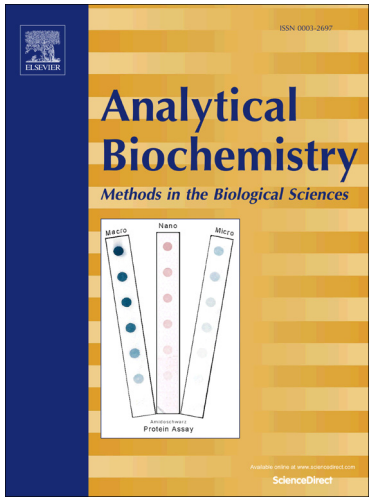


Notes & tips

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*For Analytical Biochemistry Notes & T*ips

# Comparison of colorimetric methods for the quantification of model proteins in aqueous two-phase systems

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**Subject Category:** Protein Structure and Analysis

**Short Title:** Colorimetric protein quantifications in ATPS

**Abstract**

In the present study, the quantification of different model proteins in the presence of typical aqueous two-phase system components was investigated by using the Bradford and BCA assay. Each phase-forming component above 1 and 5 wt. % had considerable effects on the protein quantification in both assays, respectively, resulting in diminished protein recoveries/absorption values by increasing PEG/salt concentration and PEG molecular weight. Therefore, a convenient dilution of both components (up to 1 and 5 wt. %) before protein quantification is recommended in both assays, respectively, in which the BCA assay is favored in comparison to the Bradford assay.

*Keywords:* Aqueous two-phase system, Protein quantification, Colorimetric analysis, Bradford assay, BCA assay.

Aqueous two-phase systems (ATPS)[1] are aqueous biphasic systems which are generally formed by mixing of either two incompatible water-soluble polymers like poly(ethylene glycol) (PEG) and dextran or a polymer and a salt in water beyond critical conditions, like concentration and temperature, resulting in two coexisting immiscible phases. Both phases are composed mainly of water and each one is enriched in a different component. Due to their high water amount, ATPS can provide a gentle aqueous environment for the extraction and recovery of sensitive biological molecules like proteins. Therefore, these systems have been utilized since the mid-1950s as a mild liquid-liquid bioseparation method in biochemistry, biotechnology and cell biology for the separation and purification of large biomolecules, like proteins and enzymes [1–3]. PEG–salt ATPS are preferred compared to systems composed of two polymers because of their low cost, low viscosity and short separation time [1, 4–8].

Protein quantifications in PEG–salt ATPS are frequently influenced by high concentrated polymers/salts and polymer molecular weights [8–11]. Therefore, the evaluation of the influence of ATPS components on protein quantifications is required for the development of ATPS for protein extractions. Several model proteins have been determined in different PEG–salt ATPS by two colorimetric methods, *viz.,* the Bradford [12] and bicinchoninic acid (BCA) [13] assay proposed by Bradford and Smith, respectively. Both assays are based on protein-dye complex formations photometrically quantified at 595 and 562 nm, respectively, according to microplate tests of the Pierce Coomassie (Bradford)/BCA Protein Assay Kit from Thermo Fisher Scientific Inc. (Germany). However, the influence of typical polymers/salts used in ATPS on protein quantifications using the Bradford and BCA assay has neither been studied systematically nor both assays have been compared and evaluated in this context so far.

[1] Abbreviations used: ATPS, aqueous two-phase systems; PEG, poly(ethylene glycol); BCA, bicinchoninic acid; MW, molecular weight; BSA, bovine serum albumin; OVA, ovalbumin, LYZ, lysozyme; $\alpha$-CT, $\alpha$-chymotrypsin.

