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Short communication

Aqueous two-phase systems for protein separation Studies on phase inversion

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Abstract

Phase equilibrium studies were done with the PEG 400–phosphate system, obtaining equilibrium binodal lines, tie lines and phase inversion points. A method of calculation of the critical point on the binodal curve is described. The influence of the presence of NaCl in solution was studied, and the comparative results are presented. It was found that in some range of concentration the shift produced in the binodal line can be important. The rate of phase separation can be used as an indication of which of the phases is continuous. Using this method the phase inversion point can be determined in the system for each tie line. A range of ambiguity was found, where the continuity of the phases is affected not only by the composition of the mixture, but also by the fluid dynamics. Within this range, gentle agitation produces a bottom-continuous suspension, while strong agitation produces a top-continuous suspension. Two inversion points exist therefore on each tie line, delimiting on the phase equilibrium plane a region where the phase continuity depends on fluid dynamics. The convergence of this region towards the critical point can be used to control of the consistency of the experimental data. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Albertsson [1] was the first to observe, in the early 1950s, that when a protein is introduced into an aqueous system where formation of two liquid phases occurs, such as those formed by poly(ethylene glycol) (PEG) and dextran or PEG and salts (phosphate), it is distributed unevenly between those two phases. This gave the basis to analogies with liquid–liquid (organic–aqueous) extraction processes, which are quite common in chemical industries.

Aqueous two-phase systems (ATPSs) have great potential for the efficient and low-cost separation of

proteins that can be difficult to separate on a large scale. These systems have important advantages in their ability to process streams with small particulate material (whole broth extraction) and to handle large volumes in a continuous mode with short contact times. However, even though recent advances in obtaining high purification factors for separation of target proteins from their contaminants using ATPSs have been successful on the laboratory scale, these are not widely used in large scale, industrial operations. This is, in part, due to lack of basic engineering knowledge and understanding of the systems.

Conditions for good recovery yields and high purification factors have been obtained for most proteins that have been studied. This is usually done

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following a semi-empirical procedure that includes experimental study of the effects of variables as PEG molecular mass, type and concentration of ions included, pH, polymer concentration, protein properties, etc. As a typical example the separation and purification of the protein thaumatin, that has been cloned in bacteria has been recently demonstrated. A 20-fold purification and a 90–95% yield were achieved in one step using ATPSs [2]. This has also recently been done for monoclonal antibodies [3], tPA [4], VLPs, which are recombinant virus like particles cloned in yeast [5] and the purification of calf chymosin from *Aspergillus awamori* using a PEG 8000–Na₂SO₄ ATPS [6]. All these procedures were demonstrated as batch extraction processes without considering the rate of the phase separation step in the overall process.

The explanation of partitioning based on molecular properties of the protein has also been researched in some detail, and investigations into predictive partitioning are already under way both based on group contributions [7–10] and on fundamental thermodynamic theory [11,12]. Experimental data, however, remain the only basis for industrial design and applications, especially because those theoretical approaches give an approximate description of the equilibrium steady state, but none of them addresses the kinetics of the separation process.

The similarity in the properties of the phases is a main difference between ATPSs and the aqueous–organic systems traditionally used in the chemical industry. This similarity is also a key factor in the mildness of ATP operation, minimizing the damage and loss of activity of the proteins being recuperated. Conversely, a small difference in physical properties leads to difficulties in the separation of the phases. This is easy to overcome in the laboratory using centrifugation. In a large scale plant, on the other hand, the possibility of using gravitational separation should always be considered, because of the advantage of lower initial inversion in equipment, mechanical simplicity, maintenance and operation costs. This consideration is possible only if reliable basic knowledge of the mechanism of phase separation is available. The main macroscopic variable is separation time, which is closely related to phase continuity and phase inversion points. However the basic understanding of the separation will include the

phenomena of droplet rise (or fall) and coalescence, which, in turn, depend on the physical properties of the phases.

It has been shown recently [13] that data on separation time as a function of system properties can be obtained and presented in such a way as to give them general validity. Experimental results on the separation kinetics of the phases in PEG–phosphate systems showed that by representation the profile of dispersion height as a fraction of the initial height produces a generalized profile which is independent of the geometrical dimensions of the separator. Data taken in tubes and a two liter tank (200-fold scale-up) gave very similar results for most of the stability ratios studied. This generalization is extremely important for the design of large scale aqueous two-phase separators.

