Purification and concentration of cheese whey proteins through aqueous two phase extraction

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ABSTRACT

Whey is a by-product of cheese manufacture and contains proteins that can be recovered, but lactose presence limits its use. Proteins can be separated with aqueous two-phase extraction (ATPE), thus the objective was to evaluate this strategy to recuperate proteins from whey attending lactose elimination. An ATPE system with \((\text{NH}_4)_2\text{SO}_4\) and polyethylene glycol 4000 (PEG4000) was studied at 25°C. As PEG4000 diminished the volume ratio between phases diminished and a biphasic system was maintained in absence of the polymer. Minimum protein recovering and lactose elimination were 54.5% and 62.1% with mixtures having 25% PEG4000 and 12.1% \((\text{NH}_4)_2\text{SO}_4\), but values were 93.0% and 72.3%, respectively, with 0.1% PEG4000 and 34.7% \((\text{NH}_4)_2\text{SO}_4\). Therefore, whey incorporated with \((\text{NH}_4)_2\text{SO}_4\) in concentration higher than 34% and without polyethylene glycol can be used in ATPE systems to maximize lactose elimination and protein recovering and to concentrate proteins up to 2.45 times through a non-energy strategy.

1. Introduction

Whey is a by-product of cheese manufacture (Perumalsamy & Murugesan, 2012). Depending on raw material quality, between 8 and 9 kg of whey can be obtained from every 10 kg of milk (Nath et al., 2015). Due to a high chemical oxygen demand, whey represents an industrial concern from an environmental point of view (Masotti, Cattaneo, Stuknyte, & De Noni, 2016; Nath et al., 2015). This product contains soluble proteins, vitamins, and mineral salts (Kreczmann et al., 2015), which makes it attractive for food preparation (Liutkevičius et al., 2016) or food supplementation (Masotti et al., 2016). The major proteins of whey are \(\alpha\)-lactalbumin (αla), \(\beta\)-lactoglobulin (βlg), and bovine serum albumin (BSA) (Perumalsamy & Murugesan, 2012). Both, αla and βlg are excellent source of essential amino acids (Alcántara et al., 2011) and, in general, whey proteins possess high biological value, because they are almost totally absorbed by the digestive system (Kreczmann et al., 2015). However, whey contains also certain amounts of fat (Fagan, Castillo, O’Callaghan, Payne, & O’Donnell, 2009) that hinder recovery of proteins (Torkamani et al., 2016), and high concentration of lactose (Kalavani & Regupathi, 2015), which has been identified as the main problem for the use of this by-product (De Souza et al., 2010), because many people around the world suffer from lactose intolerance (Jelen & Tossavainen, 2003).

Whey protein recovering has been commonly based on membrane separation and spray drying (Anandharamakrishnan, Rielly, & Stapley, 2007; Kreczmann et al., 2015); however, affectionation by heat treatment can occur. The use of an aqueous two phase extraction (ATPE) system is an alternative that avoids thermal procedures and is feasible to separate proteins (Asenjo & Andrews, 2012; Glyk, Scheper, & Beutel, 2015). In ATPE systems, a liquid–liquid separation occurs through mixtures of two polymers or a polymer and a salt, which in certain concentrations produce a true solution.
in a single phase, but in others they cause formation of two immiscible phases, among which the biomolecules present in the mixture are separated (Raja, Murty, Thivaharan, Rajasekar, & Ramesh, 2011). ATPE has been studied even to separate proteins in selective form (Gai, Qu, Zhang, & Zhang, 2011; Nitsawang, Hatti-Kaul, & Kanasawud, 2006).

