due to the fact that most of the R-NMe$_3^+$ groups present in the gel are bound to the sulfonic groups on the resin surface, and do not interact with the solutions which have been used for the titration of the material. If this was the case, the actual capacities would have been reduced by an amount equal to the support surface capacity of the sulfonated PS-DVB resin.

Another reason for which the measured capacities are so much less than those found in plain PS-DVB resin particles filled with AMPS gels (which are usually above 100 µmol/mL column volume) could be that part of the monomer solution that was retained by the resin after the drying step evaporated during the heating step, and then condensed on the surface of the jar in which the resin was contained: indeed, a significant amount of droplets could be seen on it.

It is well possible that both issues affected the results that were obtained.

In conclusion, the overall poor results should be attributed to the lack of control granted by the solution soaking technique: there is no way to know if the monomer solution retained by the resin before gelation will be only inside the pores, and, even in this case, there is still no way to assess whether it will stay there; it could evaporate, or even leach out of the pores and spread over the particle surface.

Therefore, the solution soaking technique was not elected as viable procedure to produce the desired material.

## 4.3 Oil dispersion

The oil dispersion technique was first devised to address the issue that was encountered while applying the solution soaking, where there was lack of control over where gelation can occur with respect to the resin particles. In order to limit the space accessible to the monomer solution it was thought to disperse the resin in a hydrophobic medium, after it

had been soaked in monomer solution. The steps in which the procedure could be subdivided would then be those depicted in Figure 4-13:
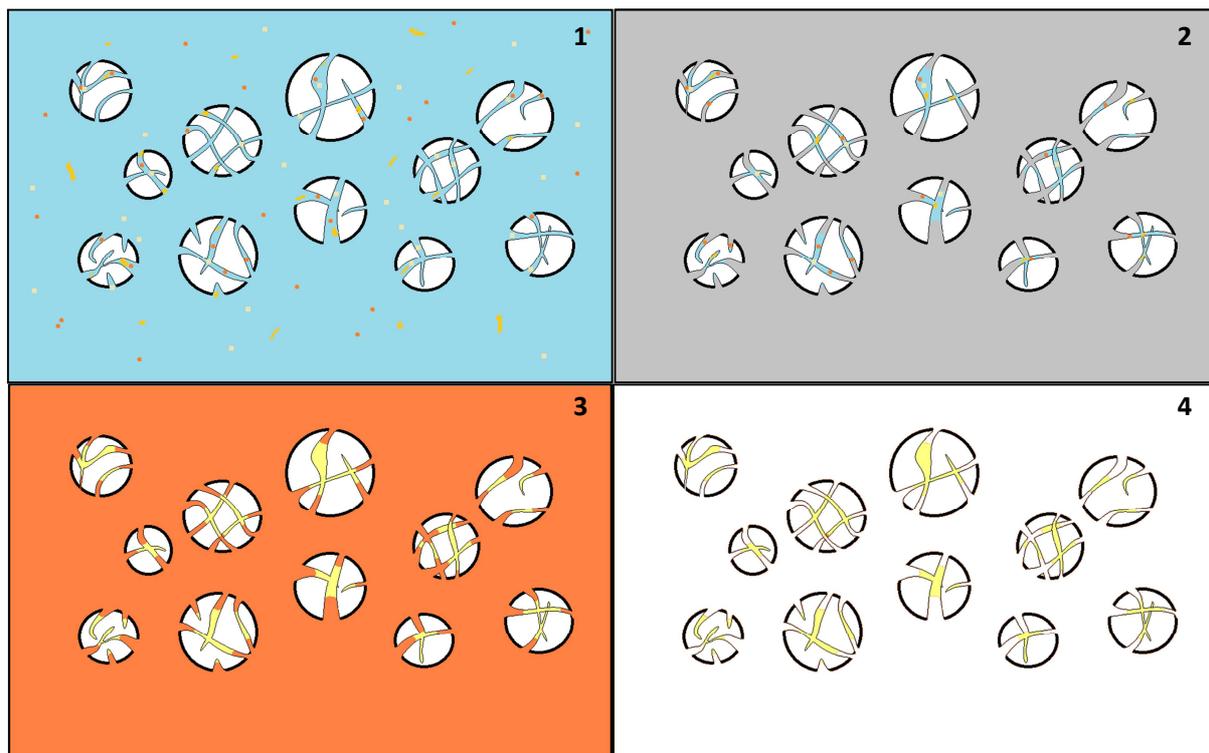


**Figure 4-13: graphic interpretation of the 4 steps involved in the oil dispersion technique: soaking in monomer solution (1), dispersion in hydrophobic medium (2), heating at 60 °C for 5 hours (3) and cleaning of the resin (4)**

In this case the critical step is the second one: the resin needs to be suspended in the continuous hydrophobic medium under stirring. This should ensure that all excess solution left on the external surface of the resin particles segregates, thanks to the centrifuge action exerted by the stirring, and forms droplets away from the resin; meanwhile, all the solution retained in the pores is still trapped in there and is not able to leave the pore volume without entering the hydrophobic phase. Note that PS-DVB resins are hydrophobic, therefore the particles will be easily wetted by the hydrophobic medium and segregation of the excess monomer solution (which is an aqueous solution)

should be thermodynamically favored by the resulting hydrophobic effect.

As hydrophobic medium, a synthetic oil was thought to be the ideal candidate: oil dispersion technique needs a very inert oil, to prevent any reaction between resin and oil during the polymerization of the gel; thermal stability and good heat-transfer properties are also desirable. Silicone oil, which is a suitable option in all these regards, causes a significant increase in the average particle size according to light scattering analysis run on the treated samples. Furthermore, inspection of the equipment used in such analysis showed traces of silicone oil on several surfaces that were in contact with the resin. Based on this observation it was assumed that the noticeable shoulder in the fine size region in Figure 4-14 is not due to very fine particles (with diameters lesser than 2 μm), but to small oil droplets.



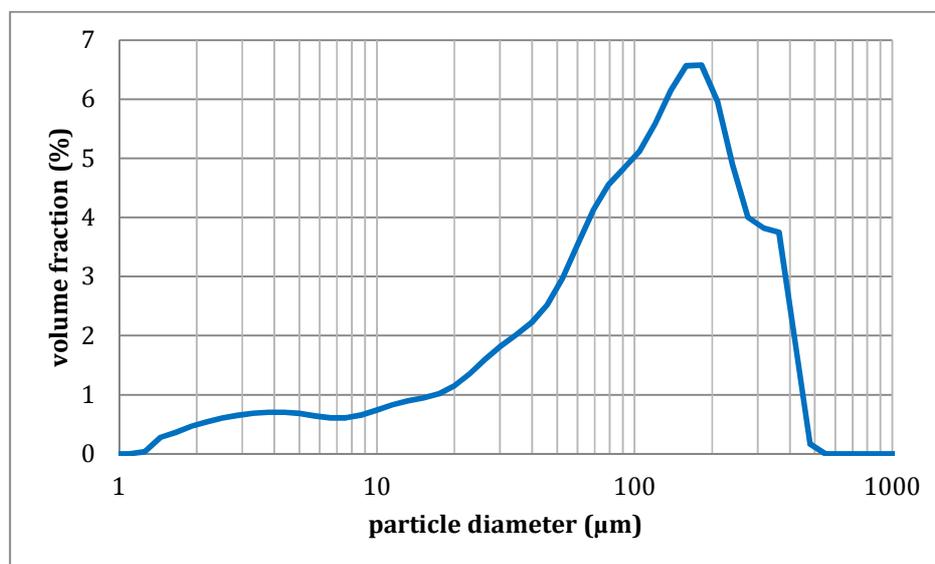Figure 4-14: PSD plot of a sample treated with the oil dispersion technique

Cleaning silicone oil requires an effort that makes the whole process unappealing for industrial production. An efficient cleaning procedure might make this technique feasible, but the tested cleaning methods did not prove efficient enough in the removal of silicon oil due to its generally bad compatibility with surfactants.