Some attention has been given in the literature to the problem of ATP separation rate [14–17] but no systematic studies have been carried out to establish the background for the development of engineering methods in the design of large scale gravitational separators for ATP systems. Such knowledge is also important in simulation schemes, to find the optimal design of a whole system, including the phase separation step.

1.1. Phase inversion point

Phase inversion is the phenomenon in which, under certain conditions the phase that was initially present as dispersed droplets coalesces and becomes continuous, while the continuous one is broken and dispersed as droplets. It has been observed that this phenomenon may become very important in aqueous–organic systems [18–20].

In the case of aqueous–organic systems, the detection of phase continuity can be done using conductivity probes [18]. In ATPSs it is difficult to perform the measurement of a physical property indicating which phase is the continuous one because the physical characteristics of the phases are quite similar. However, visual observation of the system during the separation may allow identification. Provided that droplets are large enough and the system is very calm, either ascending droplets are observed (indicating a continuous bottom phase) or descending

droplets (indicating a continuous top phase). However, the appearance of the dispersion near the interface differs, as will be explained later, so that the visual observation gives a clear indication of phase continuity. Nevertheless, it was found here that the most unequivocal method for the determination of the phase inversion point is the measurement of the phase separation time. This will be expanded later.

Several important points related to the phase inversion in a PEG 4000–phosphate system have been reported previously [13]. Phase continuity was found to have a strong influence on the rate of separation of ATPSs. Depending on the volumetric phase ratio and on which of the phases is the continuous one, the separation time may vary almost by one order of magnitude in PEG–dextran systems, and by 100% in PEG 4000–phosphate systems. Photographs corresponding to the same tie line (TL) at both sides of the phase inversion point showed the difference between a system with top-continuous phase and bottom-continuous phase [13]. In that paper a straight line was reported to represent the locus of phase inversion points for each of the TLs. It was suggested that for the specific case of PEG 4000–phosphate ATP systems, the phase plane could be divided into three regions: (1) below the binodal curve, where a single phase exists. (2) Above the binodal curve, and to the left of the phase inversion line, where the system will separate, having a continuous bottom (PEG lean) phase. (3) Above the binodal curve, and to the right of the phase inversion line, where the system will separate having a continuous top (PEG rich) phase. While the representation of the inversion point as a straight line was a close enough representation of the experimental results [13], the present data differ in this point, as will be shown later in this paper.

1.2. Phase separation mechanism

There can exist two extreme situations during the separation of an ATPS. If the rate of droplet ascent and descent is much slower than the coalescence, then the former will determine the separation rate. If in addition, droplet interaction is small (which will happen when the phase ratio is such that the dis-

persed phase is much smaller) the whole process will be governed by Stokes law. The latter systems will be characterized by a dispersion that is very homogeneous and by an undefined limit between the separated continuous phase region and the dispersion. On the other hand if the coalescence of the droplets is the controlling mechanism, the droplets will rapidly finish their “Stokes ascent/descent” (no coalescence will occur during this journey) and “queue” at the interface, waiting for coalescence. This was the case for most of the systems reported in the present paper.

2. Experimental

PEG with a molecular mass of 4000 Da was obtained from Fluka (Buchs, Switzerland). Phosphate solutions with pH 7 were prepared from mixtures of monobasic and dibasic sodium phosphate from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively, and sodium chloride from Merck. “Mother solutions” of PEG 40% and phosphate 20% were prepared and used for all the experiments in a series. The methods detailed by Kaul et al. [13] were used. The experimental determination of the binodal curves was done by the “cloud point” method, using a volumetric method when PEG-lean phase or water was added, and a gravimetric method when more viscous PEG-rich solution was added.

For the determination of the TLs, a simple gravimetric method was used, which makes unnecessary any chemical analysis. The method is the following: a point is selected in the phase-plane, through which the TL will pass. The components required are then weighed and mixed thoroughly. The mixture is left at 20°C in a decanter overnight, and then the two fractions are weighed. Calling M, T, and B the points representing the mixture, the top phase and the bottom phase, respectively, X the weight fraction of phosphate and Y the weight fraction of PEG the following mass balance equations can be written:

$$Y_T = (Y_M/\alpha) - ((1 - \alpha)/\alpha)Y_B \quad (1)$$

$$X_T = (X_M/\alpha) - ((1 - \alpha)/\alpha)X_B \quad (2)$$

$$Y_T = f(X_T) \quad (3)$$

$$Y_B = f(X_B) \quad (4)$$

where $f(X)$ is the function representing the binodal giving Y as a function of X , and α is the following measured ratio:

$$\alpha = (\text{weight of the top phase}) / (\text{weight of the mixture}) \quad (5)$$

This is a system of four equations with four unknowns that can readily be solved, and the equation for the TLs can be obtained:

$$Y = Y_o + SX \quad (6)$$

where the slope S is given by:

$$S = (Y_T - Y_B) / (X_T - X_B) \quad (7)$$

and Y_o is the point corresponding to $X=0$.