In the case of whey, mixtures based on polyethylene glycol (PEG) and potassium phosphate or sodium citrate have shown potential to separate α-lactalbumin in the polymeric phase and β-lactoglobulin in the saline one (Alcántara et al., 2011; Giraldo, Reis, & Minim, 2001; Kalaivani & Regupathi, 2015). On the other hand, Anandharamakrishnan, Raghavendra, Barhate, Hanumesh, and Raghavara (2005) showed that fat may be separated using also ATPE, which makes the method attractive to be used with whey. However, the case of lactose elimination through ATPE has not been studied, but based on works of Chethana, Nayak, and Raghavara (2007) with betalains, such component could be retained also in the saline phase of an ATPE system, but it would remain in mixture with β-lg making difficult its purification. The present work is focused on the recuperation of whey proteins attending the elimination of lactose, in order to prepare a raw material for further fractioning of αlactalbumin and β-lactoglobulin. Anandharamakrishnan et al. (2005) studied the system PEG6000/ammonium sulfate (AS) and found that as the mixture was far away from the equilibrium monophasic/biphasic condition the recovery can be increased, although with small separation yields, but the study did not consider a clear evaluation of the volume ratio effect between phases. The AS is an auxiliary compound in protein quantification methods due to its ability to cause protein precipitation (Fujita et al., 1993; Jinn, Yeh, Chen, & Lin, 1989) and, due to this, the present work was conducted under the premise that a more in-depth study would be necessary to favor or rule out its use in ATPE systems oriented to separate proteins from whey, attending the lactose elimination. The development of ATPE systems is often supported by phase diagrams that provide data about the binodal curve that separates concentrations that produce a monophasic system from those that cause a biphasic one, and about concentrations of phase components in top and bottom phases, and phase volume ratios (Raja et al., 2011). Data corresponding to phase diagrams for the PEG/AS system are available in literature (González-Amado, Rodil, Arce, Soto, & Rodríguez, 2011) and this can facilitate an evaluation of using such salt in ATPE systems. The objective of the present work was to evaluate the use of an ATPE system based on PEG and AS to eliminate lactose and separate proteins from whey.

2. Materials and methods

2.1. Cheese whey

Basket cheese was made from whole milk produced by Holstein cattle with the method described by Lobato-Calleros, Lozano-Castañeda, and Vernon-Carter (2009). Whey was separated and subjected to evaluation of total sugars, protein, and fat contents (see sub-section 2.5).

2.2. Phase binodal diagram

Data corresponding to phase diagrams for the mixture of PEG [HO-(CH₂CH₂O)n-CH₃OH], poly(ethane-1,2-diol) 4000 (PEG4000) with AS (NH₄)₂SO₄ were used. According to González-Amado et al. (2016), the binodal curve for this system is represented by the Merckhu model (Merchu, Andrews, & Asenjo, 1998) in the form of Equation (1), where $x_{\text{AS}}^\text{bin}$ and $y_{\text{P}}^\text{bin}$ are concentrations (%) of (NH₄)₂SO₄ and PEG4000, and $k_1$ (%), $k_2$ (% 0.5), and $k_3$ (% 3) are regression constants with values of 63.18, −0.3036, and 0.0006845 at 5°C, 69.02, −0.3388, and 0.0008589 at 20°C, and 81.88, −0.4486, and 0.001118 at 35°C, respectively. Lagrange polynomials (Burden & Faires, 2010) were applied and constants were interpolated to have values of 72.53%, −0.3671%, 0.5%, and 0.000936%, respectively, for a temperature of 25°C, which is representative of environmental conditions in many cheese production regions.

$$y_{\text{P}}^\text{bin} = k_1 \exp\left(k_2 \left(x_{\text{AS}}^\text{bin}\right)^{0.5} - k_3 \left(x_{\text{AS}}^\text{bin}\right)^3\right)$$

(1)

In order to identify limit operation conditions, solutions with 40% PEG4000 (Sigma-Aldrich, Co., Germany) and 40% ammonium sulfate (J. Bayer, Mexico) were prepared using deionized water as solvent. The cloud point method (Li, He, Liu, Li, & Liu, 2005) was used to identify points corresponding to the highest concentrations of (NH₄)₂SO₄ ($x_{\text{AS}}^\text{max}$, $y_{\text{P}}^\text{max}$) and PEG4000 ($x_{\text{AS}}^\text{max}$, $y_{\text{P}}^\text{max}$) on the binodal curve. A straight line with slope $k_4$ (Equation 2) was fitted with such points and was plotted together with Equation (1), constituting the main tie line (TLmain) of the diagram.