Nevertheless, in the only instance where this approach was successful, titration by Stone-Carta method yielded 190.54 μmol/mL column volume as a result for ion capacity, as can be inferred from Figure 4-15.
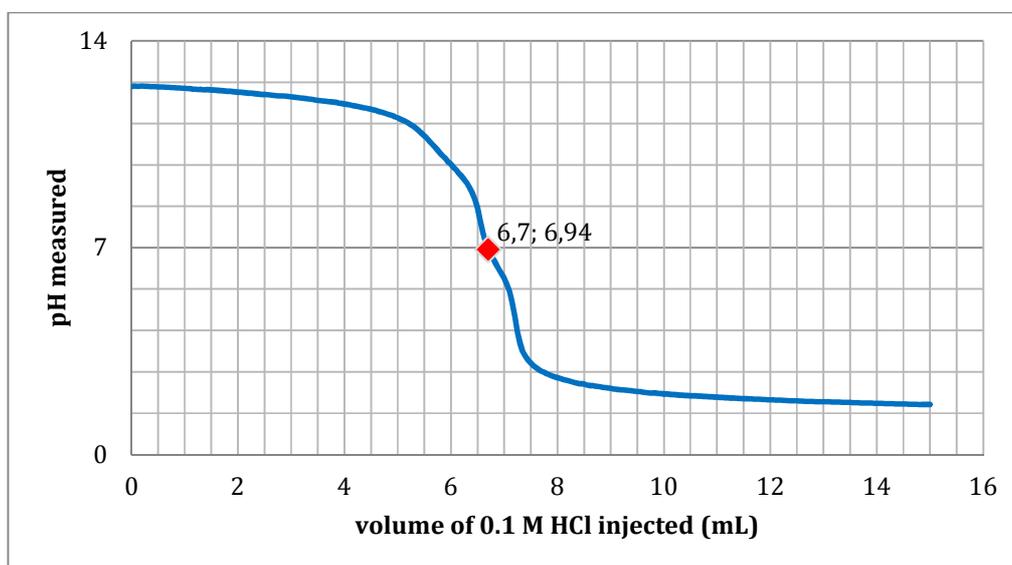


**Figure 4-15: pH measurements obtained by titration of a resin sample subjected to oil dispersion technique with an AMPS gel**

At the end of the preparation procedure for the titration of the sample, an unusual turbidity was observed in the supernatant. This was regarded as a consequence of the presence of traces of silicone oil remaining in the resin even after the thorough washing. It is believed that the leftover traces of oil might have screened some of the functional groups during the preparation procedure described in section 3.2.3, making them inaccessible to the NaOH molecules and therefore undetectable by titration with HCl. Hence, it is possible that the ion capacity of the sample was actually higher than what was measured; unfortunately, while the sample was being prepared in order to repeat the titration, the column became permanently clogged.

# 4.4 Liquid penetrant

This technique was originally inspired by a procedure often used in surface analysis, which is called "dye penetrant inspection". The procedure consists of 4 steps, summed up in Figure 4-16 below: first, the specimen to be inspected must be carefully cleaned; then, a liquid characterized by a bright colour (usually red) is applied on the surface of the specimen so that it can penetrate any present crack due to capillary suction; after a proper penetration time, the surface is cleaned once again while leaving the dye inside the cracks (for example a with a water jet hitting the surface of the specimen in a tangent direction); finally a proper "developer" is applied over the whole surface, so that it can absorb the dye left inside the cracks therefore revealing their presence.
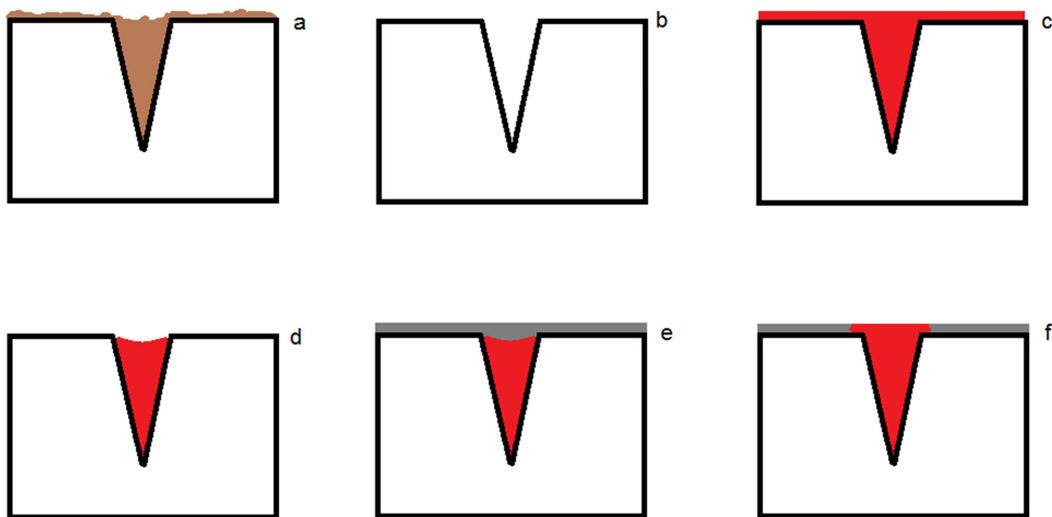


**Figure 4-16: schematization of the dye penetrant inspection procedure in 6 steps: crack concealed by dirt (a), precleaning (b), application of the dye (c), intermediate cleaning (d), application of the developer (e), crack revelation (f)**