In the present study, several PEG–potassium phosphate/sodium citrate buffer (pH 7) ATPS were investigated, composed of PEGs with varying molecular weight (MW) (2000, 4000, 6000 and 8000 g/mol), polymer/salt concentrations (0.5–30 wt. %), 0–1 mg/mL model proteins (bovine serum albumin (BSA), ovalbumin (OVA), lysozyme (LYZ) and $\alpha$-chymotrypsin ($\alpha$-CT)). Since ATPS polymers/salts can influence protein determinations [8, 9 and 11], various protein recoveries and calibration curves/absorption values were determined in the presence of PEG/salt concentrations (0.5–30 wt. %) and PEG MWs (2000–8000 g/mol) to evaluate their influence on protein quantifications. In this context, reduced protein recoveries/absorption values >10 % were considered as significant influences in both assays. The least influences/highest protein recoveries were determined in the presence of PEGs/salts diluted up to 1 and 5 wt. % in Bradford/BCA assays, respectively (Fig. S1 in the Supplementary Data). Solutions consisting of 1 mg/mL protein and pure deionized water ($\triangleq$ 100 % protein recovery) were exemplary used as reference standards for all protein recovery calculations. Thereby, the percent recovery of the samples containing a solution of 1mg/mL protein in deionized water with polymer/salt was compared to that of the same 1mg/mL protein-water solution without polymer/salt. Furthermore, deionized water or ATPS components were utilized as reagent blanks for calibration curves, respectively. All measurements were performed in triplicate and the average results are reported.

Each ATPS component provided similar considerable effects on protein quantifications using Bradford/BCA assays, as presented in Figures 1 and 2, as well as Figures S2–S9 in the Supplementary Data. For instance, considerable influences were observed for PEG 8000/phosphate/citrate concentrations above 1 and 5 wt. % in both assays, respectively, resulting in significantly reduced (>10 %) protein recoveries (Figures 1A, B and 2) and absorption values (Figures S2, S7 and S8) for exemplary quantifications of 1 mg/mL BSA/LYZ, respectively, in which the influence is increased by raising PEG/salt concentrations. Similar results were also noticed for OVA and -CT and the other polymers

(Figures S3, S4, S6 and S9). Additionally, the protein recoveries/absorption values were gradually decreased by increasing PEG MW in both assays. For instance, significant effects were noticed for 30 wt. % PEG 2000–8000 on determinations of 1 mg/mL BSA/LYZ, in which PEG 8000 is causing significantly higher reduced protein recoveries (Figures 1C and D) and absorption values (Fig. S5) in both assays, respectively, in comparison to the other PEG MWs. Thus, the influence of PEG MW can be ranked in the order PEG 8000 > 6000 > 4000 > 2000 for both assays, indicating that the protein recovery is decreased by increasing PEG MW. A similar behavior was also observed for OVA and -CT (Fig. S6).

The influence of PEG is attributed to the ability of polymers to compete with proteins for binding and sequestering the Coomassie dye in the Bradford assay [11]. Similarly, there might be a competition between PEG and proteins for binding and chelation of the corresponding reagents in the BCA assay. Hence, reduced protein recoveries/absorption values with increasing PEG concentration and PEG MW or molecule length might be explained by an increasing binding of PEG to proteins, leading to a steric shielding effect of protein interaction sites for the adsorptive staining which may affect protein-dye interactions or rather dye-based protein assay performances [8, 9], resulting in significant influences in protein quantifications using colorimetric dye-based methods. Additionally, the adsorptive binding to proteins is enhanced by an increasing viscosity due to enhanced PEG MWs, thus, decreasing protein recoveries/absorption values. Furthermore, diminished protein recoveries/absorption values by increasing PEG MW and concentration may be attributed to protein precipitation, influencing the quantitative protein determination in solution [9, 14 and 15]. Although no proteins were precipitated under the investigated conditions a similar mechanism should take place. Here, decreased protein recoveries/absorption values may be caused by an excluded volume effect. Accordingly, the proteins are sterically excluded from solvent regions occupied by linear PEG chains [9, 14]. Thus, the proteins are concentrated and the dye has much less volume for protein binding with a consequently reduction of absorption [9].

