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PARTITIONIONIG OF LYSOZYME AND BACTERIOPHAGES IN AQUEOUS TWO-PHASE SYSTEM

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Abstract

The objective of the thesis is to illustrate the multiple applications of ATPS in the biotechnological industry and to learn the methodologies applied for the partition of biomacromolecules using these systems. To achieve these objectives several experiments have been performed to find the most appropriate aqueous two-phase system to partition the chosen molecules. A methodology has been designed and applied to characterize and perform the separation with the ATPS. The results extracted followed the expected values validating the methodology imposed throughout experimentation. The conclusions are that this is, indeed, a highly appropriate separation option for the tested macromolecules and that the future of biotechnology industry could be highly bound to this mild separation methods.

I. Index

Index of figures

Index of tables

Index of equations

Eq. 1 $\Delta G_m = \Delta H_m - T \cdot \Delta S_m$

Eq. 2 $\Delta w_{12} = w_{12} - \frac{1}{2}(w_{11} + w_{22})$

Eq. 3 $\Delta H_m = \Delta w_{12}\, z\, n_1\, \phi_2$ (3)

Eq. 4 $\chi_{12} = \frac{\Delta w_{12}\, z}{kT}$

Eq. 5 $\phi_2 = \frac{n_2 P_2}{n_1 + n_2 P_2}$

Eq. 6 $\Delta S_m = k \ln W$

Eq. 7 $\Delta S_m = -k(n_1 ln\phi_1 + n_2 ln\phi_2)$

Eq. 8 $\Delta G_m = kT(n_1 ln\phi_1 + n_2 ln\phi_2 + \chi_{12}\, n_1\, \phi_2)$

Eq. 9 $\Delta G_m = kT[n_1 ln\phi_1 + n_2 ln\phi_2 + n_3 ln\phi_3 + (n_1 + n_2 P_2 + n_3 P_3)(\phi_1\phi_2\chi_{12} + \phi 1\phi 3\chi 13 + \phi 2\phi 3\chi 23]$

Eq. 10 $\phi_i = \frac{n_i P_i}{n_1 + n_2 P_2 + n_3 P_3}$

Eq. 11 $\chi_{ij} = \frac{\Delta w_{ij}\, z}{kT}$

Eq.12 $\Delta w_{ij} = w_{ij} - \frac{1}{2}(w_{ii} + w_{jj})$

Eq. 13 $\mu_i - \mu_i^0 = N_A \left(\frac{\partial \Delta G_m}{\partial n_i}\right)_{n_j}$

Eq. 14 $\phi_{2c} = \phi_{3c} = (1 - \phi_{1c})/2$

Eq. 15 $\chi_{23c} = 1/P_2\phi_{2c}$

Eq. 16 $\frac{\mu_4 - \mu_4^0}{kT} = 1 + ln\phi_4 + P_4\left[-\phi_1 + \phi_2\left(\chi_{24} - \frac{1}{P_2}\right) + \phi_3\left(\chi_{34} - \frac{1}{P_3}\right) + \chi_{12}(\phi_1 - \phi_1\phi_2 - \phi 1\phi 3 - \chi 23\phi 2\phi 3\right.$

Eq. 17 $K_4 = \exp P_4\left[(\phi_1^T - \phi_1^B)(1 - \chi_{14}) + (\phi_2^T - \phi_2^B)\left(\frac{1}{P_2} - \chi_{24}\right) + (\phi_3^T - \phi_3^B)\left(\frac{1}{P_3} - \chi_{34}\right)\right]$

Eq. 18 $TLL[\%w/w] = \{([P_{1T}]_W - [P_{1B}]_W)^2 + ([P_{2T}]_W - [P_{2B}]_W)^2\}^{1/2}$

Eq. 19 $-\log(K) = aM^{2/3}$

Eq. 20 $\log K = \frac{\lambda}{R\,T} M^{2/3}$

Eq. 21 $K = K^0 \cdot K_{el} \cdot K_{hphob} \cdot K_{bios} \cdot K_{size} \cdot \ldots$

Eq. 22 Phos = 0,00504751Cond^2 + 0,51873691Cond – 0.1151,416

Eq. 23 Cond = -0,00920888 Phos^2 + 1,65031792 Phos + 0,93762484

Eq. 24 $\mu = \mu_0 \cdot \frac{t \cdot \rho}{t_0 \cdot \rho_0}$

Eq. 25 $\rho = 0{,}00017 \cdot \mathrm{PEG} + 0{,}00079 \cdot \mathrm{Phos} + 0{,}99865$

Eq. 26 $Conductivity = 0{,}00920888 \cdot phosp^2 + 1{,}65031792 \cdot phosp + 0{,}93762484 - 0.002932 \cdot phosp \cdot PEG$

Eq. 27 $\mathrm{Viscosity} = 0.0002998 \cdot \mathrm{PEG}^2 - 0.00022520 \cdot \mathrm{PEG} + 1.67719104 + \frac{0.25}{50} \cdot \mathrm{phosp}$

Eq. 28 $Zero = \mathrm{ABS}(-0{,}00921 \cdot \mathrm{Phos}^2 + 1{,}6503 \cdot \mathrm{Phos} + 0{,}938 - 0{,}002932 \cdot \mathrm{PEG} \cdot \mathrm{Phos} - \mathrm{Cond}) + \mathrm{ABS}(0{,}0003 \cdot \mathrm{PEG}^2 - 0{,}000225 \cdot \mathrm{PEG} + 1{,}677 + 0{,}25/50 \cdot \mathrm{Phos} - \mathrm{Visc})$

Eq. 29 $recovery\ (\%) = \frac{total\ mg\ phages - mg\ phages\ Up\ layer}{total\ mg\ phages} \cdot 100$

Abbreviations

ATPS	aqueous two-phase systems
PEG	polyethylene glycol
Phos	phosphate
Dx	dextran
MW	molecular weight (g/mol)
pI or pH(I)	isoelectric point
CCD	counter-current distribution
PA	phage assay
PFU/ml	plaque-forming units
TEM	transmission electron microscopy
TBS	tris-buffer
MSDSs	material safety data sheets

Symbols

G	molar Gibbs free energy [kJ/mol]
H	molar enthalpy [kJ/mol]
K	partition coefficient
R	universal gas constant [8.314472 J/mol K]
S	entropy [J/K]
T	temperature [ºC or K]
ρ	density [g/cm^3]
σ	interfacial tension [N/m]

Introduction

The aqueous two-phase systems (ATPS from now on) consist on a three component system composed of water and two organic components separating into two phases. Both components are miscible in water but not with each other which is the reason why both phases appear. They have been known for a lot of time, nevertheless it was not until the increasing development of the bioprocesses that ATPS have assumed such an importance in the recent years (Ray, 2008).

This biotechnological industry or "white biotechnology" consists of applying the biological resources into large-scale industrial productions of several products from expensive highly complex molecules to bulk chemicals. Some examples of this industry are the production of chemical products by microorganisms (yeasts and bacteria) or enzymes as catalysts. These catalysts can increase the production's yield or contribute at destroying hazard chemical contaminants (Xu, 2005). The main objectives of these industries are to create easily biodegradable products, to reduce the energy necessary for its production and to generate less waste than in the traditional processes (Condiciones & Trabajo, n.d.).

In this scenario it is when the importance of the aqueous two-phase systems becomes especially important. These systems are a great choice for the separation of product from the associated cell debris (Ray, 2008)("Extraccion y Purificacion de Proteinas a Nivel Industrial," n.d.). About 70 papers are published each year and now there are more than 750 publications about the ATPS and their applications. The advantages of using these systems are the decrease of cost, the scalability and effectiveness (Hatti-Kaul, 2000; Zaslavsky, 1994).

The ATPS were discovered in 1896 by M. Beijernick, a Dutch microbiologist who noticed the formation of two phases when mixing solubilising gelatin and agar on starch in water (Beijerinck, 1897). Albertsson later rediscovered the ATPS in 1956 and wrote a book about the phase separation technique giving publicity to a method that would later be applied in various fields like the mild separation of cell membranes and organelles and for the purification of proteins and enzymes (Walter & Johansson, 1994; Walter, Brook's, 2012).

The challenges met when coming to protein purification are that the main objectives of the process, which are an efficient and economical purification and high purity and quantity, stand in the way of each other. Many times the purity required is imposed by the legislation of each sector (nutritional products, pharmaceutical products and industrial and diagnostic enzymes) and its compliance implies a costlier process. Obviously, the purity requirements of pharmaceutical proteins are the highest, exceeding 99% (Walsh & Headon, 1994).

The common troubles the process design has to face are low feed concentration, complex feed mixtures, presence of critical contaminants, poor feed characterization, denaturation, product stability, purity requirements and difficulty of process optimization. The impurities present in the broth may have similar properties to the product making the separation process harder. Also, because they include so many compounds the physicochemical, thermodynamic and flow properties are unknown. In this subject it is important to distinguish different impurities: critical contaminants that have to be removed and the ones that can be tolerated.

The proteins are thermolabile and sensitive to extreme pH values as well as surfactants and any other compound that can alter its conformation and thus be denaturated. All of these factors the process has to guarantee cause the optimization problem: a lot of variables change the yield, they sometimes interact between each other and also a lot of conditions have to be satisfied.

To sum up, the great difference between this type of separation and other methods is that these systems form a safe environment for biomolecules because of the high amount of water and low interfacial tension. Another advantage is that all the steps mentioned before for the protein partition is that with this kind of liquid-liquid extraction all these steps become one. However, it has not been used in the industrial scale mainly because of the lack of knowledge about the mechanism governing the compound's partition. For these reasons it is really important to study a model that describes the behaviour of these molecules in ATPS (María Paz Cortés Burgos (Universidad de Chile, Facultad de Ciencias Físicas y Matemáticas, 2008).

Scope of work

In order to evaluate the possibilities of the ATPS formed by polyethylene glycol and phosphate for the purification of protein and phages several experiments were performed. The thesis can be separated in two parts. The first step planed was the elaboration of the phase diagrams of different molecular weights of the PEG and different pH of the phosphate buffer to gather an idea of which system would be the most appropriate for the compounds that afterwards were going to be partitioned.

Secondly, a proper characterization of the system was performed before adding any sample to be capable to develop a consistent method for the analytical measurements of the concentration of each phase-forming compound as well as the substance to separate. With these methods several two-phase systems had to be analyzed to elaborate the tie-line length Figure.

After this first part of the investigation, the second objective was to be achieved by performing separations of lysozyme and then of some phages. After a first step of purification by ATPS partition of the samples, a simple precipitation allows to recover the compound of interest.

Finally, a general consideration of the competitive capacity of PEG-salt system for the separation of the mentioned compound in the industrial scale will close the thesis. It is also important to mention that the purpose of this thesis is to give a general idea of the possibility of the application of these systems in large-scale processes and therefore the precision and accuracy are relegated to the background to give more importance to the simplicity of the developed methods.

1. Literature review

1.1. Aqueous two-phase system

Manufacturing of bioproducts typically involves the processing of large, dilute, multiphase fermentation broths which require a significant number of downstream separation trains to obtain a marketable concentration. Traditional methods to purify biomolecules involve several steps, such as dialysis, ionic and affinity chromatography and they are therefore time consuming, costly and not applicable for large-scale operations (Scopes, 1994). Additionally, the composition and volume of the material handled during a given protein purification process continuously change and thus require changing purification steps. In order to combine several features of the early processing steps, liquid-liquid extractions have evolved as an interesting purification alternative. Advantages lie in the simplicity, the low costs and the ease of scale up of these systems. Problems such as protein denaturation and loss of enzymatic activity are less found in these systems because of the mild environment they provide.

The technique is inexpensive and meets requirements of purity and selectivity. The simplicity of the process and the low cost of phase-forming materials make it feasible for large-scale protein purification using appropriate scale-up techniques (Hustedt et al. 1985; Hustedt et al. 1988).

Aqueous two-phase systems provide mild environment with content in water of around 70% (w/w), high selectivity and good results in purification of fragile biomaterials without affecting their chemical or biological characteristics. Partitioning in ATPS is an energy efficient and easily scalable operation with a rapid mass transfer due to low interfacial tension(Pyle, 1990).

These two-phase systems occur by mixing a polymer such as PEG or dextran, usually with a phosphate to a fermentation product. The way in which the phases separate is determined by the structure and molecular weight of the polymer, and its concentration. A good separation is achieved by operating under conditions where the tie-line on the phase equilibrium diagram is long. One of the inevitable problems with a system in which both interfacial tension and difference in density of the phases are low lies in the difficulty of obtaining a sharp separation of the liquid layers (Ray, 2008).