Furthermore, having obtained several TLs, an expression of the slope of the family of TLs, $g(X_B)$ can be obtained in terms of X_B . The point where the derivative of the binodal equals this slope can be obtained now from:

$$g(X) = df(X)/d(X) \quad (8)$$

The value of phosphate concentration X_c that fulfils this equation corresponds to the critical point, which is obtained therefore without any other experiments.

For the experimental determination of the TL, a straight line passing through the origin was drawn arbitrarily on a graph representing the binodal obtained. A series of points was chosen in the two-phase region at regular intervals and was used to define the TLs. The compositions representing those points were prepared from the mother solutions of PEG and phosphate and water, agitated thoroughly and left overnight for complete separation. The top and bottom solutions were separated and used in different proportions to prepare mixtures corresponding to the different points along the TL. In each of these mixtures, the separation time was measured by visual inspection.

3. Results

Fig. 1 shows experimental equilibrium data obtained for the system PEG–phosphate, without addition of NaCl. The binodal line calculated by regression, using the mathematical form used elsewhere [13]. For this line, the following expression is obtained with $R=0.999$:

$$Y = 71.7 \exp(-0.62X^{0.5} - 0.00123X^3) \quad (9)$$

The TLs were obtained by the gravimetric method as explained above. The sensitivity of the obtained TLs to the experimental error in the determination was inspected. Fig. 2 shows how X_B , the concentration of phosphate in the bottom phase depends on the value of α , the ratio of mass of top phase to total mass. From the slope, and considering that the error in the determination of α is of the order of 10^{-3} , it can be concluded that the error in phosphate concentration is of the order of $4 \cdot 10^{-3}$, which is satisfactory. The TLs obtained are a family of straight lines that can be represented by:

$$Y = Y_o + SX \quad (10)$$

where S is the slope of the line given in Eq. (7).

As can be seen in Fig. 1, the slope of the TLs is not constant, as sometimes assumed, but increases

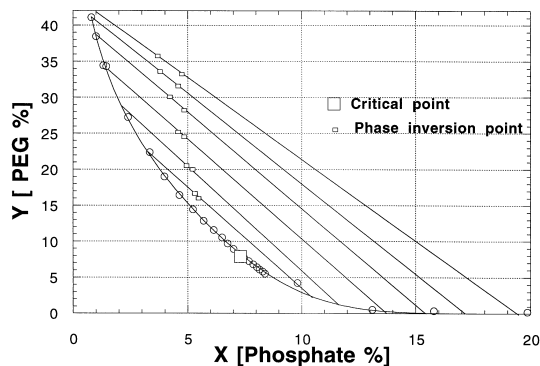


Fig. 1. Experimental equilibrium data obtained for the system PEG 4000–phosphate. The binodal line shown was calculated by regression. The TLs were obtained by the gravimetric method. The phase inversion points and the critical point obtained by the methods described in Section 2 are shown on each TL. The mass % is indicated on the axes, but in all the mathematical expressions X and Y are given in mass fraction.

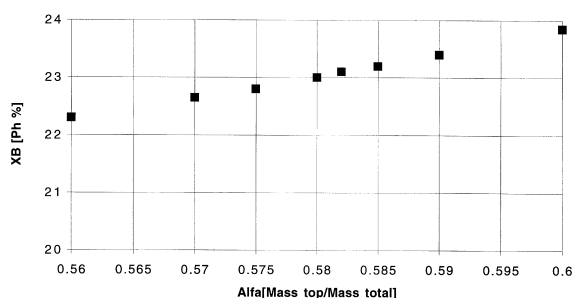


Fig. 2. Concentration of phosphate in the bottom phase, X_B , as a function of α , the ratio of mass of top phase to total mass. From the slope, and considering that the error in the determination of α is of the order of 10^{-3} , it can be concluded that the error in phosphate concentration is of the order of $4 \cdot 10^{-3}$.

slightly as the stability ratio (SR) decreases. SR is defined as the ratio of two distances on a straight line passing through the origin: the distance from a point to the binodal line, and the distance to the origin.

