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### Notes & tips

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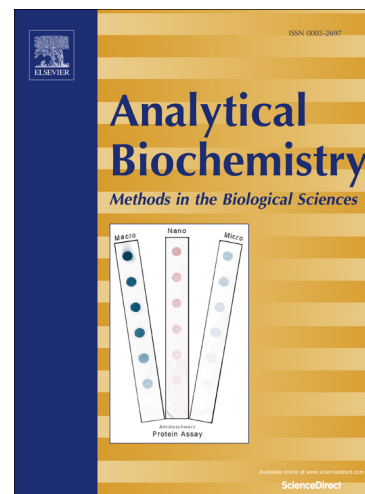
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*For Analytical Biochemistry Notes & Tips*

Determination of aqueous two-phase system  
phase-forming components in the presence of  
bovine serum albumin

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**ABSTRACT**

In the present work, the quantification of different poly(ethylene glycol) (PEG) + potassium phosphate / sodium citrate aqueous two-phase system (ATPS) phase-forming components was investigated by using conductivity and refractive index measurements. For this purpose, refractive index and conductivity calibration curves were obtained for ATPS at different pH values in the presence of different bovine serum albumin (BSA) concentrations. While BSA had no effect on the conductivity, it had a considerable effect on the refractive index. Finally, a convenient dilution of the samples prior to the ATPS constituent determination is needed to ensure no significant influence from BSA.

*Keywords:* Aqueous two-phase system, Refractive index measurement, Conductivity measurement.

Aqueous two-phase systems (ATPS)<sup>1</sup> are generally obtained by mixing a hydrophilic polymer, such as poly(ethylene glycol) (PEG) with either a suitable polymer like dextran or a salt, in water beyond a critical concentration, resulting in two immiscible phases. Both phases are composed predominantly of water, and each one is enriched with one of the different component.

These systems have been used since the mid-1950s as a mild bioseparation method of a wide applicability in cell biology, biochemistry and biotechnology for the separation and purification of large biomolecules like proteins and enzymes [1-3]. ATPS have several advantages compared to conventional downstream methods for isolation and purification of biomolecules, such as non-toxicity, high capacity, and the possibility of application on a large scale. Moreover, the required time for phase separation is relatively short and the gentle separating environment enables to maintain biological activities. Of all ATPS suitable for bioseparation, PEG–salt ATPS are favored regarding their low cost and low viscosity [1, 4].

The selective purification of biomolecules by using PEG–salt ATPS is influenced by different factors like PEG molecular weight, type of salt, pH, and system composition [1]. Therefore, the characterization of ATPS components is required for the development of ATPS, thus evaluating the influences in quantification of ATPS constituents [5]. Many polymer–salt ATPS have been analyzed by conductivity and refractive index measurements in order to determine the concentration of PEG and salt [1, 6–11]. However, the effect of the model protein bovine serum albumin (BSA) on the quantification of the phase-forming components has never been investigated systematically and reported so far.

In the present study, several PEG–potassium phosphate / sodium citrate ATPS were studied, composed of PEG's with different molecular weights (2,000; 4,000; 6,000; and 8,000 g·mol<sup>-1</sup>), pH values (5, 6, 7, 8, and 9), as well as BSA concentrations (0, 0.25, 0.5, 1, 1.5, and 2 wt. %). Various conductivity and refractive index calibration curves are reported. Each calibration

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<sup>1</sup> Abbreviations used: ATPS, aqueous two-phase systems; PEG, poly(ethylene glycol); BSA, bovine serum albumin, RMSE, root mean square error.

curve was compared with the one obtained for ATPS components in water. PEG and salt concentrations in both ATPS phases were determined using conductivity and refractive index method, respectively. All measurements were performed in triplicate, and the average results are reported.

