Practical Aspects of the Automated Preparation of Aqueous Two Phase Systems for the Analysis of Biological Macromolecules

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Highlights:

- Robotic liquid handling methods are developed for aqueous two-phase systems.
- The importance of control of aspiration/dispense speeds and delay times is stressed.
- The % bias accuracy of system preparation is assessed by gravimetric methods.
- The critical effect of the geometry of the binodal on this accuracy is reported.
- The methods are demonstrated as applied to the analysis of ovalbumin isoforms.

Abstract:

A robust strategy for the automated preparation of aqueous two-phase systems (ATPS) using a liquid handling sample processor was developed using gravimetric methods to determine the accuracy of preparation. The major robotic control parameters requiring adjustment were; speed of aspiration and dispense; delay times following aspiration and dispense alongside measures to control cross-contamination during phase sampling. In general mixture compositions of both polymer / polymer and polymer / salt mixtures could be prepared with a target bias accuracy of less than 5%. However we found that the bias accuracy with which systems of defined TLL and MR could be constructed was highly dependent on the tie line length of the ATPS and the geometrical form of the ATPS co-existence curve. For systems with a very low degree of curvature (PEG / salt systems here) increases in bias (accuracy) are appreciable at relatively long tie line lengths. Where the degree of curvature is more pronounced (PEG/dextran systems) closer approach to the critical point was possible without major effect on bias/accuracy. Application of the strategy to the measurement of the partitioning of phosphorylated and dephosphorylated forms of the model protein ovalbumin are reported. Differences in partition of phosphorylated (native) forms and dephosphorylated forms could be demonstrated. In a PEG/salt system this was manifest as a substantial decrease in solubility based on overall protein recovery derived from accurate knowledge of the system mass ratio. In a PEG/dextran system differences in partition coefficient could be demonstrated between phosphorylated and dephosphorylated forms.

Keywords:

Aqueous Two-Phase Systems, automated sample preparation, liquid handling robotics, partition, ovalbumin isoforms, phosphorylation.

1. Introduction:

Partitioning in aqueous two phase systems (ATPS) has been widely used as an extraction and purification method for the recovery of biological macromolecules and particles as well as an analytical method for the study of proteins and other biomolecules. Aqueous two-phase systems (ATPS) form when two polymers or one polymer and a salt are mixed in appropriate amounts resulting in the formation of two immiscible phases. Since both phases are aqueous, biological functions are generally maintained [1]. Differential partition of added solutes may then be exploited preparatively and analytically. Some examples of recent preparative applications include: the continuous extraction of monoclonal antibodies using a multistage process [2], and the use of ATPS to improve the purification of Porcine Parvovirus Vaccine (PPV) in a PEG–citrate system [3]. Examples of analytical applications include the measurement of biomolecule interactions [4], the analysis of protein isoforms [5] and the pre-concentration of virus to improve assay detection limits [6].

Since the partitioning method involves analyte distribution between completely liquid phases, the method may easily be adapted to conventional automated liquid handling techniques available in many laboratories. This approach has been taken in a number of studies, for instance in the screening of conditions for preparative process design employing ATPS [7,8] and for the automated preparation of analytical phase partitioning systems [9-12].

However, a few details have been reported of the practical development of automated methods for the preparation of ATPS, which involve the handling of relatively concentrated and viscous solutions of polymers and salts, nor is there much detail on the accuracy and reproducibility of their delivery. Accurate and reproducible phase system construction and subsequent sampling is a sine qua non for both analytical applications and screening for process development.

We aimed in this work to develop a robust strategy for the preparation and sampling of aqueous two phase systems to minimize the % Bias (accuracy) and maximize the reproducibility of sample preparation. In so doing we found that the accuracy with which the final system composition could be prepared was dependent on the form of the co-existence curve of the biphasic system.

2. Materials and methods:

2.1. Materials

Polyethylene glycol Mw=1000 (PEG1000) (Lot A0319044), Polyethylene glycol (Lot 1356267) Mw=8000 (PEG8000) (Lot SLBC9317V), Ammonium sulfate $(NH_4)_2SO_4$, di-potassium hydrogen orthophosphate K_2HPO_4, potassium di-hydrogen orthophosphate KH_2PO_4, Tris (hydroxymethyl)-aminomethane (Tris) were purchased from Fisher Scientific UK (Leicester, UK). Dextran500 from Leuconostoc mesenteroides (Lot BCBJ7122V) weight-average molecular weight (Mw) 450,000-650,000 and Ovalbumin from lyophilized powder, >98% (Lot SLBD2312V), Endoglycosidase F1 from *Elizabethkingia miricola*; recombinant, expressed in *E.coli*, ≥16U/mg buffered aqueous solution (Lot SLBK8022V), Neuraminidase from *Clostridium perfringens* (C. welchii) type V from lyophilized powder 0.32 mg solid 7.9U/mg (N2876-2.5UN Lot SLBF5907V), Alkaline phosphatase (ALP) from bovine intestinal mucosa-BioUltra, in buffered aqueous glycerol solution 2000-4000 DEA U/mg protein (Lot SLBF3716V) and Cibacron Blue 3G-A (product No.C9534-25G) were purchased from Sigma-Aldrich UK (Dorset, UK).

2.2. Apparatus

A Perkin Elmer MultiProbe II plus Liquid Handling Sample Processor (LHSP), fitted with a 4 tip pipetting arm, controlled by WinPREP® applications software (PerkinElmer Life and Analytical Sciences, CT, USA) was used to prepare the ATPS. Either a Sartorius Mechatronics analytical balance 1601A MP8-1 (Epsom, UK) or a Denver instruments M-220D balance (NY, USA) both having a readability of 0.1mg were used for the gravimetric measurements.

Analytical HPLC was performed using an Waters Alliance 2695 HPLC system with 2996 PDA Detector (Waters Corporation, Milford USA). Size Exclusion Chromatography was performed using a Shimadzu Isocratic HPLC system consisting of SCL-10A VP system controller, SPD-10A Liquid chromatography pump and LC-10AT UV-VIS detector with data collection by Shimadzu EZStart chromatography software version 7.3 (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan).

2.3. Preparation of ATPS Stock Solutions

PEG1000 and $(NH_4)_2SO_4$ stock solutions were prepared by weight to final compositions of 40 % w/w PEG1000 and 40 % w/w salt in 0.15 M potassium phosphate buffer pH 7.4. Stock

solutions of PEG8000 and Dextran500 were prepared by weight with compositions of 25 % w/w Dextran and 20 % w/w of PEG in 50 mM potassium phosphate buffer pH 7.4.

Enzymatic dephosphorylation was performed by incubating ovalbumin with ALP [Addition of 400 µL of 0.7 units/ µL ALP to 5 mL of protein at a concentration of 10 mg/mL] in 50 mM Tris buffer (pH 9) at 23°C overnight.

Neuraminidase treatment: 20mg of ovalbumin was dissolved in 10 mL of 10mM phosphate buffer pH 6 to which 100µL Neuraminidase stock solution was added and the mixture was incubated at room temperature. Then samples were taken approximately every hour and analysed by Ion Exchange Chromatography (IEX). Neuraminidase stock solution was made up as follows: 0.32 mg neuraminidase in 1 ml 100 mM sodium acetate buffer pH 6, with 20 mM $CaCl_2$.

Enzymatic deglycosylation was attempted by incubating 2 µL Endo F1 with 37.5 µL ovalbumin at a concentration of 5.3 mg/mL in 10 µL of reaction buffer (provided with the enzyme kit (Endoglycosidase F1 from Elizabethkingia miricola)) at 37 °C overnight.

2.4. Construction of the ATPS phase diagrams

For the systems PEG8000-Dextran500 and PEG1000- $(NH_4)_2SO_4$ phase diagrams were constructed by turbidimetric titration [13]. Tie lines connecting coexisting equilibrium phases were constructed for selected phase systems within the biphasic region following the method of Merchuk et al. [14]. The slope of the tie line (STL) was determined as the ratio STL = (ΔPEG)/ (ΔDextran) where Δ represents the difference in the concentration of each polymer in the two coexisting phases.

2.5. Preparation of phase systems using the robotic system

The LHSP was used to prepare ATPS having final masses of 3.5 g for PEG1000-$(NH_4)_2SO_4$ ATPS and 2 g for PEG8000-Dextran 500 ATPS. Stock solutions of polymers and salt were dispensed from the LHSP by volume and weighed on an analytical balance at each stage to provide calibration and performance data. Final compositions were achieved by addition of appropriate amounts of buffer or buffer containing analyte (Ovalbumin) and weighed as before.

The stock solutions used to prepare the aqueous two-phase system selected for analysis of native ovalbumin in Tris (10 mM, pH 9) and ovalbumin treated with alkaline phosphatase in the same buffer after incubation for 24h at room temperature contained 25 % w/w PEG-3350, 30 % w/w Dextran500, and 10 mM potassium phosphate buffer pH 9. While the compositions of the stock

solutions for the preparation of a PEG1000-$(NH4)_2SO_4$ system were 40 %w/w PEG1000, 40 %w/w $(NH_4)_2SO_4$ and 150 mM potassium phosphate buffer pH 8.5.

Partitioning experiments were performed using a set of eight different and increasing concentrations of protein added to a series of ATPS having the same overall polymer composition using a method outlined by Zaslavsky [15]. Systems were dispensed into test tubes and vortexed using a digital mini vortexer (3300rpm/sec, USA) for 10 sec, and centrifuged for 20 min at 5000 rpm (Jouan DBV, France) to accelerate settling of the phases. After centrifugation, the tubes were returned to the deck of the LHSP. The LHSP was then used to prepare samples of 200 µL which were diluted with 800 µL of potassium phosphate buffer. Spectrophotometric absorbance at 280 nm (A280) was used to calculate protein concentration using an Extinction coefficient for ovalbumin of 30,590 cm-1 M-1 and a Molar Mass of 42.7 kDa [16] and the partition coefficient was determined as the ratio of the sample concentration in the PEG-rich (top) phase to the sample concentration in the dextran-rich or salt-rich bottom phase.

2.6. HPLC Analysis

Strong anion exchange HPLC was used to separate ovalbumin isoforms on the basis of accessible surface charges and their corresponding electrostatic interaction with the column's stationary phase. A SOURCE 15Q 4.6/100 PE column, GE Healthcare Life sciences, was used with; Mobile Phase A: 20 mM Potassium phosphate buffer, B: 20 mM phosphate buffer containing 500 mM Sodium chloride. A flow rate of 0.5 mL/min was used with a 100 uL injection volume. Samples were eluted using a linear gradient from 0 to 100 % buffer B over 35 min.

