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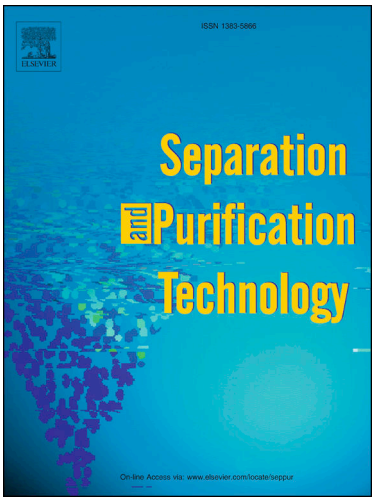

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Separation and Purification Technology

Recovery of lactose and proteins from Cheese Whey with Poly(ethylene)glycol/Sulfate Aqueous Two-Phase Systems

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Abstract

Cheese whey is an environmental problem as an effluent, but also a source of valuable raw materials, namely proteins and lactose. In this work, Aqueous Two-Phase Systems (ATPS) were studied for the recovery of lactose, BSA, β-lactoglobulin and α-lactalbumin, key components of cheese whey. ATPS formed by PEG (molecular weights: 200-8000 $g.mol^{-1}$) with sodium or ammonium sulfate were investigated. Partitioning of the selected solutes was experimentally addressed in different ATPS and pH values. Partition behavior showed that ATPS formed by PEG1500/ammonium sulfate is able to separate lactose from proteins, while PEG300/sodium sulfate ATPS may be used for protein fractionation. These separation strategies were then tested with simulated and real cheese whey. Under optimized conditions, PEG 1500/ammonium sulfate ATPS allows efficient recovery of >95% proteins (precipitate) and 80% of lactose (bottom phase), as confirmed for both simulated and real cheese whey. The results found indicate that the proposed polymer/salt ATPS can be used to design scalable and cost-effective separation strategies to apply in Cheese Whey and other related wastes.

KEYWORDS: Aqueous two-phase systems, cheese whey, bovine serum albumin, β-lactoglobulin, α-lactalbumin, separation, fractionation.

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1. Introduction

Cheese whey is a residue from cheese production and, from an environmental point of view, it constitutes a serious pollution problem. This is due to the high volumes produced and their high content in organic matter and salt. Usually, 10 kg of milk are used to produce 1 kg of cheese, which leads to 9 kg of residual whey. The high content of lactose (~4.5 %), proteins (~1%) and fats raises the chemical oxygen demand of cheese whey to 6-8 % [1,2]. Valorization and recycling of cheese whey can be done by the recovery of the key high value components, such as soluble proteins or lactose [3]. In this way, cheese whey may be converted from a residue into a raw material for new products of the dairy industry [2]. Despite the convenience of such idea, most research results focus only on the valorization of selected components of whey [1]. Among them, cheese whey proteins have the highest added value and thus the highest interest [4]. Research oriented to the recovery and/or valorization of all components is scarce [5].

Membrane filtration is the usual method employed in industry for proteins recovery, taking advantage of the large difference in molecular mass between proteins and all other components present in cheese whey dairy industry [2,4]. Recovery of lactose after membrane filtration can be carried out, for instance by crystallization, despite it has been reported scarcely [6,7]. Besides, this technology has limitations, such as the fouling and limited lifetime of membranes, the use of high pressures, and problems associated to the lack of uniformity in whey compositions. Indeed, membrane fouling by cheese whey proteins is a well-known problem for this separation process [8]. Membrane filtration capacity and product cost depend on the feed volume, and since protein concentration in cheese whey is low, the separation is an expensive process [1,3,9,10]. Chromatographic processes can also be used efficiently for protein fractionation or isolation, but in a large scale the operational cost is high due to the pressure drop required for a given flow rate and the frequent replacement of the solid phase [10,11]. Extraction with Aqueous Two-Phase Systems (ATPS), although promising, has been scarcely evaluated [10,12]. The extraction of the key components from cheese whey using Aqueous Two-Phase Systems (ATPS), even as a pre-concentration step prior to membrane filtration, would improve the separation process reducing fouling, extending membrane lifetime and allowing for the downsizing of the membrane process.

ATPS were proposed by Albertsson [13] as a separation method for a wide number of biomolecules. The high content of water present in these systems provides a biocompatible medium for proteins, which may maintain their structure and biological activity and prevent denaturation [14,15]. ATPS are based on the immiscibility between aqueous solutions of two (or more) different components under certain conditions of concentration and temperature. When equilibrium is reached, the systems form two immiscible liquid phases, and each phase is enriched in one of these phase-forming components [14-16]. Typically, two polymers or a polymer and a salt are used as phase-forming components, but currently other chemicals such as ionic liquids (ILs), saccharides and small organic molecules are also used in the creation of ATPS [17].

In this work, the separation of the main soluble proteins (BSA, lactalbumin and lactoglobulin) from lactose present in cheese whey was studied using ATPS. Since proteins are the high added-value components of cheese whey, most works in the literature focus on their recovery, either by use of ATPS or other means [4]. Nonetheless, the separation of lactose and proteins together should be the preferred strategy, although it has been studied scarcely [6,7,18]. Sulfate salts (ammonium and sodium) were employed in the ATPS composition due to their low cost and wide availability. Polyethylene glycol (PEG) was selected considering its low toxicity (it is included in FAD's GRAS List -*Generally Recognized as Safe*) and its high biocompatibility for proteins stability [19]. Although these ATPS are widely used in the literature [20-24], the characterization of the PEG 1500/ammonium sulfate ATPS at 298.15 K was required and was carried out in this work. The partitioning of the biomolecules and lactose in the various ATPS studied was determined experimentally, and the effect of different parameters such as polymer molecular weight and pH were evaluated. Based on the obtained results, different separation strategies were developed to carry out the valorization of cheese whey. Finally, these strategies were assessed using first a synthetic whey formulated with the key solutes dissolved in distilled water, and later using a real cheese whey from a local producer.

2. Materials and Methods

2.1 Materials

ATPS phase-forming components: All ATPS were formulated combining a polymer and an inorganic salt. Polymers used were polyethylene glycols (PEG) obtained from Sigma, except PEG 600 which was purchased from Merck, with different molecular weights: 8000 (BioUltra), 4000 (PhEur), 1500 (BioUltra), 600 (for synthesis), 400 (BioUltra), 300 (BioUltra) and 200 (BioUltra). Two inorganic salts were used: ammonium sulfate ($(NH_4)_2SO_4$, for molecular biology ≥99.0%) and sodium sulfate (Na_2SO_4, ACS reagent, ≥99.0%, anhydrous, granular). Both salts were purchased from Sigma.

Other chemicals: Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) were obtained from Panreac Applichem. Sulfuric acid (H_2SO_4, ACS reagent 95.5-98.0 %), citric acid ($C_6H_8O_7$, ACS reagent >99.5 %) and phosphate buffered saline (PBS, tablet, 0.01M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 ºC) were purchased from Sigma. Trifluoroacetic acid (TFA, $C_2F_3O_2H$, synthesis grade) and acetonitrile (ACN, CH_3CN, gradient HPLC grade) were obtained from Scharlau. Distilled water was used for all ATPS preparation, separation and dilution purposes.

