Towards the purification of IgY from egg yolk by centrifugal partition chromatography

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Abstract

Although their high potential as alternative biopharmaceuticals, less than 2% of the total polyclonal antibodies produced worldwide correspond to immunoglobulin Y (IgY) due to the difficulties in isolating them from egg yolk that is a complex biological matrix. In this work, the proteins water soluble fraction (WSPF) of egg yolk was first obtained and the proteins present identified by one-dimensional gel electrophoresis (SDS-PAGE) and label-free quantitative nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS). The egg yolk WSPF was then applied to create aqueous biphasic systems (ABS) composed of polyethylene glycol 1000 $g \cdot mol^{-1}$ (PEG 1000) and phosphate buffer, followed by centrifugal partition chromatography (CPC) to purify IgY. The characterization of the WSPF showed the presence of six major proteins: the target antibody IgY, serum albumin (α-livetin), ovalbumin, ovotransferrin, vitellogenin 1 and vitellogenin 2. The results obtained by ABS revealed a high affinity of all proteins to the polymer-rich phase. However, by changing the PEG and salt concentration, a higher selectivity was observed for IgY, with the remaining proteins partitioning between the two phases. The best ABS were applied in CPC, finally allowing a multi-stage partition and to the technology scale-up. The CPC operating conditions were optimized, allowing to obtain IgY with 51% of purity.

Keywords

Immunoglobulin Y; purification; aqueous biphasic systems; centrifugal partition chromatography

1. Introduction

Polymer-based aqueous biphasic systems (ABS) have been successfully used in the downstream processing of immunoglobulin (IgG) [1–5]. Rosa et al. [3] described the partitioning of immunoglobulins in conventional ABS (polymer + polymer and polymer + salt). IgG was first extracted into a PEG-rich phase, and then to a phosphate-rich phase [3]. The purification factor achieved was 2.7 and 5.9, respectively [3]. The same authors [4] reported the recovery of human IgG from Chinese hamster ovary (CHO) and hybridoma cell culture supernatants using PEG 6000 + K_2HPO_4/ NaH_2PO_4 buffer ABS. IgG with an 88% recovery yield in the polymer-rich phase was obtained [4]. In 2009, Azevedo et al. [5] studied the partitioning and purification of human IgG from a clarified hybridoma supernatant with PEG + $Na_3C_6H_5O_7$/ citric acid buffer ABS. The effect of PEG molecular weight and the addition of a neutral salt, like NaCl, on the partition of IgG, were addressed [5]. Using an ABS composed of 8 wt% PEG 3350 + 8 wt% $Na_3C_6H_5O_7$/ citric acid buffer + 15 wt% NaCl at pH 6, IgG was recovered with a 99% yield, 44% of purity and with an

IgG/protein ratio of 0.9 [5]. Later, Mao et al. [6] investigated the extraction of antibodies from host cell proteins (HCP) from clarified cell culture media using PEG + $Na_3C_6H_5O_7$/ citric acid buffer ABS. An optimal ABS composed of 14 wt% PEG + 8.4 wt% $Na_3C_6H_5O_7$/ citric acid buffer + 7.2 wt% NaCl at pH 7.2 resulted in a product yield of 89%, a 7.6-fold reduction in HCP levels relative to the clarified cell culture fluid before extraction, and overall purity of 70% [6]. However, in these works the use of techniques such as centrifugal partition chromatography (CPC) or countercurrent chromatography (CCC), is scarce [7,8].

CPC is a type of liquid-liquid chromatography requiring two immiscible liquid phases, one of which acts as the stationary phase and the other acts as the mobile phase. The CPC uses centrifugal force in order to hold the stationary liquid phase [9]. The high volume of the stationary phase that can be loaded into the CPC is a significant advantage, making this technique suitable for industrial applications [10]. The CPC works by the exploration of the partition trend of different compounds between the two immiscible solvents/phases, as long as their densities are sufficiently different [9]. The CPC apparatus can be used in two operation modes, ascending and descending, with the key difference being the density of the phase being used as the mobile phase: in the ascending mode the lightest phase is the mobile phase, flowing upwards through the heavier stationary phase, while the opposite occurs in the descending mode [10]. The main advantage of using CPC comes from the liquid-liquid nature of this process, making it unnecessary to use solid chromatographic supports, therefore guaranteeing almost 100% of the target compound recovery and easy recyclability of the solvents [9]. This technique also avoids the need to acquire, maintain and clean high-cost solid columns [9]. While this adaptability is seen as an advantage in terms of potential applications, it should be stressed the importance of a careful choice of the solvents/phases, which should be defined according to the following criteria: easiness of two-phase formation; capacity to be retained by the CPC; and separation/purification effectiveness [10]. Despite all the CPC advantages, the application of polymer + salt ABS in CPC for the separation/purification of antibodies has not been much explored. To the best of our knowledge, only a few works were reported [7,8]. Oelmeier et al. [7] describe the use of PEG + NaH_2PO_4/ K_2HPO_4 buffer at pH 6.0 ABS for the separation of four monoclonal antibodies from HCP, and an upscale from 650 µL batch systems to a 500 mL CPC was successfully demonstrated. The influence of process parameters on HCP clearance and target protein dilution were investigated. Using the CPC, HCP clearance was improved more than threefold compared to batch separations with recoveries of the target protein above 98%. Later, the same group [8], described CPC as an alternative separation step that combined with other precipitation and resolubilization techniques allow to purify monoclonal antibodies. The researchers found that after removing the cells from culture supernatant, either via regular

means like centrifugation or by using liquid-liquid separation approaches by ABS and performing a CPC run in dual-mode, an upper phase rich in antibodies could be obtained while removing host cell proteins.

Given the drawbacks associated with the purification of immunoglobulin (IgY), the reports using polymer + salt ABS for the extraction and purification of antibodies, and the advantages of CPC as an easily scalable technique, ABS formed by polymers and salts combined with CPC were here investigated to purify IgY from the water-soluble proteins fraction (WSPF) of egg yolk. Initially the WSPF was characterized by one-dimensional gel electrophoresis (SDS-PAGE) and label-free quantitative nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) using an Orbitrap high-resolution instrument, and then ABS formed by PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer were studied. The ternary phase diagrams of ABS were determined, and their selective extractive performance for IgY evaluated. The most promising ABS were applied in CPC aiming the purification of IgY.