This ATPS, therefore, can appear in several types when mixing two miscible components with water that at higher concentrations become immiscible (*Partitioning In Aqueous Two – Phase System: Theory, Methods, Uses, And Applications To Biotechnology*, 2012):

- One polymer slightly soluble in water can form a two-phase system below a critical temperature (theta Tº is the temperature at which the polymer solution behaves ideally even at high concentrations).
- When two molecular weight fractions of the same polymer are present they can form up to three phases.
- When a low molecular weight solute is added to a solution of polymer in a good solvent some sequential protein separation schemes can be performed. This case implies a reduction in the cost and an easier handling.
- Two different polymers as Dextran and PEG (Dx/PEG) or Dx/Fi can also form two phases. These systems are widely used at the moment for the purification of different materials.
- Complex coacervation occurs when two polymers segments bond strongly to each other. Then in one phase the concentration of polymers is negligible and the other is the polymer-rich phase. This phenomena has is origin in the electrostatic interaction and one example can be Dx/PEG/Fi.

The partition of proteins and other compounds in ATPS is influenced by a large number of parameters including the types of polymer composing the two-phase system, the mean molecular weight of the polymers, the molecular weight distribution of the polymers, the length of a tie-line, the types of ions in the system, ionic strength, pH and temperature.

Several investigations have been published to try to predict the partition even though for the moment usually the conditions for a desired partition have to be determined experimentally because of the several factors that influence the partition. The main problem to establish a scientific prediction of the partition is that the factors usually do not act independently. Nevertheless, Brooks et al. published a thermodynamic interpretation which will be discussed further on.

If the desired separation of different substances in an aqueous two-phase system cannot be achieved in a single step, a multi-stage procedure (countercurrent distribution or CCD) is used. The equipment consists of a series of compartments containing defined volumes of top and bottom phases in which the component partitions itself. Phase-mixing is followed by phase-settling, and moving the top phases to the adjacent bottom phases.

Other contributions to this matter have been made by Hustedt et al. who developed a continuous cross-current extraction unit. Mattiasson and Kaul proposed that a centrifugal separation step could be introduced to reduce the phase settling time. According to Albertsson (1971) about the separation of the ATPS "it depends not only on the differences in density between the phases and their viscosities but also on the time needed for the small droplets formed during shaking to coalesce into larger drops" and therefore takes a significant time.

The comparison of large-scale ATPS partitioning with other separation processes show that partitioning requires larger amounts of chemicals when no recycling was applied. Kroner et al. however, stands that the labour and investment costs are considerable lower (Ray, 2008).

About the problem that surged from the necessity of highly purified dextran for many separations Kroner et al. investigated the use of crude dextran as a cheaper alternative and found only a small change in the partition coefficients for a number of enzymes, all of which were recovered with high yields. Another thing that was found was that the residence time was reduced by the use of the crude dextran, phenomena that derived into a reduction of the operating costs. PEG-salt systems are preferred for large-scale enzyme extractions even though most proteins partition strongly into the salt-rich bottom phase.

Protein isolation and purification are now carried out on a large-scale and Hudstedt and Papamichael have developed a two-stage aqueous two-phase extraction system for the isolation of b-D-Galactosidase form Esterichia Coli. Woodrow and Quirk have investigated the partitioning behaviour of acylamidase using a polyethylene glycol-dextran system. Other extractive bioconversions and fermentations with aqueous-phase systems include the production of toxin factor, production of butanol and acetone, cyclic fermentation production of alcohol, the production of glucose-6-phsphate from glucose, the production of L-methionine from racemic mixtures, the production of glucose from starch, deacetylation of penicillin-G, saccharification of cellulose and the production of ethanol and fermentable sugars from cellulose (Ray, 2008).

The general recommendations settle the composition of the ATPS as far from the critical point as possible and keeping a similar volume of the upper and lower phase. The different studies show that partition becomes more one-sided far from the critical point. Also when decreasing the molecular weight of one polymer the partition of the solute increases into the phase where that polymer has higher concentration. (Walter, Brook's, 2012)

Other considerations to bear in mind are that in case of charged and ionic macromolecules a special attention has to be paid to the pH, which is relative to the solute pH(I), and the type of salt concentrations. When the polymer is uncharged or nonionic is composition is not important. Studies of how the type and salt concentration affect the partition coefficient are

found in several literature (Johansson, 1994; Walter & Johansson, 1994; Walter, Brook's, 2012).

1.2. Choice of ATPS components

The common thumb rule when coming to choose the proper aqueous two-phase system is by means of any of these three options. The first possibility is to select several two polymer or polymer/salt systems to rapidly acknowledge the partitioning characteristics of the materials of interest. Other options are to perform single step partition experiments in a tube or to systematically adjust the two-phase system composition until the best result is obtained.

Other important factors are the requirements the substance for partitioning imposes. Some delicate components may require of a certain tonicity, serum, vitamins, a neutral pH, higher temperatures and other conditions. The influence of all of these factors and their influence in the partition are studied in several investigations and articles.

In this particular case a PEG/phosphate system was chosen for several reasons. The most important one was that these reagents are easily available and affordable. When coming to large-scale biotechnological processes the amounts of material needed are quite large so the cost and availability are of high importance.

Another thing to bear in mind is that some substances are extremely delicate, especially when coming to macromolecules, cells and so on, and can be denaturalized by excessive agitation or acute interfacial tensions found in PEG/salt systems (Walter, Brook's, 2012).

1.3. Theoretical aspects of phase formation

The process of formation a two-phase system from a mixed phase system is extremely complex. The separation depends on the difference of densities of the phases, the viscosities and the interfacial tension of the boundary between the phases. The mechanisms of this process also depend on the distance of the phase composition from the critical point on the phase diagram.

Flory (1941) and Huggins (1941) developed the statistical mechanical treatment which best describes the basic features of phase separation. The theory explains the phase separation describing the basic physics of polymer solutions and provides a qualitative description of the phenomena. Several modifications have been added to try to describe the specific details of particular systems but the original theory is already quite successful in explaining the basics of the process.

What the theory tries is to obtain an expression for the free energy associated to the mixing of pure components (ΔG_m). The Gibbs energy follows the usual relationship:

Eq. 1 $$\Delta G_m = \Delta H_m - T \cdot \Delta S_m$$

Where, T is the absolute temperature, ΔH_m is the sum of the enthalpy of mixing and ΔS_m is the entropy of mixing.

Firstly, only a solution of polymer is provided to better understand the approach the theory makes of the system. The polymer solution is seen as a lattice of sites, which can be occupied either by a solvent molecule or a polymer segment. At the same time, each lattice has z contacting faces with adjacent sites. In this case, the enthalpy of mixing is the individual net enthalpy changes caused by the formation of contacts between polymer segments and solvent when breaking contacts between components in the pure state.

This energy change per contact Δw_{12} is expressed in the following way:

Eq. 2 $$\Delta w_{12} = w_{12} - \frac{1}{2}(w_{11} + w_{22})$$

Where w_{iu} is the energy associated with the contact between both components i and u or the contact between the same component. This energy will be positive if the contact is repulsive or negative if attraction occurs.

For a mixture of a solvent (component 1) with a polymer (component 2) the enthalpy of the mixture can be calculated as the energy change per contact multiplied by the average number of contacts on the lattice.

Eq. 3 $$\Delta H_m = \Delta w_{12}\, z\, n_1\, \phi_2 \; (3)$$

Where z is the lattice coordination number, $n_1$the number of solvent molecules on the lattice and $\phi_2$the fraction of lattice sites occupied by polymer segments. χ_{12} is called the Flory interaction parameter and represents the maximum interaction energy in units of kT that a solvent molecule can have in a mixture when it is completely surrounded by z polymer segments.

Eq. 4 $$\chi_{12} = \frac{\Delta w_{12}\, z}{kT}$$

At the same time, ϕ_2 can be described with the following expression:

Eq. 5 $$\phi_2 = \frac{n_2 P_2}{n_1 + n_2 P_2}$$

Where $n_2$is the number of polymer molecules on the lattice and P the number of segments per polymer molecule. ϕ_i is the fraction of lattice sites occupied by the component i.

The entropy is calculated from the fundamental equation:

Eq. 6 $$\Delta S_m = k \ln W$$

Where W is the total number of distinguishable ways of arranging n_1 solvent molecules and n_2 polymer molecules on the lattice. By rewriting the equation the following expression is obtained:

Eq. 7 $$\Delta S_m = -k(n_1 ln\phi_1 + n_2 ln\phi_2)$$

Combining those equations the free energy of mixing is expressed in the following way:

Eq. 8 $\Delta G_m = kT(n_1 ln\phi_1 + n_2 ln\phi_2 + \chi_{12}\, n_1\, \phi_2)$

The concentration units that appear are the volume fractions ϕ_1 and ϕ_2 occupied by solvent and polymer. The most restrictive feature of the theory is that no volume change can happen when mixing. This assumption is caused by P_2 parameter, which is defined as the number of segments in the polymer of the same volume as a solvent molecule and thus it is assumed that solvent and segments can be interchanged with no change in the lattice.

Once the most simple scenario has been determined, the general equations for a system of i components are the following.

Eq. 9 $\Delta G_m = kT[n_1 ln\phi_1 + n_2 ln\phi_2 + n_3 ln\phi_3 + (n_1 + n_2 P_2 + n_3 P_3)(\phi_1\phi_2\chi_{12} + \phi 1\phi 3\chi 13 + \phi 2\phi 3\chi 23]$

Where component 1 is the solvent and components 2 and 3 are the two polymer species characterized by molecular weights parameters P_2 and P_3.

Eq. 10 $\phi_i = \frac{n_i P_i}{n_1 + n_2 P_2 + n_3 P_3}$

Where n_i is the number of molecules of component i on the lattice. Other equations from the theory are the following:

Eq. 11 $\chi_{ij} = \frac{\Delta w_{ij}\, z}{kT}$

Eq.12 $\Delta w_{ij} = w_{ij} - \frac{1}{2}(w_{ii} + w_{jj})$

The chemical potential of any of the three species can be calculated from:

Eq. 13 $\mu_i - \mu_i^0 = N_A \left(\frac{\partial \Delta G_m}{\partial n_i}\right)_{n_j}$

Where N_A is the Avogadro's number, μ_i is the chemical potential of I when its concentration is ϕ_i and μ_i^0 is the standard state chemical potential of i in the pure state when ϕ_i=1. The derivative is taken at constant temperature and pressure.

All these equations form the theory that explains that when two phases coexist the chemical potential of each component is the same in both phases. This is Figureally expressed when plotting μ_i vs ϕ_i: ϕ_2 varies from 0 to 1 and there have to be two values of ϕ_2 giving the same value of μ_1. With the general equation of Gibbs energy and the following expressions the critical values of ϕ_i and χ_{ij} can be calculated. Assuming that P_2=P_3 and that both polymers are equally soluble in component 1 the critical conditions can be calculated with the following expression:

Eq. 14 $\phi_{2c} = \phi_{3c} = (1 - \phi_{1c})/2$

Eq. 15 $\chi_{23c} = 1/P_2\phi_{2c}$

The conclusions that are extracted by these equations are that the phase separation will result if the interaction energy between the two types of polymer segments is slightly positive or unfavourable. The higher the molecular weight of the polymer the faster the two-phase separation will occur. Another important point is that χ_{12}or χ_{13} are not in the equations so the polymer-solvent interactions are not important when coming to explain the phase separation.

- Polymer partition

To determine the partition coefficient of a polymer added to the two-phase system only another component has to be added to the Flory-Huggins theory. The assumption that is being made again is that all components are equally soluble in the solvent ($\chi_{12} = \chi_{13} = \chi_{14}$).

Eq. 16 $\frac{\mu_4 - \mu_4^0}{kT} = 1 + ln\phi_4 + P_4\left[-\phi_1 + \phi_2\left(\chi_{24} - \frac{1}{P_2}\right) + \phi_3\left(\chi_{34} - \frac{1}{P_3}\right) + \chi_{12}(\phi_1 - \phi_1\phi_2 - \phi 1\phi 3 - \chi 23\phi 2\phi 3\right.$

Eq. 17 $K_4 = \exp P_4\left[(\phi_1^T - \phi_1^B)(1 - \chi_{14}) + (\phi_2^T - \phi_2^B)\left(\frac{1}{P_2} - \chi_{24}\right) + (\phi_3^T - \phi_3^B)\left(\frac{1}{P_3} - \chi_{34}\right)\right]$

Being $K_4 = {\phi_4^T}/{\phi_4^B}$

This expression of the partition coefficient of the protein illustrates some important features of the separation phenomena. First of all, the partition coefficient depends exponentially on the properties of the system and of the component. Also, the molecular weight of the component being separated and the difference in the concentrations of components 2 and 3 between the two phases have a great influence on the partition.

Another factor of influence on the partition is the interaction of the partitioned component with the phase-forming components. The energy of interaction of component 4 with the solvent can have influence if the difference of volume fraction of the solvent in both phases is not negligible. The equation also states that partition increases into the phase enriched with the polymer when its molecular weight is reduced as has been widely observed in many studies (Albertsson, 1971).