The idea was to exploit the same principle used by the dye penetrant inspection, in which capillary action makes the dye penetrate a crack and ensures that it does not

leave it during the subsequent cleaning step (provided that the cleaning is not excessively thorough); the procedure would then consist of 5 steps, sketched in Figure 4-17:
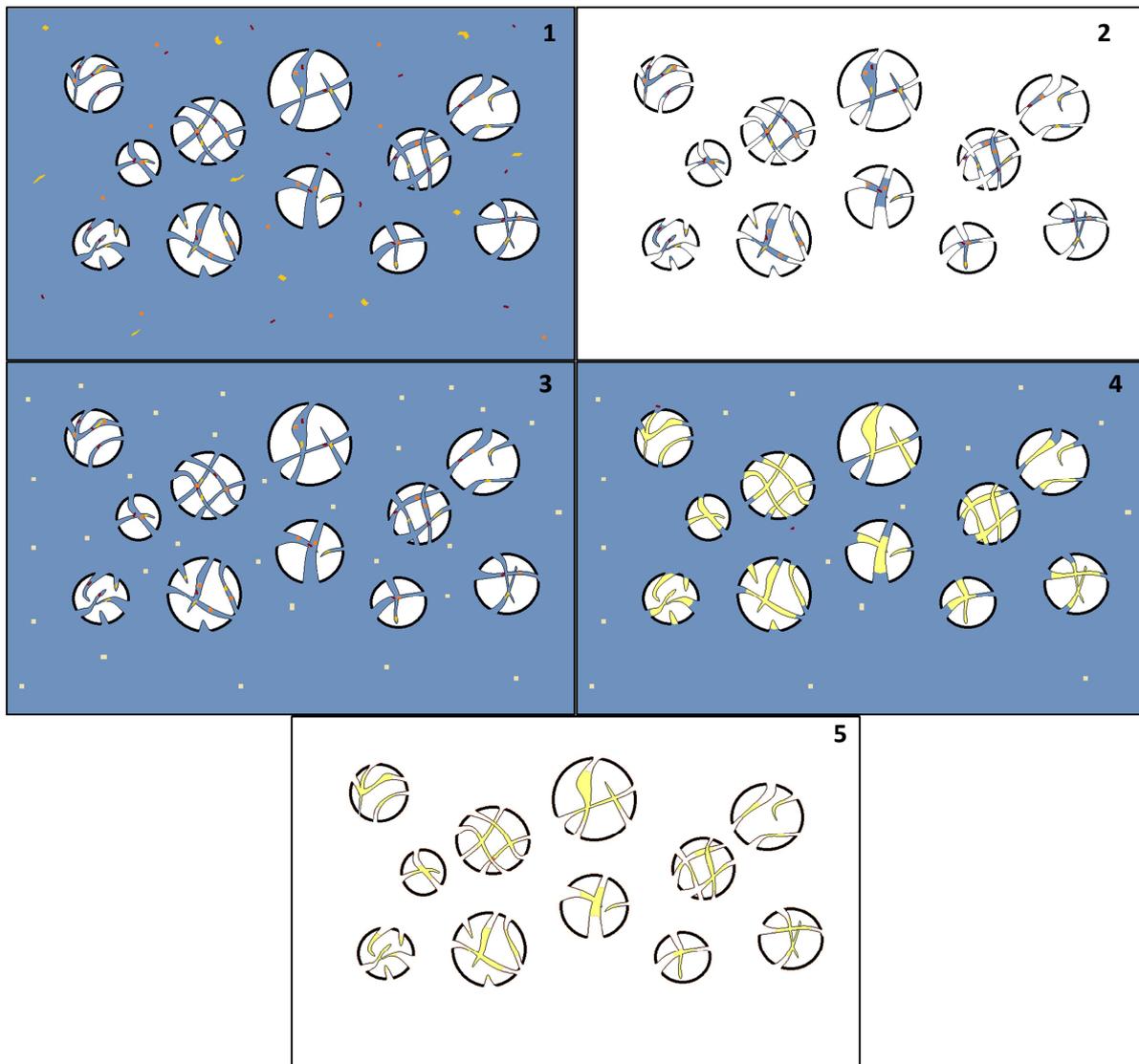


Figure 4-17: graphic interpretation of the 5 steps involved in the liquid penetrant technique: soaking in solution containing monomer, cross-linker and TEMED (1), thorough removal of excess solution (2), re-dispersion of the resin in salt solution containing APS (3), stirring of the suspension (4), drying of the resin (5)

The critical step in this procedure would be the second one: the resin must be dried so that no monomer solution can be found outside the pores in the particles, but some solution must remain inside those pores, otherwise no functionalization would be obtained. If this is done correctly, polymerization should happen at step 4: as soon as the monomer solution in the pores and the initiator from the external solution mix, the reaction starts. Being that both solutions are aqueous and that the external one is more diluted, mass transport will take place according to Fick's first law. The flux of monomer going out of the pores will be greater than that of initiator entering the same pores because the volume of the external phase is much greater than that of the pores, so a great amount of monomer molecules needs to leave the pores to reach equilibrium, while only a relatively low number of initiator molecules needs to enter the pores to make their concentration uniform in the whole system: therefore, it is expected that polymerization will tend to start out of the pores. Since this is undesirable, as already mentioned in section 1.3, the drying in step 2 must be very accurate, so that the pores are left only partially filled with monomer solution. If polymerization starts inside the pores, a polymer network will quickly form due to the fast reaction kinetics in presence of TEMED (taking only a few seconds, cf. section 4.1.2): once that happens, the polymer network will significantly hinder diffusion of monomer outside the pores and the polymerization will continue primarily inside the pores. In fact, the propagation reaction only needs monomer, which should mainly be found there; monomer units that already diffused out of the pores may still contribute to polymerization outwards, but their amount should be negligible with respect to that inside the pores.

This mechanistic picture was purely theoretical and the experimental procedure was designed to test its soundness: of course the actual outcome largely depends on the first drying step, before the resin is re-dispersed in the TEMED solution. In order to have good control over that, it would be necessary to deal with very narrow particle size distribution, well-defined pore size distribution and perfect control over the pressure used to sieve out the excess monomer solution.

All columns clogged while the resin treated following this procedure was being packed: after two minutes while a 20% ethanol solution was flowing through at 1 mL/min rate, a significant backpressure would quickly arise. Even packing at a very low flow rate of 0.5 mL/min resulted in the column blocking after passing 5 column volumes of eluent.

Being that the treated resin could not be packed into a column, an alternative to the method defined in chapter 3.2.3 had to be designed. Following the titration procedure described in section 3.5, a significant increase in ionic capacity was revealed, amounting to 166.95 µmol/mL column volume. The complete results of the titration test are displayed in Figure 4-18.
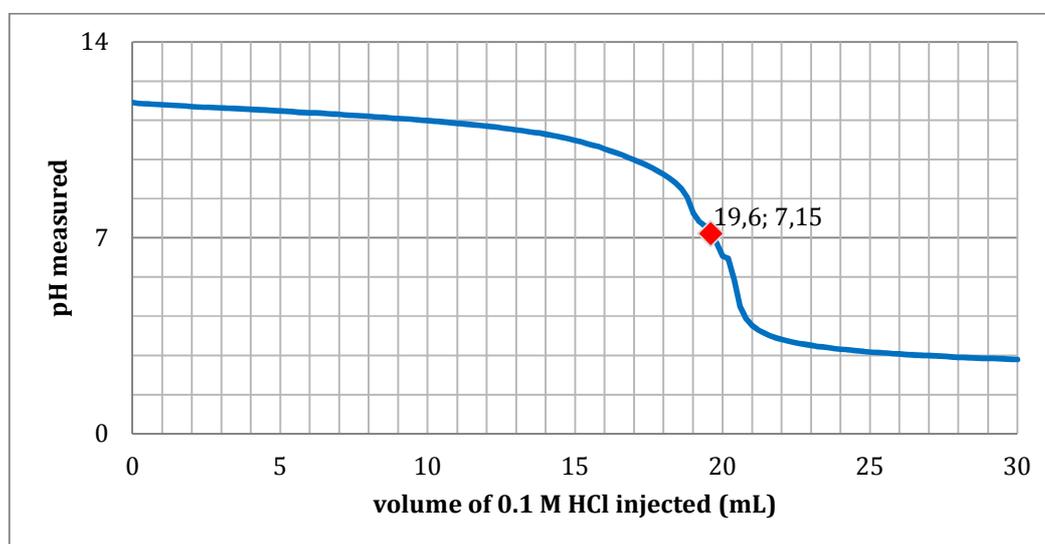