Cheese whey and biomolecules: α-Lactose monohydrate ($C_{12}H_{12}O_{11} \cdot H_2O$ ≥99% total lactose basis), bovine serum albumin (BSA, ≥98%, lyophilized powder), β-lactoglobulin (β-LG, from bovine milk, ≥85%, lyophilized powder) and α-lactalbumin (α-LA, from bovine milk, Type I, ≥85%, lyophilized powder) were obtained from Sigma. Real cheese whey from cow milk (pH 6.5) was kindly provided by a local cheese producer, Queizúar S.L. (Galicia, Spain).

2.2 ATPS phase diagram

The screening of different ATPS for the separation of the key components of cheese whey was based on phase diagrams available in the literature [20-24]. Liquid-liquid equilibrium data for the ATPS based on PEG 1500/ammonium sulfate were however not available, and the phase diagram with tie-lines was obtained experimentally. Stocks solutions for PEG 1500 (*ca*. 40 % wt) and ammonium sulfate (*ca*. 38 % wt) in distilled water were prepared using a

Mettler Toledo balance model XPE205 precise within ±0.01 mg. Polymer was dried at 40 ºC and salts were dried at 140 ºC prior to use for at least 24 h to eliminate adsorbed water.

Tie-lines were determined experimentally at 25 ºC by the addition of suitable amounts of water, salt and polymer stock solutions to obtain mixtures within the heterogeneous region of the phase diagram. A jacketed equilibrium cell made of glass was used in the experiments, and the cell jacket was connected to a thermostatic bath for temperature control (Julabo F12-EH). The system was agitated using magnetic stirrers (Labinco L23) for 30 min and then allowed to rest for *ca.* 24 h until the two equilibrium phases were completely separated and clear. Preliminary tests showed that these times were long enough to guarantee thermodynamic equilibrium. Then, samples from the top and bottom phases were taken and diluted appropriately. Two replicates with different dilutions were prepared for each sample.

Polymer and salt compositions were obtained from the measurement of two physical properties: density and refractive index. This technique has been used satisfactorily in the past by our group [22,25] and others [26,27]. In order to obtain the compositions, calibration equations were previously determined. Homogeneous aqueous binary and ternary mixtures of polymer and/or salt with compositions from 0 to 10 wt % (total solute composition) were prepared by weight, and then density and refractive index were measured at 25 ºC. Density was measured in a densimeter Anton Paar DSA 48 with an uncertainty of 0.1 kg/m^3. Refractive index was measured using a refractometer Atago RX 5000, with an uncertainty of $4 \cdot 10^{-5}$. Experimental data were fitted to a first order polynomial by least-squares, according to Eq. (1):

$$Z = a + b \cdot w_P + c \cdot w_S \qquad (1)$$

where Z is the physical property (density or refractive index), w_P and w_S are the mass fractions of polymer and salt, respectively, and *a, b* and *c* are fitting parameters. The experimental data of density and refractive index of the corresponding binary and ternary mixtures are shown in Tables S1-S2 in the Supplementary Information. Table S3 in the Supplementary Information presents the fitting parameters and deviations obtained for each physical property.

2.3 Solute partitioning in ATPS

ATPS were prepared gravimetrically adding suitable amounts of polymer, salt, water and each solute in Eppendorf tubes of 2 mL. Low solute concentrations were used to prevent effects on ATPS behavior. These concentrations were set to 1.17 mg/mL for systems with sodium sulfate and 0.7 mg/mL for systems with ammonium sulfate to allow comparison among different ATPS, due to differences in tie-lines' phase-forming components concentrations. Bulk polymer and salt were added (no use of stock solution), while for the different key solutes stock solutions (*ca*. 2 mg/mL) were used. To adjust the pH, buffered solutions instead of distilled water were employed for all stock solutions and ATPS preparation. Sodium phosphate-citric acid buffer and sodium phosphate buffer were used for pH 4-5 and for pH 6-8, respectively. Two replicates of a given ATPS, with the same feed composition, were prepared in all partition experiments. The systems were vigorously vortex-mixed for at least one minute and left to rest one hour in a thermostatic bath (Julabo F12-EH) at the corresponding equilibrium temperature (293.15 or 298.15 K depending on the system). Then, the tubes were centrifuged at 13000 rpm during 5 min using a centrifuge Ortoalresa series Digicen 21. Samples of the top and bottom phase were collected and diluted (1:10) with water for analysis by HPLC, using the methods described below.

The partition coefficient (*K*) of each biomolecule in the ATPS is defined as the ratio of its concentrations in the equilibrium top and bottom phases [15,28]. *K* values larger than 1 indicate solute preference for the top phase (PEG-rich phase), while values below 1 indicate the preference for the bottom phase (salt-rich phase). When there is solute precipitation to a significant extent, partition coefficients are not enough to show the behavior of the compounds. For this reason, the recovery yield (Y_P) on each phase is more meaningful to evaluate the efficiency of the solute recovery towards each phase. The Y_P was calculated according to eq. (2) [28]:

$$Y_P\,(\%) = \frac{C_P \cdot V_P}{C_i \cdot V_i} \cdot 100 \qquad (2)$$

where C and V represent the concentration and the volume of each phase, respectively, and subscripts *i* and *P* indicate the initial *i* or final *P* phase (*P* = *T*-Top or *B*-Bottom). Yields for the top and bottom phase were directly obtained from the concentrations measured experimentally by HPLC, while yield in the precipitate was obtained by mass balance.

2.4 Protein and lactose quantification by HPLC

Proteins quantification was carried out on Agilent series 1100 high-performance liquid chromatographs (HPLC) equipped with a diode array detector (DAD) Agilent 1100 series. Any of the following equivalent columns were used: a) Shodex Protein KW-800, 8 x 300 mm, absorbance measured at a wavelength of 280 nm. b) Phenomenex Yarra SEC 2000, 7.8 x 300 mm and pore size 145 Å, absorbance measured at a wavelength of 214 nm. The chromatographic method is the same independently of the column brand: column oven worked at 323.15 K with an injection volume of 25 μL. A gradient method was applied: Solution A composed of 72.3 % distilled water with 0.1 % TFA and 27.7 % acetonitrile with 0.1 % TFA, whilst Solution B is constituted by acetonitrile with 0.1% TFA. Protein quantification was calibrated in the concentration range from 0.05 to 6.4 mg/mL (BSA) and from 0.05 to 4.4 mg/mL (LG and LA).

Lactose quantification was carried out using a refraction index detector (RID) Agilent 1260. The detector is installed in series with the DAD in the HPLC, since they are both non-destructive. Two different columns have been used for lactose quantification: a) Bio-Rad Aminex HPK-87H, 7.8 x 300 mm, particle size 9 μm. This column worked at 323.15 K, with an injection volume of 20 μL. The mobile phase (0.6 mL/min) was 5 mM sulfuric acid in distilled water. b) When samples contained proteins (simulated or real cheese whey samples), a different column was used: Phenomenex Yarra SEC 2000, 7.8 x 300 mm with a pore size 145 Å. This column worked at 323.15 K, with an injection volume of 25 μL. The mobile phase is a mixture of two solutions: 79.3% of a solution A (composed of 72.3 % distilled water with 0.1 % TFA and 27.7 % acetonitrile with 0.1 % TFA) and 20.7% of a solution B (composed of 0.1% TFA in acetonitrile). Lactose quantification was calibrated in the concentration range from 0.05 to 4 mg/mL.