Protein samples were analysed by Size Exclusion Chromatography (SEC) using a TSKgel G2000 SW column; (5µm, 7.8 x 300 mm; Tosoh Bioscience purchased from HiChrom, Reading, UK). 0.05 M potassium phosphate buffer pH 7.0 containing 0.3 M NaCl was used to equilibrate the column at a flow rate of 0.4 mL/min with UV detection at 280 & 260 nm.

The SEC column was calibrated from the retention times of a Bio-Rad Gel Filtration Standard (Catalog 151-1901) having molecular weight markers ranging from 1,350 to 670,000 Da.

3. Results and discussion:

3.1 Phase System Preparation

The performance of all liquid-handling steps of the LHSP are controlled by an instrumental performance file which can be used to adjust a wide range of parameters as the speed and the delay time of dispensing and aspirating as well as the height of the probe tips. We found that the most important parameters were control of (decrease from the instrument default settings) the speed of delivery during both aspiration and dispense and the time delay following both aspiration and dispense. Aspiration rate for the PEG1000 stock solution (40 %w/w) for volumes in the range 100 - 2000 µL was set at 5 µL/s, and for the PEG8000 stock solutions (20 %w/w) in the range 100 - 2000 µL was set at 10 µL/s. For the Dextran stock solution (25 % w/w) for volumes of >100, >250, >400, and 900-2000 µL) the aspiration speed was set at 5, 10, 12.5 and 15 µL/s respectively. Finally, for stock salt solutions (40 %w/w) for volumes of >100, and 250 - 2000 µL the aspiration speed was set at 6 and 10 µL/s respectively. The speed of dispense was also reduced, for PEG 1000 the speed of dispense was 15 µL/s over the same volume range as above, whilst for salt the dispense speed was set at 100 µL/s.

For PEG8000 the dispense speed over the volume range >100, and 250-2000 µL was set at 400 and 300 µL/s while for Dextran500 this was set at 100 and 30 µL/s over the same range of volumes.

The height of the probe tips was also adjusted; during aspiration this was set so that the tips entered 2 mm below the liquid surface to avoid air entry and during dispense the tips were positioned either 1 mm below the liquid surface or 5 % above the tube bottom so that any drops remaining on the tips after dispense were removed by contact with the liquid surface.

Finally, the time delays between the end of aspiration and dispense and tip withdrawal was extended to ensure that the procedure was complete. The aspiration delay for PEG1000, PEG8000 and salt stock solutions for volumes in the range 100 - 400 µL was set at 200 ms. Whilst for volumes of 400-2000 µL this was set at 300 ms. For Dextran500 stock solutions this was further extended to 800 ms. We also found it necessary to adjust LHSP performance parameters and to calibrate the sampling procedure for phase sampling in the same way as previously described for system preparation.

Conservatively we used the PEG8000 performance parameters for sampling top phases and the Dextran500 performance parameters for sampling bottom phases with both PEG-Dextran and PEG-salt systems.

We found that cross-contamination of the phases was problematic during phase sampling since the probe had to pass through the upper phase in order to sample the lower phase. This was

visualised during exploratory experiments by including a dye (Cibacron Blue 3G-A) in the ATPS which strongly partitioned to the upper PEG phase and was readily visible in lower phase when cross-contamination occurred. This was overcome by creating the following three-step sampling procedure; 1. Sample 400µL of each top and bottom phase, 2. Centrifuge these samples to separate any residual contaminating phase, 3. Re-sample 200µL from each separated phase.

The following gravimetric procedure was used to pre-calibrate the LHSP for the delivery of ATPS components. For each stock solution the relationship between demanded volume and delivered mass was measured gravimetrically. Delivery of a range of volumes between 50 µL and 1.4 mL was examined for each stock solution without any volume compensation. Thus at this stage a linear relationship between delivered mass and demanded volume was assumed having a slope of 1 and offset value of 0 as defined in Eq. (1).

$$W = V.x + C \quad (1)$$

Where W is the mass delivered, V is volume demanded, x is the slope and C is the intercept value. The true relationship between the volume demanded and the weight delivered was determined from gravimetric determinations of the delivered mass and a new slope and offset calculated. These values were then used to set the volume compensation of the LHSP. Note that this is equivalent to a determination of the density of the stock solutions and that a similar procedure could be implemented through performance files in the absence of a volume compensation parameter. This procedure which was repeated for the delivery of all stock solutions required to form the ATPS may be summarized as; 1. Dispense the first component without volume compensation, 2. Gravimetrically determine the delivered mass, 3. Calibrate the LHSP with this delivered vs demanded relationship, 4. Confirm the accuracy of the calibration, 5. Repeat for each additional component.

Once the above pre-calibration procedure had been completed for all components of the ATPS the performance of the LHSP in delivering a specific ATPS composition of defined Tie Line Length (TLL) was examined.

To give a specific example, 8 replicates of a PEG1000-$(NH_4)_2SO_4$ system (TLL: 38.9 % w/w composed of 16.18 % w/w PEG1000 and 17.48 % w/w $(NH_4)_2SO_4$) and a PEG8000-Dextran500 system (TLL: 24.1 % w/w composed of 5.67 % w/w PEG8000 and 10.84 % w/w Dextran500) were constructed using the volume compensations previously established for each component. The appropriate amount of each component required to give the final composition

was dispensed in turn and the results examined gravimetrically. The whole procedure was replicated three times. The results are shown in Fig. 1 and 2.

Insert Figure 1

Insert Figure 2

Although the composition of each of the components of each system was within 1 % of the target composition there was systematic deviation from the target. For the PEG -salt system, the PEG concentration was lower than required and vice versa for the salt, while for the PEG-Dextran system the PEG concentration was higher than required and the Dextran concentration varied above and below the target. Several reasons may be adduced for this; first the regression is the best fit through the calibration data and may not perfectly describe all individual compositions within the range. Secondly, the final fractional composition of the system is a function of each added component, since:

$$T_m = X_m + Y_m + Z_m \tag{2}$$

$$X_F = \frac{X_m}{T_m} \tag{3}$$

$$Y_F = \frac{Y_m}{T_m} \tag{4}$$

Where T_m is the total system mass, X_m, Y_m, Z_m are the mass of the three major components of the ATPS (salt or dextran, PEG, and buffer respectively) and X_F, Y_F are the mass fractions of each component.

From these results the %Bias (accuracy) in the delivery was determined for each component. % Bias was determined from the actual mass dispensed relative to the intended mass as shown in the Eq. (5). Where M_A is actual mass dispensed and M_O is the intended mass [17].

$$\%\text{Bias (accuracy)} = \left(\frac{(M_A - M_0)}{M_0}\right) * 100 \tag{5}$$

In an attempt to correct these systematic deviations we elected to use the mean % Bias converted to a volume basis as a correction factor applied to the demanded volume of PEG and salt or PEG and Dextran. For best results we found it necessary to use this correction factor for both components.

As a result of applying this correction, the mean composition of the systems was brought much closer to the target value; however the range was not generally improved as shown in Fig.3 (see also Table S1 in the Supplementary material).

Insert Figure 3

Subsequently we constructed a PEG-salt system (8 replicates as before) at a reduced TLL (25.57 % w/w compared to the previous TLL of 38.9 % w/w). We found that the % Bias in the compositions of the PEG and salt was comparable at each TLL as shown in Table 1.

Insert Table 1

For each of the eight systems constructed at each nominal TLL, the actual TLL of the delivered systems was calculated from their gravimetric compositions. Under the assumption that the slope of each tie line (STL) is the same for all tie lines lying very close together, the TLL for each constructed system was calculated by numerical methods from its slope and intersection with the binodal curve. From this estimate of the TLL and the system composition the mass ratio (MR) of the each of the eight systems could be obtained along with the % Bias of both TLL and MR.

The mean % Bias of the mass ratio (MR) and TLL was found to be greater at shorter TLL. This must affect the variability (error in determination) of the partition coefficient (K) of added solutes at shorter TLL since K is a function of TLL [1]. In addition the increase in % Bias (accuracy) of MR of systems lying closer to the critical point will similarly compromise the calculation of the mass balance of the ATPS. This is an important consideration when systems approach the analyte solubility limit or where molecular association occurs.

On the basis of this finding we used the range in system compositions already found to be delivered by LHSP to estimate the %Bias of TLL and MR for a series of tie lines chosen to lie increasingly close to the critical point for each PEG-salt and PEG-Dextran ATPS (see Tables 2 & 3). For each TLL examined in both of these phase systems the compositions of four hypothetical systems were calculated. The composition of these systems was assigned such that they lay two standard deviations (SD) (based on the distribution of the data already found for the PEG/salt system (TLL 38.9 % w/w) and the PEG Dextran system (TLL 24.1 % w/w)) above and below the phase compositions of a system having mass ratio of 1 at each TLL, thus encompassing 95% of the previously determined experimental variability. The compositions of these hypothetical systems may be expressed as X+2SD Salt, Y+2SD PEG; X-2SD Salt, Y-2SD PEG; X+2SD Salt, Y-2SD PEG; X-2SD Salt, Y+2SD PEG; and similarly for the PEG-

Dextran system. Note that these systems form a rectangle surrounding the selected target composition with one pair (X-2SD Salt or Dextran, Y+2SD PEG and X+2SD Salt or Dextran, Y-2SD PEG) lying approximately parallel to the slope of the TLs and the opposite pair lying approximately orthogonal to this slope.

Insert Table 2

Insert Table 3

The effect of the variability in system range (SR) composition on the % bias in TLL and MR for the PEG8000-Dextran500 system is shown in Fig. 4 A&B. The % Bias (accuracy) of TLL length is within ±1% of the target value at long TLL and even at the shortest TLL examined only increases to about ± 2%. Similar results were found for the effect of variability in system composition on the % Bias (accuracy) of MR in this system. These results seem quite acceptable in the context of automated system preparation; erroneous results due to excessive variability of TLL and its effect on the partition coefficient or due to large changes in MR and its effect on mass balance calculations are unlikely at any but the very shortest tie line lengths say below 13 % w/w.

Insert Figure 4

It seems to be accepted that for automated sample preparation in analytical applications % Bias should be within 5%, although this criterion appears to be more relaxed for very high throughput minimal volume systems such as may be used in pharmaceutical lead analysis [18].