**Figure 4-18: pH measurements obtained by titration of a resin sample subjected to liquid penetrant technique with AMPS gel**

While this increase in capacity is considerable, the inability to pack the resin into a column is a serious issue. The initial expectation that this technique was not sound appear to be true: the hydrogel tends to form out of the pores, which leads to fragments of hydrogel being eroded by the eluent flow used during the packing. These fragments can drift inside the column until they fill the interstitial spaces through which the mobile phase is supposed to flow, thus clogging the column. This might be happening because the amount of monomer solution retained inside the pores of the particles after the first filtration has moved away from the centre of the particles due to the pressure exerted by the vacuum pump: this causes the reaction between monomer and initiator to happen in the proximity of the external surface of the particles, which leads to

formation of hydrogel outside of the pores.

If this was actually the cause of the problem, there could be a few solutions for it, such as producing PS-DVB resin particles with smaller pores and a narrower pore size distribution (cfr. Equation 15); even then none of them would grant that no reaction at all happens outside of the pores, or in the proximity of their end; as was already stated, AMPS hydrogels can have a strong tendency to swelling, meaning that even a small amount of gel sticking outside of a pore can lead to column blocking. This technique was therefore decided to be unfit for the purpose of this research.

## 4.5 Solution flow-through

The solution flow-through technique was designed to overcome the limitations of all previously explored techniques: in particular, it was decided to established better control over the drying step than that achieved in both solution soaking and liquid penetrant technique; furthermore, it was decided to bypass the issue of the polymerization of the hydrogel outside the pores by letting the reaction happen in the pores of resin particles after packing them into the column. This lead to a procedure that can be summarized in the 4 steps shown in Figure 4-19:
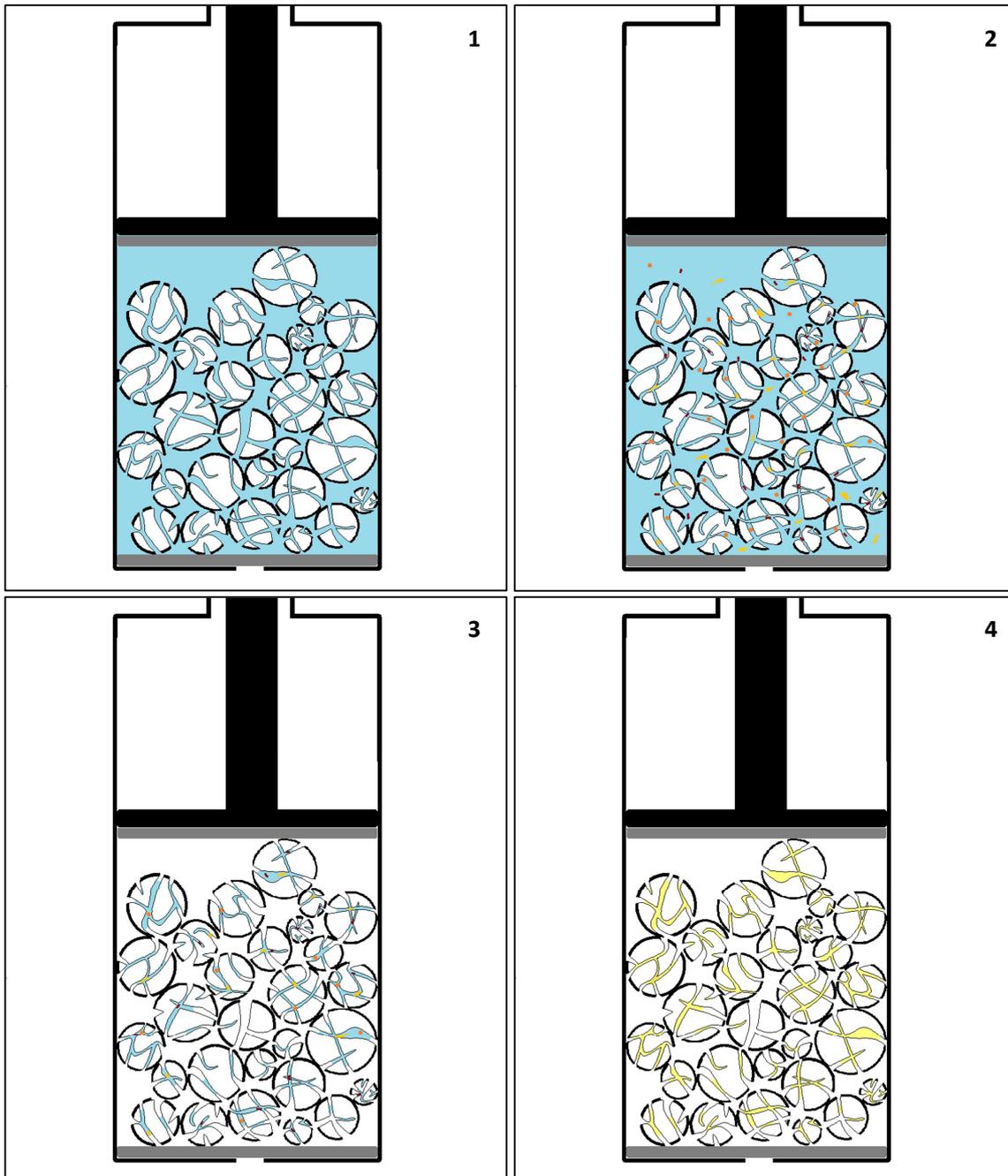
**Figure 4-19: graphic interpretation of the 4 steps involved in the solution flow-through technique: column-packing of the resin (1), injection of monomer solution into the column (2), removal of excess solution (3) and heating of the column at 60 °C for 5 hours (4)**

The critical step in this procedure is the third one: only the excess solution must be ejected, but the rest must be left inside the particles. On the other hand, the distinction between pores and interstitial spaces is not as important as in the other techniques, because the formation of hydrogel outside the pores cannot cause issues during the packing of the resin, since the resin is already packed into a column when polymerization happen. Removal of the excess solution is only necessary to prevent the formation of a continuous, massive layer of solid hydrogel across the cross section of the column that would halt the flow of the mobile phase.

A considerable advantage of this procedure is that the resin particles are confined in a small known volume: since it is required that the columns involved are able to withstand a temperature of 60 °C for 5 hours, only PEEK columns could be used. The ones used in this work had a 4 mm internal diameter and measured 50 mm in length: the internal volume would then be 628 µL. A typical value for the external porosity of a column packed with spherical particles is 35-40% [14]; since the porosity of the PS-DVB particles used in this thesis measured by mercury intrusion was determined to be 83%, the total porosity of the column is approximately 89%. The excess solution is filling the interstitial spaces among the particles: the volume of those spaces is given by the column volume times the external porosity of the column, which in this case is equal to 235 µL. Hence, in order to measure such volume, it was decided to weigh the amount of solution ejected during the drying step.