There are some general thermodynamic expressions that also allow predicting and analyzing the experimental behaviour to avoid the assumptions of the Flory and Huggins theory of no volume change and not severe dilute solutions. They are based in the chemical potential of the material being distributed and the activity coefficient. However, in this thesis the objective is far from the theoretical application of these equations to predict the system so no further description of other theoretical approaches will be done in this work (For further information: Walter, Brook's, 2012).

1.4. Phase diagram

Phase diagrams are used for the characterization of ATPS; they are the representation of the concentrations at which the phases appear. The binodial is the boundary between the one-phase from the two-phase area. They are usually represented in %w/w which corresponds to the weight of one individual polymer per 100 weight unities of the mixture in percentage.

The vertical axis belongs to the polymer enriched in the top phase. The two points that form the concentrations of the top (A) and bottom phases (B) are connected to show the tie-line. When lowering the polymer concentrations the tie line decreases until it is no longer a line but a single point: that is the critical point (C). When approaching that point the difference between the two phases diminishes. C is defined as the point in the phase diagram where composition and volume of the two phases are equal. The straight lines connecting two nodes at the binodial are called the tie lines.

The phase volume ratio is also related to the ratio of distance between the points representing the bulk composition and each of the phase composition of the phase diagram.

Eq. 18 $TLL[\%w/w] = \{([P_{1T}]_W - [P_{1B}]_W)^2 + ([P_{2T}]_W - [P_{2B}]_W)^2\}^{1/2}$

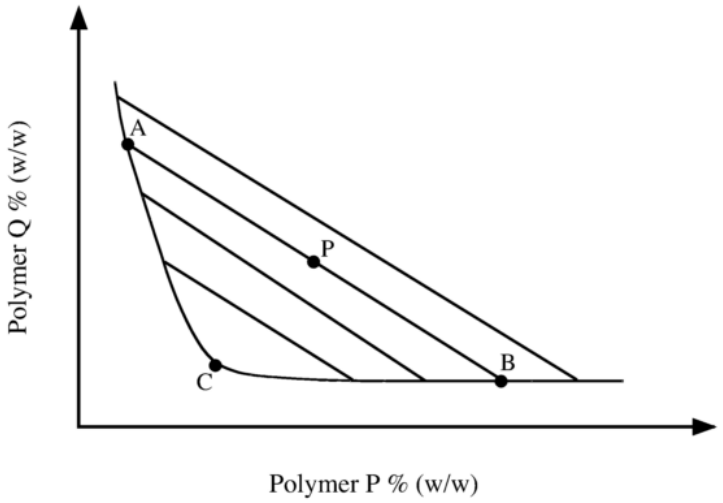

Figure 1. Phase diagram for a two-polymer system. P arbitrary mixing point. A, B concentration of both phases in equilibrium. C critical point.(Nilsson, 2002)

It is important to notice the fact that one can obtain the same partition coefficient (K) with different volume ratios as when moving in the same tie line the partition is maintained changing only the volume ratio. This phenomenon has a clear application allowing concentrating the sample at the same time purification is being made.

1.5. Variables influencing partitioning

The partition behaviour of a bioproduct can be influenced by different parameters like molecular weight of polymer, density, viscosity, interfacial tension, hydrophobicity, ionic strength and ionic composition, addition of affinity molecules, addition of a neutral salt, pH,

temperature and biomass present in the broth. Here the explanation of the effect of the parameter that can be more directly changed follows:

- Effect of salts.

Phosphate and other polyvalent anions change partitioning by shifting the critical point and the whole binodial towards a lower polymer concentration (Bamberger et al, 1984a; Brooks et al, 1984). Some constants to measure this change in the protein partitioning can be found in the Handbook of Chemistry and Physics.

Sodium or potassium phosphates as well as potassium or magnesium sulphates are widely used; also a biodegradable alternative can be the citrate. The higher the valencies of the ions, the lower the concentration of the salt is needed to achieve phase separation. The salts affect the partitioning of charged biomolecules by direct partitioning of negatively charged substances to the PEG rich top phase. By the addition of NaCl the partition can also be altered.

- Polymer molecular weight.

By decreasing the molecular weight the binodial moves to higher concentration, but on the other hand it can be desirable for the viscosity. Therefore, to get a system with the same characteristics with a lower molecular weight more concentration of the polymers will be needed to equal the TLL and equally the interfacial tension.

By lowering the molecular weight of the polymer, the partition towards the phase enriched in this polymer is increased. This is caused because the molecules are smaller resulting in an increase in the water content. Thus, the partition is entropically favoured towards this phase. The partition of low molecular mass substances is not as affected of change in polymer molecular weight as much as substances with higher molecular mass (Nilsson, 2002).

- Concentration of the phase-forming components.

When a substance partitions towards one phase, the partitioning will be enhanced when polymer concentrations are increased, since the chemical differences between the phases increases. A higher polymer molecular weight also increases the difference as well as the TLL.

- Effect of temperature.

By decreasing this parameter the binodial moves towards lower concentrations allowing a longer tie line length for the same system. Also one has to bear in mind that the closer to the critical point the system is the more it is influenced by the temperature, thus it is important to avoid working closer to the critical point to guarantee a robust system. Changes in the temperature are the cause of different slopes of the TLL.

The solubility of thermoseparating polymers in water decreases when the temperature is increased and then the LCST (lower critical solution temperature) decreases. This phenomenon is explained by the increase of polymer hydrophobicity with the increase of the temperature. In PEG/salt systems the hydrophobicity difference between the two phases is larger, allowing a more extreme partitioning. PEG-rich top phase contains around 20-30% (w/w) of PEG plus salt

while the salt-rich bottom phase composition is around 10-15% (w/w) salt. This is caused by the larger number of molecules in salt phase that provokes the partitioning towards the bottom phase to be entropically favoured (Nilsson, 2002).

1.6. Physicochemical properties of the ATPS

The other variables that condition the partition behaviour are here explained. These properties also affect the partition of the bioproduct in the ATPS but they usually are harder to modify as they depend on more than one factor.

- Interfacial tension

The interfacial tension in these systems is quite low (from 0.1 to 100μN/m) and it can only be measured with a couple of methods that are further explained in the pointed literature: rotating drop method (Vonnegut, 1942), pendant drop method (Schürch, Gerson, & McIver, 1981) and sessile drop method (Schürch, 1982).

- Electrostatic Potential Differences

The electroneutrality requirement of each phase causes the salt ions to have different relative affinities for both phases, phenomena that results in a Donnan-type of electrostatic potential difference. It is never recommended to measure the potential of one or other phase to interpolate the other. Because of all the interactions and variables the only important data is the difference of potential between the phases.

The ionic strength is proportional to the square of the net charge and would favour the partition into the phase with higher salt concentration. The increase in the phosphate concentration increases the TLL as well as the interfacial tension, leading to a higher partition.

- Phase viscosities

The viscosity is highly related to the settling time. Higher viscosities correspond to high molecular weight systems and to high concentrations as well. Moreover, the more difference in the densities between the phases the longer the lie-line is. As it was mentioned before, with a higher molecular weight an equivalent ATPS can be prepared with lower concentrations. It is said that two systems are equivalent when the tie-line lengths are the same.

- Hydrophobicity

The difference in hydrophobicity can be measured as the energy necessary to transfer an ethylene group between the phases, by means of the partition of a homologous series of alkyl chain detergents. Albertsson (1971) established that the solutions differ in this property but it has not been proved that it is related to partition.

1.7. Protein partitioning

Protein partition started in the early 50s when Craig searched for two-phase systems useful for protein separation in his countercurrent distribution apparatus. He selected organic solvent-water type with organic substances like butanol-water, ethanol-aqueous salt and several others. He added tricloroacetic acid or p-toluene sulfonic acid to increase the protein solubility. These studies can be found in Morris & Morris (1964). Then it was Albertsson (1958) who rediscovered the ATPS phenomena but with both phases with high concentration of water. By this change he avoided problems such as the denaturalization and precipitation and the extreme partition of the phases.

A correlation was found between the molecular weight of the proteins (M) and the partition coefficient (K), being *a* a constant that depends on the concentration of the polymer:

Eq. 19 $-\log(K) = aM^{2/3}$

Bronsted (1931) formulated a relation applicable to globular proteins which can be comparable, being λ a factor that depends on the system and the protein:

Eq. 20 $\log K = \frac{\lambda}{R\,T} M^{2/3}$

Afterwards, Albertsson and Nyns (1959) performed some experiments with Dx/PEG system and obtained distribution curves with a Craig glass CCD apparatus close to theoretical values, proving that the partitioning is only dependent on the protein concentration. The problems they faced were mainly the difficulty for analyzing proteins in presence of polymers and the removal of polymers from the separated protein fractions.

The partition of macromolecules, unlike the partition of cells and particulates, does never occur on the interface but on the top or bottom layers. The samples dissolved in a phase buffer are added to a suitable concentrated phase system such that polymer dilution due to sample addition brings the system to the desired composition.

One must be especially careful with the agitation, since denaturation and accumulation at the air or phase interface can occur resulting into the loss of material or the apparition of foam. After the equilibration of the phases, aliquots from the top and bottom phases are taken to analyze the concentration of the material distribution. Take care into not only measuring one phase concentration because some material may have been lost through adsorption to tube walls, at the interface or in other step.

Protein purification proposed by Petrides et al. and modified by Nfor et al. (Nfor et al., 2008) can be conducted in four process stages: recovery, concentration, purification and product formulation. Each stage at the same time consists on different steps which change in the case of intracellular or extracellular product and the purity and final form of the product. These separations occur because of differences in the physical and chemical proprieties of the several compounds of the broth (Dreyer, 2008).

Usually the first step accomplishes a separation of the proteins from the non-proteins components. Some examples of physical separation methods used in this step are filtration and centrifugation which could denature the protein. Direct extraction, another separation process also partially purifies the product if the selectivity of the technique is high enough.

The second step is to concentrate the dilute complex mixture of contaminant proteins that was obtained in the recovery by for example performing ultrafiltration or extraction. When the concentration is sufficient the solution can skip this step.

The purification consists of the following sequence: The capture, which implies the isolation, concentration and volume reduction of the concentrated broth, followed by the intermediate purification that achieves the removal of bulk impurities or most of the protein contaminants and finally, polishing. This last step removes trace impurities and protein aggregates. Chromatography is a very common purification method that has proved its extreme efficacy. Membrane filtration and aqueous two-phase extraction are the new alternatives due to the reduction of costs and its applicability in large-scale processes.

Lastly, the product formulation involves getting the protein in the desired final form the consumer demands. Additives are added in this stage to achieve this final form and to provide the product of all the functions the consumer may want.

The protein chosen for the partition is the lysozyme which is a protein that protects the body against bacterial infection by breaking the carbohydrate chains that form the bacteria cell wall. They are commonly found in mucus and tears of the human body to protect the most exposed areas. It has not been medically used because of its structure since the protein is too large to travel between cells. This structure consists of a long active site cleft which is the one that binds to the bacterial carbohydrate chain.

Illustration 1. Real-space refinement of the structure of hen egg-white lysozyme (Diamond, 1974)**.**

1.7.1. Protein properties affecting partitioning.

- Protein surface (Nilsson, 2002)

The protein partition coefficient can be divided into different contributions:

Eq. 21 $K = K^0 \cdot K_{el} \cdot K_{hphob} \cdot K_{bios} \cdot K_{size} \cdot \ldots$

Considering this, it is evident that the hydrophobicity of the protein will have an impact in the global partition coefficient. Several studies have been published about different experiments that try to show how this variable can affect the partition. The hydrophobic contribution to

protein partitioning was studied by chemical modification of proteins with alkyl groups to make the proteins more hydrophobic. The effect of hydrophobicity affecting the partition of several PEG/salt and PEG/dextran systems was determined by plotting the logarithms of the different partitioning coefficients for the modified proteins against their hydrophobicity measured by the hydrophobic interaction chromatography, HIC, obtaining a linear correlation.

- Salt composition.

As has already been mentioned, the requirements for electroneutrality forces anions and cations to partition together. Different affinities of the ions for the two phases will generate an electrochemical driving force between the phases. The anion or cation can use the protein as a counter ion depending of the net charge of the protein, and so direct partitioning of the protein towards one of the phases. Also, it is remarkable that the larger the net change present in the system the larger the salt effect will be.

1.8. Phages partitioning

The phages partitioning could be considered quite similar to the protein partitioning as these viruses can be considered an agglomeration of proteins. The T4 bacteriophages have sometimes been used to perform the trial and error experimentations that would determine the characteristics of an ATPS system adequate to a similar biological system. For example, some of studies of gene therapy with viral vectors that have been published recently explain that a first step of defining the two-phase system with the T4 phage and the BSA were performed before starting with the viral vectors samples, due to the limiting amounts.