2. Experimental Section

2.1. Materials

Fresh eggs were periodically provided by Dr. Ricardo Pires from Biocant, located in Cantanhede, Portugal. IgY to establish the SE-HPLC calibration curve was purified using the Pierce® Chicken IgY Purification Kit (Thermo Scientific, EUA). The reagents used in the preparation of the gels for the SDS-PAGE analysis were supplied by Bio-Rad. Laemmli buffer, Coomassie Brilliant Blue G-250 were purchased from Sigma-Aldrich. Trypsin was purchased from Promega. The ABS studied in this work were formed by using phosphate buffer salts composed of potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$, purity > 99%) and monopotassium phosphate (KH_2PO_4, purity > 99.5%), acquired from PanReac and Sigma-Aldrich, respectively. Poly(ethylene)glycol (PEG) with a molecular weight of 1000 $g \cdot mol^{-1}$ (PEG 1000) was supplied from Fluka. The solvents/salts required to perform the SE-HPLC analysis comprise sodium phosphate monobasic (NaH_2PO_4, purity: 99 – 100.5%), and sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$, purity: 98.2 – 102.0%) and sodium chloride (NaCl), obtained from Sigma–Aldrich. The water employed was double distilled, passed through a reverse osmosis system and treated with a Milli-Q plus 185 water purification apparatus.

2.2. Water-soluble proteins fractionation (WSPF) from egg yolk

The WSPF of egg yolk was prepared from fresh eggs, following the protocol described by Liu et al. [11]. This method was selected based on preliminary experimental studies carried out by us

and results described in the literature. This protocol includes a dilution of egg yolk with distilled water (1:6, v:v) followed by a pH adjustment (pH 5.0), a freeze (overnight)/thaw and a final step of centrifugation. Size exclusion-high performance liquid chromatography (SE-HPLC) was applied to separate the proteins present in the WSPF by size. An ÄKTA pure instrument was used for this purpose, with a GE Healthcare High-Performance column Superdex^{TM} 200 10/300 GL. A 100 mM phosphate buffer with NaCl 0.3 M was run isocratically with a flow rate of 0.5 mL·min^{-1}. The injection volume was 25 µL. The protein content on collected samples was quantified at 280 nm using a NanoDrop ND-100 Spectrophotometer.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile of the coexisting phases was investigated by SDS-PAGE using a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell from Bio-Rad. The protein concentration was quantified at 280 nm using a NanoDrop ND-100 Spectrophotometer, diluted in order to achieve a total protein content of 0.003 mg, and further mixed with the Laemmli buffer (2:1, v:v). The samples were then subjected to SDS-PAGE in 15% polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue G-250 overnight and then distained at room temperature. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

2.4. Tryptic digestion, mass spectrometry analysis, and protein identification

Tryptic digestion was performed according to [12], with a few modifications. Protein bands were manually excised from the gel and transferred to eppendorf tubes. Replicate bands were excised and also identified. The gel pieces were washed three times with 25 mM ammonium bicarbonate/ 50% acetonitrile and one time with acetonitrile. The protein's cysteine residues were reduced with 6.5 mM 1,4-dithiothreitol (DTT) and alkylated with 54 mM iodoacetamide. Gel pieces were dried in a SpeedVac (Thermo Savant) and rehydrated in digestion buffer containing 12.5 µg·mL^{-1} sequence grade modified porcine trypsin in 25 mM ammonium bicarbonate. After 90 min, the supernatant was removed and discarded, 100 µL of 25 mM ammonium bicarbonate was added, and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by the addition of 10% formic acid/ 50% acetonitrile three times and finally with acetonitrile. Tryptic peptides were lyophilized in a SpeedVac (Thermo Savant) and resuspended in 5% acetonitrile/ 0.1% formic acid solution. The samples were analyzed with a QExactive Orbitrap (Thermo Fisher Scientific) that was coupled to an Ultimate 3000 (Dionex, Sunnyvale) HPLC system. The trap (5 mm × 300 µm I.D.) and analytical (150 mm × 75 µm I.D.) columns used were C18 Pepmap100 (Dionex, LC Packings), the latter having a particle size of 3 µm. Peptides were trapped at 30 µl/ min in 95% solvent A (0.1% formic

acid: 5% acetonitrile, v:v). Elution was achieved with the solvent B (0.1% formic acid: 100% acetonitrile, v:v) at 300 nL/ min. The 50 min gradient used was as follows: 0 - 3 min, 95% solvent A; 3 - 35 min, 5 - 45% solvent B; 35 - 38 min, 45 - 80% solvent B; 38 - 39 min, 80% solvent B; 39 - 40 min, 20 - 95% solvent A; 40 - 50 min, 95% solvent A. Nanospray was achieved using an uncoated fused silica emitter (New Objective) (o.d. 360 µm; i.d. 50 µm, tip i.d. 15 µm) biased to 1.8 kV. The mass spectrometer was operated in the data-dependent acquisition mode. An MS2 method was used with an FT survey scan from 375 to 1600 m/z (resolution 35,000; AGC target 3E6). The 10 most intense peaks were subjected to HCD fragmentation (resolution 17,500; AGC target 5E4, NCE 25%, max. injection time 120 ms, dynamic exclusion 35 s). Spectra were processed and analyzed using Proteome Discoverer (version 2.0, Thermo), with the MS Amanda search engine (version 2.1.4.3751, University of Applied Sciences Upper Austria, Research Institute of Molecular Pathology). Uniprot (TrEMBL and Swiss-Prot) protein sequence database (version of January 2016) was used for all searches under Gallus gallus. Database search parameters were as follows: carbamidomethylation and carboxymethyl of cysteine as a variable modification as well as oxidation of methionine, and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 10 ppm, and fragment ion mass tolerance was 0.05 Da. To achieve a 1% false discovery rate, the Percolator (version 2.0, Thermo) node was implemented for a decoy database search strategy and peptides were filtered for high confidence and a minimum length of 6 amino acids, and proteins were filtered for a minimum number of peptide sequences of 2 and only rank 1 peptides.

2.5. ABS phase diagrams and tie-lines

The binodal curves of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO4 buffer at different pH values were determined through the cloud point titration method at (25 ± 1)°C and atmospheric pressure [13]. Aqueous solutions of PEG 1000 at 60 wt% and K_2HPO_4/ KH_2PO_4 buffer at 25 - 40% were used in the determination of the phase diagrams. The phosphate buffer was prepared at different pH values - 5.5 (±0.22), 6.0 (±0.16), 6.5 (±0.16), 7.5 (±0.02) and 8.0 (±0.05) - using different molar ratios of the two phosphate-based salts. The composition of each buffer solution is given in **Table S1** of Supporting Information.

Tie-lines (TLs) were determined by a gravimetric method originally described by Merchuk et al. [14]. A mixture at the biphasic region was gravimetrically prepared with PEG + salt + water, vigorously stirred, and allowed to reach equilibrium by separation into the two phases over at least 12 h at 25 °C. After the separation of the coexisting phases, the phases were further weighted. Finally, each individual TL was determined by the application of the lever-arm rule to

the relationship between the weight of the top and bottom phases and the overall system composition. Details for their determination are provided in the Supporting Information.