On the other hand, the influence of salt is related to the nature of salts and their ability to change the specific pH of the assays due to their alkaline behavior in this study [8, 11]. The Bradford assay is performed at an acidic pH which may be changed by added alkaline salts, thus, significantly influencing its performance and protein determination [11]. A pH modification is resulting in a composition change of the Coomassie dye solution, in which different ionic forms can be present, thus, reducing protein-dye binding and protein recoveries/absorption values by increasing salt concentrations [16]. Similarly, protein-dye interactions or protein recoveries/absorption values may be decreased in the BCA assay with increasing salt concentrations due to increasing pH variations by the addition of salts. Since the BCA assay is performed at an alkaline pH, the added alkaline salts may have less influence on protein determinations in this assay than in the Bradford assay. Therefore, protein recoveries were generally higher in the BCA assay than in the Bradford assay.

In conclusion, protein quantifications using the Bradford and BCA assay can be considerably influenced by increasing PEG/salt concentrations, as well as PEG MWs. All investigated ATPS components yielded to suitable concentrations up to 1 and 5 wt. % in both assays, respectively. Hence, convenient dilutions of ATPS components according to the proposed concentration limits are recommended for both assays, respectively, to obtain minimum influences/maximum protein recoveries. Thereby, the obtained results revealed that the BCA assay is favored and recommended in comparison to the Bradford assay due to higher, more reliable and accurate protein recoveries. Although the BCA assay is more time-consuming than the Bradford assay, it is the better assay regarding sensitivity and robustness against ATPS components. From an experimental point of view the BCA assay is also advantageous over the Bradford assay because it has a larger protein concentration working range. On the whole, all obtained results can facilitate protein quantifications using colorimetric Bradford and BCA assays in the presence of typical ATPS phase-forming components in order to establish ATPS as a liquid-liquid extraction technology for proteins.

**References**

[1] P.A. Albertsson, Partition of Cell Particles and Macromolecules, third ed., John Wiley, New York, 1986.

[2] B.Y. Zaslavsky, Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications, Marcel Dekker, New York, 1994.

[3] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-Phase Systems, Academic Press, New York, 1985.

[4] M.T. Zafarani-Moattar, R. Sadeghi, A.A. Hamidi, Liquid-Liquid equilibrium of an aqueous two-phase system containing polyethylene glycol and sodium citrate: experimental and correlation, Fluid Phase Equilib. 219 (2004) 149–155.

[5] M.T.H. Cavalcanti, T.S. Porto, B. de Barros Neto, J.L. Lima-Filho, A.L.F. Porto, A.P. Pessoa, Aqueous two-phase systems extraction of  -toxin from Clostridium perfringens type A, J. Chromatogr. B 833 (2006) 135–140.

[6] J. Benavides, O. Aguilar, B.H. Lapizco-Encinas, M. Rito-Palomares, Extraction and purification of bioproducts and nanoparticles using aqueous two-phase systems strategies, Chem. Eng. Technol. 31 (2008) 838–845.

[7] J. Benavides, M. Rito-Palomares, Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products, J. Chem. Technol. Biotechnol. 83 (2008) 133–142.

[8] M. González-González, K. Mayolo-Deloisa, M. Rito-Palomares, R. Winkler, Colorimetric protein quantification in aqueous two-phase systems, Process Biochem. 46 (2011) 413–417.

[9] H. Barbosa, N.K.H. Slater, J.C. Marcos, Protein quantification in the presence of poly(ethylene glycol) and dextran using the Bradford method, Anal. Biochem. 395 (2009) 108–110.

[10] A.C. Dumetz, A.M. Chockla, E.W. Kaler, A.M. Lenhoff, Comparative effects of salt, organic, and polymer precipitants on protein phase behavior and implications for vapor diffusion, Cryst. Growth Des. 9 (2009) 682–691.

[11] S.C. Silvério, S. Moreira, A.M.F. Milagres, E.A. Macedo, J.A. Teixeira, Interference of some aqueous two-phase system phase-forming components in protein determination by the Bradford method, Anal. Biochem. 421 (2012) 719–724.

[12] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[13] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Frovenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.