The manipulations should be performed under sterile conditions in laminar flow, equally to the protein partition experiments. PEG-salt systems have been proved to be useful for these particulates. The usual procedure is to prepare a concentrated sterile stock solution so when the feedstock or broth is added the concentrations achieved are the desired ones. When the broth has been added the distribution of the phases is achieved by turning the tubes around 20 times in a very gentle manner to avoid the damage of the T4 fiber. A mild centrifugation of about 1200 x g for 5 minutes follows to accelerate the phase separation. Evidently, depending on the volume of the sample the method might change.

One important aspect in this case is that the phage has to preserve its infectivity during the whole process the same way a protein has to maintain its structure to maintain its functions (Negrete, Ling, & Lyddiatt, 2007).

The surface properties of microorganisms determine their interaction with other bacterial and animal cells. Owing to the adaptability of the microorganisms, they can form surface structures which promote or counteract adhesion. Adhesion is a prerequisite for colonization and survival. Anti-adhesive properties are also important in order to prevent engulfment by phagocytizing cells (Walter, Brook's, 2012).

Traditional techniques to measure the surface properties have been electrophoresis and phase partitioning. Nevertheless, these techniques are being replaced by polymer aqueous two-

phase partitioning together with gel chromatography for this kind of analytical measurements (Edebo et al, 1980). However, in general these phase systems have been used mainly for the separation of cells and to a lesser extent for analytical purposes.

1.8.1. Separation of phages with ATPS

Albertsson and Frick (1960), Philipsson et al. (1960), Norrby and Albertsson (1960) and Frik (1961) determined that phages could be separated and purified by aqueous phase partitioning while Bengtsson et al. (1962) and Bengtsson an Philipsson (1963) found that mutants of poliovirus could be fractionated by CCD. This CCD apparatuses have to be used when the sample contains a complex mixture of cells and cellular debris.

When coming to face the virus isolation and detection three problems will appear: separation and concentration of virus from cultures of large volume, separation and characterization of different types of mutants of virus and the effect of antiviral antibodies. Albertsson (1971a, 1974) and Philipsson (1969) provided of suitable choices of ATPS for the partitioning of virus.

1.8.2. Quantification of bacteriophages

Traditionally, there are three methods to quantify bacteriophages: plaque counts on agar plates seeded with the bacteria in which the phages can propagate, a dilution method, where bacterial lysis is used as an indicator of phage presence and measuring the length of time required to lysis a standardized bacterial suspension.

Only the first method (Herelle, 1917) has been useful to determine actual phage titers, and it has been used in numerous studies. The basis of that traditional phage assay (PA) involves the interaction of only one lytic phage particle and a permissive bacterium, which leads to the host bacterium's lysis and the release of new formed phage progeny. When phages and their bacterial host cells are poured onto the surface of nutrient-containing agar supporting bacterial growth, the host cells continue their growth.

In the areas where phages are present, the phage progeny will lyse neighboring bacteria and produce a growing zone of liberated phages, which eventually becomes visible to the naked eye as a clear circular area or “plaque”. The plaques are counted, and the phage concentration/titer is then expressed as the number of plaque-forming units (PFU)/ml.

Although the PA is one of the best methods nowadays for determining phage concentrations, it still has many disadvantages: poor reproducibility and long process of quantification.

1.9. Application of ATPS in industry and limitations

The application that has been largely mentioned until this point has been the large-scale extraction application. This means to use the ATPS as a primary recover step in a biomolecule purification process. As has been mentioned, these systems allow a very straightforward to scale up because the same partition behaviours will be obtained when scaling-up.

Also, the great advantage of this process is that a concentration of the compound can be achieved simultaneously by changing the concentration of the two-phase system forming components along the same tie line to change the volume ratio. After this step has been finished the isolation of the protein from the phase-forming chemicals can be easily done in different ways. For example, using an ion-change chromatography as the protein is changed and the polymer is uncharged. To recycle the salts and the polymers an ultrafiltration can be performed. Another method can be by back extraction.

2. Materials and methods

In the following pages the methodology that has been applied during all experiments will be explained in detail. It is again important to highlight the fact that the instrumentation used is for educational purposes and thus more importance to the simplicity of the methodology was imposed, leading to simple analytical instrumentation. Regarding the methodology, the order in which everything is explained was the same order in which the experiments were performed.

2.1. Materials

The instruments that have been used along the experiments are the ones detailed below:

- Balance: to measure the weight of the phase-forming components a Mettler Toledo AG245 Analytical Balance and a Mettler Toledo PB3002-S DeltaRange Balance were used, depending on the precision required in each measurement.
- pH adjustment: Mettler Toledo MP220 Basic pH-meter was used to measure and adjust the pH of the phosphate solution.
- Conductivity: the conductivity meter used was a Mettler Toledo SevenEasy model AG8603 with a Mettler Toledo InLab 741 ISM conductivity probe with ATC. To maintain the desired temperature (30ºC) a Julabo ED -5 thermostatic bath with open tray was used.
- Viscosity: for the viscosity measurements two Canon-Fenske viscometers (K=0.0079[1], K=0.0084[3]) were submerged inside a water tank with a thermostat open-bath immersion circulator Julabo MD-33. To measure the time a chronometer TFA Dostmann 38.2015 HiTrax was used.
- Density: the density instrument is formed by four modules: the data printer MD2 AP Paar, the AP Paar Density Measuring Cell DMA 602, the Density Meter DMA 60 and the Hetofrig InterMed refrigerator (Heto Brikerød, Danmaark).
- Centrifuge: Heraeus.
- HPLC: Column is strong cation exchange CIM disk monolithic column bearing SO_3 moieties, mobile phase A is 20mM TRIS (pH=7.2) and mobile phase B is 20mM TRIS+0.6mM NaCl (pH=7.2). The settled flow is 1ml/min.

Some other instruments used were the pipette eppendorf Research plus 1 ml, beakers of different volumes, jars of 10 and 15 ml. Also a magnetic stirrer Tehtnica MM-530 was used to dissolve the solutions. The ultrasonic cleaner was used too. Finally the absorbance instrument

was used even though in the end its measures were not useful (Infinite 200 PRO Tecan multimode reader).

The compounds that have been used have been PEG 6000, PEG 8000, monobasic dihydrated sodium phosphate, dibasic sodium phosphate, hydrochloride acid and sodium hydroxide whose MSDSs are found in the Annex.

2.2. Methods

2.2.1. Equilibrium curves

The first step of the experimental work was an approach to the system and therefore the realization of the equilibrium curves. For this purpose, several solutions were prepared to elaborate a curve that separates the two-phase area from the one-phase.

First of all, two concentrated solutions of PEG and phosphate were prepared by measuring certain mass and adding water until a certain volume. After dissolving the solid components, the pH of the phosphate had to be adjusted. In the case of the dibasic phosphate (pK≈12) some chloride acid had to be added to low the pH while for the monobasic phosphate (pK≈4) sodium hydroxide was added. This adjustment was done with the above specified pH-meter and the final volume was measured to calculate the concentration.

Solutions of different concentrations of PEG 6000 and phosphate were prepared to try to find the point of the equilibrium line that separates the one-phase system from the two-phase system. Also its absorbance was scanned to try to see if it could be useful as an analytical method. However, it failed to draw any conclusions so both absorbance and fluorescence analytical methods were discarded. In the Annex different Figures of equilibrium lines obtained under different conditions are attached.

In the Figure 2 it can be seen the equilibrium line of the chosen system for the following experiments. It is so because a neutral pH was needed for the sensitive macromolecules that later on were going to be partitioned and the difference between the PEG 8000 and 6000 were not especially outstanding.

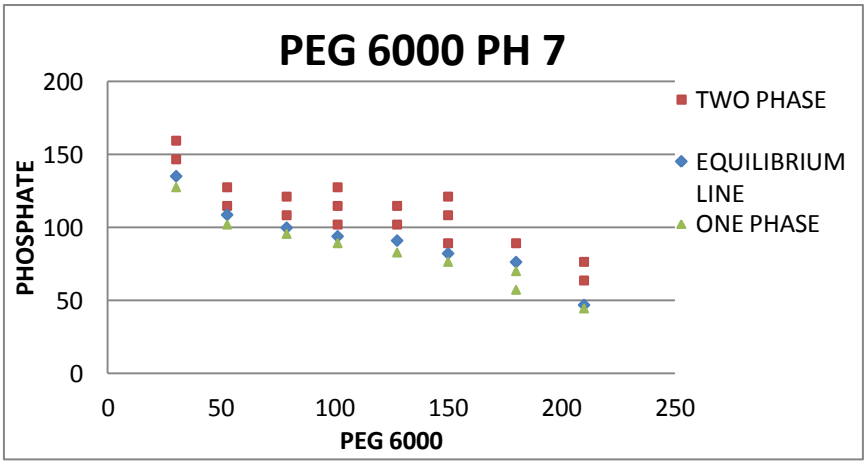

Figure 2. Equilibrium line of the PEG 6000-phosphate system at pH 7.

2.2.2.Analytics of phase-forming compounds

Once the system had been chosen and having its phase diagram the following step was to determine an appropriate method to analytically measure the concentration of the compounds in the two-phase system to analyze the partition coefficient of the phase-forming components as well as the tie-lines. The protocol in these cases is always the same: select an analytical method for each component, elaborate a calibration curve and finally analyze the influence of other components to the measure.

- **Calibration curve for phosphate analytics**

To measure the phosphate concentration the chosen analytical method was the conductivity and the procedure which was followed is described in the following pages. The temperature at which all the measurements of conductivity were done was 30ºC.

First of all, several solutions with different phosphate concentration were prepared to measure its conductivity to get the calibration curve.

Table 1. Solutions prepared of different concentrations of phosphate

mg/ml phosphate	Conductivity	mg/ml phosphate	Conductivity
121,723	87,3	9,738	16,49
81,149	80,0	7,912	13,77
60,862	68,5	6,663	11,79
48,689	59,6	5,754	10,37
24,345	35,0	4,603	8,44
18,727	28,3	3,634	6,72
15,215	24,0	2,869	5,41
12,813	20,8	2,049	3,92
11,066	18,37	1,655	3,19

A linear zone with a positive trend was obtained in the zone of lower concentrations. With that linear equation obtained the concentration of the phosphate in each phase could be calculated. Nevertheless, a non-linear equation was preferred due to the clear non-linear nature of the data and to reduce discrepancies in further calculations. Also, another advantage is that the range in which the measurements can be done is larger. The calibration curve is shown below with both variables isolated.

Eq. 22 $phos = 0.00504751 \cdot cond^2 + 0.51873691 \cdot cond - 0.1151416$

Eq. 23 $cond = -0.00920888 \cdot phos^2 + 1{,}65031792 \cdot phos + 0{,}93762484$

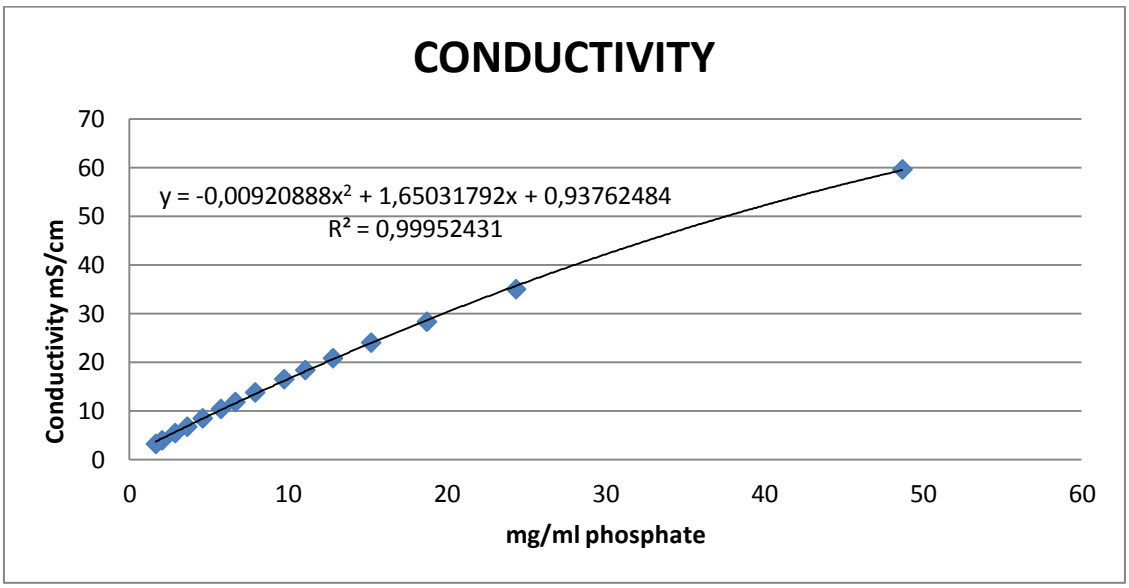

Figure 3. Calibration curve of phosphate concentration.

- Calibration curve for PEG measurement

The measurement of viscosity was the analytical method to calculate the PEG concentration in the samples. It was measured with a Cannon-Fenske viscometer. The temperature (20ºC) was maintained with the thermostat indicated above.