Detailed values for the parameters of the gradient methods are summarized in the Supplementary Information, Table S4.

2.5 Application of synthetic and real cheese whey

Synthetic cheese whey was prepared in distilled water combining the model solutes described in Table 1. Real cheese whey was supplied by a local cheese producer, Queizúar S.L. (A

Coruña, Spain). It is an acid whey with a pH value of 6.5. The supplied whey was stored frozen into falcon tubes at -20 ºC,. For use, frozen samples were melted overnight into a refrigerator. Composition of the real cheese whey was analyzed by HPLC as described in the previous section, and results are presented in Table 1. Real cheese whey was pre-treated by adjusting pH to the desired value (for ATPS at a fixed pH) and vacuum-filtered to remove any solid residues.

In order to study the partition and separation of proteins and lactose using synthetic and real cheese whey, the ATPS were prepared as described in section 2.3, using 2 mL Eppendorf tubes. Bulk polymer and salt were used to get the desired final ATPS concentrations, in order to maximize whey load in the ATPS. The tubes were vigorously vortex-mixed for at least one minute, allowed to rest for one hour in a thermostatic bath at 25 ºC and centrifuged at 13000 rpm during 5 min. Then, two samples were withdrawn from each phase (top and bottom) for quantification of lactose and proteins, respectively. The HPLC methods used to quantify lactose and proteins are described in detail in section 2.4.

Table 1. Synthetic and real cheese whey composition.

	Synthetic Whey (mg/mL)	Real whey (mg/mL)
BSA	0.40	0.46
β-LG	2.52	3.20
α-LA	1.06	1.20
Lactose	50	38

3. Results and Discussion

3.1. Aqueous Two-Phase Systems (ATPS)

PEG/Sulfate ATPS were used for the separation of the key components of cheese whey. PEG is probably the most used polymer in ATPS formulations [16]. Among the salts used in polymer/salt ATPS, phosphates and sulfates are also among the most used species [16]. This is due to their low cost and ability to form ATPS at low salt concentration, which is linked to their high valence or high Gibbs energy of hydration [29]. Under these considerations, PEG and sodium and ammonium sulfates were selected as phase-forming components to test ATPS for cheese whey valorization. A wide set of PEG/(sodium or ammonium) sulfate ATPS

phase diagrams are available in the literature [20-24]. In order to perform a comparison of different PEG molecular weights, the phase diagram for PEG 1500/ammonium sulfate ATPS at 25 ºC was obtained experimentally in this work. Feed and tie-lines' compositions are presented in the Supplementary Information (Table S5). The length of the tie-lines, TLL, was calculated according to eq (3):

$$TLL = (\Delta P^2 + \Delta S^2)^{1/2} \quad (3)$$

where ΔP and ΔS are the differences of the polymer and salt mass fractions in the coexisting phases, respectively. The slopes of the tie-lines (STL) were obtained by linear regression of their feed, top and bottom compositions, with $r^2 > 0.999$ in all cases. The binodal curve (the curve that separates the single phase from two-phase region of the phase diagram) is defined by the locus of the end points of the tie-lines. The experimental data were correlated with the equation proposed by Merchuk and co-workers [30]:

$$w_{PEG} = a \cdot exp\left[b \cdot (w_{salt})^{0.5} - c \cdot (w_{salt})^3\right] \quad (4)$$

where w_{PEG} and w_{salt} are the polymer and salt compositions in mass fraction, respectively, and a, b and c are fitting parameters obtained by nonlinear regression. These fitting parameters are given in the Supplementary Information (Table S6). Figure 1 presents the experimental obtained phase diagram (tie-lines and correlated binodal curve).

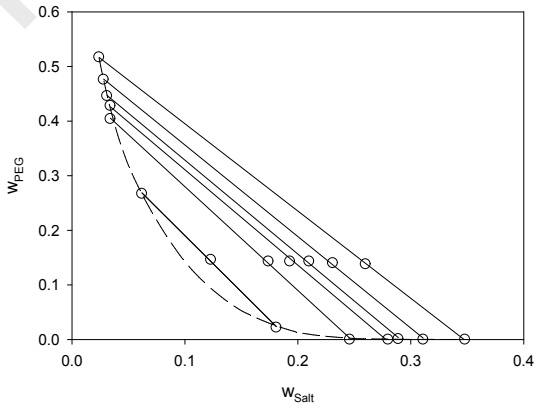

Fig.1. Experimental tie-lines and Merchuk correlation for the binodal curve of PEG 1500/ammonium sulfate ATPS at 298.15 K. Compositions in mass fraction. Symbols: Tie-

Line feed and end points (○). Lines: tie-lines (—) and binodal curve (---). See Supplementary Information (Tables S5 and S6) for details.

3.2. PEG molecular weight screening for biomolecules partition

Four key model solutes have been considered for the valorization of cheese whey: lactose, BSA, β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). The effect of the PEG molecular weight on their individual partition behavior was evaluated in PEG/sulfate ATPS. Table 2 presents the tie-lines selected from the corresponding dataset of PEG/(sodium or ammonium) sulfate ATPS. These tie-lines were selected considering similar tie-line length and reduced composition of phase-forming components. In order to facilitate the discussion of the effect of PEG molecular weight using different tie-lines and salts, the different tie-lines used in partitioning experiments are shown in Figure 2. It is important to note that Figure 2 is not a phase diagram, but a comparison of different ATPS systems: Each tie-line corresponds to a different PEG molecular weight and salt.

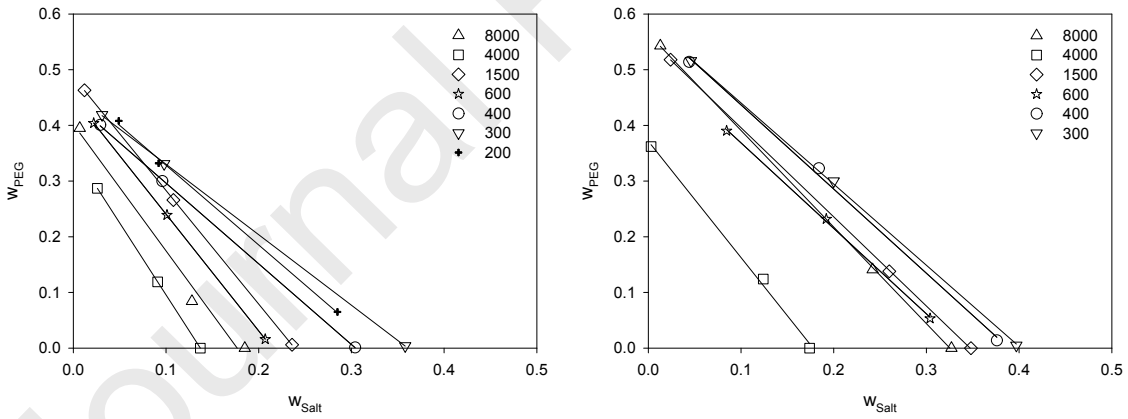

Figure 2: Position of the tie-lines evaluated in the partitioning experiments. PEG molecular weight used on each tie-line is indicated in the inserts. Left: PEG/sodium sulfate; right: PEG/ammonium sulfate. For ATPS composition [20-24], see Table 2.