This dependence can be expressed in this particular case as:

$$S = -1.754 - 18.75X_B + 85X_B^2 \quad R = 0.984 \quad (11)$$

The slope S can also be expressed as a function of other variables, as the tie line length (TLL)

$$S = -2.99 - 4.64(\text{TLL}) + 10.5(\text{TLL})^2 \quad (12)$$

or the stability ratio (SR). In the present experiments, the SR for the points selected for the definition of the TLs varied from 0.06 to 0.44. The slope of the TL can now be represented by

$$S = -2.782 - 0.659(\text{SR}) + 3.934(\text{SR})^2 \quad (13)$$

In Eq. (10), Y_o is the ordinate in the origin that would be obtained by extending the TL. In the present case it can be expressed as a function of X_B :

$$Y_o = -0.935 + 14.23X_B - 38.18X_B^2 \quad R = 0.989 \quad (14)$$

The value of S can be used to find the critical point on the binodal. The point where S , (Eq. (9)), equals the slope of the binodal (the derivative of Eq. (7)) gives for the critical point the coordinates $Y_c = 7.14$, $X_c = 8.73$. The slope of the binodal was obtained by derivation of the mathematical expression representing the binodal curve, Eq. (9) in the present case.

3.1. Experimental results on fluid dynamics and phase inversion

It has been shown before, on the basis of experimental evidence and thermodynamic analysis [18], that the fluid dynamics of the system may have an influence on phase continuity. In experiments reported here it was found that this phenomenon occurs also in ATPSs. Experiments were performed by measuring the time to obtain macroscopic separation of a dispersion obtained by mixing different proportions of top and bottom phase along a given TL. A sample result obtained for the system PEG–phosphate on the TL passing through the point PEG=15.6%, Ph=11%, are shown in Fig. 3. The Figure displays the separation time, t_s , as a function of ϕ , the phase-ratio (the mass ratio of top to bottom phase). Two lines can be seen. The lower one, representing the separation time measured after gently shaking the tube for 30 s, corresponds to bottom phase continuous mixtures. The upper line when the top phase is continuous was obtained by vortex mixing the tube for 1 min.

The two types of dispersions (top-continuous and bottom-continuous) were easy to identify: the top-continuous phase mixtures had an opaque, cloudy appearance, while the bottom-continuous mixtures were extremely shining and relatively large droplets were observed. The coalescence process was easily visualized, with large droplets appearing at the interface and coalescing into the upper phase. Fig. 3 shows that a range of ϕ exists, where, depending on the intensity of mixing, either top-continuous or bottom-continuous systems may be obtained each leading to quite different separation times. This is not a real hysteresis, since the loop is not generated because of the direction of changes in the variable ϕ . Rather, there are two lines, one of separation time after gentle mixing, and the other after intense mixing, which coincide at high and low ϕ . If mild mixing is used, the mixing time remains almost constant as the top-phase fraction ϕ increases, until $\phi = 0.81$ is reached. At this point, a sudden jump in the separation time can be seen, indicating that the suspension had become top-phase continuous. This change in the separation behavior coincided with the change in the appearance of the suspension, which becomes cloudy and milky. If vigorous mixing is