To calculate the viscosity the formula is:

Eq. 24 $\mu = \mu_0 \cdot \frac{t \cdot \rho}{t_0 \cdot \rho_0}$ [mPa·s]

Being $_0$ referred to the reference liquid which is MilliQ water: $\mu_0 = 1.002\ \mathrm{mPa \cdot s}$, $\rho_0 = 0.998203\ \mathrm{g/ml}$ and t_0= 2.145791 min for the viscometer 1 and t_0= 1.976125 for the viscometer 3. The density took part in the equation so it was measured with a DMA 600 in several samples. Afterwards, a calibration curve of the density was done.

First of all, for the calibration curve of the density different solutions with different PEG concentrations were measured with the instrumentation mentioned in the materials paragraph and plotted as seen in Figure 4. A clear trend between the density and the PEG concentration was observed.

Secondly, three samples of each PEG concentration (100, 50 and 20 mg/ml of PEG) with different concentrations of phosphate were prepared. Its density was measured and the results were plotted as seen in the Figure 5 and similar trends were visualized. An average of this trend was calculated.

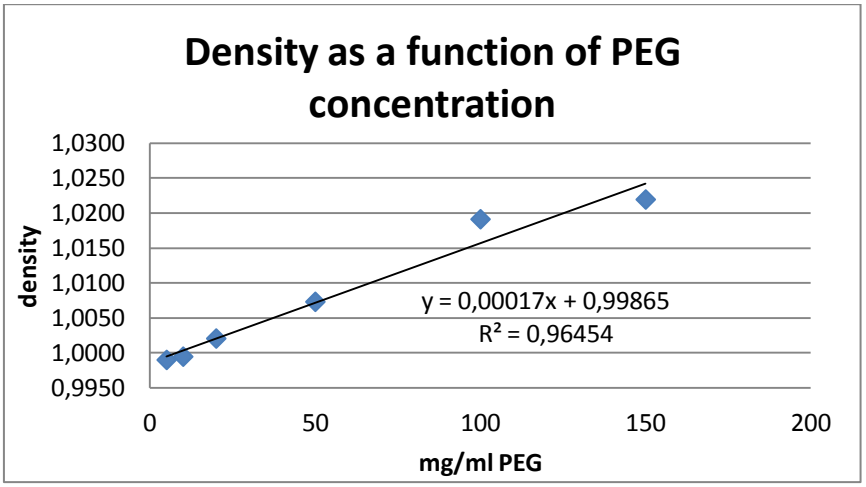

Figure 4. Density calibration as a function of the PEG concentration.

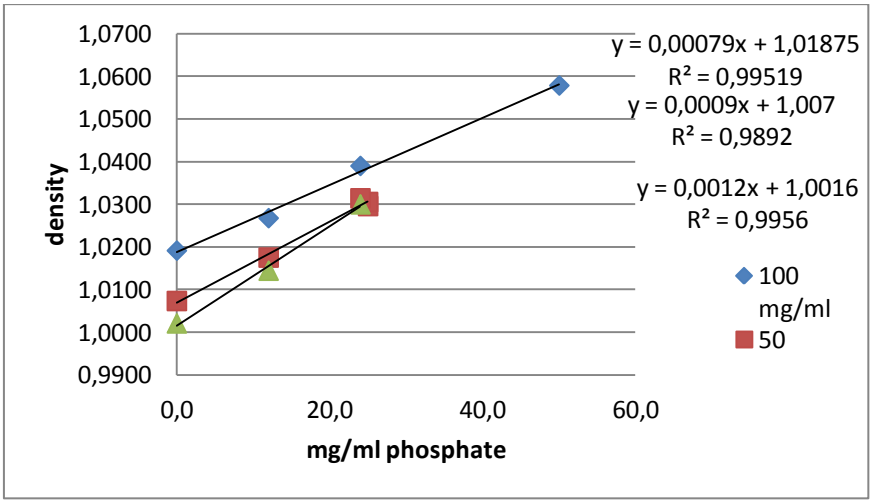

Figure 5. Density calibration: the influence of phosphate in density.

Finally, the combination of both creates the following equation for the calculation of the density and with this equation the densities of all the samples is calculated.

Eq. 25 $\rho = 0{,}00017 \cdot \mathrm{PEG} + 0{,}00079 \cdot \mathrm{Phos} + 0{,}99865$

Illustration 2. Density-meter DMA 60.

Once this equation was achieved the calibration for the PEG was the next objective. Several different concentrations of PEG were prepared to measure its viscosity to get the calibration curve. In table 2 the concentration of the samples can be observed as well as the instrumentation in the illustration 3.

Table 2. Calibration curve samples.

mg/ml PEG	measured time	viscosity
120	11,3743	5,42732
106,7	9,6153	4,58803
96	8,5058	4,05862
87,3	7,6382	3,64461
73,8	6,4167	3,03174
64	5,5912	2,64171
53,3	4,8440	2,28869
45,7	4,5167	2,13403
35,6	3,8483	1,81826
26,7	3,2195	1,50609
21,3	3,0038	1,40520
17,8	2,6364	1,23332
27,5	2,9865	1,51704

Illustration 3. Cannon-Fenske viscometer.

The results of the calibration are seen below. Also some samples with 50 mg/ml of phosphate are plotted so that one can appreciate the differences that appear in the measurements because of the presence of the other phase-forming component.

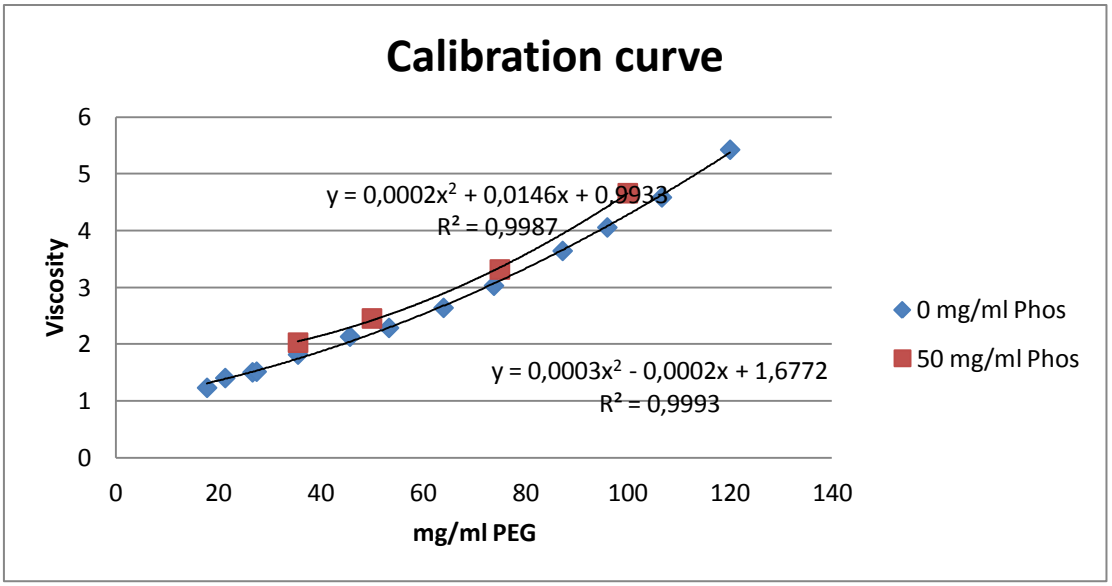

Figure 6. Calibration curve for PEG and change of the calibration curve by the influence of phosphate.

- Effect of PEG in conductivity

To analyze if the presence of PEG would have some impact in the measured conductivity the samples described in the table 3 were prepared. The idea was to maintain a concentration of phosphate and compare the conductivity measured with different amounts of PEG.

Table 3. Samples prepared to compare different the same concentration of phosphate with different concentrations of PEG and its conductivity.

	mg/ml		mS/cm		mg/ml		mS/cm		mg/ml		mS/cm
	PEG	PHOS			PEG	PHOS			PEG	PHOS	
P1	150	12	11,22	P7	150	24	19,72	P13	150	0	0,0562
P2	100	12	13,01	P8	100	24	23	P14	100	0	0,0505
P3	50	12	15,15	P9	50	24	25,7	P15	50	0	0,0322
P4	20	12	16,21	P10	20	24	28,3	P16	20	0	0,01658
P5	10	12	16,93	P11	10	24	29,5	P17	10	0	0,01045
P6	5	12	17,18	P12	5	24	30,3	P18	5	0	0,00557

The conductivities of the samples indicated above are plotted in the Figure 7. As it can be appreciated, a decreasing trend can be appreciated when increasing the PEG concentration. For this reason a compensation factor had to be added to the calibration curve equation.

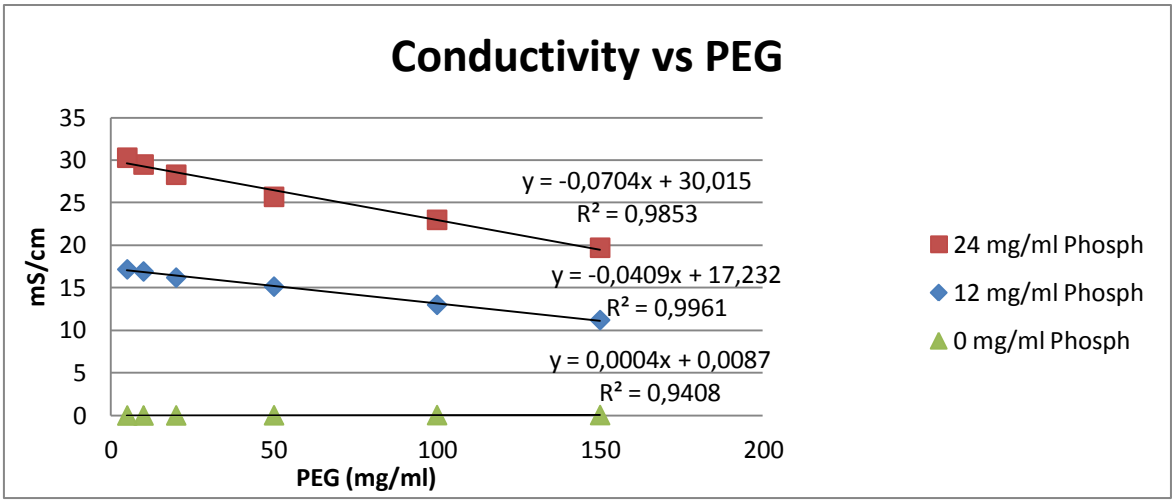

Figure 7. Comparison of the conductivity values obtained with the theoretical values we should expect by the conductivity and the calibration curve.

The theoretical values plotted in Figure 7 were calculated with the calibration curve equation and the compensation factor that were obtained with these experiments.

Table 4. Compensation factor data.

PEG (mg/ml)	decreasing slope
0	0
12	-0,0409
24	-0,0704

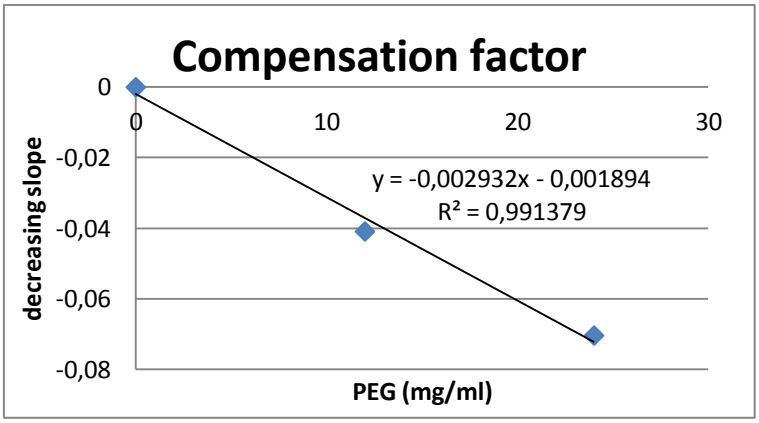

Figure 8. Compensation factor.

Therefore, the compensation factor is the trend shown in Figure 8 (-0.0029·PEG) but as seen before it also depends on phosphate so the factor added to the conductivity calibration curve is finally -0.0029·PEG·phosphate. The final calibration curve for the phosphate is expressed in the equation 26.

Eq. 26 $Conductivity = 0{,}00920888 \cdot phosp^2 + 1{,}65031792 \cdot phosp + 0{,}93762484 - 0.002932 \cdot phosp \cdot PEG$

- Effect of phosphate on viscosity

As it was seen in the Figure 6 of the calibration curve of viscosity, an average of the distance between both curves has been approximated as 0.25. This distance divided by the concentration of phosphate that was present in those solutions gives the proportion in which the distance between the curves happens. By multiplying this factor by the phosphate concentration of each sample the compensation factor is obtained.