Since the conductivity of ATPS phases depends on salt concentration [11], calibration curves of conductivity versus salt concentration were prepared for different pH values. Typical linear conductivity calibration curves for water with potassium phosphate or sodium citrate at various pH values, and 25 °C are shown in Figs. 1 A and B, respectively. The salt concentration ranged from 0–3 wt. %. The linear calibration ranges of all illustrated calibration curves in this paper were determined by the least-squares regression method. Furthermore, linearity was verified by using Mandel's fitting test with a confidence level of 95 %. As shown in Figs. 1 A and B, the conductivity rose with increasing salt concentration, as well as pH value. Conductivity is directly related to the number of ions in the solution. The higher the ion concentration is, the higher is the conductivity. Therefore, conductivity is increased due to more free moving ions in higher concentrated salt solutions at higher pH values. The conductivity is expressed by the following equation (eq 1), previously reported by Govindarajan [8]

$$K = b_0 + b_1w_s \quad (1)$$

where  $K$  represents the conductivity ( $\text{mS} \cdot \text{cm}^{-1}$ ), while  $b_0$  and  $b_1$  are the fitting parameters ( $b_0$ : intercept,  $b_1$ : slope) obtained by regression analysis from linear calibration plots of conductivity measurements of aqueous potassium phosphate (Fig. 1 A) and sodium citrate (Fig. 1 B) solutions at different pH values. Here,  $b_0$  values of potassium phosphate aqueous solutions are  $0.46 \pm 0.23$  (pH 6),  $0.57 \pm 0.29$  (pH 7),  $0.67 \pm 0.34$  (pH 8), and  $0.71 \pm 0.35$  (pH 9), while  $b_0$  values of sodium citrate aqueous solutions are  $0.39 \pm 0.20$  (pH 5),  $0.46 \pm 0.23$  (pH 6),  $0.49 \pm 0.25$  (pH 7), and  $0.52 \pm 0.26$  (pH 8). Furthermore, aqueous phosphate solutions have the following  $b_1$  values:  $7.43 \pm 0.12$  (pH 6),  $8.58 \pm 0.16$  (pH 7),  $9.35 \pm 0.18$  (pH 8), and

$9.61 \pm 0.19$  (pH 8), whereas aqueous citrate solutions have the following  $b_1$  values:  $5.86 \pm 0.11$  (pH 5),  $6.55 \pm 0.12$  (pH 6),  $6.85 \pm 0.13$  (pH 7), and  $7.06 \pm 0.14$  (pH 8). Finally, after determining the conductivity of the phases, the mass fraction of salt,  $w_s$  can be calculated by rearranging of eq 1 as follows (eq 2)

$$w_s = (K - b_0) / b_1 \quad (2)$$

It should be noted, that eqs 1 and 2 are only valid for diluted salt solutions in the concentration range of 0–3 wt. %. The root mean square error (RMSE) of  $w_s$  was  $\pm 0.0002$ . Additionally, the conductivity of PEG water solutions of varying molecular weights, as well as BSA aqueous solution was investigated, in order to evaluate these effects on conductivity. Hence, in Fig. 1 C and D linear calibration curves for PEG water systems at different molecular weights and BSA aqueous systems at different BSA concentrations are illustrated, respectively. The PEG concentration varied between 0–3 wt. %, whereas the concentration of BSA varied between 0–2 wt. %. In Fig. 1 C the conductivity of PEG is slightly increased by increasing of PEG concentration, and slightly diminished by enhancing of PEG molecular weight according to literature previously reported [11], while in Fig. 1 D a small increase of conductivity is presented by increasing of BSA concentration. The conductivity of PEG water systems is decreased with increasing of the polymerization degree of PEG due to a decreasing of conductible ions at higher polymerized PEG. Furthermore, the free moving speed of conductible ions is restricted by viscosity of PEG leading to decreased conductivity. In the case of BSA water solution the slightly increasing conductivity can also be explained by a higher amount of free moving ions. However, PEG and BSA had no appreciable effect on conductivity. Therefore, the conductivity of these components can be neglected in comparison to aqueous salt solutions.

Since the refractive index of ATPS phases depends on PEG and salt concentration [10, 11] calibration plots of refractive index versus PEG concentration were obtained at different salt concentrations. As an example, linear refractive index calibration plots for PEG 8000 +

potassium phosphate / sodium citrate systems at different salt concentrations, pH 7, and 25 °C are illustrated in Figs. 2 A and B, respectively. The concentration of PEG varied between 0–10 wt. %, while the salt concentration ranged from 0–5 wt. %. As shown in Figs. 2 A and B the refractive index of ATPS phases increased with increasing of PEG concentration, as well as with increasing of salt concentration. The reason is that with increasing of PEG and salt concentration, the density of the solution is also increased resulting in an increasing of the refractive index. The composition of PEG was determined, once known the salt concentration from conductivity measurements, by using refractive index measurements. The refractive index,  $n_D$ , and the polymer mass fraction,  $w_p$ , and  $w_s$  were related by the following equation (eq 3) proposed by Cheluget et al. [6]