Similar results showing the effect on % Bias (accuracy) of TLL in the preparation of the PEG-1000 Ammonium sulphate system using parameters derived from the LHSP performance data for the target range of TLLs given in Table 3 are shown in Fig. 5A. The % Bias in TLL of the shortest TLL examined (12.2 % w/w) for systems lying orthogonal to the TLs were found to be very much greater than 5% (+42% and -100%). The latter because this TL composition lies outside the co-existence curve and would fail to form a biphasic system. Similar results were found for MR (see Figure 5B), at the shortest TLL, % Bias in MR is in excess of 10% and the % Bias of one system reported as 0% is meaningless as it again lies outside the binodal curve. Increase in TLL to 25 % w/w results in a reduction in % Bias (accuracy) of both TLL and MR; however % Bias (accuracy) of TLL is close to ±10% and greater than 5% for MR.

Insert Figure 5

Note that system pairs lying approximately parallel to the target tie line lead to the greatest error in MR and for those lying orthogonal to the Tie line to the greatest error in TLL. By 32 % w/w TLL the error associated with TLL and MR is only marginally acceptable being still somewhat greater than 5% at 2SD from the mean. At longer tie line lengths % bias accuracy is reduced to more acceptable levels.

In conclusion, in systems where % Bias (accuracy) is much greater than 10 %, partitioning measurements could only be accurately made with complete knowledge of system composition obtained gravimetrically or photometrically [8] or perhaps by the exhaustive determination of numerous replicates.

The % Bias accuracy of TLL and MR increases more rapidly in PEG-salt systems than in PEG/Dextran systems as tie line length is reduced. The reasons behind this difference are related to differences in the form of the co-existence curve for these systems. In the PEG-salt system the curvature of the co-existence curve is very low and the TLs run almost parallel to the co-existence curve with the result that the TLL increases rapidly with distance from the critical point. Figure 6 shows the phase diagrams for the systems used here along with the instantaneous radius of curvature of the binodal curve which may be calculated from the curvature defined as:

$$\kappa = \frac{|y''|}{[1+(y')^2]^{\frac{3}{2}}} \qquad [6],$$

where *y* represents a function, which describes the binodal curve [19]. It is immediately apparent that the curvature of the PEG-salt system is very low and the radius is consequently very high in comparison to the PEG-dextran system. This is likely to be true for many, but perhaps not all, relatively high Mw. PEG-salt systems. In some PEG-salt systems in the region of the critical point the curvature of the co-existence curve approaches zero i.e. is almost a straight line see for instance the many phase diagrams given in [20].

Insert Figure 6

On the other hand in the PEG8000-Dextran500 system examined here, this effect is much less pronounced and the co-existence curve has by comparison considerable curvature; this is likely to be the case for many, but not all, polymer-polymer systems. As a consequence the relative increase in TLL (equivalent to the chord length) is more modest as the distance from the critical point increases and the impact of error in system preparation is consequently much reduced. In establishing robotic sample handling systems for the preparation of ATPS attention to the

curvature and disposition of the tie lines of the system is important for their accurate preparation particularly when attempting to work close to the critical point.

3.2. Analytical Example of the Application of the Automated Procedure

The automated phase system preparation and sampling procedure was applied to the analytical determination of the isoforms of a model protein, ovalbumin, since in the native state it consists of several isoforms having differences in glycosylation [21] and phosphorylation [22].

If the effect of these post translational modifications (PTMs) on the partition coefficient of the model protein could be quantified it would be possible to develop the partitioning technique as a simple method to detect and quantify particular PTMs, for example in the detection of clinical biomarkers or as quality control attributes during industrial bioprocessing.

To this end we elected to compare the partitioning of native ovalbumin to dephosphorylated and deglycosylated forms of the protein prepared enzymatically in vitro. Neuraminidase was used in an attempt to prepare ovalbumin depleted in nueraminic acid residues [21] and the endoglycosidase Endo F was used to prepare substantially deglycosylated forms [23]. Unfortunately neither of these methods was successful in creating isoforms substantially different from the native form when analysed by IEX (data not shown) despite the fact that other workers have had at least partial success in generating substantially deglyosylated forms [21].

On the other hand alkaline phosphatase was successfully used to prepare a substantially dephosphorylated form of native ovalbumin (see materials and methods). Analytical IEX was used to demonstrate that ovalbumin was substantially dephosphorylated post ALP treatment (see Supplementary materials Fig. S1 and S2). SEC was used to show that the resulting de-phosphorylated ovalbumin was in other respects identical to the native form in terms of the amount of multimeric and aggregated species present. Size Exclusion Chromatography (SEC) was used to assess the effect of dephosphorylation on the molecular integrity and degree of aggregation of ovalbumin. Removal of phosphate groups was found to produce a change in the normalised retention time of the phosphorylated and de-phosphorylated species. This could be interpreted as a change in molecular size with ALP-treated ovalbumin having an apparent molar mass of 40.9 kDa compared to 43.6 kDa of the native state as illustrated in the separation of Bio-Rad standard in Fig. S3 and the effect of dephosphorylation in Fig. S4 in Supplementary materials. No other gross changes between the two species were observed such as proteolytic

degradation or extent of aggregation or the presence of multimeric species (See Supplementary materials Fig. S4 and Table S2).

Phosphorylation affects the surface charge and charge density of the proteins and may induce conformational change and changes in hydrophobicity which should be reflected in changed interaction parameters with the ATPS components and hence in the partition coefficient.

The partitioning behaviour in ATPS of ALP treated and native untreated ovalbumin was examined in order to determine the influence of dephosphorylation on the partition coefficient using the previously established experimental design.

Initially, a number of systems were screened for their ability to discriminate between ALP treated and native ovalbumin. These systems were: PEG600-Na2SO4, PEG600-$(NH_4)_2SO_4$, PEG1000-$(NH_4)_2SO_4$, PEG8000-Dextran500, PEG4600-Dextran500 and PEG3350-Dextran500. Each of the PEG-salt systems was found to have its own shortcomings for instance: in the PEG600-Na_2SO_4 system the maximum solubility of the salt was found to be 14 % w/w which in preparing systems from stock solutions left little room for sample addition. Also the Low molecular weight PEG600-$(NH_4)_2SO_4$ system amplified the partition coefficient (K=8.5) precluded accurate determination of lower phase concentration by A280. In the PEG1000-$(NH_4)_2SO_4$ system dephosphorylated ovalbumin was found to have greatly reduced solubility compared to the native form which led to accumulation at the interface and a failure to close the mass balance (Fig. 7 A&B) compared to the PEG3350-Dextran500 system (Fig. 8 A&B).

Insert Figure 7

Insert Figure 8

Little difference in partitioning behaviour was found for the PEG-Dextran systems mentioned above except in the PEG3350-Dextran500 system. For each of the aforementioned PEG-Dextran systems, A range of systems were screened containing 10mM phosphate buffer but differing in pH (4.6, 5.5, 7.4, and 9) and with respect to different added salts (100mM $NaClO_4$, 100mM KCl, 50mM K_2SO_4) (data not shown). Over this limited survey the best result (greatest difference in K between ALP treated and native (untreated) ovalbumin was found in a PEG3350-Dextran500 system containing 10mM phosphate buffer pH 9 containing 50mM potassium sulfate salt. This result is shown in Fig. 8 A&B. The slope of the relationship between the concentration in the top and bottom phase corresponds to the partition coefficient [15] and this was shown to be significantly different by analysis of covariance (Table S3 in Supplementary materials).

Conclusions:

Since ATPS are composed of entirely liquid phases they lend themselves to the application of automated liquid handling techniques for their preparation and thus to applications in extensive screening programmes for example to rapidly screen systems for optimal recovery conditions in bioprocessing or to detect the occurrence of process variants or isoforms during bioprocess development or in clinical analysis. In attempting to develop a robust liquid handling strategy for these viscous liquid systems which minimised bias and maximised reproducibility we found the following control parameters of particular importance. It was necessary to considerably reduce speeds of aspiration and dispense compared to those conventionally used and recommended as standard. In addition delay times of aspiration and dispense had also to be extended. It was also necessary to ensure that dispensed droplets were fully disengaged from the liquid handling probes and that steps were taken to eliminate carry over and cross contamination of phases during sampling. Such adjustments to the operation of LHSP devices should be widely applicable and maybe implemented on most liquid handling systems. With these precautions in place it was possible to construct polymer / polymer and polymer / salt mixtures whose bias accuracy of final composition fell well within target values of 5%. However we also found that for the accurate preparation of ATPS close to the critical point the geometrical form of the phase diagram co-existence curve was of crucial importance since this directly affected the accuracy with which systems of defined TLL and MR could be constructed. For systems with a very low degree of curvature, PEG / salt systems in this example, increases in bias (accuracy) are appreciable at relatively long tie line lengths. Where the degree of curvature is more pronounced, PEG/dextran systems here, closer approach to the critical point is possible without major effect on bias/accuracy. These findings are not dependent on the liquid handling system used but are a consequence of the nature of the ATPS phase diagram. Using the robotic methods that were developed we examined the partition of the model protein ovalbumin. A strategy involving dephosphorylation and deglycosylation was used to prepare substantially different isoforms. Deglycosylation proved unsuccessful but differences in partition of phosphorylated (native) forms and dephosphorylated forms could be demonstrated. In a PEG/salt system we examined this was manifest as a substantial decrease in solubility based on overall protein recovery derived from accurate knowledge of the system mass ratio. In a PEG/dextran system we examined differences in partition coefficient could be demonstrated since systems could be prepared at a constant tie line length.

Acknowledgments:

The first author especially would like to thank Iraqi Ministry of High Education & Scientific Research and University of Karbala for funding this project as part of her PhD (Ref. No. 643 from 10/01/2013). Furthermore, the authors are grateful to Prof Derek Fisher for support and inspiration for this work as well as Dr Ian Garrard for technical support and advice on using LHSP.

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Figure 1. Box Plots of the Phase compositions of PEG1000 – $(NH_4)_2SO_4$ system in terms of (A) % w/w PEG1000 and (B) % w/w $(NH_4)_2SO_4$, following a pre-calibration procedure (No. 1-3) and a post calibration adjustment (No. 4-6). The black Line within the box represents the mean of data; the red line represents the median.