For the first set of experiments, testing the effect of the pressure of the inlet nitrogen flow during the flushing of the excess solution, only one of the samples could be successfully titrated: the one in which the nitrogen flow had a 400 mbar pressure. When titrated by Stone-Carta method, the sample revealed a capacity of 225.81 µmol/mL column volume. The data from which this result was inferred are given in Figure 4-20.
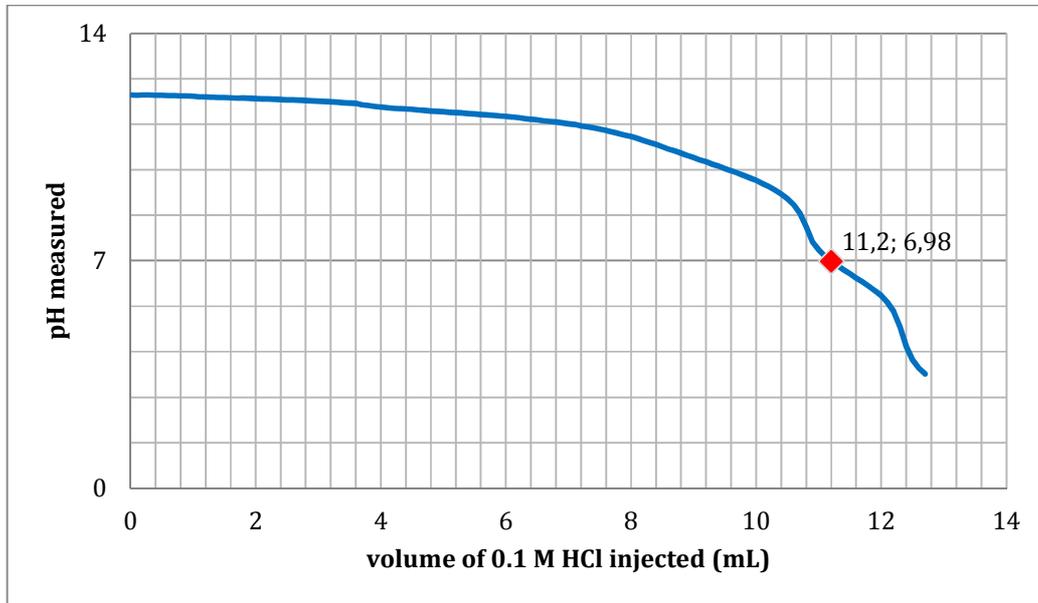
**Figure 4-20: pH measurements obtained by titration of a resin sample subjected to solution flow-through technique with AMPS gel**

The column prepared using this sample was later tested on a HPLC unit, to evaluate its chromatographic performance. 0.1 mg of lysozyme were dissolved in 20 μL of a 20 mM phosphate buffer (pH = 6) and were injected into the column over the course of 1 minute and the recorded chromatogram is shown in Figure 4-21.
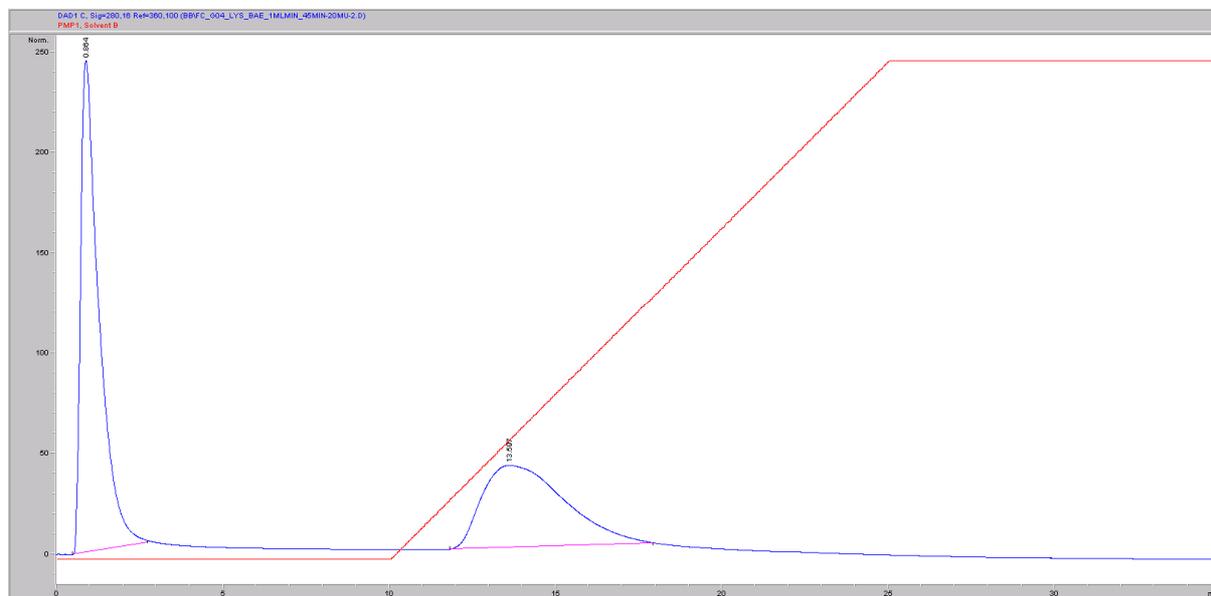
**Figure 4-21: chromatogram for a 20 μL injection of lysozyme on the sample of Figure 4-20**

By integrating the eluted peak, it was determined that 10.80 mg of lysozyme were retained by the column: hence, a capacity of only 1.19 μmol of lysozyme per mL column volume, or 17.20 mg/mL was calculated (i.e. 25% of the dynamic binging capacity of Fractogel resins for the same protein [37]). The difference between the two values measured by the two different titration methods was assumed to be due to the different nature of the charge carriers involved: in other words, the charged species in the hydrogel network are too densely packed, resulting in one single protein occupying many of the sites that can bind each small ion that is injected in the column on a one-to-one basis. This is a known issue in column chromatography, generally referred to as "shielding" of the charges.

The next set of experiments aimed at determining the optimal pressure for the nitrogen flow during the removal of excess solution with more precision. Results are reported in Table 4-2, and a graphical interpretation is given in Figure 4-22: graphical representation of the data presented in Figure 4-22 for a better understanding.