[14] D.H. Atha, K.C. Ingham, Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume, J. Biol. Chem. 256 (1981) 12108–12117.

[15] K.C. Ingham, Precipitation of proteins with polyethylene glycol, in: P.D. Murray (Ed.) Methods in Enzymology, Academic Press, San Diego, 1990, pp. 301–306.

[16] S.J. Compton, C.G. Jones, Mechanism of dye response and interference on the Bradford protein assay, Anal. Biochem. 151 (1985) 369–374.

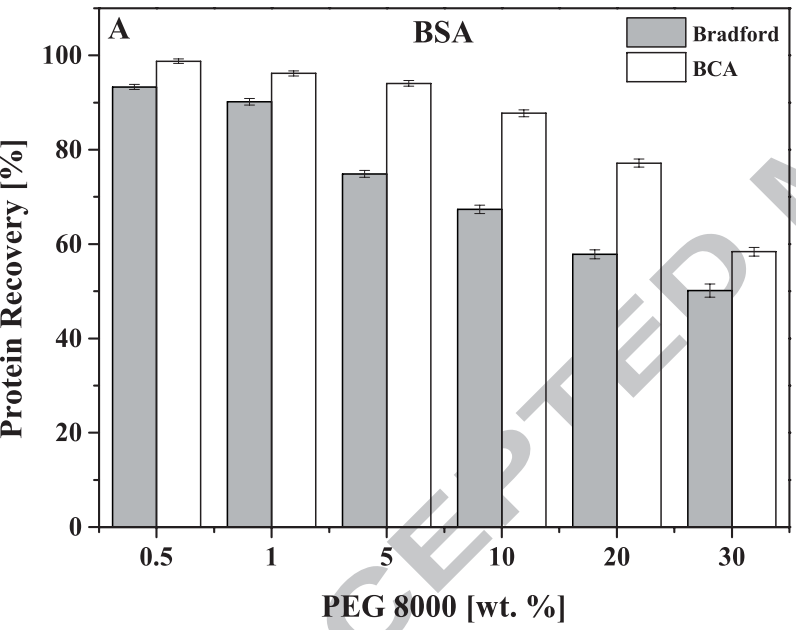
**Figure Captions**

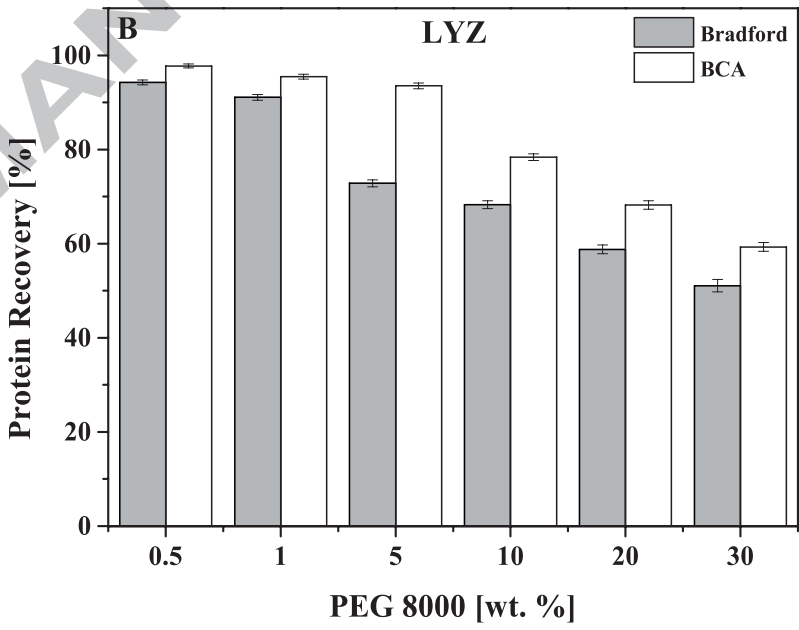
**Figure 1.** Influence of increasing PEG 8000 concentration (A, B) and 30 wt. % PEG MW (C, D) on the protein recovery of 1 mg/mL BSA (A, C) and LYZ (B, D) using the Bradford and BCA assay, respectively. Reference: 1 mg/mL protein in pure deionized water ≙ 100 %.