2.6. Extraction and purification of IgY from the egg yolk WSPF using ABS

The WSPF of egg yolk was prepared from fresh eggs, following the protocol PREVIOSLY described and then applied in the ABS composition. The ternary mixtures compositions to be applied as ABS to purify IgY from the WSPF were chosen based on the phase diagrams determined for each PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer systems at different pH values. A common ternary mixture composition was prepared using 18 wt% PEG 1000 + 15 wt% K_2HPO_4/ KH_2PO_4 buffer + WSPF. **Table 1** describes the mixture compositions and TLL of each ternary mixture determined in this work to address the pH value effect. With the optimum pH value identified (6.0), different concentrations of PEG and K2HPO4/ KH2PO4 buffer were then studied (4 mixture points). These experimental mixture compositions are presented in **Table 2**.

Table 1. Ternary mixture composition of ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer for the purification of IgY from the WSPF, and respective TLL.

pH	[PEG]/ wt%	[K_2HPO_4/ KH_2PO_4 buffer]/ wt%	[WSPF]/ wt%	TLL≈
5.5	18.0	15.0	67.0	28.9
6.0	18.0	15.0	67.0	38.3
6.5	18.0	15.0	67.0	38.6
7.5	18.0	15.0	67.0	41.4
8.0	18.0	15.0	67.0	42.3

Table 2. Ternary mixture composition of ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer pH 6.0 for the purification of IgY from the WSPF, and respective TLL.

	[PEG 1000]/ wt%	[K_2HPO_4/ KH_2PO_4 buffer]/ wt%	[WSPF]/ wt%	TLL≈
pH 6.0	18.0	15.0	67.0	42.3
	18.0	13.0	69.0	29.9
	21.0	16.0	63.0	47.6
	21.0	14.0	65.0	42.5

Each mixture was vigorously stirred and left to equilibrate for at least 4 h, at 25 °C, to achieve the complete IgY partitioning and other impurities between the two phases. In all the ternary

mixtures evaluated, the polymer-rich aqueous phase is the top phase, while the bottom phase is mainly composed of salt and water.

2.7. pH measurements

The pH values of both the PEG-rich and salt-rich aqueous phases were measured at (25 ± 1) °C using a METTLER TOLEDO SevenMulti pH meter within an uncertainty of ± 0.02. The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00).

2.8. IgY quantification in the ABS's phases

After a careful separation of the phases, SE-HPLC was used with the aim of quantifying IgY in each phase and inferring its purity. A calibration curve for IgY was determined for this purpose. A phosphate buffer solution (1000 mL) was prepared using 47 mL of a Solution A (27.8 g NaH_2PO_4), 203 mL of a Solution B (53.65 g $Na_2HPO_4{\cdot}7H_2O$) and 35 g of NaCl. Each phase was diluted at a 1:9 (v:v) ratio in the phosphate buffer solution before injection. A Chromaster HPLC (VWR Hitachi) was used for IgY quantification. The SE-HPLC was performed on an analytical column Shodex Protein KW- 802.5 (8 mm x 300 mm). A 100 mM phosphate buffer + NaCl 0.3 M was run isocratically with a flow rate of 0.5 $mL{\cdot}min^{-1}$. The column oven and autosampler temperatures were kept at 25 °C and at 10 °C, respectively. The injection volume was 25 µL. The wavelength was set at 280 nm using a DAD detector. The obtained chromatograms were treated and analyzed using the PeakFit (version 4) software.

The extraction efficiency, *EE*%, of the studied systems for IgY from the WSPF was determined according to equation 1,

$$EE\% = \frac{w_{IgY}^{\text{top phase}}}{w_{IgY}^{\text{top phase}} + w_{IgY}^{\text{bottom phase}}} \times 100 \quad (1)$$

where $w_{IgY}^{\text{top phase}}$ and $w_{IgY}^{\text{bottom phase}}$ are the weight of IgY in the PEG-rich (top phase) and in the salt-rich (bottom phase) aqueous phases, respectively.

The partition coefficients of IgY and protein impurities in the WSPF, $K_{IgY,}$ and $K_{\text{protein impurities}}$, were determined according to equations 2 and 3, respectively,

$$K_{IgY} = \frac{\text{peak area of IgY}_{\text{top phase}}}{\text{peak area of IgY}_{\text{bottom phase}}} \quad (2)$$

$$K_{\text{protein impurities}} = \frac{\text{peak area of protein impurities}_{\text{top phase}}}{\text{peak area of protein impurities}_{\text{bottom phase}}} \quad (3)$$

where peak area of IgY/protein impurities$_{\text{top phase}}$ and peak area of IgY/protein impurities$_{\textit{bottom phase}}$ are the peak area of the IgY or protein impurities in PEG-rich (top phase) and in the salt-rich (bottom phase) aqueous phases, respectively.

The purity of IgY, Purity%, was determined according to equation 4,

$$\text{Purity\%} = \frac{\text{peak area of IgY}_{\text{top phase}}}{\text{peaks area of protein impurities + peak area of IgY}_{\text{top phase}}} \quad (4)$$

where, peak area of IgY$_{\text{top phase}}$ and peak area of cprotein impurities + peak area of IgY$_{\text{top phase}}$ are the peak area of IgY and peak areas of protein impurities and IgY in PEG-rich (top phase) aqueous phase, respectively.

The recovery yield of IgY was determined by the ratio between the amount of IgY in the PEG-rich phase (top phase) and the total amount of IgY present in the WSPF added to each system, by the application of equation 5,

$$\text{Yield (\%)} = \frac{\text{WIgY}_{\text{top phase}}}{\text{WIgY}_{\text{WSPF}}} \times 100 \quad (5)$$

At least three individual experiments were carried out for each ABS, allowing the determination of the average extraction efficiencies, partition coefficients, purity and recovery yield, and respective standard deviations. Control or “blank” solutions at the same mixture point used for the extraction studies (with no WSPF/proteins added) were used in all systems.

2.9. Centrifugal partition chromatography (CPC)

A Fast Centrifugal Partition Chromatography (FCPC)® system, model FCPC-C, from Kromaton Rousselet-Robatel (Annonay, France), was used for the IgY purification from the WSPF by applying ABS. The equipment design comprises a pattern of cells interconnected by ducts and dug in a stainless-steel disk. The cell design, also called twin cells, contains a restriction in the middle ducts of the canal creating two superimposed chambers. The total cell volume is about 50 mL, with 10 mL or 20 % of the column volume corresponding to the connecting ducts. The maximum theoretical liquid stationary phase retention factor (S_f = VS/VC) is 80% since 20% of connecting ducts volume can only contain the mobile phase. The maximum rotor rotation is 3000 rpm, generating a maximum centrifugal field of ~ 1500 G. Two rotating seals are displayed at the rotor entrance and exit, and they can withstand a maximum pressure of 70 bar (7 MPa or 1000 psi). The FCPC system was connected to an ECOM ECB2004 Gradient box with degasser,

an ECOM ECP2010 Analytical HPLC pump, an ECOM Flash 14 DAD detector (four wavelengths are simultaneously being analyzed), and to a continuous scan (ECOM spol. S.r.o., Czech Republic). Several fractions were collected with an ADVANTEC® Super Fraction Collector CHF122SC (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Each sample was injected manually using a Rheodyne valve model 3055-023 through a 10 mL sample loop. Analogical detector signals were processed using the ECOMAC software (ECOM spol. S.r.o., Czech Republic).