Table 2. Tie-line compositions (feed, top and bottom phases, in weight fraction) used to evaluate solute partitioning in PEG/sulfate ATPS.

		Feed		Top phase		Bottom phase		
PEG Mw	T (ºC)	PEG	Salt	PEG	Salt	PEG	Salt	Ref.
PEG/sodium sulfate ATPS								
8000	20	0.128	0.084	0.395	0.007	0.000	0.185	22
4000	20	0.119	0.091	0.287	0.026	0.000	0.137	22
1500	25	0.266	0.108	0.463	0.012	0.006	0.236	23
600	25	0.239	0.101	0.404	0.022	0.016	0.207	20
400	25	0.300	0.096	0.401	0.029	0.0013	0.304	20
300	25	0.331	0.098	0.419	0.031	0.004	0.358	20
200	25	0.332	0.092	0.408	0.049	0.065	0.285	20
PEG/ammonium sulfate ATPS								
8000	20	0.141	0.242	0.543	0.013	0.000	0.327	22
4000	20	0.124	0.124	0.362	0.003	0.000	0.174	22
1500	25	0.138	0.260	0.518	0.024	0.000	0.348	*
600	25	0.232	0.192	0.390	0.085	0.053	0.304	21
400	25	0.323	0.184	0.514	0.044	0.014	0.376	24
300	25	0.300	0.200	0.516	0.046	0.005	0.397	*

* These data were experimentally determined by our group following the methodology indicated above (see Fig.1 and Table S5).

Since there was some solute precipitation in most systems, partition coefficients between ATPS phases do not provide the whole picture of solute behavior. The solute yield recovered in each phase is presented in Figures 3 and 4 for PEG/ammonium sulfate and PEG/sodium sulfate ATPS, respectively, for the four key compounds (BSA, α-Lactalbumin, β-Lactoglobulin, and lactose). The complete data set of results, together with partition coefficients when they could be calculated, is given in the Supplementary Information (Tables S7 and S8).

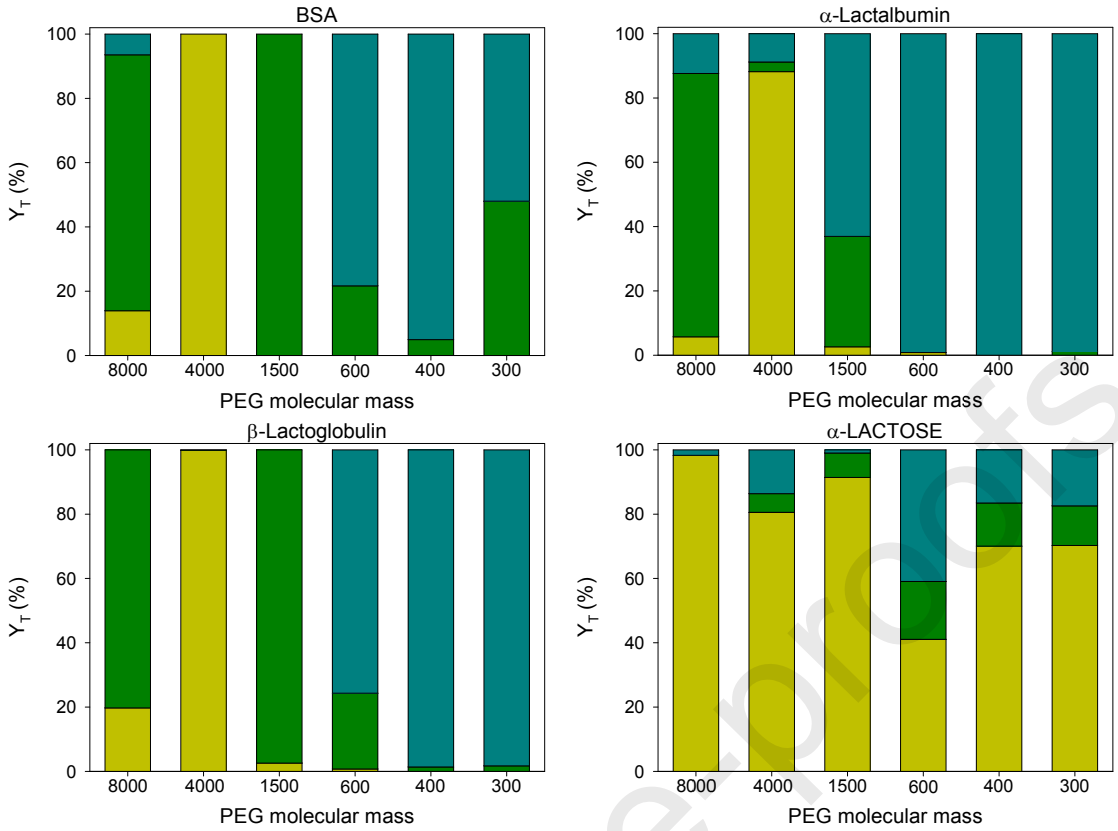

Figure 3: Effect of PEG molecular weight on the recovery yield for BSA, α-LA, β-LG, and lactose in PEG/ammonium sulfate ATPS. For ATPS composition see Table 2. Blue: top phase; yellow: bottom phase; green: precipitate.

Figures 3 and 4 demonstrate the significant effect of the polymer molecular weight on the recovery yield of each biomolecule. The large range of PEG molecular weights investigated (200-8000 g/mol) is the reason for such large effect. Proteins have a higher affinity for the top, polymer-rich, phase at lower PEG molecular weights, while for higher PEG molecular weights the affinity changes towards the bottom phase. This behavior, for both salts, is due to the hydrophilic character of these proteins and the increase of the hydrophobicity with the molecular weight increase, which leads to a preferential partition of the proteins to the salt rich phase. Overall, proteins partition yields move from the bottom to the top phase as the PEG molecular weight decreases. Besides, protein precipitation is more likely at higher PEG molecular weights in ammonium sulfate ATPS, but at lower or intermediate molecular weights in sodium sulfate ATPS. On the other hand, lactose shows a higher affinity to the bottom phase, which decreases with the PEG molecular weight. This behavior is more evident for sodium sulfate ATPS (Figure 4) than for ammonium sulfate ATPS (Figure 3).