$$y_{\text{P}} = k_4 \left(x_{\text{AS}} - x_{\text{AS}}^\text{bin}\right) + y_{\text{P}}^\text{max}$$

(2)

Secondary tie lines (TLsec), parallel to TLmain, were constructed. To do that, the critical point (C), defined as the condition where a tie line have zero length (Raja et al., 2011), was located deriving Equation (1) and the result was matched with the slope $k_4$ (Equation 3), from which the abscissa $x_C$ was obtained, whose substitution in Equation (1) allowed to obtain the ordinate $y_C$.

$$\frac{dy_{\text{P}}^\text{bin}}{dx_{\text{AS}}^\text{bin}} = \frac{d}{dx_{\text{AS}}} \left[k_1 \exp\left(k_2 \left(x_{\text{AS}}^\text{bin}\right)^{0.5} - k_3 \left(x_{\text{AS}}^\text{bin}\right)^3\right)\right] = k_4$$

(3)

From the point $C(x_C, y_C)$ a perpendicular to TLmain with slope $−1/k_4$ (Riddle, 1995), was drawn. The intersection between both (denoted as D) was determined and five equidistant points (E, F, G, H, and I) were located on such perpendicular, through which TLsec were drawn. Equation (4) represents the secondary tie line passing through the point $E(x_{\text{AS}}^\text{max}, y_{\text{P}}^\text{max})$.

$$y_{\text{P}} = k_4 \left(x_{\text{AS}} - x_{\text{AS}}^\text{max}\right) + y_{\text{P}}^\text{max}$$

(4)

The intersections of each TLsec with the binodal curve (Equation 1) were determined with a substitution procedure and the length (TLu, %) was determined for each line through the Pythagorean theorem (Raja et al., 2011).

2.3. Volume ratio analysis

The volume ratio ($V_t$) between top ($V_t$) and bottom ($V_b$) phases of ATPE systems was defined in the form of Equation (5) and was evaluated at the middle point of TLmain at the point D, and at other six points of the same line, with PEG4000 concentration of 30%, 25%, 15%, 5%, 0.5%, and 0.1%.
\[ V_r = \frac{V_t}{V_b} \]  

The \( V_r \) value was correlated with PEG4000 and (NH\(_4\))\(_2\)SO\(_4\) concentrations and the state that allowed for equivalent volume ratio (\( V_r = 1 \)) was determined. A similar procedure was performed with \( T_{\text{LB}} \). All mixtures were prepared at 25°C with deionized water as solvent.

### 2.4. ATPE systems based on (NH\(_4\))\(_2\)SO\(_4\)-PEG4000-whey

Mixtures with 30%, 25%, 15%, 5%, 0.5%, and 0.1% PEG4000 were again prepared at 25°C, but with whey as solvent instead of water. Phases were evaluated in terms of \( V_r \) and protein and lactose concentrations. A partition coefficient (\( K \)) and separation yields (\( Y_r, Y_b \)) were determined for each component with Equations (6) and (7), where \( c_r, c_b \), and \( c_p \) are concentrations in top (\( T \)) and bottom (\( B \)) phases, and in the original whey volume (\( V_0 \)), respectively.

\[ K = \frac{c_r}{c_b} \]  
\[ Y_r (\%) = \left( \frac{c_r \times V_t}{c_r \times V_0} \right) \times 100; \quad Y_b (\%) = \left( \frac{c_p \times V_b}{c_p \times V_0} \right) \times 100 \]  

### 2.5. Response variables

Protein concentration was determined with the absorption method at 220 nm (Kamizake, Gonçalves, Zaia, & Zaia, 2003), with a Hach DR 5000 UV-Vis spectrophotometer (Hach, Mexico). Concentrations were expressed as mg equivalents of BSA per mL (mg mL\(^{-1}\)) with the aid of a standard curve based on BSA in a range from 10 to 80 mg mL\(^{-1}\). Lactose concentration was quantified with the phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), with a spectrophotometer with a microplate reader (Biotek, Sinergy 2, BioTeck Instruments, U.S.A). A lactose standard curve in the range from 10 to 200 mg mL\(^{-1}\) was used to evaluate concentrations. In addition, fat content in the original whey was measured with a Milko Scan FT1 (Foss, Denmark).