The CPC assays were carried out using the PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer at pH 6.0 ABS. The systems were set to work in the ascending mode. The rotor was entirely filled with the bottom-rich phase (salt-rich phase) at 600 rpm to achieve the homogeneous solvent re-equilibration on the rotor. Then, the rotation was set to the intended speed, needed for the appropriate stationary phase retention. After the working rotational speed was set up, the PEG-rich phase (top phase) was pumped through the stationary phase to reach the equilibrium, *i.e.,* when only the mobile phase came out of the column, and the signal baseline is stabilized. The stationary phase retention, S_f%, was calculated by the ratio of the stationary phase volume (VS) and the column volume (VC), as described in equation 6,

$$S_f\% = \frac{VS}{VC} \times 100 \quad (6)$$

3. Results and Discussion

The WSPF of egg yolk was obtained according to the protocol described by Liu et al. [11], where the lipids and lipoproteins are removed as a precipitated fraction. **Figure 1** shows the SE-HPLC chromatogram of the egg yolk WSPF, which includes 3 major peaks (A, B and C). There is a small peak before peak A (higher molecular weight proteins) that was not considered in the analysis and discussion of the results regarding the identification of the several proteins present since it is related with an aggregation phenomenon [15]. According to the literature, there are three classes of livetins in egg yolk: γ-livetins (180 kDa), α-livetins (80 kDa) and β-livetins (45 kDa) [16]. The ratio between them in egg yolk is 3:2:5, being in close agreement with the SE-HPLC results and the identified peaks A, B, and C in **Figure 1**. The γ-livetin fraction is predominantly constituted by IgY as described in the literature [17,18]; however, only a few reports describe the specific proteins that constitute α-livetins and β-livetins, namely serum albumin and α2-glycoprotein, respectively [17]. In order to identify the proteins in the WSPF, this matrix was initially fractionated by SE-HPLC, where the proteins are separated according to their molecular weight. For each peak (A, B and C), two fractions were collected, with a total of 6 collected samples (A, A', B, B', and C, C'). These samples were then analyzed by SDS-PAGE, with SDS-gels

of 15%, for better separation and refinement of individual proteins separated by molecular weight.

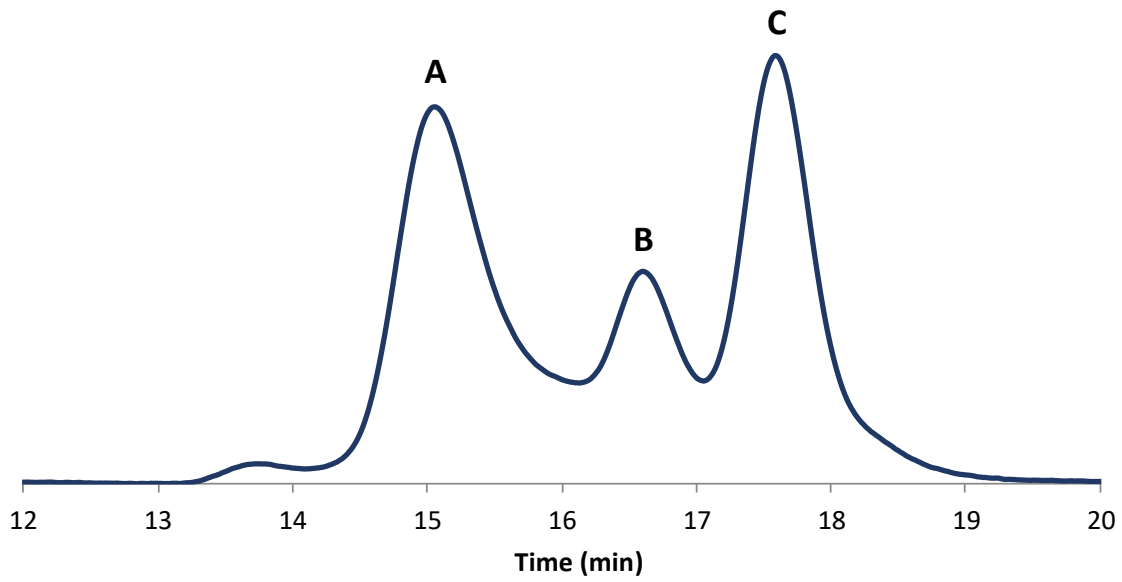

Figure 1. SE-HPLC chromatogram of the WSPF from egg yolk.

Figure 2 shows the proteins profile of each sample collected from the initial gel filtration by SE-HPLC. These SDS-PAGE results (obtained under reducing conditions), show that there are two major bands corresponding to peak A (samples A and A'), and which correspond to the heavy (≈70 kDa) and light (≈25 kDa) chains of IgY. These results agree with the information previously reported in the literature describing IgY as the major constituent of γ-livetins [17]. Although the main protein of peak A was identified, also based on literature data [17,18], peaks B and C are composed of several non-identified small size proteins (< 48 kDa). In order to identify them by mass spectrometry (MS), each band/spot was excised (identified by the orange squares in **Figure 2**). A total of 27 bands/spots were subjected to trypsin digestion and peptide extraction, and the digested peptides were analyzed by nano-LC-M/MS. In this setup, peptides were first separated in a nano-HPLC system, and tandem mass spectrometric analysis was carried out in a QExactive instrument (Thermo Scientific).