Figure 2. Box Plots of the Phase compositions of PEG8000 – Dextran500 system in terms of (A) % w/w PEG8000 and (B) % w/w Dextran500, following a pre-calibration procedure (No. 1-3) and a post calibration adjustment (No. 4-6).

Figure 3. (A) PEG1000-$(NH_4)_2SO_4$, (B) PEG8000 –Dextran500 systems show the accuracy of the delivery of the final system composition following pre and post calibration procedures, (●) Demand system, (▲) Pre-Calibration systems, (◆) Post-Calibration systems.

Figure 4. Modelled variability in the composition of a PEG8000-Dextran500 system in terms of (A) the %Bias (accuracy) of the TLL and (B) the %Bias (accuracy) of the MR. Each symbol represents the modelled system range of known TLL (●) TLL1 (▲)TLL2 (⬢)TLL3 (◆)TLL4 (■)TLL5 , illustrating the effect of TLL on the % Bias accuracy in TLL and MR in the construction of ATPS. . (PEG 8000-Dextran500 system TLL were listed in Table 3)

Figure 5. Modelled variability in the composition of a PEG1000- $(NH_4)_2SO_4$ system in terms of the (A) %Bias (accuracy) of the TLL and (B) %Bias (accuracy) of the MR. Each symbol represents the modelled system range of known TLL (●) TLL1 (⬢) TLL2 (◆) TLL3 (▼) TLL4 (■) TLL5 (▲) TLL6 (◐) TLL7, illustrating the effect of TLL on the % Bias accuracy in TLL and MR in the construction of ATPS. (PEG1000-$(NH_4)_2SO_4$ system TLL were listed in Table 2).

Figure 6. Phase diagrams of (A) the PEG8000–Dextran500 system and (B) the PEG1000-$(NH_4)_2SO_4$ system showing the coexistence curves and disposition of the tie lines and illustrating the considerable difference in curvature of their co-existence curves.

Figure 7. Partition of (A) native and (B) ALP treated ovalbumin in the PEG1000- $(NH_4)_2SO_4$ system showing the concentration in the top (●) and bottom (■) phases and the overall recovery from both phases (▲).

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Fig. 1A

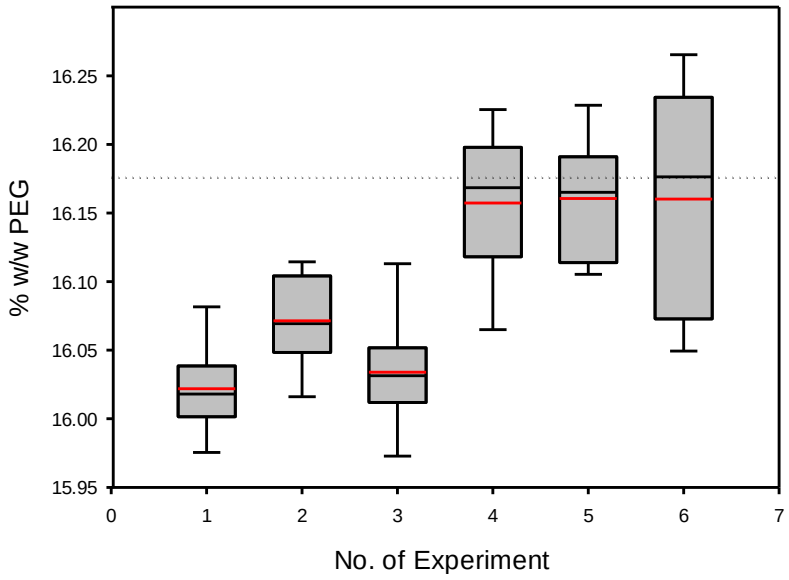

Fig. 1B

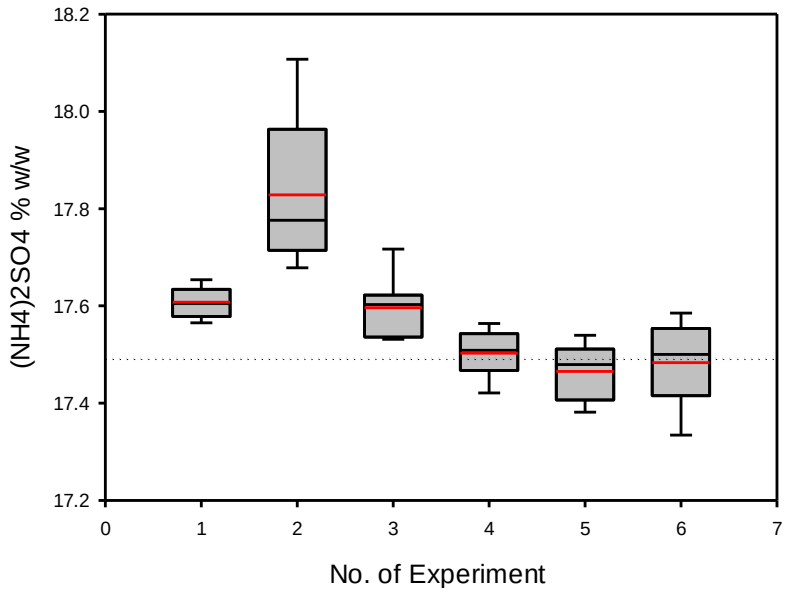

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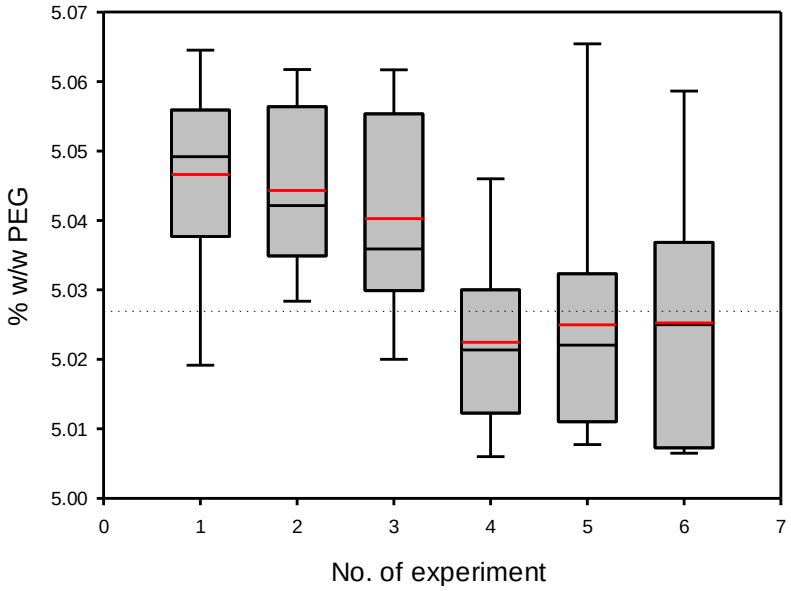
Fig. 2A



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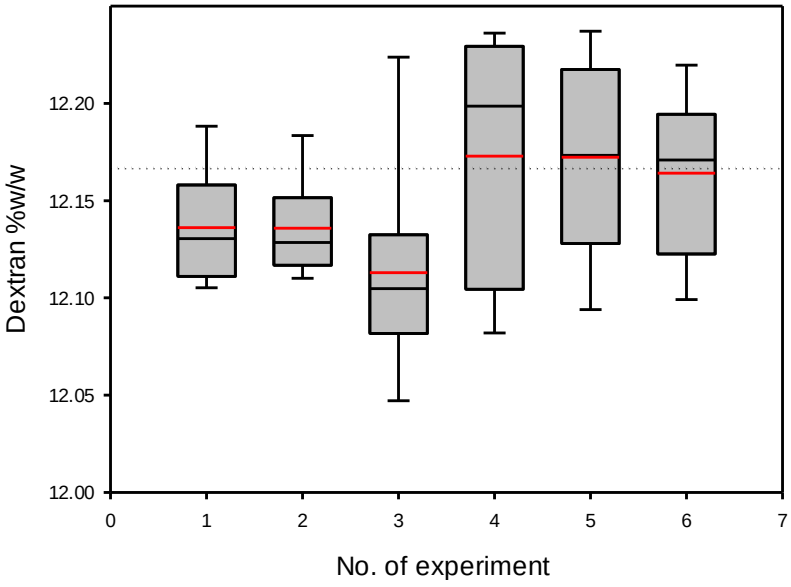
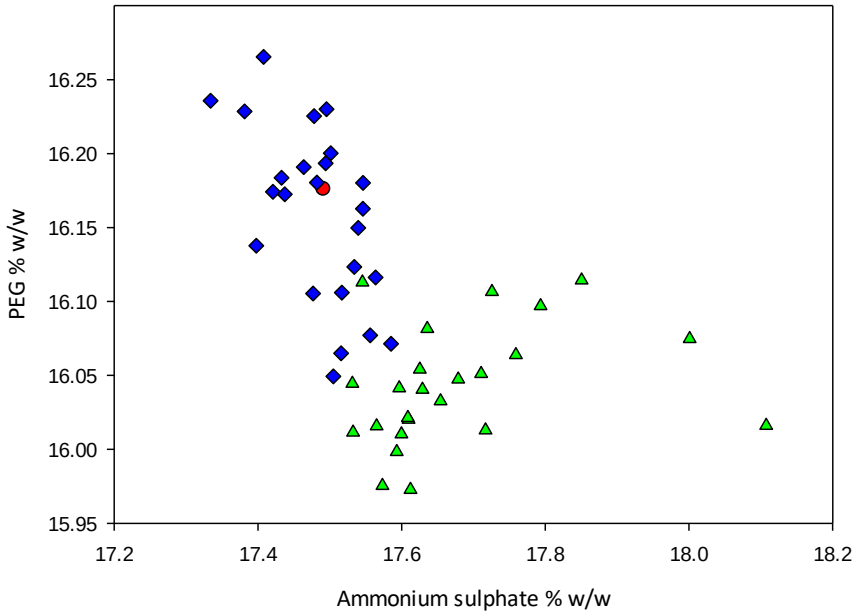


Figure 2. Box Plots of the Phase compositions of PEG8000 – Dextran500 system in terms of (A) % w/w PEG8000 and (B) % w/w Dextran500, following a pre-calibration procedure (No. 1-3) and a post calibration adjustment (No. 4-6).