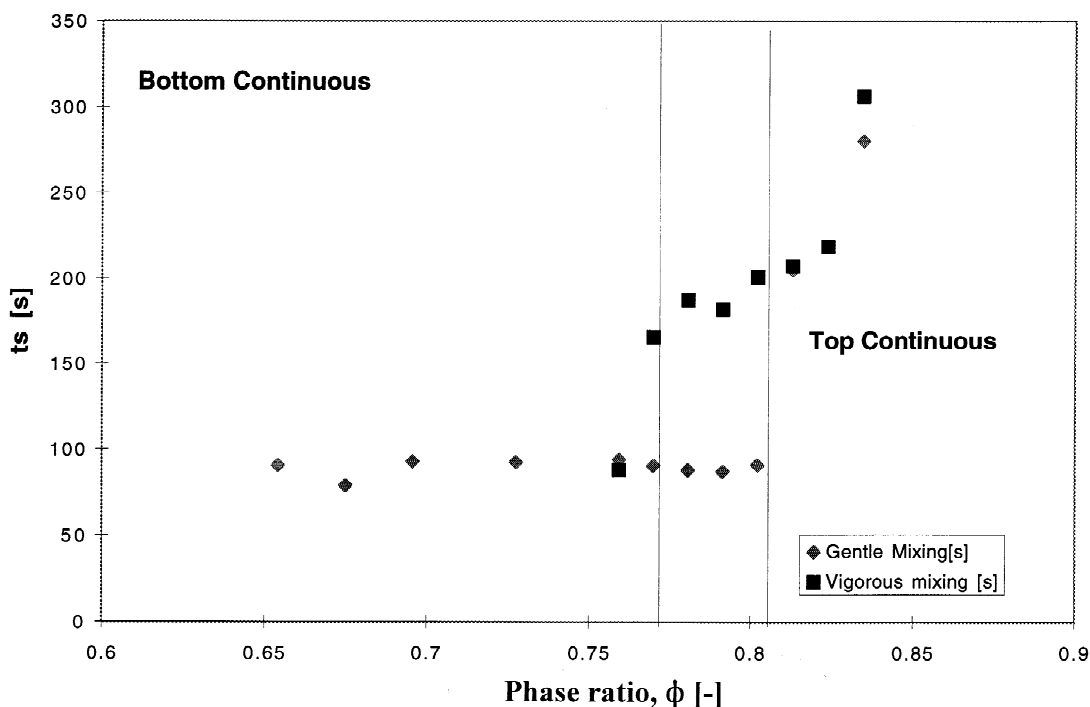


Fig. 3. Separation time, t_s , as a function of ϕ , the phase-ratio i.e., the mass ratio of top to bottom phase. The lower line represents the separation time measured after shaking gently the tube during 30 s, and corresponds to bottom phase continuous mixtures. The upper line where the top phase is continuous was obtained by vortex mixing the tube during 1 min.

applied, the jump is detected earlier, at $\phi = 0.77$.

This observation, which has not been reported until now, may have important consequences in the engineering considerations for scale-up of a ATPS.

This procedure was repeated for each of the TLs. The points of phase inversion obtained are shown on Fig. 1. Both the upper and the lower inversion points are shown on each TL. The pairs of inversion points for the six TLs presented illustrate a region where the phase continuity of the dispersion depends not only on the properties of the phases. Phase continuity remains ambiguous unless fluid dynamics are defined. The zone of ambiguity becomes narrow as TLL of SR decrease.

The critical point can be defined as the point where the top and the bottom phase coincide. Obviously, the phase inversion point, which must be located between the top-continuous and the bottom-continuous states, must also fall at the same point.

This fully concurs with the critical point previously obtained, as can be seen in Fig. 1. It is worth stressing that the critical point was obtained from

completely independent experiments. The critical point falls on the line representing all inversion points, which is an additional proof of the consistency of the experimental data.

3.2. Effect of NaCl concentration

The application of ATPSs to continuous processes for protein recovery requires that conditions be changed from stage to stage to be able to direct the desired product either to the top, PEG-rich phase, or to the bottom phosphate-rich phase. This is possible by manipulation of variables such as pH or NaCl concentration, which have a strong influence on the partition constant of the proteins. It is, however, important to be aware that those changes in composition may affect the phase equilibrium itself (binodal and TLs).

To explore this problem, a series of experiments was run by adding enough NaCl to reach the concentration of 2% and 10% in all mixtures. The addition was made in the mother solutions of PEG

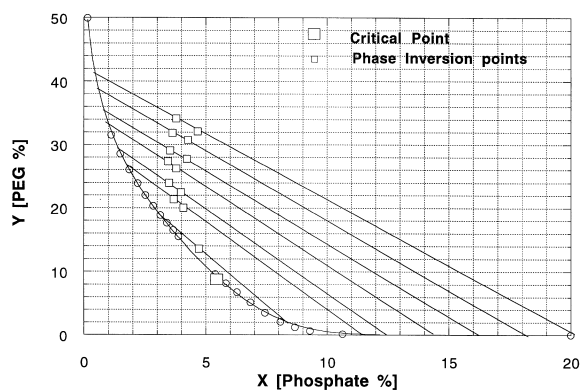


Fig. 4. Experimental equilibrium data obtained for the system PEG 4000–phosphate with the addition of 2% NaCl. The binodal line shown was calculated by regression. The TLs were obtained by the gravimetric method. The phase inversion points and the critical point obtained by the methods described in Section 2 are shown on each TL. The mass % is indicated on the axes, but in all the mathematical expressions X and Y are given in mass fraction.

and phosphate and in the water to be used in the determination of the binodal line by the cloud point method. The binodal lines for both concentrations of NaCl are shown in Figs. 4 and 5.