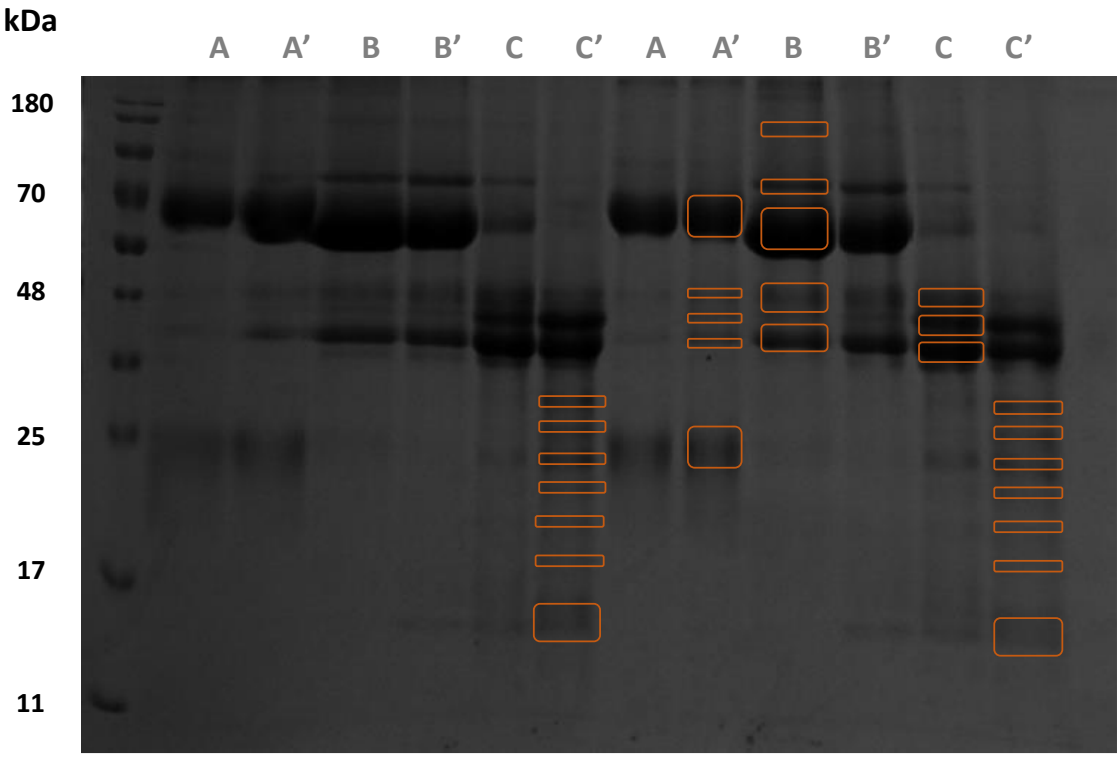

Figure 2. SDS-PAGE stained with Coomassie blue for each fraction of the WSPF collected from gel filtration chromatographic separation.

The number of peptides and identification of the correspondent proteins of samples A, B and C are provided in **Table 3**. Two proteins were identified in the sample corresponding to peak A: the target protein IgY (identified as Ig lambda chain C region) and chicken serum albumin usually denominated as α-livetin [17,19]. By the analysis of **Table 3**, it is notable that the identification of IgY occurs only in peak A, which is in agreement with **Figure 2** (SDS-PAGE), where the bands corresponding to IgY are not present in samples B and C. The proteins identified in peaks B and C are also present in peak A. Two main proteins were identified in the sample corresponding to peak B, namely serum albumin and ovotransferrin (a glycoprotein of egg white that can be transferred to the yolk). In peak C a high number of peptides identifying vitellogenin 1 and 2 (precursors from which are derived the smaller and well-characterized yolk proteins lipovitellin 1 and 2 and phosvitin) were observed. Serum Albumin was also identified in peak C.

Table 3. Identified proteins in the egg yolk WSPF using the *Gallus gallus* proteome in the UniProt databank [20].

	Accession number	Protein	Number of peptides
Peak A	P20763	Serum albumin OS=Gallus gallus GN=ALB PE=1 SV=2	10
	P19121	Ig lambda chain C region OS=Gallus gallus PE=4 SV=1	10
	P01012	Ovalbumin OS=Gallus gallus GN=SERPINB14 PE=1 SV=2	7
	P02845	Vitellogenin-1 OS=Gallus gallus GN=VTG1 PE=1 SV=1	5
	P87498	Vitellogenin-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1	4
Peak B	P19121	Serum albumin OS=Gallus gallus GN=ALB PE=1 SV=2	81
	P02789	Ovotransferrin OS=Gallus gallus PE=1 SV=2	41
	P02845	Vitellogenin-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1	8
	P87498	Vitellogenin-1 OS=Gallus gallus GN=VTG1 PE=1 SV=1	4
	P01012	Ovalbumin OS=Gallus gallus GN=SERPINB14 PE=1 SV=2	4
Peak C	P87498	Vitellogenin-1 OS=Gallus gallus GN=VTG1 PE=1 SV=1	34
	P19121	Serum albumin OS=Gallus gallus GN=ALB PE=1 SV=2	29
	P02845	Vitellogenin-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1	23
	P01012	Ovalbumin OS=Gallus gallus GN=SERPINB14 PE=1 SV=2	12
	P02789	Ovotransferrin OS=Gallus gallus PE=1 SV=2	10

The proteins identified in this work agree with those previously identified by other authors, such as Mann and Mann [21] and Nilsson et al. [22]. The first authors [21] identified some plasma proteins in egg yolk, in agreement with our results that identified the presence of serum albumin. Furthermore, egg white proteins (ovalbumin, ovomucoid, ovotransferrin, cystatin, and ovoinhibitor) were identified by Man and Man [21] and by us (ovalbumin and ovotransferrin) in the WSPF obtained from egg yolk. Some of these proteins display relevant biological activities. For instance, ovotransferrin and cystatin have antibacterial activity, whereas ovomucoid and ovoinhibitor have antiviral activity [17]. Nilsson et al. [22] also reported other egg white proteins in the WSPF of egg yolks, such as fibrinogen, hemopexin, and coagulation factor IX. In addition, lipoproteins of the egg yolk have also been suggested to have an antibacterial effect [17], and several apolipoproteins were detected in the WSPF: apolipoprotein A1, apolipoprotein B, vitellogenins 1 and 2, and apovitellenin 1 by Nilsson et al. [22], and vitellogenins 1 and 2 by us.

Based on the MS results and literature data [23,24], **Figure 3** depicts the identification of each major protein present in each spot resolved by SDS-PAGE. In summary, by using different

techniques we were able to identify the main proteins presents in the WSPF obtained from egg yolk, namely the target IgY in the sample corresponding to peak A, serum albumin (α-livetin), ovotransferrin and vitellogenin 2 in the sample corresponding to peak B, and vitellogenin 1, vitellogenin 2 and ovalbumin in the sample corresponding to peak C.