Nevertheless, the effect of polymer molecular weight cannot be separated from the effect of the tie-lines' compositions, presented in Table 2: For each PEG molecular weight a tie-line is selected, and that tie-line has specific feed, top and bottom phase compositions. The different tie-lines selected are represented in Figure 2. It can be seen that, in general, ATPS with higher molecular weight PEG (1500-8000 $g.mol^{-1}$) used lower feed (polymer and/or salt) compositions than ATPS with lower molecular weight PEG (200-600 $g.mol^{-1}$). Besides, Figure 2 shows that there is little effect of PEG molecular weight on the slope of the tie-lines in PEG/ammonium sulfate ATPS, while there is a clear effect on sodium sulfate ATPS: The slope of tie-lines becomes steeper with PEG molecular weight. This means that in PEG/sodium sulfate ATPS the PEG composition in top phase increases and/or salt concentration in bottom phase reduces when PEG molecular weight is increased. This effect enhances the differences between the two types of ATPS at low PEG molecular weights. These differences in tie-line composition will affect the solute partitioning.

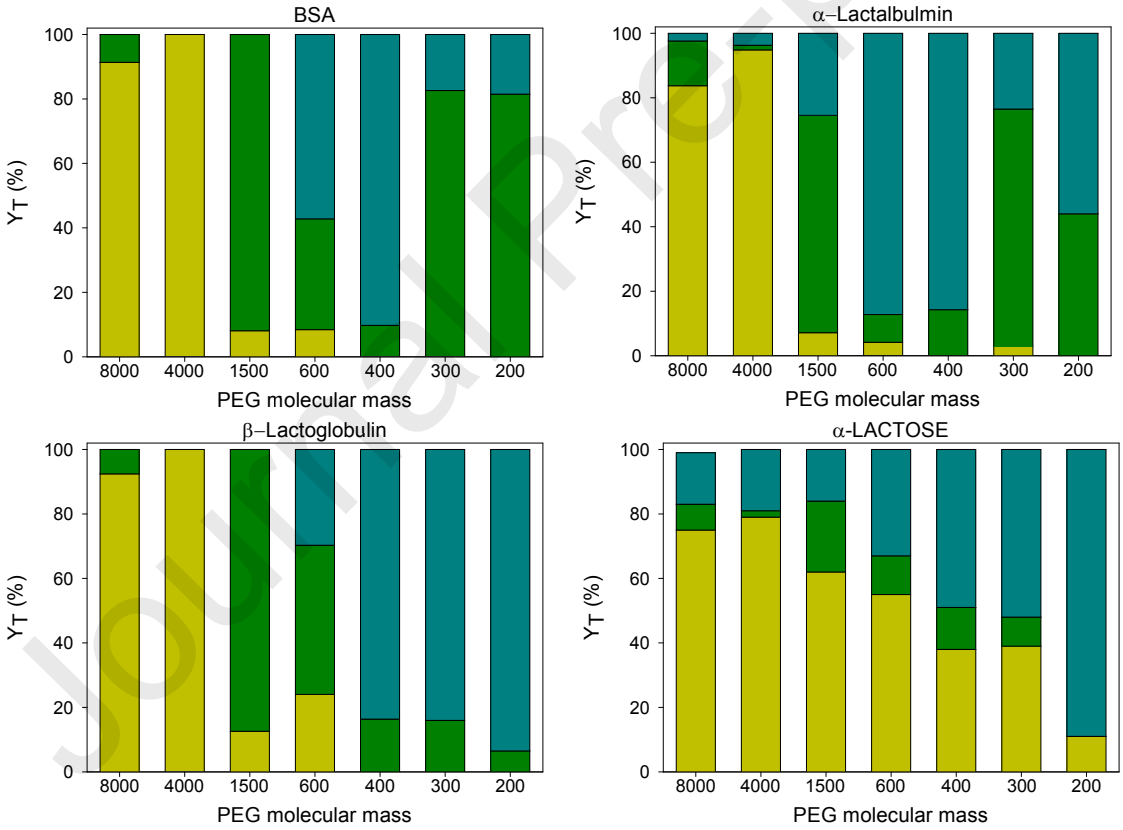

Figure 4: Effect of PEG molecular weight on the recovery yield for the key solutes in PEG/sodium sulfate ATPS. For ATPS composition see Table 2. Blue: top phase; yellow: bottom phase; green: precipitate.

In terms of separation of the biomolecules, some important insights can be drawn from the data obtained. Lactose has a higher affinity to the bottom, salt-rich, phase of the ATPS. In ammonium sulfate ATPS, the affinity is almost independent of the polymer molecular weight. Moreover, the lactose precipitated is less than 20 % in all cases. Thus, separation of the proteins from lactose may be possible if they are recovered in the top, polymer-rich, phase, or precipitated at the interface.

Overall, PEG 8000 and 4000 can be discarded for all ATPS because all proteins and lactose are mostly in the salt-rich phase. For the sodium sulfate ATPS, low molecular weight PEG (200-400 $g.mol^{-1}$) ATPS can be discarded due to the high affinity of all solutes to the top phase, while with PEGs of intermediate molecular weight (600-1500 $g.mol^{-1}$) lactose would cross-contaminate all phases despite proteins partition between the top phase and precipitate. Nevertheless, ammonium sulfate ATPS provide promising results with PEGs of low and intermediate molecular weight (300-1500 $g.mol^{-1}$). In these systems, lactose concentrates preferentially in the bottom (salt-rich) phase, while proteins concentrate in the top phase and precipitate. Among these results, the ATPS composed of PEG 1500 provides some protein loss to the bottom phase, which can be avoided using PEGs with lower molecular weights.

On the other hand, sodium sulfate ATPS do not show the possibility of separating lactose from the proteins. Moreover, some degree of protein fractionation may be possible with PEGs of low molecular weight (200-300 $g.mol^{-1}$), since BSA mostly precipitates and β-LG concentrates in the top phase, while α-LA splits between the top-phase and the precipitate.

Comparing the sugar/protein separation, the ATPS formed by ammonium sulfate seem the most promising. For PEGs with lower molecular weight, about 20% of the sugar was present in the top phase with proteins. Using PEG 8000 almost all lactose preferentially partitions to the bottom phase, but 20 % β-LG, 14 % BSA and 6% α-LA are also in the bottom phase. PEG 1500 shows the best results, because a small amount of proteins, 3 % of β-LG and 3 % of α-LA are in the bottom phase.

3.3. pH screening

Considering the important effect that pH plays on protein behavior, especially when the isoelectric point (pI) is crossed, the effect of pH on the partitioning behavior of the target compounds was further evaluated. Based on the results discussed above, the PEG

1500/ammonium sulfate and PEG 300/sodium sulfate ATPS were chosen for this study, and the pH was varied from 4 to 8. As the proteins have pIs about 4.5-5.0 [31], the selected pH screening crosses the pI of all of them. Lactose was included in the first ATPS system, but as expected it is not affected by pH (see Figure 5). Thus, lactose was not included in the PEG 300/sodium sulfate ATPS pH screening. The results obtained for PEG1500/ammonium sulfate and PEG300/sodium sulfate ATPS are shown in Figures 5 and 6, respectively, together with the results without pH control (no buffer).