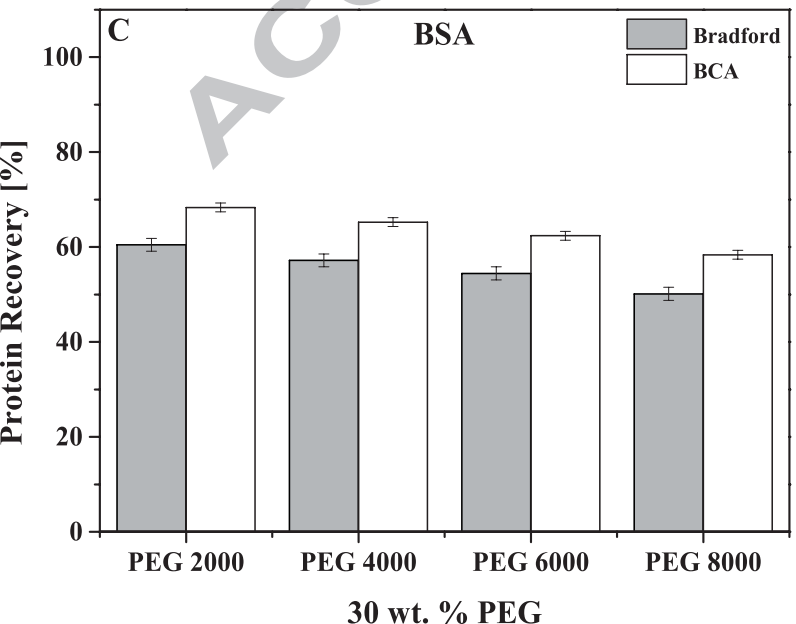
**Figure 2.** Influence of increasing phosphate (A, B) and citrate (C, D) buffer concentration (pH 7) on the protein recovery of 1 mg/mL BSA (A, C) and LYZ (B, D) using the Bradford and BCA assay, respectively. Reference: 1 mg/mL protein in pure deionized water ≙ 100 %.

Figure 1





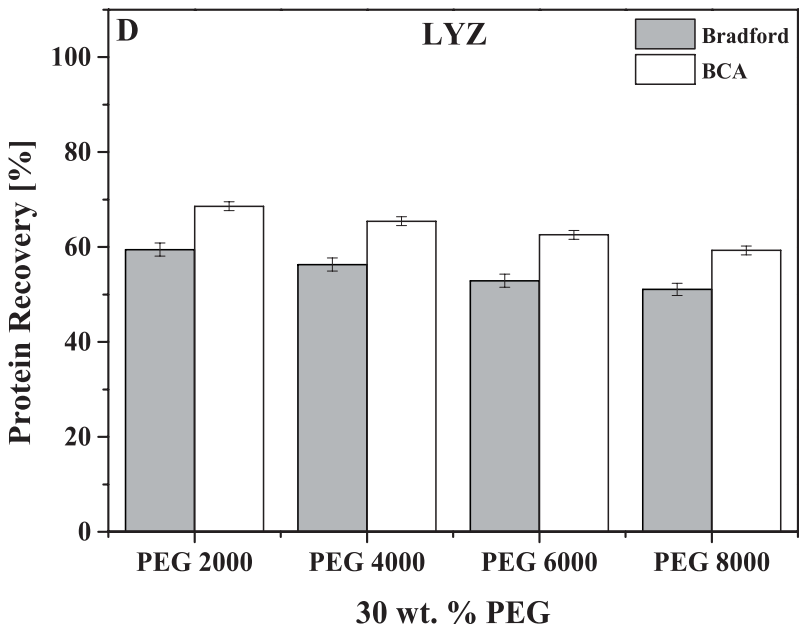



Figure 2