The same formalism was used for the mathematical representation of the three binodal curves:

$$Y = M_1 \exp(M_2 X^{0.5} + M_3 X^3) \quad (15)$$

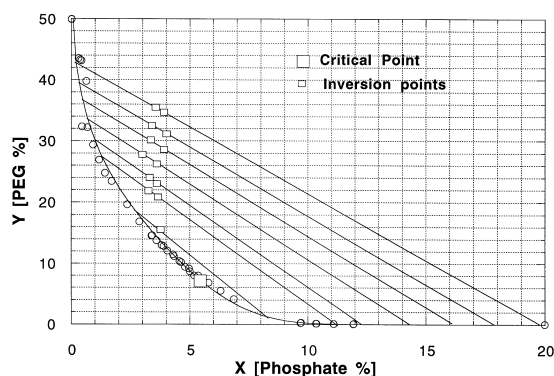


Fig. 5. Experimental equilibrium data obtained for the system PEG 4000–phosphate with the addition of 10% NaCl. The binodal line shown was calculated by regression. The TLs were obtained by the gravimetric method. The phase inversion points and the critical point obtained by the methods described in Section 2 are shown on each TL. The mass percentage is indicated on the axes, but in all the mathematical expressions X and Y are given in mass fraction.

Table 1
Binodal line parameters

NaCl %	M_1	M_2	M_3
0	71.7	−0.062	−0.00123
2	63.69	−0.6438	−0.00268
10	57.43	−0.644	−0.0035

where the respective values of the constants are given in Table 1.

It was found that the coefficients M_1 , M_2 and M_3 could be represented as follows:

$$M_1 = 64.552N^{(-0.009)} \quad R = 0.995 \quad (16)$$

$$M_2 = -0.608 - 0.0254N \quad R = 1 \quad (17)$$

$$M_3 = 0.00145 - 0.0004743N \quad R = 0.995 \quad (18)$$

where N is the concentration of NaCl in percentage units. The curves for the three concentrations can be compared in Fig. 6. It can be seen that the addition of NaCl produces a shift of the binodal line to the left. The line of 2% NaCl almost coincides with that of 10% at low values of PEG concentration, and is closer to that of water at high PEG concentrations. The figure shows that the shift in the binodal can be considerable, and should not be neglected in simulations and design calculations.

In Figs. 4 and 5 the TLs were obtained experimentally by the method described above. The equations that describe the TLs for the system PEG 4000–phosphate with the addition of 2% NaCl are the following:

$$S = -4.416 + 16.62X_B - 30.47X_B^2 \quad R = 0.998 \quad (19)$$

$$Y_o = -0.504 + 8.787X_B - 21.44X_B^2 \quad R = 0.998 \quad (20)$$

and for the 10% NaCl addition, the following:

$$S = -3.686 + 7.99X_B \quad R = 0.979 \quad (21)$$

$$Y_o = 0.154 + 1.40X_B \quad R = 0.999 \quad (22)$$

where X_B is the mass fraction of phosphate solution in the bottom.

The inversion point was investigated for each TL by the method of separation time described above. Two points are indicated on each TL. The section between those points indicates the zone where the