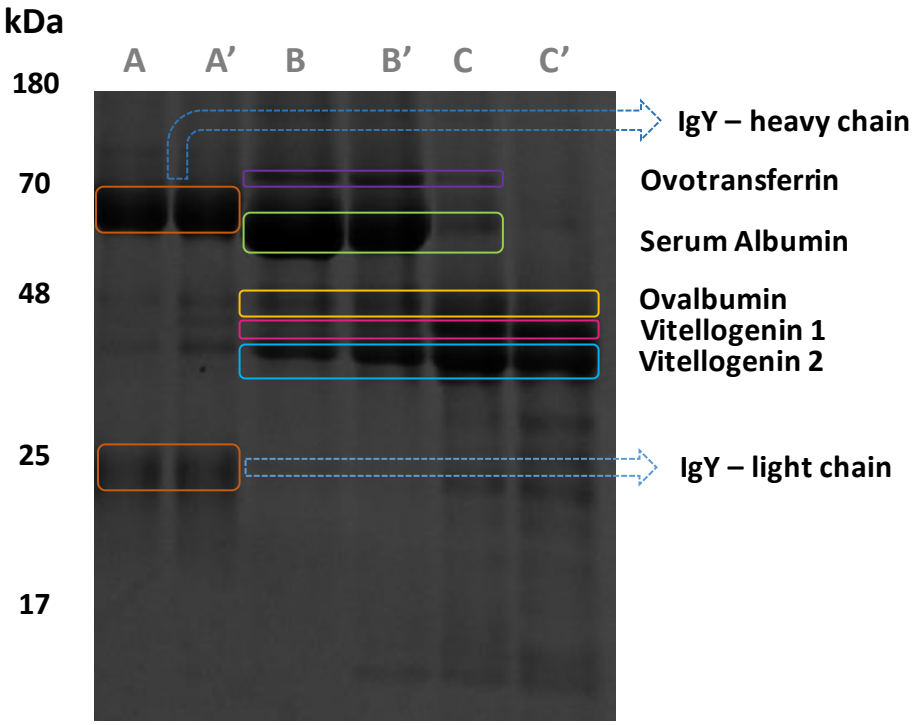

Figure 3. SDS-PAGE stained with Coomassie blue for each fraction collected from the WSPF by gel filtration chromatographic separation.

With the proteins in the WSPF identified, the studies on the use of ABS combined with CPC aiming the complete purification of IgY from the WSPF were initiated. To select a proper ABS to be used in the CPC equipment, three main parameters should be used, namely the easiness of two-phase formation, the retention capacity in the CPC rotor, and the separation/purification effectiveness [10]. In order to move on with an adequate system composition, a literature review was carried out to identify systems which could be applied in CPC. Foucault et al. [25] compared several polymers + salt and polymer + polymer ABS in CPC assays, concluding that while all of the systems analyzed have an effective retention capacity, the major problem is the mass transfer effect, which requires significant reductions in the apparatus' flow-rate to overcome this fact. Advances in CPC technology [26] have allowed the use of higher flow rates with polymer + salt ABS, as demonstrated by Sutherland et al. [27], using ABS composed of 12.5 wt% of PEG 1000 + 12.5 wt% of K_2HPO_4, at a 10.0 mL/min flow-rate, with an effective retention capacity.

Based on the effectiveness of the PEG 1000 + K_2HPO_4 ABS, when considering the retention capacity of the phases in CPC [27], the remarkable results provided by this system for the purification of enzymes [28] and IgG antibodies [29], this system was selected and investigated here in an attempt to purify IgY from the WSPF by CPC. To initially evaluate the effectiveness of the 12.5 wt% PEG 1000 + 12.5 wt% K_2HPO_4 + 75 wt% H_2O ABS in the CPC apparatus, several operating conditions were tested (rotation speed, flow-rate, and mode - **Table 4**). In general, high retention values were obtained with stationary phase retention (S_f%) above 40%. Although, the increase of the flow-rate decreases the stationary phase retention, these lower values are not significant since high stationary phase retention was obtained. Thus, this system has an effective retention in CPC, enabling its use in this work for the purification of IgY from the egg yolk WSPF.

Table 4. Stationary phase retention (S_f %) achieved for the CPC assays with the 12.5 wt% PEG 1000 + 12.5 wt% K_2HPO_4 + 75 wt% H_2O ABS, and respective operating conditions.

CPC operation mode	Rotation speed/ (rpm)	Flow rate/ (mL/min)	S_f (%)
Descending	2000	1.5	48.52
Descending	2500	2.0	44.90
Ascending	2000	1.5	47.30
Ascending	2500	2.0	46.00

After demonstrating that the PEG 1000 + K_2HPO_4 + water ABS had sufficient retention to be used in CPC assays, further optimizations were carried out in order to purify IgY. Since the pH is a crucial parameter when dealing with the extraction of proteins, K_2HPO_4/ KH_2PO_4 buffer solutions at different pH values were prepared, namely 5.5, 6.0, 6.5, 7.5 and 8.0, to appraise the effect of the pH on these ABS extraction capacity and selectivity for IgY. The respective ternary ABS phase diagrams were determined at (25 ± 1) °C and atmospheric pressure, and are illustrated in **Figure 4**. The experimental weight fraction data are given in Supporting Information (**Tables S4 to S6**). In the studied ABS, the top phase is the PEG-rich phase, while the bottom phase is primarily composed of salt and water.

Regarding the phase diagrams presented in **Figure 4**, there is an increase in the biphasic region area with the increase of pH, meaning that the capacity to form ABS follows the trend: pH 5.5 < 6.0 < 6.5 < 7.5 ≈ 8.0. This behavior is due to the salting-out of the polymer, in which ions that are more easily hydrated are more able to induce phase separation. With the increase of pH in the system there is a higher concentration of K_2HPO_4, and as such, higher concentrations of HPO_4^{2-},

a more strongly hydrated anion according to the Hofmeister series [30], causing an increase of the salting-out effect. Similar trends have been observed in other ABS composed of polymers and salts [31,32].