559 Fig. 3A



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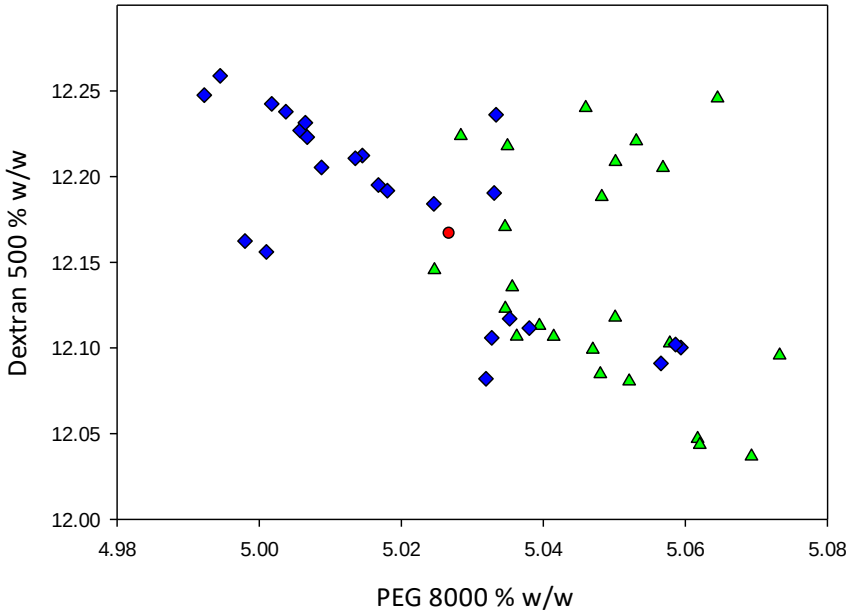
561 Fig. 3B



562

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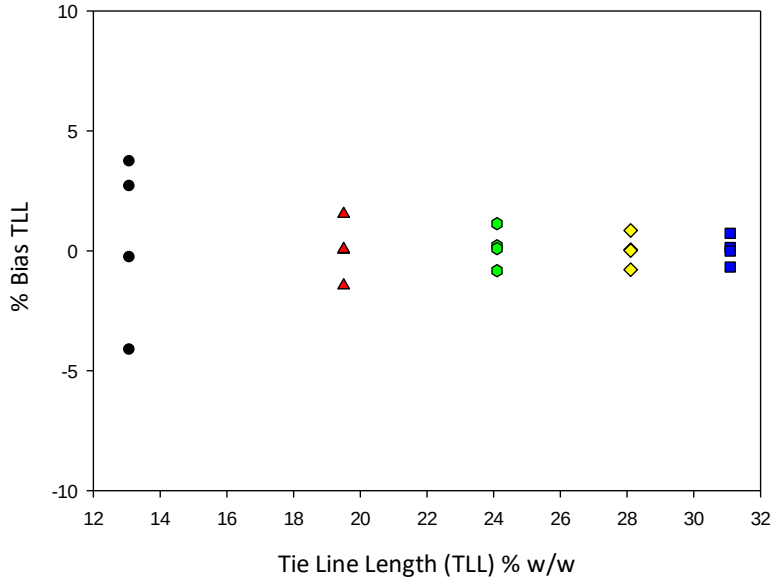
566

567 Fig. 4A



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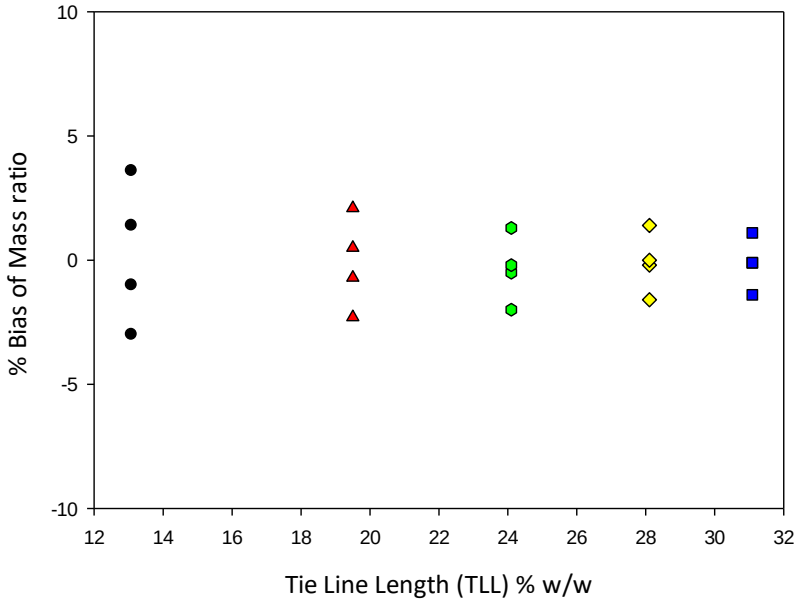
569 Fig. 4B



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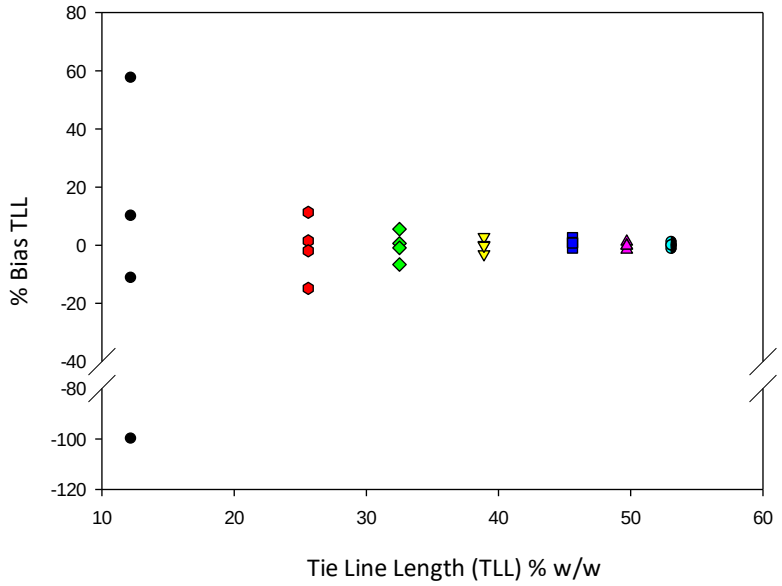
576

577 Fig. 5A



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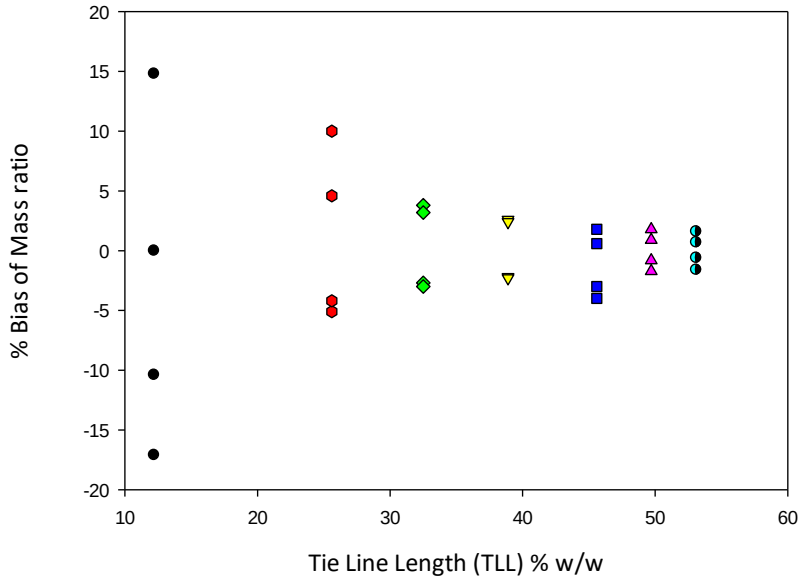
579 Fig. 5B



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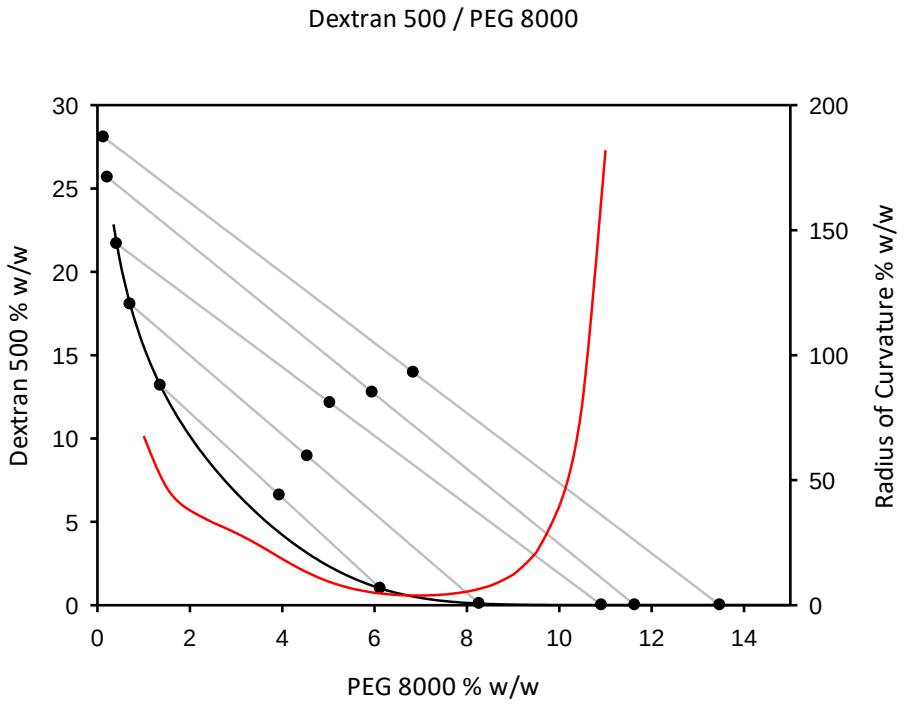
587 Fig. 6A