Therefore, the calibration curve that is left is the following:

Eq. 27 $$\text{Viscosity} = 0.0002998 \cdot \text{PEG}^2 - 0.00022520 \cdot \text{PEG} + 1.67719104 + \frac{0.25}{50} \cdot \text{phosp}$$

- Methodology of calculation

In the end, the best approximation that could be done with the experimental data after measuring many samples leads to obtaining these two equations. The problem that arises next is that both equations depend on two variables and thus the system is non-linear. To solve this system the procedure chosen is to create a matrix with different concentrations of PEG and phosphate.

The first step is to build the matrix so that each cell has a certain value of PEG and phosphate concentration assigned. Then, a range from 0-200 mg/ml with a step of 1.5 mg/ml of concentrations of PEG in the first column and a range of the same step of concentrations of phosphate in the first row of the matrix are placed.

The second step is to calculate in each cell the solution to the following equation searching the zero. Each cell has a concentration of PEG and phosphate assigned, as was mentioned, so by substitution of the concentration of the phase-forming components in the equation a number is calculated.

Eq. 28 $$Zero = \text{ABS}(-0{,}00921 \cdot \text{Phos}^2 + 1{,}6503 \cdot \text{Phos} + 0{,}938 - 0{,}002932 \cdot \text{PEG} \cdot \text{Phos} - \text{Cond}) + \text{ABS}(0{,}0003 \cdot \text{PEG}^2 - 0{,}000225 \cdot \text{PEG} + 1{,}677 + 0{,}25/50 \cdot \text{Phos} - \text{Visc})$$

Being, Cond the conductivity measured, Visc the viscosity measured and the zero the number calculated in each cell of the matrix for different PEG and phosphate values to try to find the minimum.

The advantage of this method is that all the possibilities are taken into account and the whole possible rank is tested. Once the first approach is done with the matrix, a second approach is done in the rank where the best solution was found with a step of 0.01 approximately to find a solution of the equation as precise as possible. The order of the solution of the previous equation is about 0.001.

This calculation has to be made for each phase of the sample separately and only by changing the conductivity and viscosity of the sample tested in the excel sheet prepared for this matter the whole matrix changes its results. After the values of PEG and phosphate concentration of

the samples tested are calculated, to obtain the real concentration of the original two-phase system the dilution factor of each measurement has to be taken into account.

- Two-phase system samples

Once the calibration curves were prepared some two-phase system samples were prepared. They were prepared by mixing two concentrated solutions of PEG 6000 and dibasic sodium phosphate. Then, each phase was separated by extracting the lower phase with the pipette. Therefore, the only volume measured in this particular experiment was the volume of the lower phase, being the other one calculated.

Table 5. Samples of two-phase systems.

	mg/ml PEG	mg/ml phosphate	ml UP	ml DOWN		mg/ml PEG	mg/ml phosphate	ml UP	ml DOWN
3	46,3	190,1	1,125	8,875	23	152,1	102,9	6,95	6,7
7	77,0	155,3	2,44	7,56	24	60,0	100,0	2,3	7,61
9	92,4	155,3	2,79	7,21	25	60,0	60,0	3,08	6,82
14	135,7	116,6	4,4	5,6	26	40,0	60,0	2,15	7,73
15	143,7	109,3	4,8	5,2	H	46,0	150,0	1	6,5
20	30,4	158,8	0,6	13,73	I	77,0	140,0	1,4	5,95
21	76,0	123,5	3,21	11,5	J	60,0	100,0	1,5	6,15
22	106,5	141,1	4,18	10,77	K	88,7	107,5	1,8	5,53

- Conductivity

Once the phases were separated, a dilution had to me made in order to reduce the conductivity to make sure the concentration was in the linear zone.

In the following table the dilution factors are shown as well as the conductivity measured:

Table 6. Conductivity measurements and dilution factor applied.

	DILUTION FACTOR		CONDUCTIVITY	
	UP	DOWN	mS/cm UP	mS/cm DOWN
3	0,2195	0,1498	20,91	37,10
7	0,3789	0,1472	23,2	30,7
9	0,4109	0,1464	23,4	34,4
14	0,5238	0,1414	20,9	25,8
15	0,5455	0,1398	20,3	25,3
20	0,0789	0,1667	6,77	29,3
21	0,3910	0,1667	16,9	25,6
22	0,5110	0,1667	25,5	27,7
23	0,6347	0,1667	25,4	24,4

24	0,3151	0,1964	5,48	32,5
25	0,4350	0,2222	8,61	23,7
26	0,3007	0,2222	9,5	23,9
H	0,1333	0,1667	16,58	26,5
I	0,1867	0,1667	14,07	24,2
J	0,2000	0,1667	14,06	22
K	0,2400	0,1667	15,03	18,27

- Viscosity

After the conductivity was measured, the viscosity of the samples mentioned before were measured.

Table 7. Viscosity results of the two phase systems.

	DILUTION FACTOR		TIME MEASURED		VISCOSITY CALCULATED	
	UP	DOWN	UP	DOWN	UP	DOWN
3	0,6308	1,0000	5,218	4,589	2,8332	2,4804
7	0,7630	0,8954	3,808	3,614	1,8727	2,0789
9	0,6936	0,7062	3,685	3,182	1,9635	1,8556
14	0,7368	0,6512	4,342	3,037	2,1114	1,5869
15	0,7458	0,6341	4,436	2,964	2,3389	1,5473
20	0,0789	1,0000	3,073	4,113	1,5159	2,1440
21	0,3910	1,0000	3,358	3,615	1,7754	2,0222
22	0,3299	1,0000	8,882	4,577	4,3431	2,3740
23	0,6347	1,0000	9,360	3,346	4,5536	1,8645
24	0,2473	0,8839	3,442	4,621	1,6507	2,3979
25	0,3392	0,8721	3,308	3,185	1,7265	1,7405
26	0,2350	0,8855	2,978	3,297	1,4391	1,8024
H	0,1333	0,6842	4,146	3,404	1,9360	1,5897
I	0,1867	0,6263	5,529	3,070	2,8034	1,5567
J	0,2000	0,8200	5,071	2,942	2,3680	1,4919
K	0,2400	0,5821	5,950	2,990	3,0172	1,3962

With the values of conductivity and viscosity of each phase of each sample introduced in the matrix explained in the methodology of calculation with the equation 29. Afterwards, the dilution factor is reversed and then the following concentrations were obtained.

Table 8. Concentration of two-phase system samples calculated from the calibration curve.

	UP		DOWN	
	PEG (mg/ml)	PHOS (mg/ml)	PEG (mg/ml)	PHOS (mg/ml)
3	432,828	68,379	18,800	125,510
7	209,616	44,869	25,129	146,063
9	91,654	38,331	109,327	163,836
14	78,556	26,651	193,194	207,193
15	110,133	26,492	197,419	120,972
20	45,980	42,813	31,120	126,000
21	72,125	27,878	33,300	106,800
22	281,264	40,841	44,960	120,840
23	152,040	33,086	19,000	97,680
24	61,057	9,204	54,194	125,756
25	39,209	11,034	30,386	72,000
26	39,153	17,958	25,298	72,000
H	189,000	79,800	10,377	104,400
I	191,786	48,214	15,008	100,800
J	230,000	46,000	35,732	88,800
K	251,250	42,917	46,383	73,200

To have an idea of the accuracy of the calculated concentrations a %error was calculated from the mass balance. The comparison was done between the theoretical total mass of each phase-forming compound (calculated as the concentration multiplied by the total volume) and the calculated measured analytically mass calculated as the sum of the concentration of each phase multiplied by its volume. In table 9 the %errors are shown.

Table 9. % Error calculated for each two-phase system sample detailed before.

	PHOS			PEG		
	THEORETICAL	MEASURED	%ERROR	THEORETICAL	MEASURED	%ERROR
3	1900,82	1190,83	37,35	462,70	653,78	-41,30
7	1397,51	1213,72	-15,14	693,04	701,44	1,21
9	1397,51	1288,20	7,82	831,85	1043,96	-25,50
14	1166,35	1277,54	-9,53	1221,07	1427,53	-16,91
15	983,98	756,21	23,15	1293,10	1555,22	-20,27
20	2047,52	1755,67	14,25	435,89	454,87	-4,35
21	1634,75	1317,69	19,39	1006,76	614,47	38,97
22	1898,76	1472,16	22,47	1591,63	1659,90	-4,29
23	1264,12	884,41	30,04	1868,43	1183,98	36,63
24	991,00	978,17	1,29	594,60	552,85	7,02
25	594,00	525,02	11,61	594,00	327,99	44,78

26	592,80	595,17	-0,40	395,20	279,73	29,22
H	1012,50	758,40	25,10	345,00	256,45	25,67
I	926,10	667,26	27,95	509,39	357,80	29,76
J	688,50	615,12	10,66	413,10	564,75	-36,71
K	709,18	482,05	32,03	585,29	708,75	-21,09

In table 10 it can be appreciated that the average error for phosphate measurements is around 15% with a standard deviation of around 15 while the PEG measurements have less %error but two times the standard deviation of the phosphate measurements.

Table 10. Statistics information of the average and standard deviation of the %error data.

	PHOS	PEG
Average value of %error	14,88	2,68
Standard deviation	15,15	28,38

- Tie Line Lengths

With the calculated concentrations of both phases of the samples the tie line were plotted in figure 9.

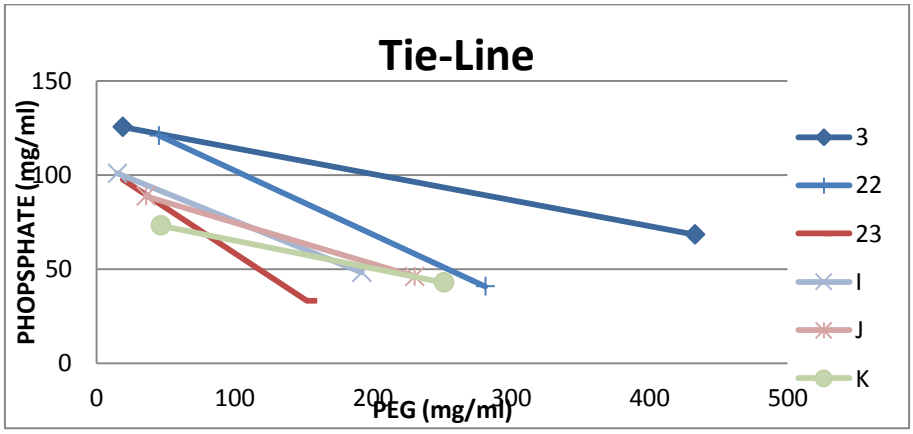

Figure 9. TLL of samples 3, 22, 23, I, J, K.

- Partition of lysozyme

The analytics method of the lysozyme was the HPLC with the previously mentioned components. First of all, a calibration curve was created with a concentrated solution of lysozyme of 2mg/ml and its dilutions. Five samples were measured and with the X-Chromgate software the area of the peaks was determined for each sample.

Table 11. Calibration curve for HPLC measurements data.

Sample	Area HPLC	mg/ml lysozyme
1	11821942	2,000
2	6759433	1,000
3	4117157	0,500
4	2678459	0,250
5	1040800	0,125

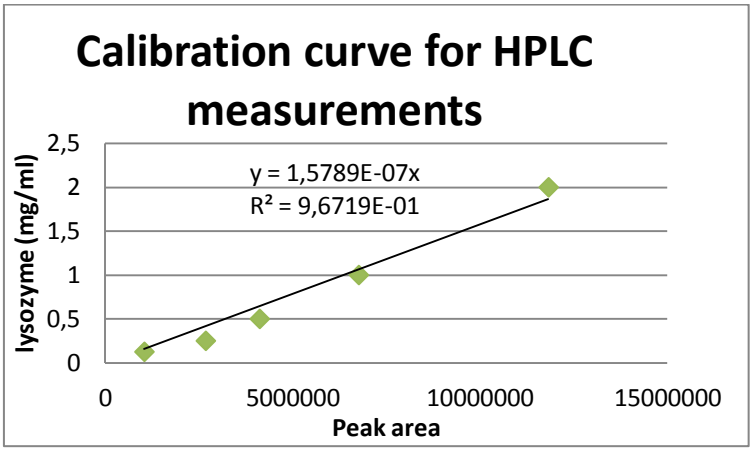

Figure 10. Calibration curve for HPLC.

To analyze the partition of this enzyme in aqueous two-phase system the samples were prepared measuring different weights of solid lysozyme and then dissolving it with a concentrated solution of PEG. Then the correct amount of a concentrated solution of phosphate and water were added to get the desired two-phase system.

The name 7, 22 and H explain the composition of the two-phase system. These three ATPS were selected to perform this experiment because of its TLL and to study very different compositions. The letters A, B and C are referred to the composition of lysozyme, being A for a composition of around 0.75 mg/ml, B for a composition of around 1.22 mg/ml and C for a composition of 2.22 mg/ml.