**Table 4-2: collected volume of excess solution as a function of the inlet pressure of the nitrogen flow**

| Nitrogen Pressure (mbar) | 250 | 300 | 350 | 400 | 450 |
|---|---|---|---|---|---|
| Collected volume (mL) | 182.91 | 378.20 | 332.88 | 289.41 | 63.74 |
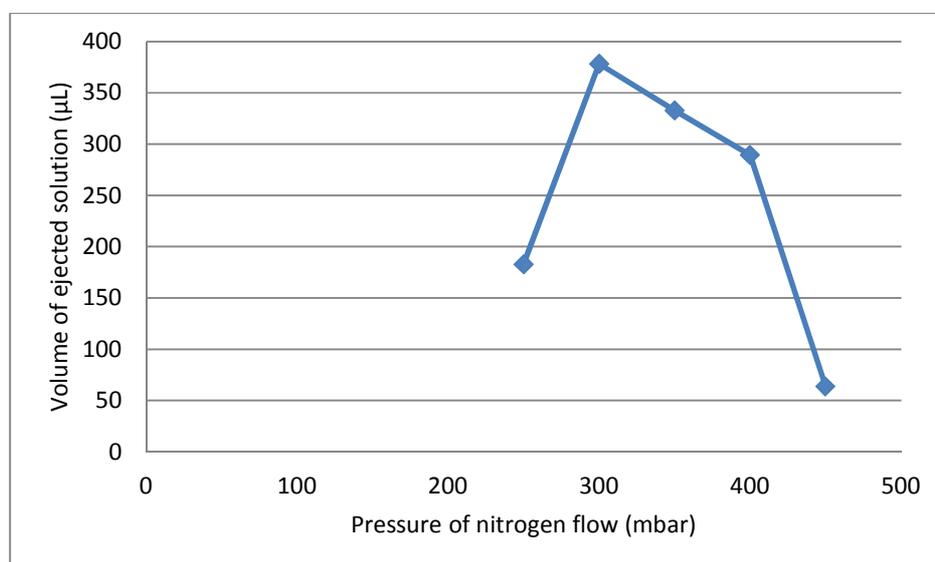


**Figure 4-22: graphical representation of the data presented in Table 4-2**

Since the flow rate of the monomer solution leaving the column was very low for every investigated nitrogen pressure, there was no issue of accuracy. The results indicate that the liquid removal increases at increasing inlet pressure. However, a maximum of removal is quickly established, most probably because of strong channeling at higher pressures. The optimal pressure would be the one resulting in the removal of the external liquid only, while leaving the pores filled with the monomer solution (in average, capillary forces should be stronger inside the pores than among the resin particles). Since the volume of external liquid was estimated around 235 μL, pressure values from 300 to 400 mbar appear to be suitable. Therefore, the pressure which

allowed collecting the target amount in the minimum time was elected to be the optimal pressure, in this case 300 mbar.

None of the specimens made in order to seek the optimal monomer content for the solution to be injected could be titrated, except for the one with the least monomer content (sample HG 4 with reference to Table 3-7): all the other columns clogged at some point during the titration process. Upon inspection of their content, they all revealed a significant volume of particles glued together, nearly at the end of each column. The length of these "plugs" of gelled resin changed in every instance, from approximately 7 mm to 35 mm, which would amount to 14-70% in terms of column length. A picture of one of these plugs is given in Figure 4-23.



**Figure 4-23: gelled resin "plug" found in specimen HG 3**

The plug in sample HG 1 was placed in a 15 mL vial and soaked in a 20% ethanol solution. After only a few seconds, the plug doubled its volume, then it continued swelling at significantly slower rate. It reached a volume of about 15 mL in the end, which would be equal to approximately 24 times the initial volume.

As for the one specimen that could actually be titrated by Stone-Carta method, the resulting capacity for ions was 316.67 µmol/mL column volume.

Regarding the experiments on AETAC gels, it could be easily noted that the sulfonated resin does not need a packing procedure as long and precise as that used for standard PS-DVB resin: the reduced hydrophobicity of the functionalized resin means that its particles are much easier to pack.

An ionic capacity as high as 208.33 µmol/mL column volume was measured by titration of the samples obtained, as can be deducted from Figure 4-24.
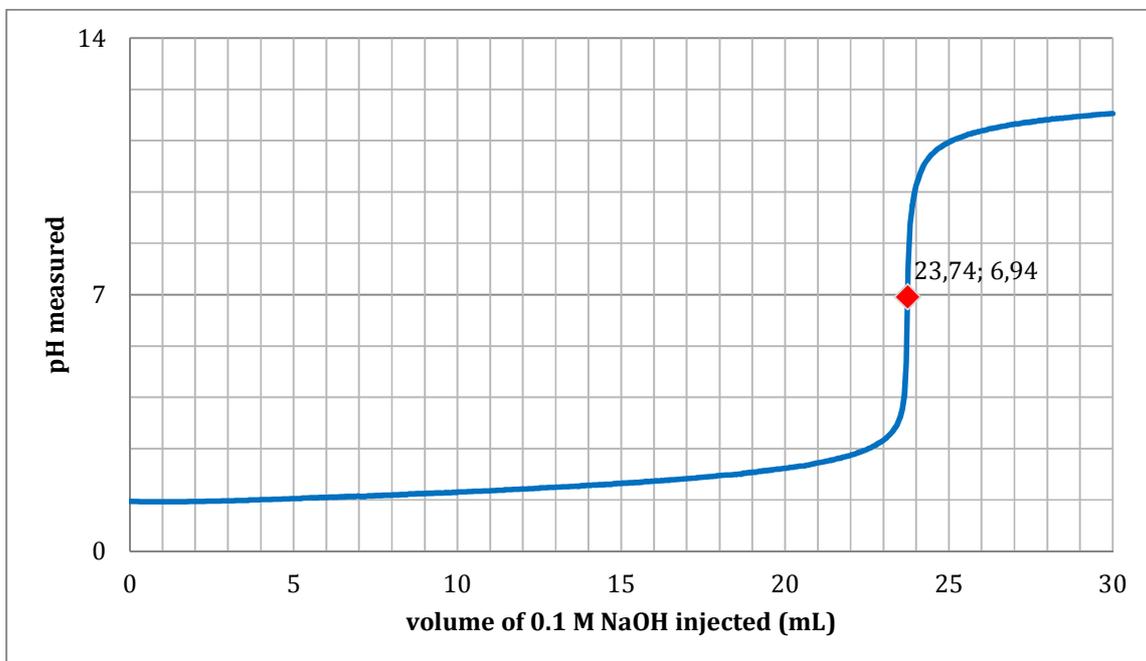


**Figure 4-24: pH measurements obtained by titration of a resin sample subjected to solution flow-through technique with AETAC gel**

This sample was also tested for protein capacity. This time, it was decided to measure it by ultraviolet-visible spectrophotometry: this analysis measures the equilibrium binding capacity of a material, meaning that kinetic effects are neglected. This was done in order to be able to discern whether the reduction in capacity encountered when separating proteins was in fact due to screening effects, or if it was actually an issue with protein diffusion being excessively slow in the hydrogel medium.

The spectra measured by the spectrometer showed a peak at 278 nm for all reference samples: the absorbance of each peak was noted and is reported in Table 4-3.