$$n_D = a_0 + a_1 w_p + a_2 w_s \quad (3)$$

where  $a_0$  is the refractive index of pure water, while  $a_1$  and  $a_2$  are the fitting parameters ( $a_1 / a_2$  : slope) corresponding to polymer and salt obtained by regression analysis from linear calibration plots of refractive index measurements of only PEG–water, and only salt–water systems, respectively. Here,  $a_0$  is 1.3330,  $a_1$  is  $1.375 \cdot 10^{-3} \pm 5.940 \cdot 10^{-6}$ , and  $a_2$  is  $1.420 \cdot 10^{-3} \pm 5.927 \cdot 10^{-6}$  (for potassium phosphate), as well as  $1.745 \cdot 10^{-3} \pm 8.344 \cdot 10^{-6}$  (for sodium citrate). Finally, after measuring the refractive index, and previously determined  $w_s$ ,  $w_p$  can be calculated by rearranging of eq 3 in the following (eq 4)

$$w_p = (n_D - a_0 - a_2 w_s) / a_1 \quad (4)$$

The RMSE of  $w_p$  which was achieved by using eqs 3 and 4 was better than  $\pm 0.002$ . The coefficients  $a_1$  and  $a_2$  were independent of polymer molecular weight, which was also reported earlier for other PEG + salt systems [9]. Furthermore, these coefficients were independent of pH. For different polymer / salt aqueous systems  $a_0$ ,  $a_1$ , and  $a_2$  remain unchanged. However, it should be pointed out, that eqs 3 and 4 are only valid for diluted solutions up to concentrations of 10 wt. % polymer and 5 wt. % salt, for which linear refractive index calibration curves are obtained [11]. Beyond these concentrations, linearity is

not maintained because of the proximity to the two-phase region [9]. Thus, before refractive index measurements, it was necessary to dilute the samples to the above mentioned mass fraction range. In addition, the effect of BSA on refractive index of PEG–salt APTS was investigated. Therefore, refractive index calibration curves of PEG–phosphate / citrate in the presence of different BSA concentrations (0–2 wt. %) are presented in Figs. 2 C and D, respectively. Here, the salt concentration is fixed (5 wt. %), while the PEG concentration is increased as before. As shown in these figures, BSA had a considerable effect on the refractive index, in which a substantial increase of the refractive index was observed by increasing of BSA concentration due to a higher density. So, before refractive index measurements, the phases should be diluted conveniently to BSA concentrations  $\leq 0.25$  wt. % in order to diminish BSA influence on PEG determination.

In conclusion, the determination of salt concentration in APTS using conductivity measurements can be strongly influenced by increasing of salt concentration and pH, while an increase of PEG and BSA concentration in diluted aqueous solutions had no appreciable effect, and therefore these concentrations are negligible. It is recommended that salt and PEG concentration should be in the range of 0–3 wt. % in order to obtain linear calibration plots, whereas linearity is obtained for BSA in the range of 0–2 wt. %. Furthermore, the determination of PEG concentration in APTS using refractive index measurements can be significantly influenced by increasing of PEG, salt, as well as BSA concentration. Here, linearity of calibration curves is only valid for concentration ranges of 0–10 wt. % PEG, 0–5 wt. % salt, and 0–2 wt. % BSA. Hence, the phases should be diluted conveniently, yielding to a diminishment of the influence of salt and BSA on PEG determination. Thus, the obtained results can be used for a convenient dilution of both APTS phases in order to ensure no significant influences from BSA and phase-forming components on the quantification methods.



On the whole, with these simple and fast analytical methods it is possible to determine phase-forming components of diluted ATPS in the presence of BSA in order to characterize and establish ATPS for extraction of biomolecules like proteins.

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#### FIGURE LEGENDS

Figure 1. Conductivity Calibration Curves for Salt–Water Systems at different pH Values and 25 °C containing different Concentrations of (A) Potassium Phosphate, (B) Sodium Citrate, (C) PEG 2000–8000, and (D) BSA.

Figure 2. Refractive Index Calibration Curves for PEG 8000–Water Systems at pH 7 and 20 °C containing different Concentrations of (A) Potassium Phosphate, (B) Sodium Citrate, (C) BSA + Potassium Phosphate (5 wt. %), and (D) BSA + Sodium Citrate (5 wt. %).