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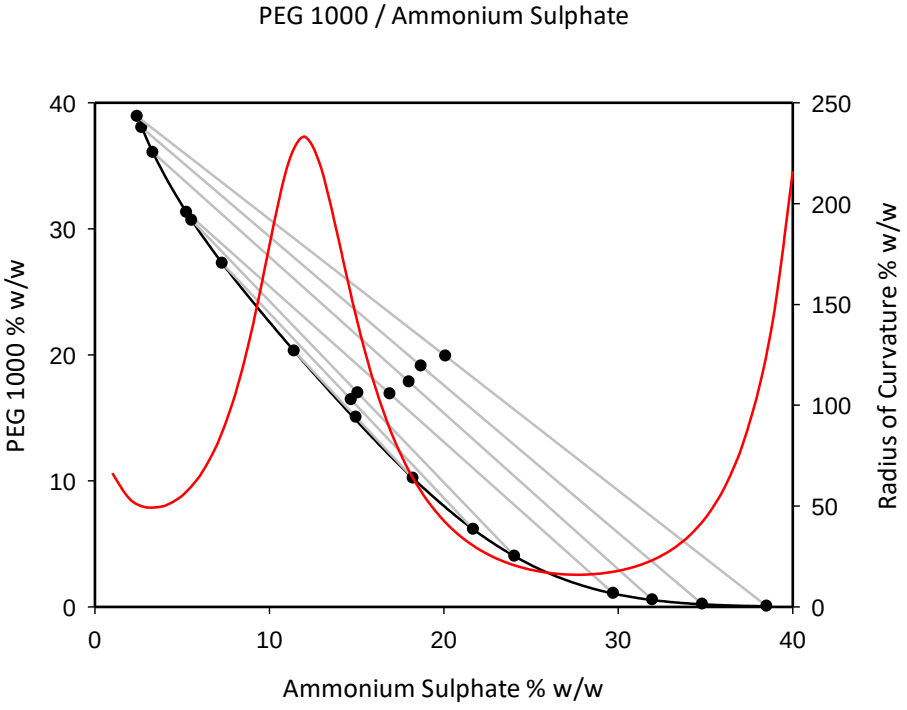
589 Fig. 6B



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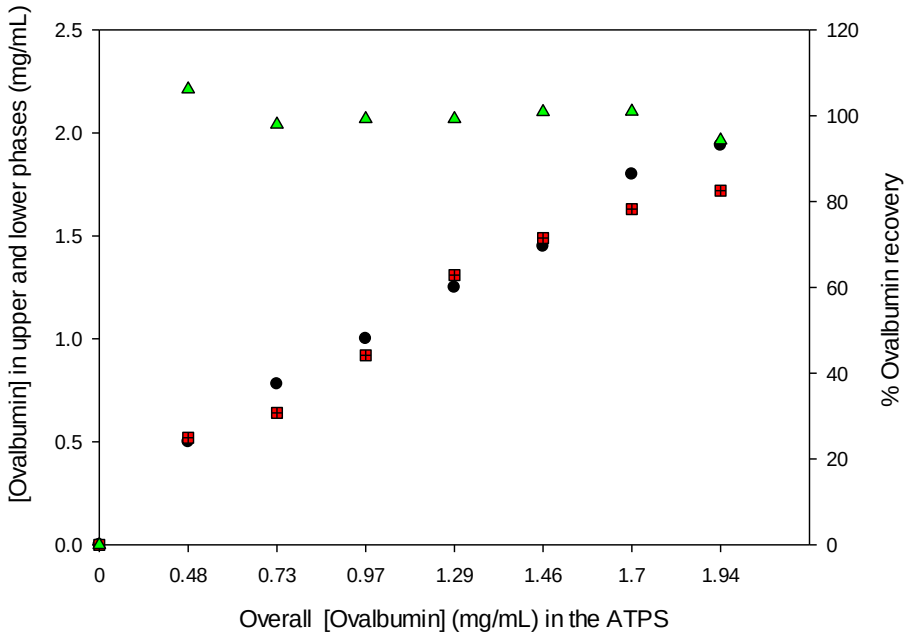
594

595 Fig. 7A



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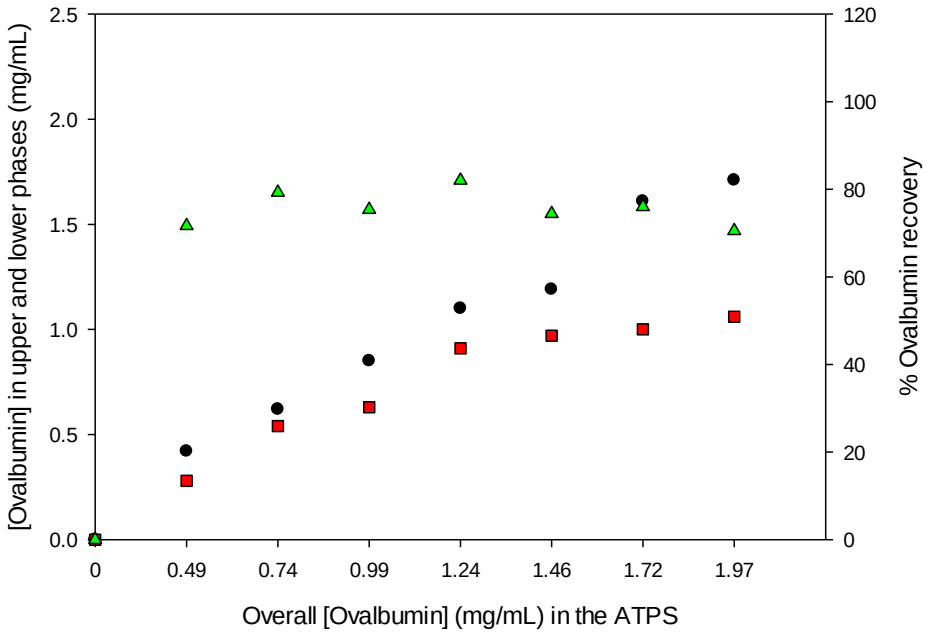

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600 system showing the concentration in the top (●) and bottom (■) phases and the overall
601 recovery from both phases (▲).

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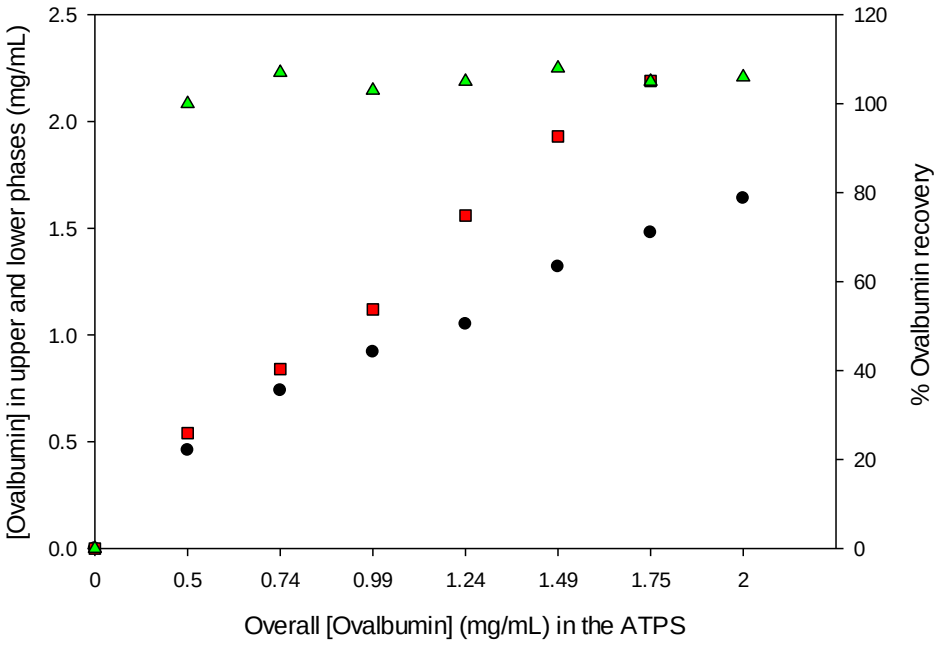
603 Fig. 8A



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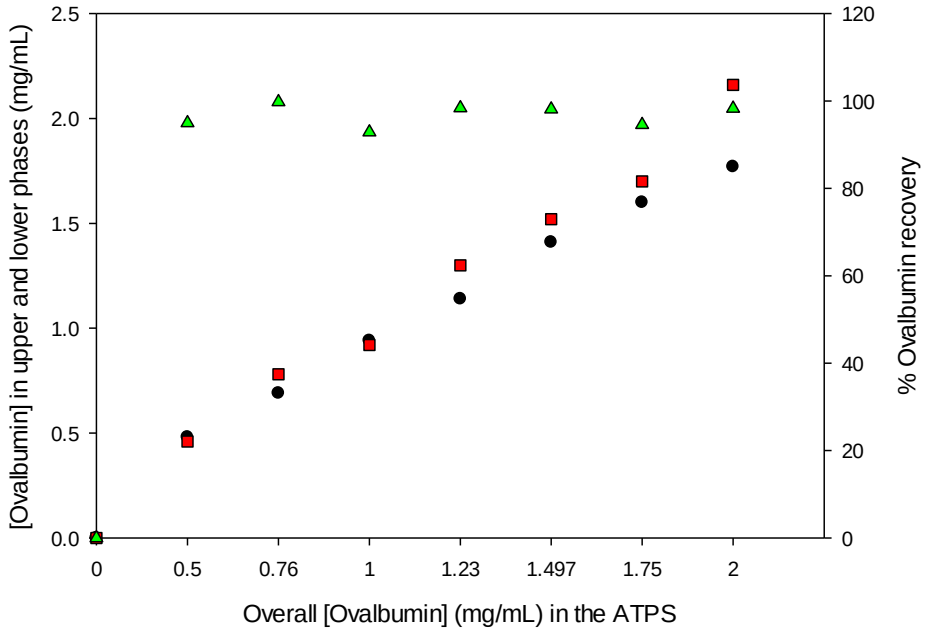

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607 Figure 8. Partition of (A) native and (B) ALP treated ovalbumin in the PEG3350-Dextran500
608 system showing the concentration in the top (●) and bottom (■) phases and the overall
609 recovery from both phases (▲).