### 2.6. Data analysis

The relationship between volume ratio (\( V_r \)), protein and lactose concentration, separation yields (\( Y_r, Y_b \)), and partition coefficient (\( K \)), with PEG4000 and (NH\(_4\))\(_2\)SO\(_4\) concentrations, was analyzed. Data were submitted to regression routines and the determination coefficient (\( r^2 \)) was taken as indicator of the significance of change of behavior.

### 3. Results and discussion

#### 3.1. Whey cheese composition

Whey had 1.02 (±0.01) % fat, 10.68 (±0.077) mg mL\(^{-1}\) protein, and 53.90 (±0.29) mg mL\(^{-1}\) lactose. Protein and lactose contents were of the same magnitude order as data reported by Kalaivani and Regupathi (2015), who found values of 5.49 and 47.20 mg mL\(^{-1}\), respectively.

#### 3.2. Phase binodal diagram

Figure 1a shows the binodal diagram obtained for the system (NH\(_4\))\(_2\)SO\(_4\)/PEG4000 with data of González-Amado et al. (2016) corresponding to the binodal curve. PEG is one of the most used polymers in aqueous two phase systems and as molecular weight increases, the required quantity to form a biphasic system is lesser (Raja et al., 2011), but with higher molecular weight the system viscosity increases and may cause increment of the required time to achieve a complete separation, thus intermediate molecular weights are recommended (Regupathi, Srikanth, & Sindhu, 2011). In this regard, Jampani and Raghavarao (2015) evaluated the recovering of anthocyanins from Brassica oleracea with ATPE systems using PEG with different molecular weights and found that the partition coefficient and the separation yield increased with an increment in molecular weight up to 4000 Da and decreased with

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**Figure 1.** (a) Phase diagram of the (NH\(_4\))\(_2\)SO\(_4\)/PEG4000 system at 25°C. Subscripts t and b correspond to top and bottom intersection points of tie lines with the binodal curve, respectively. (b) Relationship between volume ratio \( V_r \) and PEG4000 concentration for ATPE systems prepared with water and whey as solvent. MPm corresponds to the middle point of TL\(_{\text{max}}\). The (NH\(_4\))\(_2\)SO\(_4\) concentration in E\(_1\), E\(_2\), F\(_1\), F\(_2\), G\(_1\), G\(_2\), H\(_1\), H\(_2\), I\(_1\), and I\(_2\) was 9.64%, 18.38%, 8.10%, 21.65%, 6.93%, 24.90%, 5.69%, 28.25%, 4.52%, and 31.49%, respectively. An and Q: data of Anandharamakrishnan et al. (2003).

**Figura 1.** (a) Diagrama de fases del sistema (NH\(_4\))\(_2\)SO\(_4\)/PEG4000 a 25°C. Los subíndices t y b corresponden a los puntos de intersección de las líneas de operación con la curva binodal. (b) Relación de volúmenes \( V_r \) y concentración de PEG4000 para sistemas ATPE preparados con agua y suero como disolvente. MPm corresponde al punto medio de TL\(_{\text{max}}\). La concentración de (NH\(_4\))\(_2\)SO\(_4\) en E\(_1\), E\(_2\), F\(_1\), F\(_2\), G\(_1\), G\(_2\), H\(_1\), H\(_2\), I\(_1\) e I\(_2\) fue 9.64%, 18.38%, 8.10%, 21.65%, 6.93%, 24.90%, 5.69%, 28.25%, 4.52% y 31.49%, respectivamente. An y Q: datos de Anandharamakrishnan et al. (2003).
Table 1. Areas under the binodal curve corresponding to data of González-Amado et al. (2016) for the system (NH₄)₂SO₄/PEG4000.