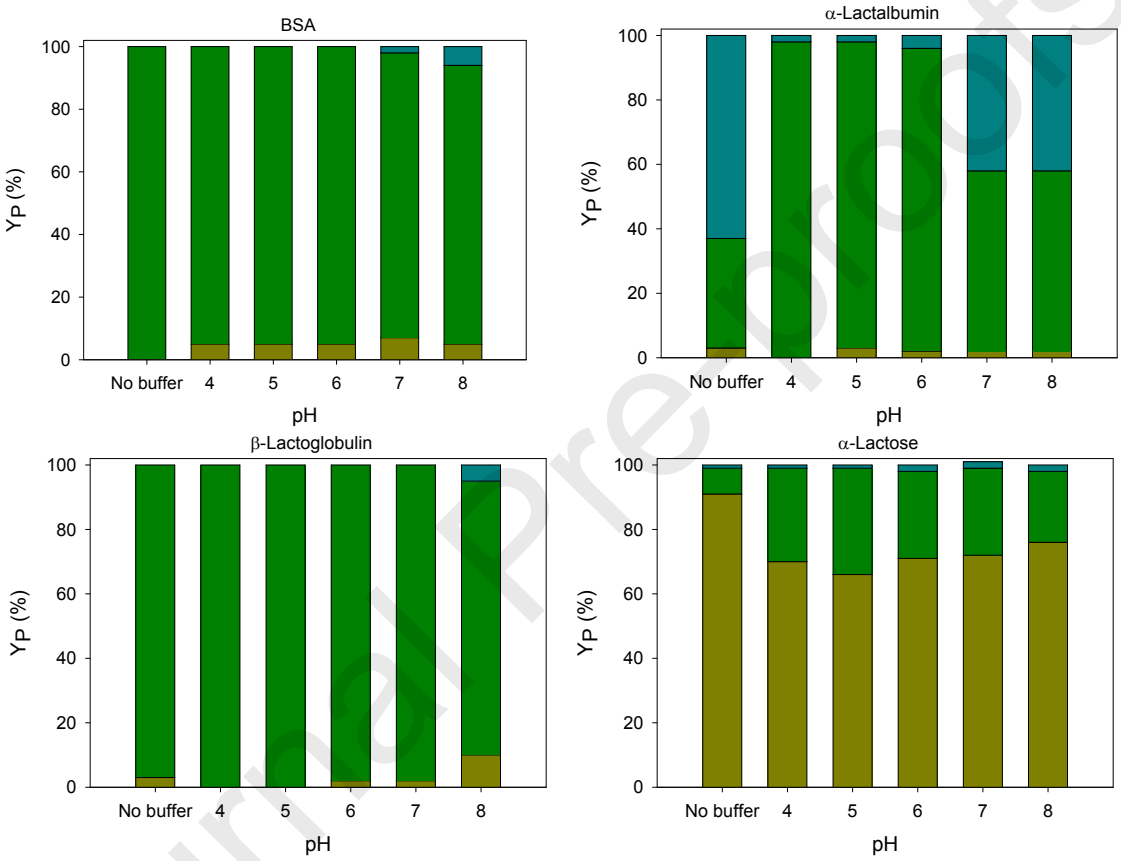

Figure 5: Effect of pH on the recovery yield for the key solutes in PEG1500/ammonium sulfate ATPS. For ATPS composition see Table 2. Blue: top phase; yellow: bottom phase; green: precipitate.

The affinity of all proteins for the top (polymer-rich) phase increases with the pH, which is in agreement with previous literature results [32]. For PEG1500/ammonium sulfate ATPS, BSA and β-LG precipitate at the interface, while α-LA increases its affinity to the top phase as pH reaches 7-8. In these conditions, about 70 % of the lactose was recovered in the bottom phase at all pH values. Even though 30 % of lactose has precipitated, the best pH was 4.0, since 70

% of the lactose was recovered in the bottom phase while >95 % of proteins are precipitated. For the PEG300/sodium sulfate ATPS, protein affinity increased for the polymer-rich phase with the increase of the pH. Besides, β-LG shows affinity for the top phase, rich in polymer, at all pH values evaluated, whereas BSA and α-LA precipitate at low pH. Thus, the manipulation of pH provides some fractionation of the whey proteins. Considering these results, a partial separation of proteins can be obtained at pH 4-5.

Comparison of solutes' partitioning with and without buffer for pH control shows that results with buffer do not exactly fit the behavior of its natural pH. The reason for that is the formulation used: buffer salts (phosphate/citrate or phosphate buffer) also have salting-out effect, and thus they slightly modify the partition behavior of all solutes.

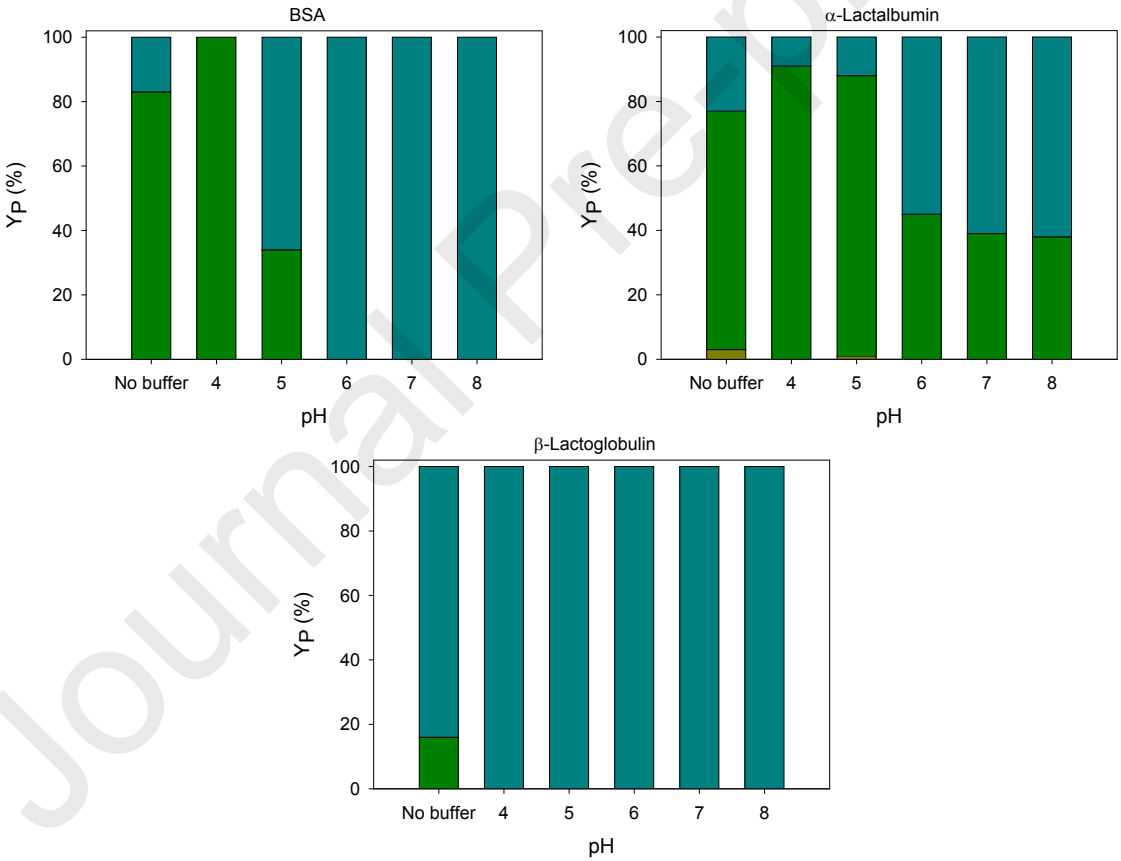

Figure 6: Effect of pH on the recovery yield for the key solutes in PEG300/sodium sulfate ATPS. For ATPS composition see Table 2. Blue: top phase; yellow: bottom phase; green: precipitate.