The samples were centrifuged to separate the phases in a quicker way and the phases were separated into different flasks measuring their volumes with a pipette. Firstly, the conductivity and viscosity were measured with the mentioned protocol and then the HPLC measurements were performed to measure the lysozyme concentration.

Table 12. Samples prepared to measure the lysozyme partition.

	CONCENTRATION (mg/ml)			VOLUME	
	PEG	PHOSP	LYSOZYME	UP	DOWN
7A	77	155	0,75	2,20	7,60
7B	77	155	1,23	1,70	7,60
7C	77	155	2,22	1,80	7,50
22A	106	141	0,72	2,95	6,75
22B	106	141	1,23	2,60	6,50
22C	106	141	2,23	2,80	6,78
HA	46	150	0,72	1,34	8,40
HB	46	150	1,22	1,36	8,00
HC	46	150	2,2	1,15	7,95

Table 13. Results of the conductivity and viscosity measurements.

	DILUTION FACTOR COND		COND		DILUTION FACTOR VISC		VISCOSITY	
	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
7A	0,440	0,132	25,30	29,30	0,244	0,792	3,668	1,990
7B	0,340	0,132	21,50	29,30	0,189	0,792	3,345	1,878
7C	0,360	0,158	21,40	32,40	0,200	0,789	3,343	1,960
22A	0,590	0,129	25,00	28,10	0,328	0,771	5,130	1,922
22B	0,520	0,113	22,80	24,30	0,289	0,565	4,387	1,610
22C	0,560	0,115	25,00	24,10	0,311	0,576	4,729	1,619
HA	0,268	0,135	17,50	27,50	0,149	0,808	1,834	1,834
HB	0,272	0,133	18,55	28,30	0,151	0,800	2,325	1,886
HC	0,230	0,133	16,34	29,20	0,128	0,799	2,135	1,970

Table 14. PEG and phosphate concentration in the two-phase system samples in both phases.

	PHOS CONCENTRATION		PEG CONCENTRATION		LYSOZYME AREA HPLC	
	UP	DOWN	UP	DOWN	UP	DOWN
7A	45,182	155,368	326,495	33,726	6046983	217113
7B	48,529	152,640	387,000	23,621	9959722	210601
7C	44,333	145,920	365,300	30,400	12811888	264273
22A	35,966	150,500	323,390	29,296	5197847	251223
22B	35,096	138,619	324,831	8,811	6662993	286728
22C	37,125	136,130	319,821	15,985	14749956	313677
HA	41,455	137,540	126,940	18,596	7818369	274763
HB	46,250	145,275	294,154	24,500	4894637	471594
HC	46,522	153,268	290,191	32,090	10709795	419015

Table 15. Calculations of concentration of lysozyme in each phase, partition coefficient, recovery and TLL.

	mg/ml Lysozyme				
	UP	DOWN	$K_{LYSOZYME}$	Recovery	TLL
7A	3,472	0,043	80,179	-1,844	312,8176
7B	7,400	0,042	176,186	-2,282	377,999
7C	8,991	0,053	170,104	27,102	349,9684
22A	2,226	0,051	43,284	8,810	315,609
22B	3,237	0,080	40,414	31,574	332,5442
22C	6,654	0,086	77,326	16,450	319,5604
HA	7,370	0,054	137,211	-37,165	144,8127
HB	4,546	0,093	48,842	49,322	287,262
HC	11,764	0,083	142,065	38,508	279,3041

As it can be appreciated in table 15, the partition coefficient is quite high and the lysozyme clearly concentrates itself in the upper layer of the two-phase system. In the lower phase the concentration calculated is negative and really close to zero so it is considered to be negligible and, therefore all the lysozyme goes to the upper layer and the amount that is lost can be in the interface or lost during the whole process. The average of the partition coefficient is 101.735 and the standard deviation is 54.929. The recovery, on the other hand is of 14.5 on average and its standard deviation is 26.2.

Another parameter that allows determining if the experiment was successful is the recovery of the separation, which was calculated with the equation 29. Also the TLL was calculated to see if it had correlation to any of the parameters that define the separation method.

Eq. 29 $recovery\ (\%) = \frac{total\ mg\ phages - mg\ phages\ Up\ layer}{total\ mg\ phages} \cdot 100$

- Partition of phages T4

To prepare the samples for this experiment, 100µl of a solution of a concentration of $1 \cdot 10^7$ PFU/ml of PC5 phages were added to a concentrated two-phase system. For these samples the PEG and phosphate concentration were not measured since the volume added and the concentration were so low that it was considered that the presence of bacteriophages would not affect the phase-forming components concentration.

The PC5 bacteriophages were measured following traditional phage assay (PA) by counting number of plaques as described previously. Results are presented in table below.

Table 16. PFU/ml measured of bacteriophages in ATPS samples 7, 20 and K, mass balance, partition coefficient, recovery and TLL calculated.

	Concentration (mg/ml)		Volume		PHOSPHATE		PEG		Titer PFU/ml	
	PEG	PHOS	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
7	77,00	155,28	2,10	7,60	44,87	146,06	209,62	25,13	4,70E+03	2,90E+03
20	30,42	158,76	0,92	8,90	42,81	126,00	45,98	31,12	8,90E+04	8,00E+03
K	88,72	107,50	3,20	6,29	42,92	73,20	251,25	46,38	2,35E+04	5,90E+02

	mass balance				
	mg measured	total mass	K_{PHAGE}	recovery	TLL
7	31910,0	103092,8	1,621	69,047	210,419
20	153080,0	101833,0	11,125	-50,325	84,503
K	78911,1	105374,1	39,831	25,113	207,093

With these results the partition coefficient was calculated for each sample being 1.62, 11.1 and 39.8 respectively. There is a clear correlation between the TLL and the K_{PHAGE} as it can be appreciated in figure 13. This could mean the phages are conditioned by the ionic strengths that are of more importance when the concentration of the phosphate is so different in both phases.

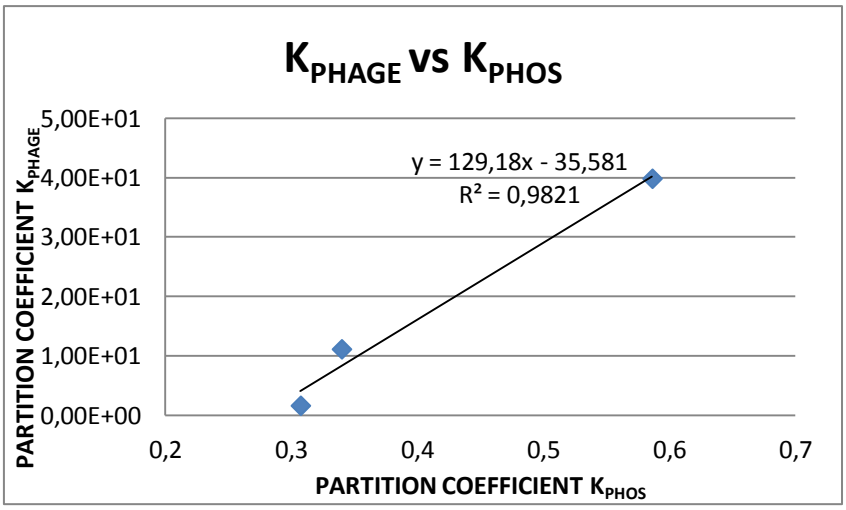

Figure 11. Correlation between K_{PHAGE} and K_{PHOS}.

3. Results and discussion

Many different experiments have been performed in order to achieve the best understanding of the phenomena of the aqueous two-phase system and its application in macromolecules. The objectives that were established have been, therefore, fulfilled since the purpose of the thesis was to get an overall impression of this separation process, the possibility of mastering as many analytical methods as possible and to acquire the knowledge and methodology necessary to work in the field of research.

The main problem faced during the calculations was the error committed with the first calibration curves. The compensation factors had to be added as well as an analysis of every step of the measurements in order to try to find the mistake source. In the end it was established that the addition of different inaccuracies were the cause to such high error percentages. For this reason the calibration curves were not a lineal approximation but a polynomial equation.

Another issue that at first was not taken into account was the density when calculating the viscosity of the samples. The rank was only between 0.99-1.04 g/ml but it induced about 7% error so a calibration curve was created to approximate its value without having to measure it in every sample because of the extra time that was consumed in those measurements.

After making these improvements to the method, and because a slight correlation between the phase-forming concentration and the %error done in the measurements was detected, the compensation factors were introduced. This was done in order to make sure the best approximation could be done with the precision that the instruments used allowed. The calculation of the compensation factors is explained in the methodology of calculation section, effect of PEG in conductivity and effect of phosphate in viscosity.

However, this incorporation led to the calculation of a non-linear system, increasing the difficulty of the calculations. This last problem was then fixed with the matrix system that allowed a simple way of calculating the phase-forming components concentration.

The protocol exposed might not be the most accurate to measure the concentration of these ATPS but it has showed to be an acceptable method for approximating the values that were tested. This is showed in the % errors that are specified in table 9.

After the protocol for measuring the PEG and phosphate concentration was set, the partition of lysozyme and bacteriophages was tested. For the lysozyme, all partition coefficients were really high and they increased when the initial concentration of lysozyme was higher. Another thing that was noticed is that between the three ATPS tested the $K_{lysozyme}$ increased in the following order: H>7>22. It can be noticed that this trend is also followed by the K_{PEG}. In the case of K_{PHOS} the differences between two-phase systems are not so remarkable.

The conclusion of this experiment then is that $K_{lysozyme}$ is positively proportional to the K_{PEG} and can also be increased by increasing the initial concentration of the enzyme added to the ATPS.

Table 17. Partition coefficients of phase-forming components.

	K_{PHOS}	K_{PEG}
7	0,307187319	8,34177744
22	0,337978251	6,25586898
H	0,764367816	18,2134915

The partition coefficients obtained for the lysozyme are quite near to the rank of expected values according to the literature, which settles the common partition coefficients between 6 and 80 (Zhang & Cundiff, 2003). It can also be pointed out that some recoveries calculated in table 15 were negative. This can be due to a failure weighting the lysozyme, measuring the volumes of both phases or to an error measuring the lysozyme concentration in the HPLC.

For the bacteriophage experiments, however, the correlation seems to be with the K_{PHOS} as it is shown in the Figure 11 although with the data available this trend is not determinant. Further samples should be tested to be able to draw any consistent conclusions. Also, not much literature is available with information about ATPS separation of bacteriophages so it can not be compared with scientific published studies. About the partition coefficients, they are quite different depending on the ATPS. For the aqueous systems 7, 20, K the K_{PHAGES} obtained have been 1.62, 11.1 and 39.8 respectively, which shows that clearly the K aqueous two-phase system is clearly the best choice for this phages.

This is probably caused because of the concentration of PEG and phosphate which are quite similar, leading to similar layer volumes. In the literature there are several references that have established that similar volumes of up and down phases lead to a higher partition coefficient.

To sum up, the conclusions about the experiments is that the results are similar to the ones expected according to the literature reviewed and that the separations performed have been successful in terms of partition coefficients and recovery percentages. These aqueous two-phase systems have been proofed to be suitable for a large-scale separation of biomolecules.

4. Summary

The aqueous two-phase system is an emerging separation method that has many advantages. It is an excellent way of separating macromolecules because of the mild environment it provides that does not create any extreme condition that could damage the conformation of the macromolecules. This point is especially important since the function of the macromolecules, which are the valuable component that needs to be purified at the end of its production process, depends directly on its conformation.

It is also an ecological alternative since the energy requirements are rather low and the phase-forming components as PEG and phosphate are not highly toxic or dangerous. Moreover, the ATPS alternative combines a few steps of the traditional separation method, leading to a decrease in chemical used and energy required.

A very extensive literature can be found on this subject and this allowed a great deep knowledge in the theoretical aspects that explain the phenomena of the phase separation and the latter molecule partition.

In the experimental part, the system of PEG/phosphate that has been tested has proved to be an adequate system to partition the macromolecules selected and has showed excellent results.

The analytical protocol to measure the concentration of PEG and phosphate was chosen to be as simple as possible and thus it is not so accurate but it led to results that were accurate enough to the purposes established.

All in all, the ATPS has showed to be a great separation method for biomolecules and the results that have been obtained along the experiments have been really positive and followed the behaviours that were expected by comparing this case with some literature. About the bacteriophages, there is fewer available information but the results of the experiments performed lead to a promising future and establish a future path for investigating further on the subject.