| Binodal system | AUC (%)³ | Difference (%)² |
|----------------|----------|-----------------|
| González-Amado et al. (2016); 5°C | 187.97 | 11.55 |
| González-Amado et al. (2016); 20°C | 168.51 | - |
| Present work; interpolation at 25°C | 159.26 | 5.49 |
| González-Amado et al. (2016); 35°C | 130.86 | 22.34 |

* AUC: Area under the curve obtained with the Simpson’s 1/3 method (Burden & Faires, 2010) in the range from 3.46% to 34.78% of ammonium sulfate.

²Difference in relation to 20°C condition.

³AUC: Area bajo la curva obtenida con el método de Simpson de 1/3 (Burden & Faires, 2010), en el rango de 3.46% a 34.78% de sulfato de amonio.

⁴Diferencia relativa a la condición de 20°C.

This behavior was expected, since in the absence of any of the components ((NH₄)₂SO₄ or PEG4000) the ATPE system should tend to a single phase solution. On the other hand, in separation operations based on liquid-vapor equilibrium, the greater the difference between phase composition the easier the separation of components (McCabe, Smith, & Harriott, 2005). By analogy, in ATPE systems, with greater distance between the binodal curve and an operating condition, it is expected to have greater stability of the biphasic system, suggesting that maximum stability can be achieved with a mixture located on TLmain and particularly on point D, which is the most away from the binodal curve (Figure 1a).

Figure 1. Relationship between tie line lengths and concentration of ammonium sulfate ((NH₄)₂SO₄) and, as TLw diminished in water-based mixtures, which indicates that the bottom phase was significantly greater than the top one. In a continuous operation, the separation of components based on ATPE can be implemented in a similar way to a continuous gravitational decantation (McCabe et al., 2005), and the phase extraction through overflows at the high and low regions of the equipment can be favored if the interface line is located at the middle of the system. However, based on Equation (8), the composition of mixtures with Vr = 1 on the main (TLmain) and also on secondary (TLsec) tie lines did not correspond to middle points, but to states located in the first upper third of tie lines when the system used water as solvent (Figure 1a) and, as TLw was smaller, the condition for Vr = 1 was closer to the binodal curve, suggesting lower system stability. In addition, the relationship between TLw and concentration of (NH₄)₂SO₄ and PEG4000 for Vr = 1 was linear (Figure 2; Equations 9 and 10) and the required amounts of PEG4000 increased at a higher rate with TLw than those of (NH₄)₂SO₄, which may be a disadvantage due to the polymer higher cost.

\[ x^{1/3} = 0.1099 TLw + 7.6807; \quad r^2 = 0.9726 \]  \hspace{1cm} (9)

\[ y^{1/3} = 5.4680 TLw + 0.3986; \quad r^2 = 0.9987 \]  \hspace{1cm} (10)

Figure 2. Relationship between tie line lengths and concentration of ammonium sulfate ((NH₄)₂SO₄) and PEG4000 to obtain volume ratio Vr = 1.
Based on these considerations and in order to evaluate the effect of PEG4000 concentration on whey protein purification, a study was made with mixtures located on the main tie line.