**Table 4-3: results of UV-Vis analysis for all reference samples**

|  | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| **Albumin concentration (mg/mL)** | 0.5 | 1.0 | 1.5 |
| **Absorbance at 278 nm (AU)** | 0.21 | 0.43 | 0.66 |

It is important to observe that these results seem to indicate a linear dependence of the measurable absorbance on the albumin concentration, as expected. This can be easily seen in Figure 4-25 below.
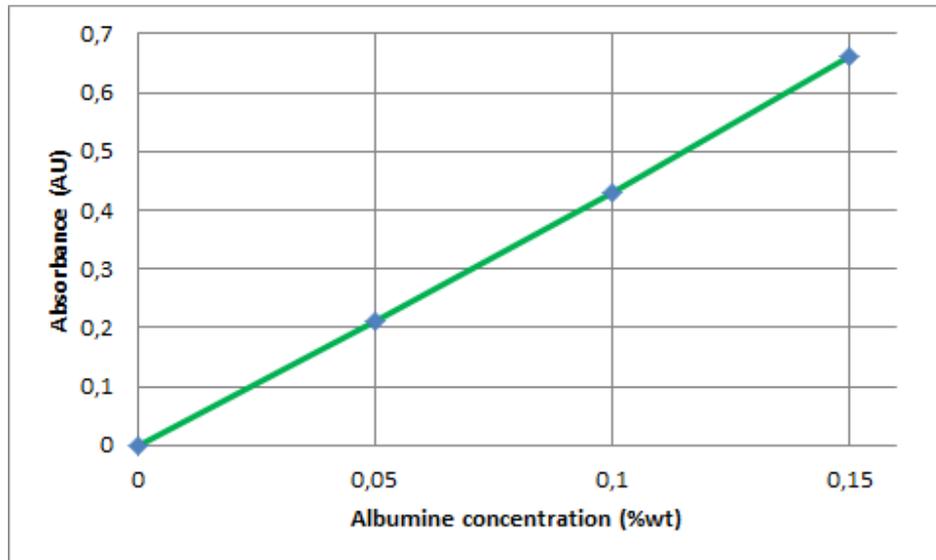
**Figure 4-25: plot of the data reported in Table 4-3**

The sample obtained from HG 2 showed a peak in its absorbance spectrum at the same wavelength, measuring 0.27 AU. Since there is a linear dependence of absorbance on albumin concentration, a proportion can be established between the two parameters of a reference sample (both known) and those of the solution that was separated by HG 2 (for which only absorbance is known): an albumin concentration of 0.64 mg/mL was thus calculated for the sample.

The solution collected by flushing sample HG 2 weighed 42.86 g: given its dilution, it can be assumed that its density is equal to that of pure water, and therefore its volume should be 42.86 mL. By multiplying this volume by the measured concentration, a value of 27.55 mg is obtained: this value is equal to the total amount of albumin present in the solution. The amount of albumin retained by sample HG 2 is given by the difference between the amount dissolved in the original solution (50 mg) and the amount left in the solution after column equilibration, i.e. equal to 22.45 (mg)

So, assuming a molar weight of 66463 g/mol for albumin, it can be said that 0.338 µmol were retained by the stationary phase inside the column. Given the column volume of a Metrosep 50 x 4 column (628 µL), a protein capacity of 0.538 µmol/mL, or 35.75 mg/mL

column volume (i.e. 36% of the capacity of Fractogel resin for a similar protein reported on its producer's website) was measured. Since this value is some orders of magnitude lower than the one that was gained for ion capacity, it can be deduced that the major issue for protein separation using this type of materials is in fact the shielding effect, more than diffusion limitations. This theory is also supported by the fact that the capacities for proteins measured by HPLC and by UV-Vis are similar: static and dynamic binding capacities are significantly different even for commercial materials, and the difference measured for the material produced with the flow-through technique is not much larger; this means that this material is possibly more susceptible to diffusion limitations than most commercial resins for biochromatography, but only so slightly that it can still offer a viable alternative to those other materials.

However, it should be pointed out that the shielding effect can only reduce the capacity for proteins (with respect to the capacity for ions measured by means of titration) so much: a single protein can have many positively charged groups interacting with a stationary phase, typically a few dozens. If the shielding effect was the sole responsible for the difference between the two measured capacities for the material, it would mean that one protein is occupying 500-1000 charges; this is an unusually large value that could only be justified by a high charge density in the hydrogel network: albumin proteins usually have a hydrodynamic radius of about 5 nm [38], so in the volume occupied by one (500 nm$^3$ ca.) there should be 500-1000 monomer units, which would translate to a charge density of 1-2 charges per nm$^3$; assuming an average value of 1.5 charges per nm$^3$ and rearranging dimensions, this value can be translated to 2490.87 µmol/mL of charge density in the hydrogel.

Titration data revealed that the total number of charges present in 1 mL of packed material produced by this procedure is 208.33 µmol. By comparing the charge density of the hydrogel with this value, it can be inferred that 8.4% of the column volume is occupied by hydrogel, i.e. 0.053 mL. This is in contrast with experimental data: given that the bed porosity is 35-40% and the pore volume of the resin measured by mercury intrusion is 83.11%, 0.554 mL is the total volume accessible to the monomer solution after the resin is packed into a column; being that 0.311 mL of solution were collected from sample HG2 during the nitrogen injection step, it is expected that 0.243 mL of the column volume are occupied by hydrogel (neglecting the effect of swelling on its volume).

This seems to indicate that only 22% of the solution left inside the column after nitrogen injection polymerized into a hydrogel. That could be explained by electrostatic interaction between monomer and resin affecting the concentration gradient of monomer in the fluid phase before polymerization: this means that the concentration of monomer in the proximity of the resin is much higher than in the rest of the fluid phase, leading to local polymerization. Note that this idea is consistent with the observation of monomer solutions with 5% monomer concentration (such as those used for sample HG2 and most specimen produced for the solution flow-through technique) not polymerizing in bulk, as reported in section 4.1.2. Another effect that can lead to incomplete polymerization is radical capture by oxygen; the procedure requires refitting the column caps after flushing with nitrogen. During this step, the packing material is exposed to air and oxygen can be absorbed into the liquid phase.

If either of these hypotheses was true, a significant amount of the monomer solution retained inside the pores would not polymerize into a hydrogel after heating, and would be eluted during the titration steps.

If this issue could be solved (e.g. by increasing the monomer and/or initiator concentration in the monomer solution, by decreasing the charge density on the functionalized resin, or by reducing the average pore diameter) and all of the retained monomer solution could be made into a hydrogel, a significant improvement could be expected.

# 5 Conclusions and outlook

In this chapter, an evaluation of all the results that were gathered over the course of this thesis is given. Possible improvements to be applied in future studies are also suggested.

The studied material needs to be improved before it can become interesting for industrial purposes. There are three main issues that were encountered during this work: limited service life, loss of capacity and charge shielding. The specific results of each of the four techniques proposed for producing the desired material are hereby summarized in Table 5-1.

Table 5-1: summary of results and comments about each of the four proposed techniques

|  | Solution soaking | Oil dispersion | Liquid penetrant | Solution flow-through |
|---|---|---|---|---|
| Success in producing a working sample with increased capacity | Yes | Yes | No | Yes |
| Highest ion capacity obtained (μmol/mL column volume) | 360 | 190 | 170 | 320 |
| Reproducibility of the result | No | No | No | Yes |
| Number of eluted column volumes before clogging | 10-50 | 100 (ca) | 5-20 | 500 (ca) |
| Loss of capacity | Yes | Yes | Yes | No |
| Specific issues encountered with produced samples | Not working | Cleaning | Clogging | Charge shielding |
| Possible improvements | None | Devising a more efficient cleaning procedure | Producing resin with narrower PSD and pore size distribution | Producing resin with narrower PSD and pore size distribution |

# 5 CONCLUSION AND OUTLOOK

Although some of the produced samples were promisingly good in terms of capacity, they seemed to lose their properties after processing less than 100 column volumes of protein solution. When the specimens were used to separate ions, they would block after 200-500 column volumes of aqueous solution processed; even before then, whenever it was possible to run multiple measurements for the capacity of the stationary phase, the value seemed to decrease.