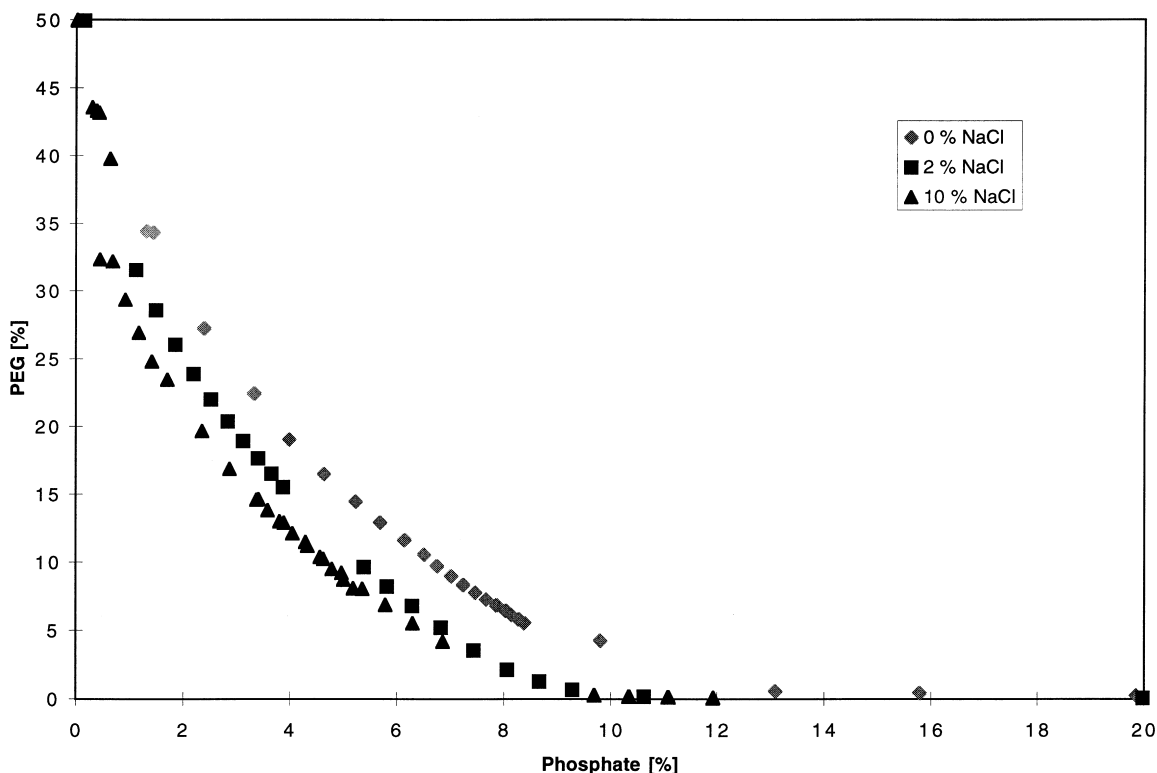


Fig. 6. Binodal lines obtained for the system PEG 4000–phosphate with the addition of 0% NaCl, 2% NaCl and 10% NaCl. The binodal lines shown were calculated by regression analysis of experimental points for each system.

continuity of the phases depends on the fluid dynamic conditions.

The critical points, obtained analytically by equaling the derivative of the function representing the binodal to the slope of the TLs (Eqs. (19) and (21)) are shown as well in Figs. 4 and 5. They correspond to (0.0514, 0.103) and (0.0489, 0.0917) in the X – Y plane. As was found before, these points become an excellent indication of the consistency of the experimental data. In both cases the critical point appears as a continuation of the zone of ambiguity of phase inversion, reassuring consistency of the data presented.

4. Conclusions

Binodal lines for the system PEG 4000–phosphate obtained with three concentrations of NaCl indicate

that the influence of the additional salt presence can not be ignored, since in a certain range of concentrations it introduces an important shift in the position of the binodal line. This can have strong influence on the downstream processing further on. TL position and slopes are also slightly changed.

The phase inversion point can be determined in the system studied (PEG 4000–phosphate) by a simple method based on measurement of separation times of samples taken along a TL. A range of ambiguity was found, where the continuity of the phases is affected not only by the composition of the mixture, but also by the fluid dynamics. Within this range, gentle agitation produces a bottom-continuous suspension, while strong agitation produces a top-continuous suspension.

The critical points were obtained by manipulation of the mathematical expressions representing the binodal and TLs. The location of this point can be

used as control of the consistency of the experimental data.

The results reported here are of relevance for the mathematical simulation of continuous processes, and for the scale up and design of large scale systems.

5. List of symbols

$f(X)$	Mathematical expression of the binodal line giving Y as function of X
$g(X)$	Mathematical expression of the slope of the binodal line
M_i	Constants in Eq. (15)
N	Concentration of NaCl, (w, %)
S	Slope of the TL, Eq. (7)
t_s	Separation time (s)
X	Concentration of phosphate (weight fraction, w, %) in the Figures
Y	Concentration of PEG (weight fraction, w, %) in the Figures

Greek symbols

α	Ratio of weights, Eq. (5)
ϕ	Weight fraction of top phase in a mixture

Subscripts

B	Bottom phase
T	Top phase
o	Origin

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