3.4. Protein and lactose separation

Mixtures for points 1, 2, and 4 to 7 of Figure 1 were prepared again, but whey was used as solvent instead of water. Mixture 1, which contained 30% PEG4000 and 7.5% ammonium sulfate, did not form a biphasic system, although it was located within the two-phase region in the binodal diagram prepared with water, suggesting that the equilibrium curve shifted to the right when whey was used as solvent. Systems 2 and 4–7 showed greater $V_r$ than systems based on water at the same concentration of PEG4000 (Figure 1b). Similarly to the case of water-based systems, $V_r$ decreased asymptotically and data fitted well ($r^2 = 0.9857$) to Equation (8), but with values in constants $k_7$, $k_8$, and $k_9$ equal to 0.3259 (dimensionless), 0.1236 (dimensionless), and 0.1053%$^{-1}$, respectively. With this support, the whey-based mixture that allowed $V_r = 1$ had concentration of 20.01% $(\text{NH}_4)_2\text{SO}_4$ and 16.13% PEG4000 (point 3; Figure 1a and 1b). This condition contrasted with that identified when water was used as solvent (13.78, 23.63%, respectively; Figure 1a) and was located near the middle point of TLmain, which strengthens the fact that the binodal curve shifted from the original. In this regard, Rito-Palomares and Hernandez (1998) studied the effect of adding whey to an ATPE system formed by phosphate/PEG1000 with water. Authors chose six points near the monophasic–biphasic equilibrium curve and gradually added whey until a monophasic mixture was obtained. Results showed that the transition condition between phases shifted to the right at high concentration of PEG and this phenomenon may be due to presence of proteins, lactose, vitamins, and mineral salts in whey (Kreczmann et al., 2015), which alter the ionic balance. Also based on Equation (8), while systems prepared with water had $V_r = 0$ when $y_\alpha = 0$, whey-based systems had $V_r = 0.3259$ at the same condition (Figure 1b). Although this result may seem unexpected, proteins are high molecular weight compounds formed by amino acids covalently linked together, forming long non-branched polymers (Nelson & Cox, 2008). In this regard, $\alpha$-lactalbumin (ala) has 123 amino acid residues with molecular weight of 14,175 Da, while $\beta$-lactoglobulin (blg) has 162 residues with molecular weight of 18,277 Da (Pihlanto, 2011). In this sense, when PEG4000 decreased, the role of the polymer in the two-phase system could be replaced by the polymeric characteristic of whey proteins and this explains that the biphasic structure of the system was maintained.

Among the most interesting characteristics of ATPE systems applied to purify proteins is the possibility of allowing a separation, in selective form, of one protein among a group, through partition between phases (Gai et al., 2011). In fact, Alcántara et al. (2011) and Kalaivani and Regupathi (2015) demonstrated the feasibility of separation of ala from blg with this type of strategy. However, if the objective is to maximize the separation of all proteins, the results of the present work suggest that the use of a mixture of whey and $(\text{NH}_4)_2\text{SO}_4$ in amounts required to form a biphasic system, could develop a non-energy strategy of protein concentration. In this regard, as PEG4000 concentration ($y_\alpha$) decreased, the protein concentration increased in the polymeric phase and decreased in the saline one (Figure 3a). In the first case, the behavior was congruent ($r^2 = 0.9821$) with a logarithmic model with the form of Equation (11), where $r$ represents protein concentration in the top phase (%) and constants $k_{10}$, $k_{11}$, and $k_{12}$ had values equal to 32.2469 mg mL$^{-1}$, 7.5125 mg mL$^{-1}$, and 2.1430%, respectively. In contrast, data of the saline phase fitted well

![Figure 3. Effect of PEG4000 concentration on protein (a) and lactose (b) concentrations, separation yield (c), and partition coefficients (d) in ATPE systems. Circled numbers identify the ATPE systems signaled in Figure 1.](image-url)
\( r^2 = 0.9895 \) to an exponential model with the form of Equation (8), where \( k_r, k_p, \) and \( k_b \) had values equal to 0.3367 mg mL\(^{-1}\), 1.0062 mg mL\(^{-1}\), and 0.0778%\(^{-1}\), respectively.

\[ v = k_{10} + k_{11} \ln (y_p - k_{12}) \]  

(11)