610

611 Table 1. Mean % bias (accuracy) of system component composition, TLL and MR for two
612 different TLLs of the PEG1000-$(NH4)_2SO_4$ system

613

Mean	PEG1000-$(NH_4)_2SO_4$ TLL=38.912		PEG1000-$(NH_4)_2SO_4$ TLL=25.56	
	PEG	$(NH_4)_2SO_4$	PEG	$(NH_4)_2SO_4$
Delivered, % w/w	16.16	17.50	16.41	14.76
%Bias	-0.12	0.07	-0.15	0.43
Delivered system TLL	38.89		26.27	
Delivered system Mass Ratio	0.998		0.93	
TLL %Bias	-0.05		2.75	
Mass Ratio %Bias	-0.19		-7.08	

614

615

616 Table 2. Systems selected for analysis of the effect of variability in construction on the TLL
617 and MR of the PEG1000 - $(NH_4)_2SO_4$ ATPS

618

No. of Tie line	PEG1000-$(NH_4)_2SO_4$ system having Mass ratio 1			
	% w/w PEG1000	% w/w $(NH_4)_2SO_4$	TLL % w/w	STL
TL1	15.03	14.98	12.19	-1.482
TL2	16.42	14.69	25.56	-1.467
TL3	16.96	15.07	32.46	-1.44
TL4	16.17	17.49	38.91	-1.236
TL5	17.84	18.02	45.62	-1.24
TL6	19.11	18.76	49.64	-1.176
TL7	20.47	19.47	53.04	-1.077

619

620

621 Table 3. Systems selected for analysis of the effect of variability in construction on the TLL
622 and MR of the PEG8000-Dextran500 ATPS

623

No. of Tie line	PEG8000–Dextran500 system having Mass Ratio 1			
	Dextran500 % w/w	PEG8000 % w/w	TLL % w/w	STL
TL1	7.097	3.74	13.08	-2.557
TL2	9.08	4.49	19.50	-2.377
TL3	10.85	5.67	24.10	-2.066
TL4	12.84	5.93	28.11	-2.249
TL5	14.04	6.81	31.09	-2.105

624

Supplementary Materials. Table S1 [A]. Comparison of the mean % Bias of ATPS prepared following pre-calibration alone and prepared following pre and post-calibration of the PEG1000-$(NH_4)_2SO_4$ System

Set of runs	Pre-calibration		Post-calibration		Mean %Bias Accuracy		Coefficient of variation CV% Pre-Calibration		Mean %Bias Accuracy		Coefficient of variation CV% Post-Calibration	
	%Bias Accuracy		%Bias Accuracy		Pre-Calibration				Post-Calibration			
	PEG	Salt	PEG	Salt	PEG	Salt	PEG	Salt	PEG	Salt	PEG	Salt
1	-0.584	0.826	0.305	-0.074	-0.953	0.665	0.002	0.002	-0.115	0.0677	0.003	0.003
2	-0.886	0.932	-0.081	0.313								
3	-0.838	0.788	0.15	0.058								
4	-0.963	0.678	0.091	-0.156								
5	-1.024	0.62	-0.369	0.414								
6	-1.098	0.583	-0.325	0.245								
7	-0.99	0.423	-0.011	-0.401								
8	-1.239	0.469	-0.686	0.141								
1	-1.008	1.291	0.026	0.315	-0.878	0.6	0.003	0.004	-0.097	-0.044	0.008	0.005
2	-0.754	0.767	0.333	0.024								
3	-0.389	0.31	0.552	-0.475								
4	-0.956	0.671	-0.611	0.372								
5	-1.256	0.693	-0.646	0.538								
6	-0.832	0.603	-0.021	-0.307								
7	-0.813	0.23	0.369	-0.897								
8	-1.017	0.236	-0.783	0.079								
1	-0.429	1.342	0.043	-0.152	-0.646	1.928	0.002	0.009	0.013	0.087	0.003	0.003
2	-0.693	1.533	-0.001	0.179								
3	-0.989	3.524	-0.342	-0.068								
4	-0.795	1.072	0.212	0.213								
5	-0.772	1.256	0.281	0.167								
6	-0.488	1.728	0.307	0.403								
7	-0.626	2.915	-0.34	0.089								
8	-0.38	2.055	-0.051	-0.134								

627 Table S1 [B]. Comparison of the mean % Bias of ATPS prepared following pre-calibration alone and prepared following pre and post calibration of the
628 PEG8000-Dextran500 system

Set of runs	Pre-calibration %Bias Accuracy		Post-calibration %Bias Accuracy		Mean %Bias Accuracy Pre-Calibration		Coefficient of variation CV% Pre-Calibration		Mean %Bias Accuracy Post-Calibration		Coefficient of variation CV% Post-Calibration	
	PEG	Dextran	PEG	Dextran	PEG	Dextran	PEG	Dextran	PEG	Dextran	PEG	Dextran
1	0.751	0.648	-0.283	-0.090	0.395	0.363	0.003	0.002	-0.035	0.044	0.004	0.004
2	0.598	0.314	-0.054	-0.070								
3	0.427	0.175	0.769	-0.599								
4	0.523	0.443	0.162	-0.396								
5	-0.150	-0.038	-0.132	0.449								
6	0.465	0.343	-0.044	0.176								
7	0.163	0.419	-0.378	0.577								
8	0.382	0.602	-0.323	0.312								
1	-0.150	-0.073	-0.512	-0.088	-0.398	0.231	0.003	0.003	-0.165	0.140	0.004	0.005
2	-0.240	-0.008	0.125	0.194								
3	-0.180	-0.029	0.648	-0.547								
4	-0.398	0.107	0.169	-0.409								
5	-0.601	0.432	-0.198	0.231								
6	-0.373	0.144	-0.685	0.663								
7	-0.299	0.401	-0.457	0.583								
8	-0.944	0.876	-0.417	0.493								
1	0.695	-0.984	0.103	-0.697	0.349	-0.442	0.002	0.004	-0.029	0.049	0.005	0.005
2	0.423	-0.674	-0.173	0.204								
3	0.157	-0.361	0.634	-0.533								
4	0.189	-0.494	0.224	-0.451								
5	0.503	-0.709	-0.397	0.461								
6	0.176	-0.256	-0.357	0.316								
7	0.617	-0.527	0.131	0.569								
8	0.032	0.467	-0.402	0.530								

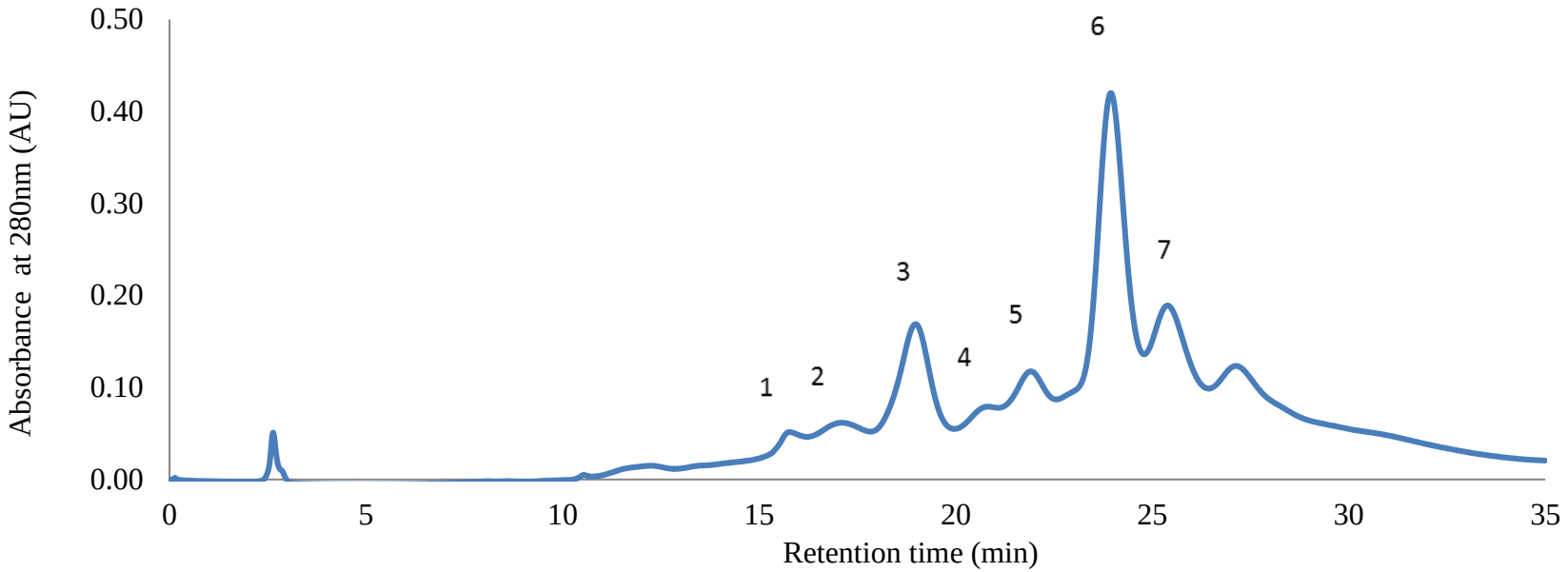

Figure S1. HPLC chromatogram of the separation of Ovalbumin isoforms using operating conditions: Strong Anion-exchange column SOURCE 15Q 4.6/100 PE, Flow Rate: 0.5 mL/min. Temperature: 25 °C .Inj. Volume: 100 µL Detection: UV, 280 nm. The peaks shown in Figure S1 and labelled as peaks 1, 2 and 3 were designated (P0G1), (P0G2) and (P0G3) respectively under the assumption (deduced from the dephosphorylation experiment shown in Figure S2) that P0 is an isoform having zero phosphorylation and G1, G2 and G3 represent glycoforms differing in charge. Peaks 4(P1G1) and 5(P1G2) represent ovalbumin with one site phosphorylated and similar glycan variants conveying additional negative charge, while peak 6(P2G1) and peak 7(P2G2) may represent ovalbumin having both phosphorylation sites modified but again with various glycan structures conveying additional negative charge