Out of the four proposed techniques, the solution flow-through process seems to be the most reliable in producing a composite material with increased capacity, at least with the materials that were used in this study; all columns produced using this procedure showed a significant increase in capacity and, with a careful design of the composition of the hydrogel, it was possible to consistently produce and titrate the material.

One of the major issues that was encountered with the first specimens produced, where columns would block after being injected with a few hundreds of column volumes, could be addressed by reducing swelling of the hydrogel: those columns in which the composition of the hydrogel was designed so that swelling would be minimal did not block even after 1000 column volumes of injection. Minimization of swelling of the hydrogel is therefore thought to be of critical importance.

To reduce swelling by adjusting the composition of the hydrogel, two possibilities exist: the first is to increase cross-linking; the second is to increase the monomer concentration. As it was stated at the end of section 4.1, the effect of increasing the concentration of cross-linker in the monomer solution was quickly vanishing, so that it is effective to add MBAA up to its solubility limit (cfr. section 4.1.5); the amount of monomer present in solution has much less effect at concentrations higher than 10% in weight on the other hand. Therefore, the optimal course of action is to produce 10% monomer solutions and use the maximum concentration of cross-linker allowed by solubility. With the monomers used in this research and MBAA as a cross-linker, a concentration of 15% in weight for the cross-linker was used and found to be working well.

Using such a composition for the hydrogel seemed to solve the issue with the limited service life of the first specimens produced, and also to significantly reduce (if not remove) the effect of loss in capacity that was often encountered with those specimens.

The third problem however proved to be more difficult to solve: shielding of the charges present on the surface of a stationary phase is a well-known issue in chromatography, and it cannot be removed without significantly reducing the overall capacity of the material. To completely suppress shielding, one would have to reduce the density of charges in the stationary phase. This means to either increase the volume occupied by electrostatically charged groups (or by gel, in the present case) while keeping the number of charged groups constant, or to reduce the number of charged groups while keeping the volume constant. While the latter approach has no practical utility (it would reduce the capacity towards ions without affecting the capacity for proteins), the former could actually improve performances of the studied material.

The ideal way to increase the volume occupied by the hydrogel would be to enhance the capillary forces acting on the monomer solution inside the pores before polymerization of the gel. This would ensure that a larger fraction of the total pore volume is actually filled by gel after polymerization, which would in turn increase the total capacity of the material. One way to achieve this would be to reduce the average pore diameter of the resin: In fact, Equation (16) states that as a first approximation the capillary pressure in a porous medium is proportional to the reciprocal of the radius of the capillary containing the fluid; also, for a very narrow pore size distribution the $\lambda$ parameter in Equation (15) tends to 1, which drastically reduces the influence of other material characteristics (i.e. pore size distribution and normalized water saturation) on the capillary pressure in the medium.

It can also be noted that Equation (15) seems to indicate that by broadening the pore size distribution the capillary forces inside the pores could be further increased: this is inaccurate, because doing so would increase the capillary forces inside the medium as a whole, but not necessarily inside the individual pores; in fact, having a broad pore size distribution would mean that large pores would be present as well as narrow ones, and capillary forces would be significantly weaker in those large pores than in the narrower ones, leading to poor liquid retention characteristics of the material, as is highlighted in Figure 5-1: in it two hypothetical pore size distributions (amounting to the same total pore volume) are plotted on the same diagram; the black line divides the pores with a diameter larger than an arbitrary value (0.5 μm), which, according to Equation (16), identifies a certain pressure for which all pores with a diameter larger than that will be emptied. The picture below makes it clear that a larger amount of liquid will be retained

by the resin having the narrower pore size distribution (the volume emptied by the arbitrary pressure is highlighted).
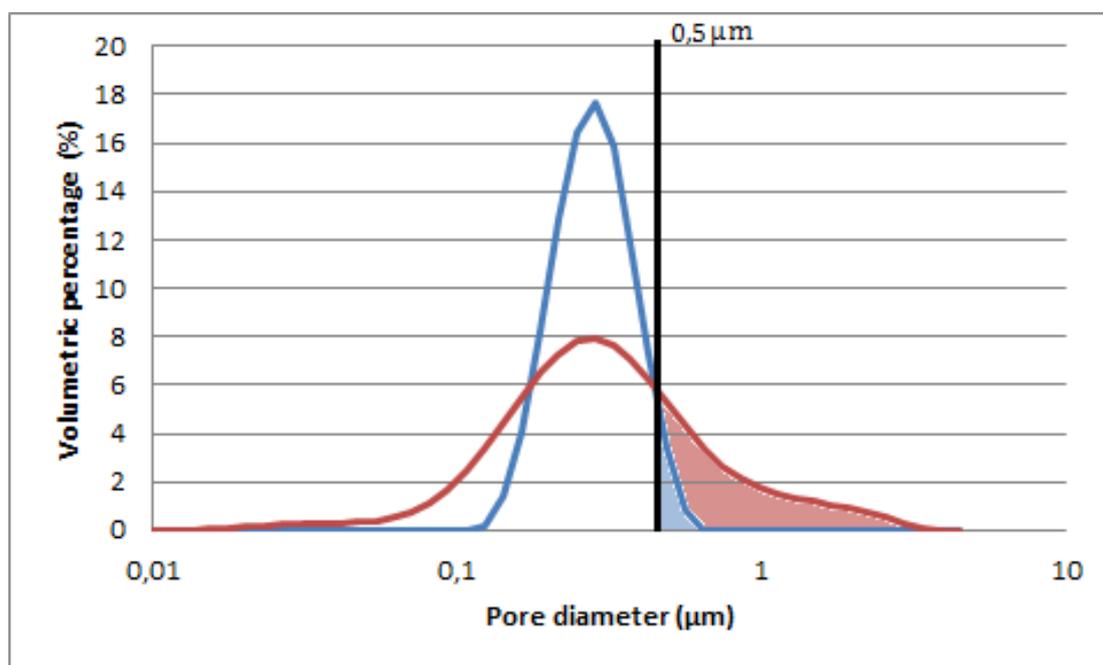


**Figure 5-1: effect of broad (red) and narrow (blue) pore size distribution on volume of retained liquid**

In conclusion, it is believed that further investigations on the presented material could make it suitable for industrial purposes: in particular, it would be advisable to assess the effect of a narrower average pore diameter of the resin particles, and to always minimize swelling of the hydrogel by carefully designing its chemical composition. Considering the values obtained for protein separation on a HPLC device, a capacity comparable with that of popular commercial materials such as Fractogel by Merck Millipore seem to be obtainable, after the suggested improvements to the material have been made.

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