However, it should be considered that \( v \), decreased with PEG4000 concentration (Figure 1b), causing that the separation yield of protein (\( Y_r \), Equation 7) increased in the upper phase as \( y_p \) decreased (Figure 3c), in congruent form with Equation (11), where \( v \) represents \( Y_r \), \( k_{10} \) = 206.5252\%, \( k_{11} \) = 37.0026\%, and \( k_{12} \) = 21.5045\%, so that when the mixture had 25% PEG4000 and 12.1% \((\text{NH}_4)_2\text{SO}_4\), \( Y_r \) was 54.45\%, but with 0.1% PEG4000 and 34.7% \((\text{NH}_4)_2\text{SO}_4\), \( Y_r \) increased to 92.99%, indicating high protein separation efficiency. The AS is an auxiliary compound in protein quantification methods due to its ability to cause protein precipitation (Fujita et al., 1993; Jinn et al., 1989). As a result, as the saline phase volume increased, a phase with high protein content was formed in the polymeric region. In system 2, which had the highest \( V_r \) value among the evaluated mixtures (Figure 1b), the protein concentration was similar between phases, whereas in system 7, where \( V_r \) had the smallest value, the greatest difference in protein concentration between phases occurred (Figure 3a). The protein concentration in the original whey was 10.68 mg mL\(^{-1}\), while value in the upper phase of system 7 was 26.18 mg mL\(^{-1}\), which means that protein was concentrated 2.45 times by a non-energy method formed by the ATPE system.

Anandharamakrishnan et al. (2005) studied the system PEG6000/\((\text{NH}_4)_2\text{SO}_4\) and found that as the mixture corresponded to a higher tie line length the protein separation yield (\( Y_r \)) increased. Figure 1 shows the mixtures prepared by these authors, where maximum \( Y_r \) was 24.6\%, which was obtained with a mixture having 17.88% PEG6000 and 14.26% \((\text{NH}_4)_2\text{SO}_4\) that produced a volume ratio (\( V_r \)) equal to 0.76 (point Q, Figure 1). Although the corresponding diagram could be slightly displaced to the left in relation to that prepared with PEG4000 (González-Amado et al., 2016), results of the present work suggest that \( V_r \) and PEG6000 concentration were not small enough and that of \((\text{NH}_4)_2\text{SO}_4\) was not high enough to obtain high protein recovering, because all mixtures showed \( V_r \), around 0.74. On the other hand, protein recovering and protein fractioning have been frequently studied using potassium phosphate in the ATPE system (Alcántara et al., 2011; Giraldoo et al., 2001). In fact, Anandharamakrishnan et al. (2005) studied the ATPE system formed with PEG6000 and potassium phosphate and showed that with \( V_r \) equal to 13.5 it can be possible to have protein recovering from whey of 85\%, although target compounds still remain much diluted, because the polymeric phase would be too large and the operation could be expensive due to the cost of the polymer.

Figure 3b shows the case of lactose behavior. As PEG4000 concentration decreased, the lactose concentration increased in the polymeric phase and decreased in the saline one, which seems to be an opposite effect to the desired goal of reducing lactose in the protein extract. The behavior was logarithmic, congruent with Equation (11), where \( v \) represents lactose concentration and \( k_{10}, k_{11}, \) and \( k_{12} \) had values equal to 59.4740 mg mL\(^{-1}\), 13.8419 mg mL\(^{-1}\), and 4.0770% in the case of top phase (\( r^2 = 0.9535 \)), and 455.5747 mg mL\(^{-1}\), 112.9901 mg mL\(^{-1}\), and 80.5589\%, in the bottom one (\( r^2 = 0.9698 \)), respectively. However, as the polymeric phase volume decreased and that of the saline phase increased with reduction of PEG4000 concentration, the lactose separation yield in the saline phase increased (Figure 3c), which fitted well (\( r^2 = 0.9686 \)) to Equation (11), with \( k_{10} = 101.1726, k_{11} = 10.5688, \) and \( k_{12} = 15.2788\% \), from which the separation of lactose in the lower phase (\( Y_p, \) Equation 7) went from 62.11\% with 25% PEG4000 to 72.29\% with 0.1\% of the polymer. The initial whey had lactose concentration of 53.90 mg mL\(^{-1}\) and, although at the almost total absence condition of PEG4000 (system 7, Figure 1) the upper phase of the ATPE system recorded 39.69% lactose (Figure 3b), the analysis of separation yield showed that, of the total, more than 70\% of the disaccharide was retained by the lower phase (Figure 3c). This suggested that the best condition for separating whey proteins is an ATPE system without PEG, formed only with the addition of AS, because it allows the concentration of protein and the elimination of lactose in high percentage.