3.4. Strategies for the separation of lactose from proteins

The results presented in Figures 3-6 were used to design strategies for the recovery of added-value components in cheese whey using ATPS. First, it is clear that ATPS composed of PEG/ammonium sulfate and low molecular weight PEGs (300-400 $g.mol^{-1}$) may provide a way of recovering most proteins to the top phase or as a precipitate, and thus separated from lactose which is recovered mostly in the bottom phase. Considering the PEG 1500/ammonium sulfate ATPS, at low pH values all proteins are recovered as a precipitate with some limited BSA loss to the bottom phase and some cross-contamination of lactose in the precipitate. This strategy is presented in Figure 7. The PEG/sodium sulfate is not able to achieve this separation.

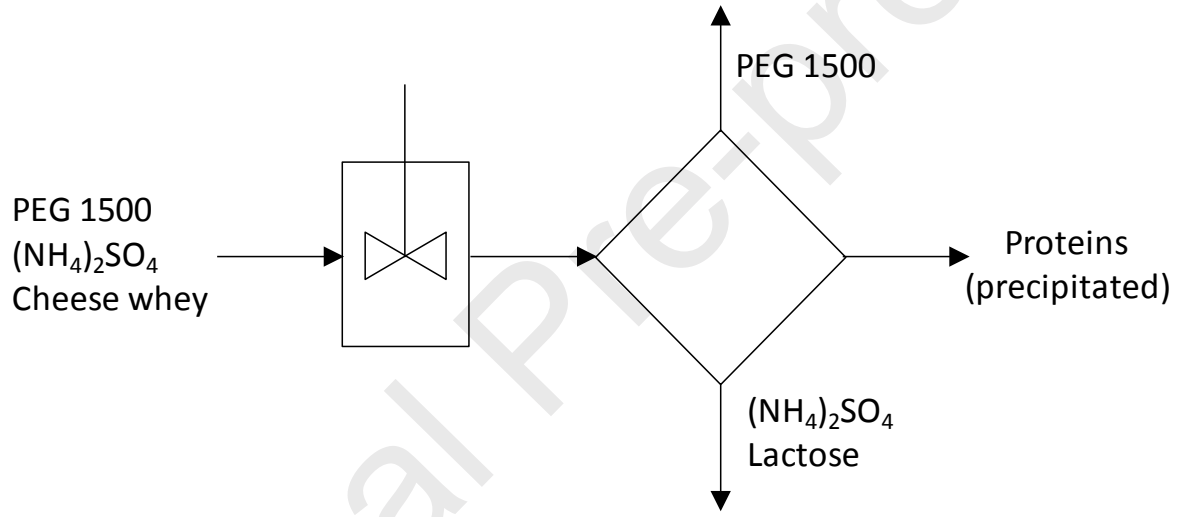

Figure 7: Flow diagram with the strategy to separate lactose and whey proteins. The PEG 1500/ammonium sulfate ATPS at pH 4 is used. Separation stage must be carried out into three phases: liquid top and bottom ATPS phases, and the solid precipitate.

The PEG 300/sodium sulfate ATPS may be used to separate the various proteins present in cheese whey, which would maximize the profit of the separation process: A first step at pH 4 may separate the β-LG in the top phase contaminated with 9 % of the α-LA, whilst most α-LA and BSA are precipitated in the interface. After, BSA and α-LA can be separated using the same phase-forming compounds at pH 5, which yields BSA in the top phase and α-LA in the precipitate. This strategy is summarized in Figure 8. The combination of the two separation strategies in series would provide the separation of lactose and the three main proteins present in cheese whey.

After the separation processes depicted in Figures 7 and 8, it would be necessary to separate the solutes extracted (final product, either proteins or lactose) from the corresponding ATPS phase-forming components (for recycling). This is beyond the purpose of this work, but it may be suggested the use of membrane filtration for proteins (taking advantage of the large molecular weight difference) or adsorption processes for lactose.

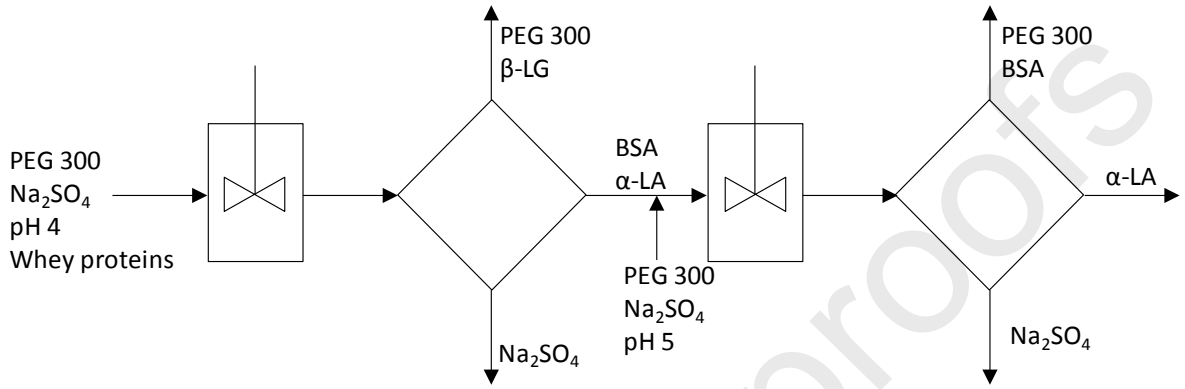

Figure 8: Flow diagram with the strategy for whey proteins' fractionation. The PEG 300/sodium sulfate ATPS is used, and proteins are separated by modification of the pH. Separation stages must be carried out into three phases: liquid top and bottom ATPS phases, and the solid precipitate.

3.5. Aqueous Two-Phase Extraction of added-value components from cheese whey

The first separation strategy described in the previous section (Figure 7) was tested with cheese whey. First, simulated whey was prepared with the model solutes in a buffered solution at the desired pH, as described in section 2.5, and results were confirmed using real cheese whey. The lactose/proteins separation was carried out using ATPS formed by PEG 1500 and ammonium sulfate at pH 4 and 25 ºC, and results are shown in Figure 9 (see also Table S9 in the Supplementary Information).

The results obtained for real cheese whey confirm the behavior of the simulated whey. The results mimic what was found for the individual solutes at pH 4 (shown in Figure 5). It has been found that for both simulated and real whey 100 % of BSA and β-LG and >96 % of α-LA can be recovered as a precipitate at the interface. This precipitate also contains 20 % of the lactose. The remaining lactose was concentrated in the bottom phase. Thus, the separation of these compounds is feasible using one step extraction.