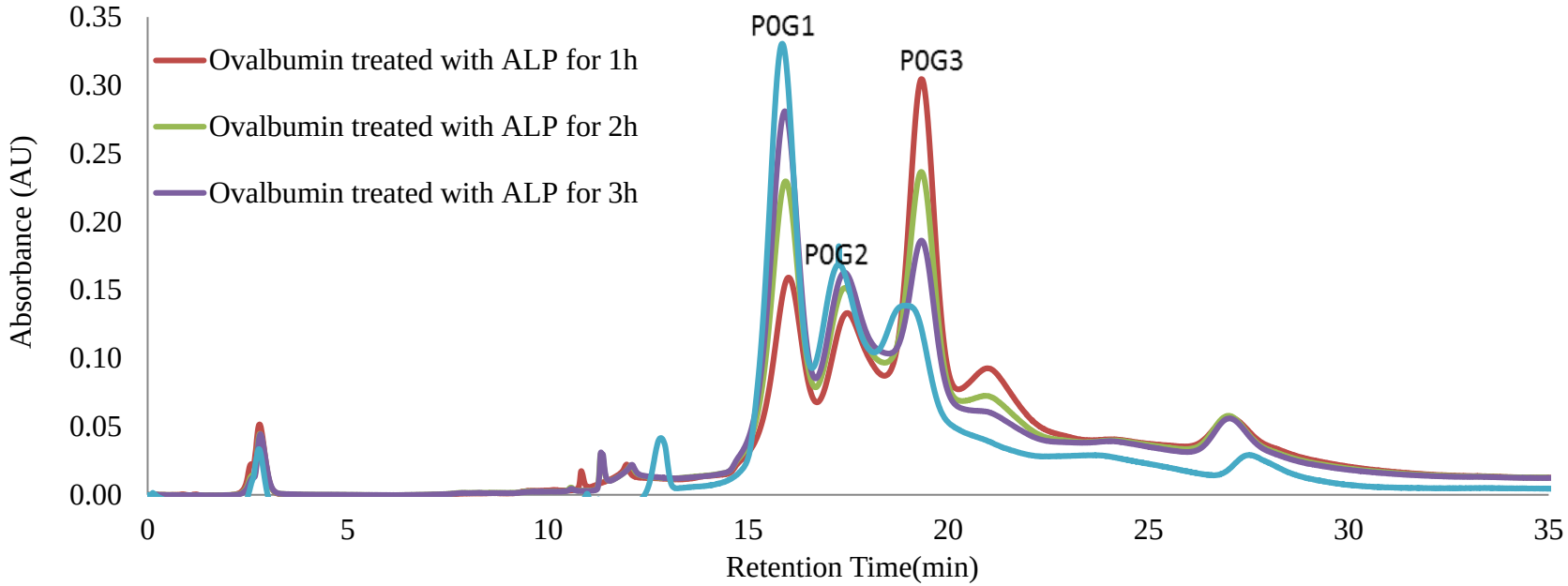

Figure S2. HPLC-chromatogram to identify the phosphorylated sites in ovalbumin using the alkaline phosphatase strategy with treatment time of 1, 2, 3 & 24 hours. The chromatogram of native ovalbumin as shown in Figure S1 indicated a variety of phosphate isoforms: peaks P0 represented dephosphorylated forms consisting of 3 different glycoforms (P0G1, P0G2, and P0G3). From which the remaining isoforms can be tentatively assigned as singly and doubly phosphorylated forms associated with similar glycoforms (P1G1, P2G1 etc)

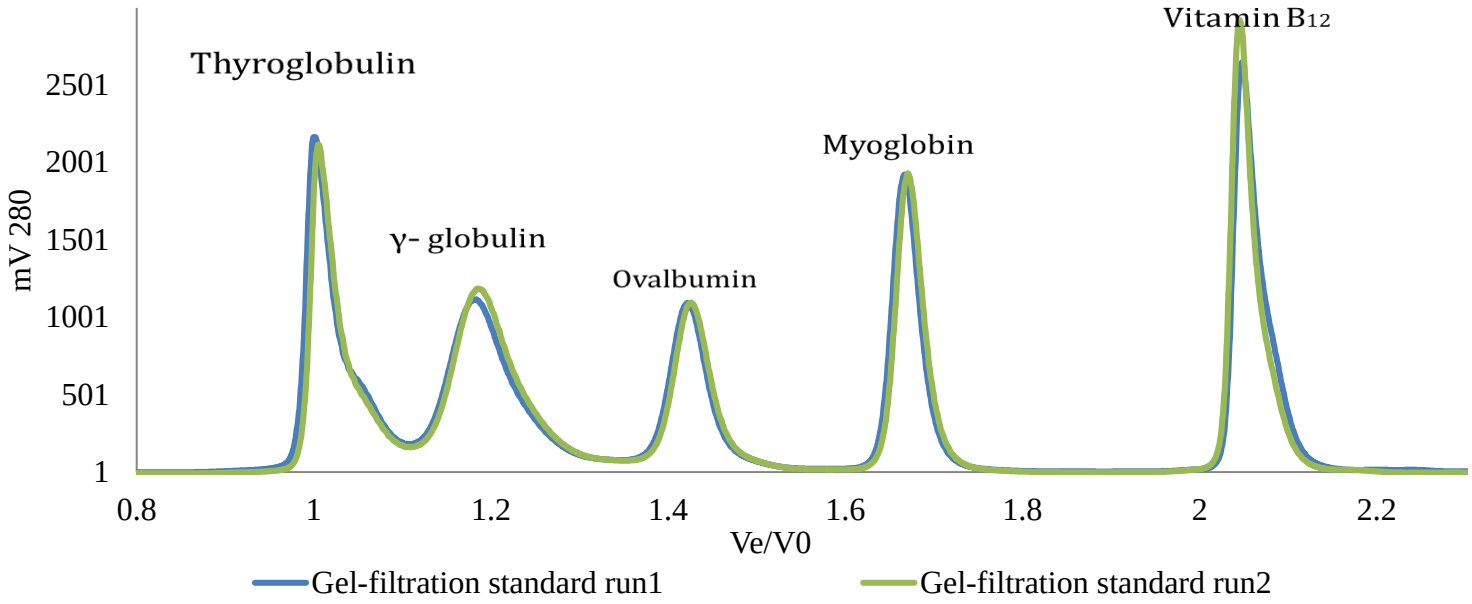

Figure S3. Separation of Bio-Rad standard during Size exclusion chromatography under the conditions: Buffer 0.05 M potassium phosphate ,0.3 M NaCl, pH 7,Column TSKgel G2000SW 300 x 7.8 mm, Flow rate 0.4 ml/min, Sample Treated & Non-Treated Ovalbumin 2mg/mL, Detection UV @ 280nm

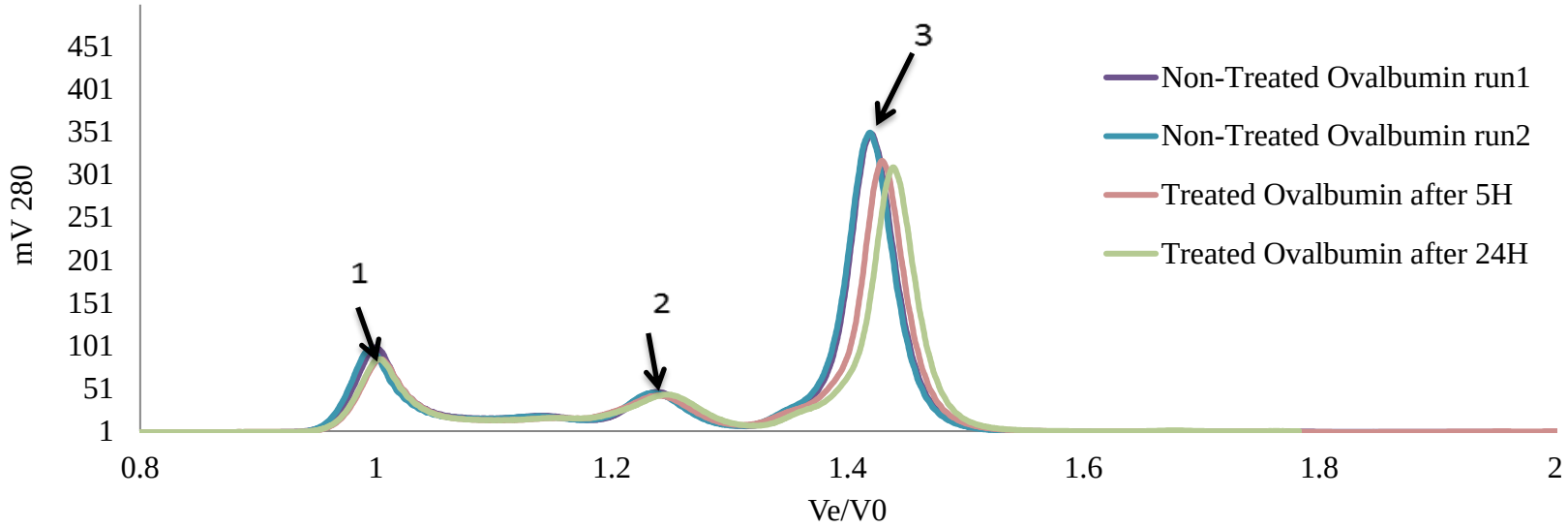

Figure S4. SEC chromatogram for ALP treated and non-treated (native) ovalbumin, where retention was defined as Ve/V_0; Ve is the elution volume of the analyte and V_0 is the column void volume [S1]. No significant changes were seen such as proteolysis and the extent of aggregation (peak1) and presence of multimeric species (peak2) remained the same and the only change was found in the molecular size of protein (peak3), were under ALP treated SEC shows an apparent reduction in molecular weight corresponding to the de-phosphorylated state

Reference:

[S1] P. Hong, S. Koza and E.S. Bouvier, A review size exclusion chromatography for the analysis of protein biotherapeutics and their aggregates, J. Liq. Chrom. Rel. Technol., 35 (2012) 2923–2950

Table S2. Molecular weight of the ovalbumin species as determined by SEC and shown in Fig S4 (above)

Non-treated ovalbumin				Treated ovalbumin		
Peak replicate	Peak 1 Mwt. KDa	Peak 2 Mwt. KDa	Peak 3 Mwt. KDa	Peak 1 Mwt. KDa	Peak 2 Mwt. KDa	Peak 3 Mwt. KDa
1	346.30	105.0	43.7	346.3	104.0	40.5
2	346.07	107.0	43.5	346.3	106.0	41.01
3	346.37	108.0	43.5	346.3	102.0	41.3

Table S3. Analysis of covariance (ANCOVA - IBM SPSS statistics version 20) was used to compare the two regression coefficients for the PEG3350 - Dextran500 system shown in Figure 8 in the associated paper. The method determines whether changes in the partition coefficient differ significantly with respect to the independent variable while considering the possible effects of the covariate. P values were found to be well below the 0.05 probability level.

Tests of Between-Subjects Effects					
Dependant Variable: Cb					
Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected Model	8.826[a]	2	4.413	137.568	0
Intercept	0.041	1	0.041	1.265	0.281
Ct	8.582	1	8.582	267.509	0
Sample	0.407	1	0.407	12.680	0.003
Error	0.417	13	0.032		
Total	33.411	16			
Corrected Total	9.243	15			

a. R squared =0.955 (Adjusted R squared =0.948)