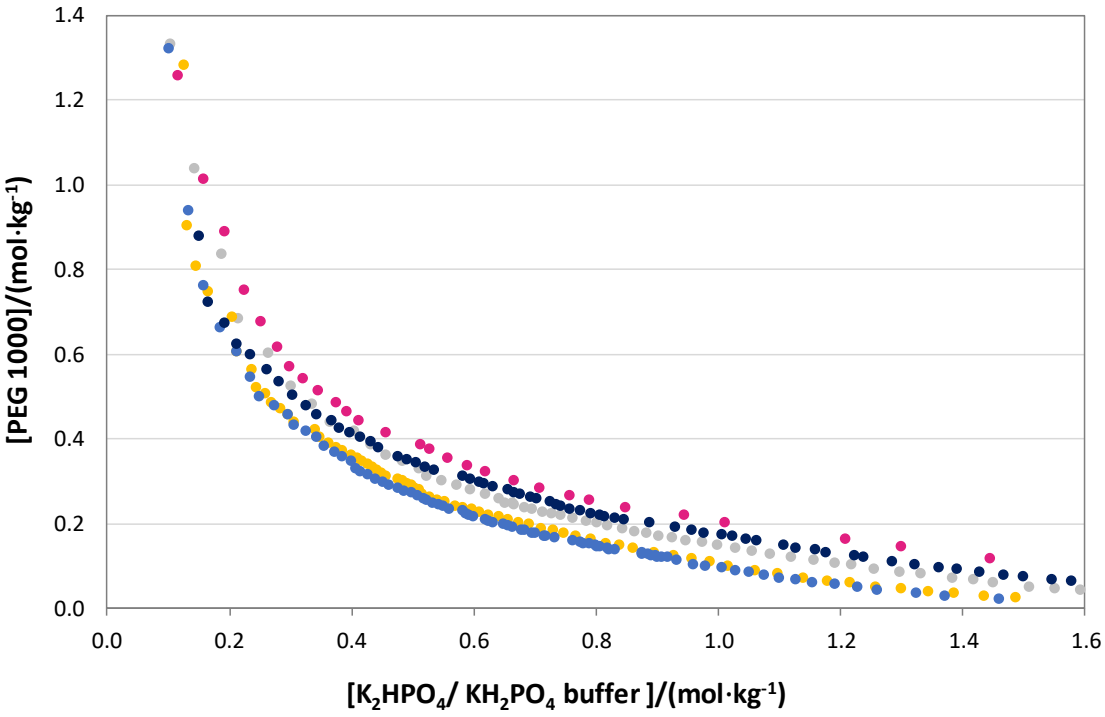

Figure 4. Phase diagrams of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer + H_2O at different pH values: 5.5 (●); 6.0 (●); 6.5 (●); 7.5 (●); 8.0 (●), at (25 ± 1) °C and atmospheric pressure.

The experimental binodal data were fitted according to the empirical relationship described by equation S1 reported in Supporting Information. The regression parameters A, B, and C, which were estimated by the least-squares regression, are provided in **Table S7** of Supporting Information, along with their corresponding standard deviations (σ). Overall, good correlation coefficients were obtained, indicating that these fittings can be used to predict data in any given region of the phase diagram, without the need to resort to experimental data. The experimental data for the TLs and their respective length (TLL) are reported in Supporting Information (**Table S8**).

After characterizing the PEG 1000 + phosphate buffer ABS by their phase diagrams, a fixed mixture point for the extraction of IgY was selected: 18 wt% PEG 1000 + 15 wt% of K_2HPO_4/ KH_2PO_4 buffer + 67 wt% of WSPF. This mixture point was selected to fit within the biphasic area of all phase diagrams, but as close as possible to the binodal curve to maximize the water content on the systems and avoid the proteins denaturation. **Figure 5** shows the macroscopic appearance of the systems prepared at different pH values. The appearance of these systems

suggests that proteins in the WSPF suffer denaturation and precipitate at the interface. In general, there is an increase in the amount of proteins precipitated by the increase of the pH values. The occurrence of proteins denaturation may be related with the strong salting-out capacity of the K_2HPO_4 salt used, as previously described [33], and with the low water content at the polymer-rich phase - as shown by the TL data given in Supporting Information. Another important macroscopic characteristic is the precipitation of salt present in the ABS at pH 5.5, as can be seen in Figure 5.

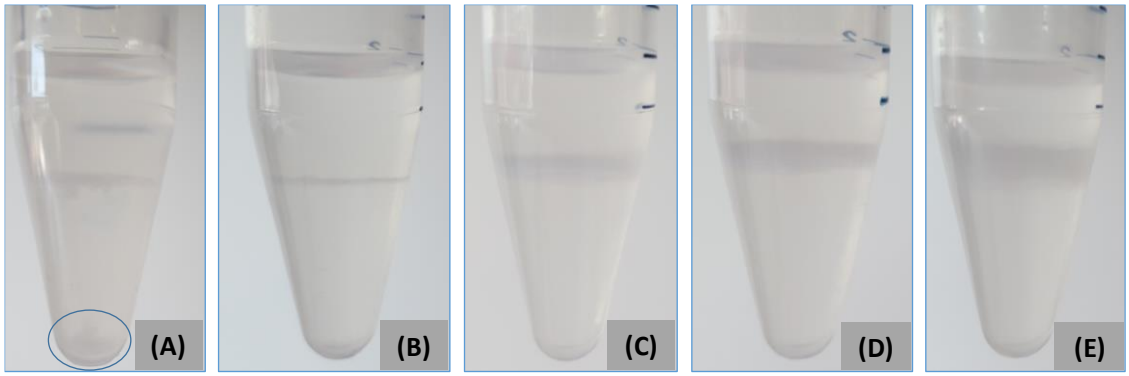

Figure 5. ABS composed of 18 wt% PEG 1000 + 15 wt% of K_2HPO_4/ KH_2PO_4 buffer + 67 wt% of WSPF at (25 ± 1) °C, at several pH values. A – pH 5.5; B – pH 6.0; C – pH 6.5; D – pH 7.5; E – pH 8.0. The blue circle highlights the presence of precipitated salt.

Despite the proteins denaturation, the purification performance of these systems cannot be ruled out, as the denatured proteins may correspond to impurities present in the WSPF. Both phases of the systems were collected and analyzed by SE-HPLC, without removing the precipitate in the interphase which was then discarded, in order to calculate the partition coefficients of the impurities, IgY recovery yield, and purity. The results obtained are shown in **Figure 6** and detailed in Supporting Information (Table S9). The partition coefficient of IgY was not established because a remarkable complete partition (selectivity) of IgY to the PEG-rich phase was observed.

The recovery yield of IgY ranges between 74.9 ± 3.4% and 89.1± 3.4%, with the lower values obtained with the systems composed of K_2HPO_4/ KH_2PO_4 buffer with higher pH values. The lower yield of IgY is due to the lower water concentration in the PEG-rich phase as detailed in experimental data for the TLs in Supporting Information (**Table S8**), leading to a WSPF proteins denaturation.

The values of the partition coefficients of the impurities to the polymer-rich phase tend to increase with the pH increase (**Figure 6**), and thus the selectivity of the systems decreases with an increase in pH. The partition coefficients of the impurities are all higher than 1, meaning that

the impurities are preferentially migrating to the top phase, which explains the low purity of IgY obtained in a single-step with all systems. Overall, the pH value and/or the K_2HPO_4/ KH_2PO_4 buffer used affects the purity of IgY, whereas extractions performed at lower pH values lead to the best results. The extractions with ABS formed with the K_2HPO_4/ KH_2PO_4 buffer at pH 5.5 and 6.0 result in similar purity levels. However, the ABS composed of phosphate buffer at 5.5 lead to some salt precipitation (**Figure 5**), making this system less adequate to work with in CPC. Therefore, the ABS buffered to pH 6.0 was selected to perform the subsequent work.