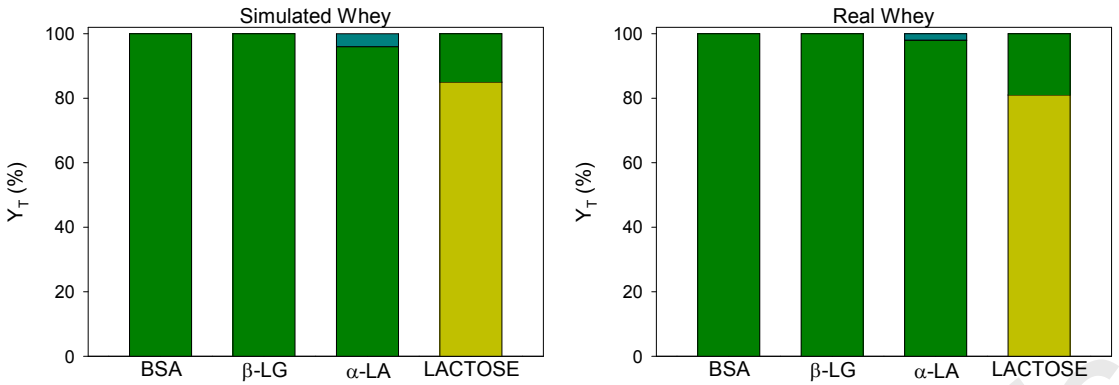

Figure 9: Application of strategy 1. Recovery yield for the key solutes: PEG1500/ammonium sulfate ATPS at pH 4 using the simulated (left) and real (right) cheese whey. For whey and ATPS compositions see Tables 1 and 2, respectively. Blue: top phase; yellow: bottom phase; green: precipitate.

The second strategy (see Figure 8) is aimed towards the fractionation of the whey proteins. When the precipitate obtained from strategy 1 was re-suspended (either in distilled water or phosphate buffer solution), all proteins precipitated in the first step of strategy 2 and no separation was obtained. Protein co-precipitation or the presence of components from the previous ATPS (PEG 1500, ammonium sulfate) may be the reasons for this behavior, different from what would be expected from the single solutes (see Figure 6).

Lactose-free whey was simulated using protein concentrations as described in Table 1, and protein fractionation was tested again. The results obtained are presented in Figure 10 (see also Table S10 in the Supplementary Information). The expected recovery of β-LG in the top phase is not obtained: 18% β-LG is lost in the precipitate, and 33% of the BSA and 37% of the α-LA are also present in the top phase. The second step (applied to the precipitated proteins) also does not reproduce the behavior of single solutes: Most of the proteins again precipitate, and no further fractionation is obtained. When the real cheese whey is used, the mass of BSA recovered as a precipitate in the interface of the ATPS is significantly lower, while β-LG and α-LA behave similarly to the simulated whey. In the second step, it is again impossible to obtain the separation of the proteins. It is clear that in these ATPS the behavior of the proteins changes in presence of other proteins (mixture), preventing a proper fractionation of the key proteins.

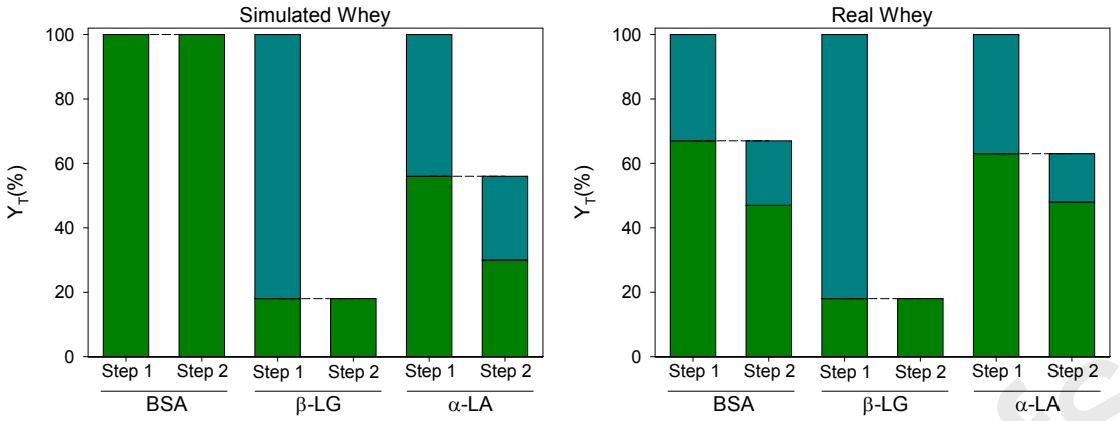

Figure 10: Application of strategy 2. Recovery yield for the proteins in PEG300/sodium sulfate ATPS at pH 4 (left, step 1) and pH 5 (right, step 2). See Figure 8 for details on the strategy 2. For whey and ATPS compositions see Tables 1 and 2, respectively. Blue: top phase; yellow: bottom phase; green: precipitate.

It has been previously explained that polymer/salt ATPS are suitable for the separation of solutes with large chemical differences, such as separation of proteins from other chemicals in their matrix [33]. That is due to the large difference between the chemical nature of the ATPS equilibrium phases. But protein fractionation will in general be difficult in polymer/salt ATPS, and polymer/polymer ATPS would perform better due to smaller or more subtle differences between the equilibrium phases [33]. This general statement has been confirmed in this work, where PEG 1500/ammonium sulfate ATPS was able to separate whey proteins from lactose, but protein fractionation of all three proteins BSA, β-LG and α-LA was not attained. Nevertheless, it is here shown that adequate ATPS formulations can be used to separate proteins from lactose, in which proteins and lactose can be used for different purposes and in different industries.

Conclusions

Polymer/salt ATPS formulated combining PEG of different molecular weights with sodium or ammonium sulfate were evaluated for the valorization of cheese whey, namely by the recovery of lactose and the proteins BSA, β-LG and α-LA. The effect of PEG molecular weight and pH in the partitioning of all these solutes was assessed experimentally.

The results of the single biomolecules partition were used to design different separation strategies to carry out the lactose/protein separation and the fractionation of the proteins. These separation strategies were then evaluated experimentally using first a simulated cheese whey formulated with the model solutes, and then confirmed using a real cheese whey from a local producer.

The results obtained with the real cheese whey confirm those of the simulated whey, demonstrating that the separation between lactose and proteins can be obtained with proper selection of the ATPS. Complete protein fractionation for the three main whey proteins was not possible, and only partial fractionations were obtained using the ATPS formulations tested.

It is important to note that large differences were obtained in the partitioning behavior of single proteins and that of their mixtures. This fact reinforces the need for measuring partitioning data of mixtures (proteins, or proteins and other solutes), combined with the use of spectroscopic techniques and suitable theoretical models to gain a better understanding of the multicomponent behavior.

Acknowledgements

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HIGHLIGHTS:

- Key components of cheese whey are recovered using Aqueous Two-Phase Extraction
- Effect pf polymer molecular weight and pH is evaluated for solute partitioning
- Separation strategies are designed attending to single solute partitioning
- Separation strategies are checked using simulated and real cheese whey