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ANNEX

1. Equilibrium phase diagrams

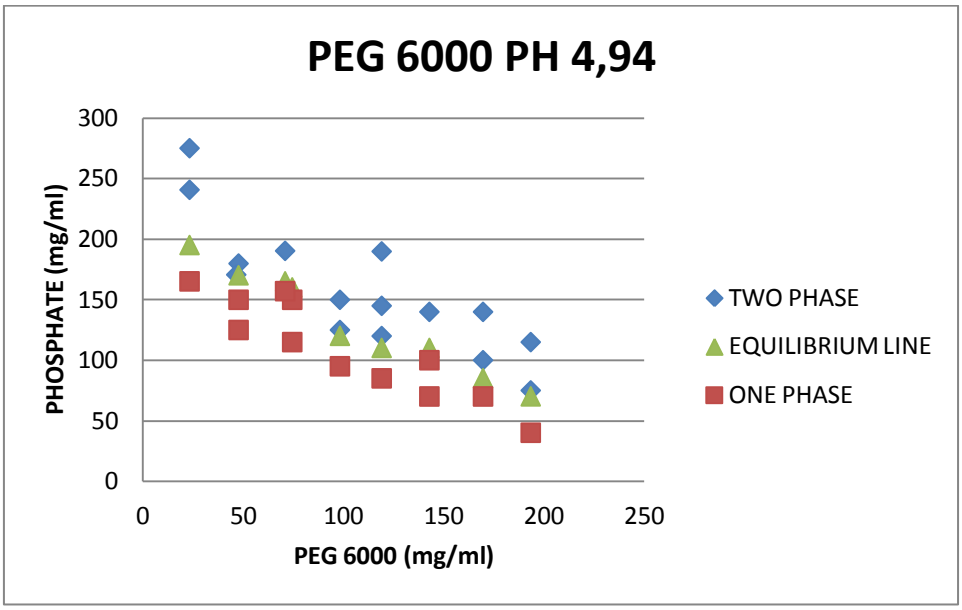


Figure 12. Equilibrium line PEG 6000 pH 5.

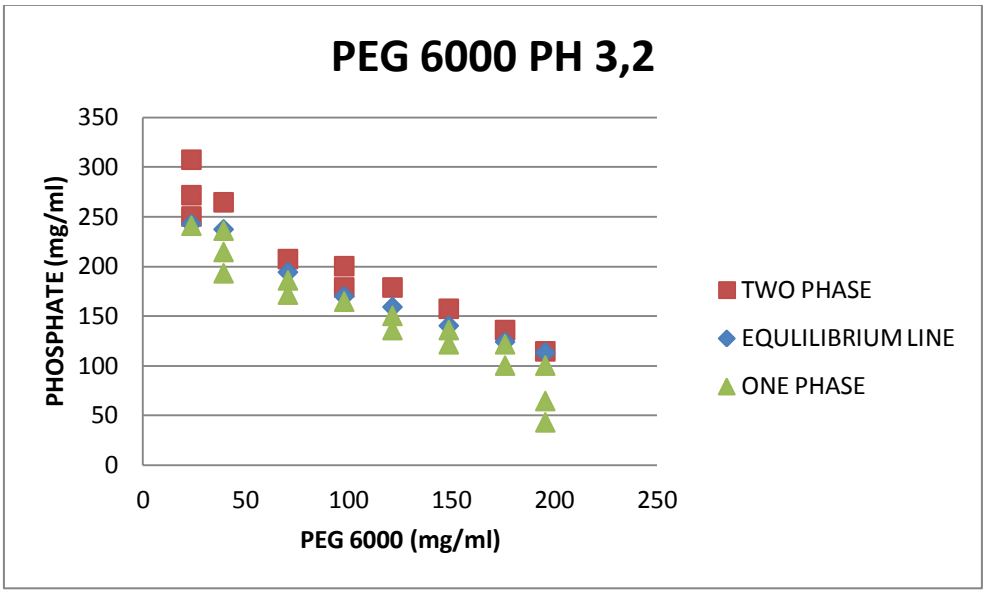


Figure 13. Equilibrium line PEG 6000 pH 3

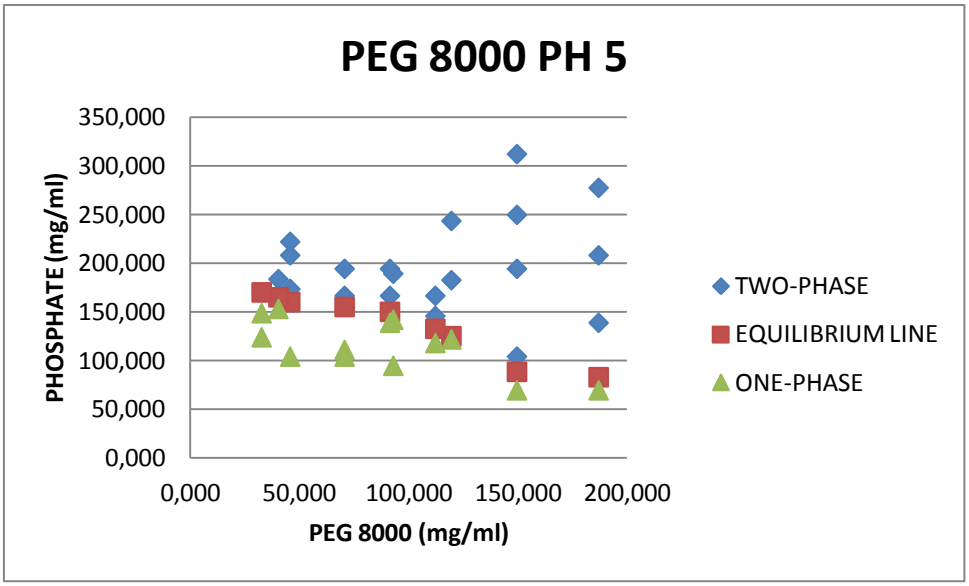


Figure 14. Equilibrium line PEG 8000 pH 5.

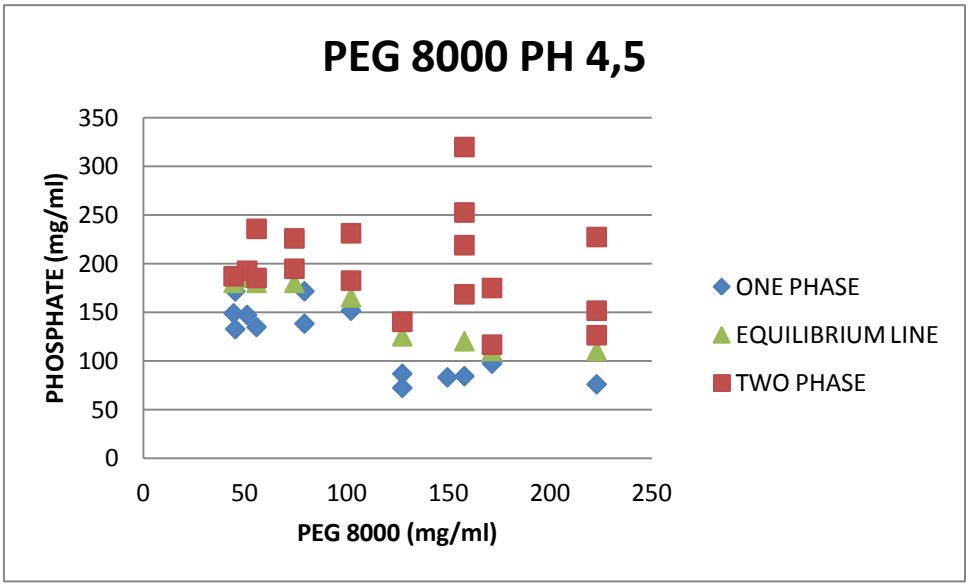


Figure 15. Equilibrium line PEG 8000 pH 4.5.

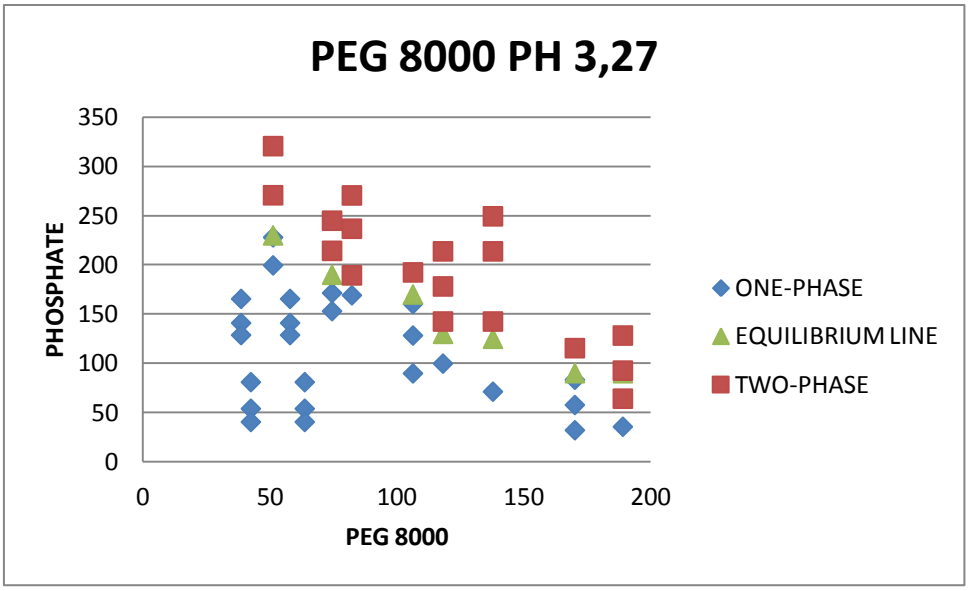
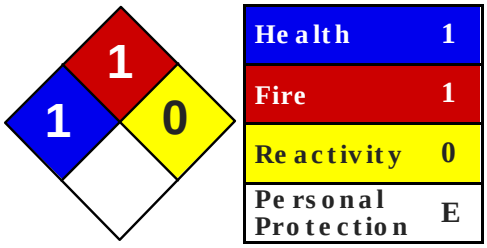


Figure 16. Equilibrium line PEG 8000 pH 3.

2. MSDSs

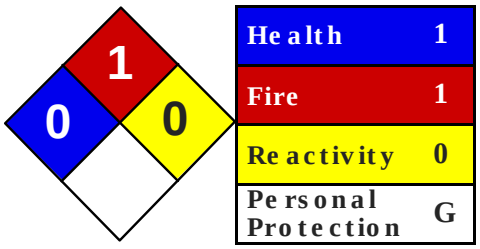
- Polyethylene Glycol 6000 (Street, 2013)



Composition and Information on Ingredients		
Composition:		
Name	**CAS #**	**% by Weight**
Polyethylene Glycol 6000	9005-08-7	100

Toxicological Data on Ingredients: Not applicable.

- Polyethylene Glycol 8000 (Science Lab, n.d.)



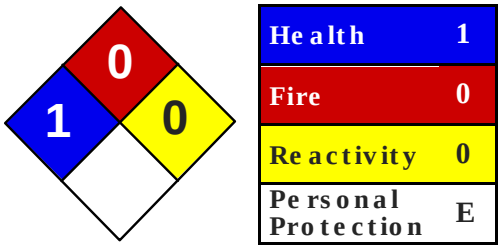
Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Polyethylene glycol 8000	25322-68-3	100

Toxicological Data on Ingredients: Polyethylene glycol 8000: ORAL (LD50): Acute: >50000 mg/kg [Rat]. DERMAL (LD50): Acute: >20000 mg/kg [Rabbit].

- Sodium phosphate dibasic monohydrate (ScienceLab.com, 2008)



Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Sodium phosphate, dibasic	7558-79-4	100

Toxicological Data on Ingredients: Not applicable.

- Sodium phosphate, monobasic dihydrate(No, Satin, & Kgaa, 2010)

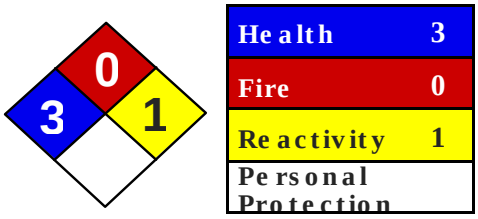
Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Sodium phosphate, monobasic dihydrate	13472-35-0	>95%

Toxicological Data on Ingredients: LD 50 ORAL: LD50 Rat 8290mg/kg
LD 50 dermal: LD50 Rabbit> 7940 mg/kg.

- Hydrochloric acid MSDS (ScienceLab.com, n.d.)



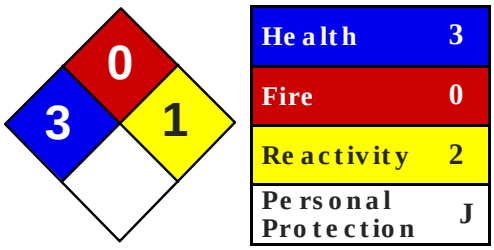
Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Hydrogen chloride	7647-01-0	20-38
Water	7732-18-5	62-80

Toxicological Data on Ingredients: Hydrogen chloride: GAS (LC50): Acute: 4701 ppm 0.5 hours [Rat].

- Sodium hydroxide(Science Lab, 2013)



Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Sodium hydroxide	1310-73-2	100

Toxicological Data on Ingredients: Sodium hydroxide LD50: Not available. LC50: Not available.