The partition coefficient \( K \) (Equation 6) had similar behavior for both protein and lactose (Figure 3d). The tendency was sigmoidal type, according to Equation (12), where regression constants \( k_{13} \) (dimensionless), \( k_{14} \) (dimensionless), \( k_{15} \) (%), and \( k_{16} \) (%) had values of 0.9735, 31.5051, 8.1000, and 2.9243 for the protein case (\( r^2 = 0.9928 \)), and 0.2070, 0.9240, 4.8594, and 3.4524 for the lactose one (\( r^2 = 0.9895 \)), respectively.

\[ K = k_{13} + k_{14}/[1 + \exp( - (y_p - k_{15})/k_{16})] \]  

(12)

A value greater than unity in \( K \) means the number of times that a compound concentration is higher in the polymeric phase than in the saline one, whereas a number lesser than unity means that the saline part has higher concentration than the polymeric. In the case of protein, the partition ranged from 1.07 to 30.56 in the transition from 25.0 to 0.1\% PEG4000, whereas for lactose the variation went from 0.21 to 0.95, indicating that the protein was mainly concentrated in the polymeric phase and the lactose in the saline one. On the other hand, whey contains fat that, although normally is present in small quantities (Fagan et al., 2009; Kreczemna et al., 2015), it has been identified as a factor that can cause fouling that hinders the recovery or proteins with processes that use ultrafiltration with membranes (Torkamani et al., 2016). In this regard and although fat elimination was not studied in the present work, Anandharamakrishnan et al. (2005) showed that it can be retained within the saline phase of the ATPE system and would be separated at the same time of lactose. Authors explained that the presence of fats reduces the solubility and dispersibility of fat and this forms big droplets in salt rich phase.

### 3.5. pH variation

The pH of the original whey was 6.90 (±0.02) and decreased linearly (\( r^2 = 0.9502 \)) at a rate of 0.0105%\(^{-1}\) as the concentration of \((\text{NH}_4)_2\text{SO}_4\) increased and that of PEG4000 decreased in ATPE systems (Figure 4). However, the difference between the original whey and the system with the highest concentration of \((\text{NH}_4)_2\text{SO}_4\) was only 0.36
units, even though this compound forms solutions with acid pH (Benavides & Rito-Palomares, 2008). Hill, Irvine, and Bullock (1985) reported a whey buffering capacity when pH was within 5.6 and 7.0, due to the content of phosphate salts, which may explain the observed behavior. The basket whey contains β-lactoglobulin, α-lactalbumin, and BSA in approximate amounts of 60.0%, 30.0%, and 6.0% (Boaglio, Bassani, Picó, & Nerli, 2006), in relation to total protein content, with isoelectric points of 5.2–5.4, 4.7–5.1, and 4.9–5.1, respectively (Capezio, Romanini, Picó, & Nerli, 2005). The pH of systems of the present work was far more than one unit from the isoelectric point for the proteins contained in whey, which may be beneficial, because the solubility of such compounds is not modified to a great extent.

4. Conclusions

The ATPE system formed with (NH₄)₂SO₄/PEG4000 allowed the separation of proteins and lactose from whey. The use of whey as dissolvent of the forming ATPE components caused displacement of the binodal curve in relation to the case that used water as dissolvent. The volume ratio (V/w) in the ATPE system affected partition between phases and separation yields of proteins and lactose. As PEG concentration diminished and that of (NH₄)₂SO₄ increased the separation yield of proteins and lactose increased, thus small V/w values are recommended to maximize target biomolecules separations. Even in conditions of absence of PEG4000 of a biphasic system was still formed, which was attributed to the polymeric structure of proteins. The handling of whey incorporated with AS can be used as a non-energy strategy of concentration and purification of proteins of this by-product.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Author Ricardo Domínguez-Puerto wishes to acknowledge the financial support received from Consejo Nacional de Ciencia y Tecnología de Mexico (CONACyT).

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