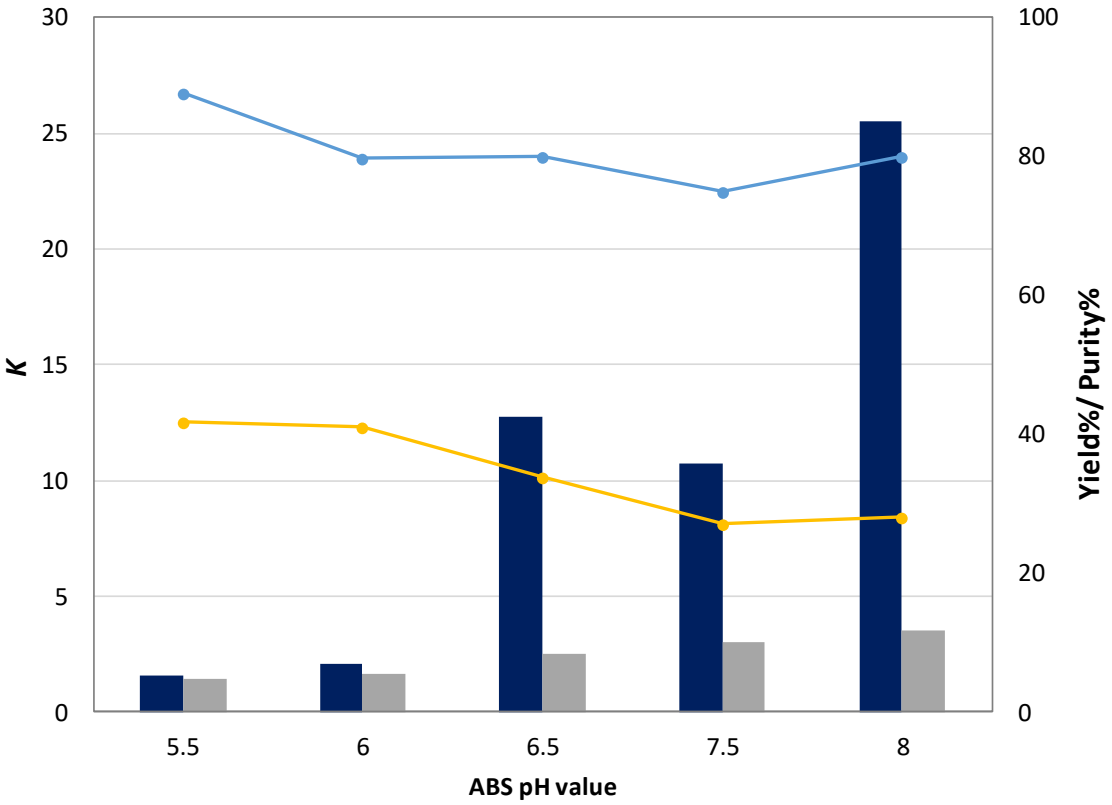

Figure 6. Yield and purity of IgY (light blue and yellow lines, respectively) and partition coefficients of protein impurity 1 (blue bar) and protein impurity 2 (grey bar), in 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer + 69 wt% WSPF systems at (25 ± 1) °C.

Taking into account that the best results are achieved with ABS with pH 6.0, and in order to decrease the protein denaturation, several mixture compositions were evaluated by changing the concentration of PEG 1000 and K_2HPO_4/ KH_2PO_4 buffer at pH 6.0. Three new mixture compositions were tested: 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer; 21 wt% PEG 1000 + 16 wt% K_2HPO_4/ KH_2PO_4 buffer and 21 wt% PEG 1000 + 14 wt% K_2HPO_4/ KH_2PO_4 buffer (**Table 2**). The extraction results, in terms of IgY purity and yield, and partition coefficient of the impurities are reported in **Table 5**.

Comparing the results obtained with the three new mixture compositions with the ABS initially studied, better results have been obtained in conditions that can to be further applied in CPC trials. In terms of purity, no mixture surpassed the ABS initialy studied (18 wt% PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer), with only the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer system achieving comparable results (41.1 ± 0.5% *versus* 39.3 ± 1.0 %). However, since the CPC will be applied to purify IgY, the purity of IgY is not a key-element to choose the ABS for further experiments. Regarding the IgY yield, the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer system clearly performs better. The yield of this system is 100.0 ± 2.4%, meaning not only that this system is partitioning IgY exclusively to its top phase, but that there is also no loss of IgY. In comparison, the first system studied (18 wt% PEG 1000 + 15 wt% K_2HPO_4/ KH_2PO_4 buffer) leads to a yield of IgY 79.8 ± 3.1%. The last parameter that demonstrates the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer system as a more suitable liquid-liquid system for CPC assays is the partition coefficient of the protein impurities. The partition coefficients of the protein impurities are related to the elution time in a chromatographic assay due to their relative affinity between the mobile and stationary phases. Therefore, a higher difference between $K_{impurity1}$ and $K_{impurity2}$ will allow easier separation of the different protein impurities by CPC. In fact, the partition coefficients of both protein impurities, 3.9 ± 0.1 and 1.8 ± 0.4, are more distinct than the those reported for first ABS studied and discussed above (2.0 ± 0.1 and 1.7 ± 0.1). It should be remarked that it was not possible to determine the partition coefficients for protein impurities 1 in the 21 wt% PEG 1000 + 16 wt% K_2HPO_4/ KH_2PO_4 buffer system, and for both protein impurities in the 21 wt% PEG 1000 + 14 wt% K_2HPO_4/ KH_2PO_4 buffer ABS since the protein impurities totally partitioned to the PEG-rich phase. In these systems, there is no selectivity for IgY, with all proteins present in the WSPF partitioning to the same (PEG-rich) phase. In summary, the 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer ABS is the best system to apply in the CPC trials due to the high extraction yield of IgY and the higher difference between the partition coefficients for the protein impurities.

Table 5. Purity and yield of IgY and partition coefficients of each protein impurity, $K_{impurity1}$ and $K_{impurity2}$, in the PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer at pH 6.0 + WSPF systems at (25 ± 1) °C.

Mixture composition	IgY		Protein impurities	
	Purity%	Yield%	$K_{impurity1}$	$K_{impurity2}$
18 wt% PEG 1000 + 15 wt% phosphate buffer	41.1 ± 0.5	79.8 ± 3.1	2.0 ± 0.1	1.7 ± 0.1
18 wt % PEG 1000 + 13 wt% phosphate buffer	39.3 ± 1.0	100.0 ± 2.4	3.9 ± 0.1	1.8 ± 0.4
21 wt% PEG 1000 + 16 wt% phosphate buffer	27.2 ± 0.1	53.8 ± 0.6	n.d.*	8.8 ± 2.7
21 wt% PEG 1000 + 14 wt% phosphate buffer	22.8 ± 2.0	35.9 ± 3.3	n.d.*	n.d.*

Based on the screening on the ABS ability to purify IgY, and after confirming their feasibility for use in CPC, the systems formed by PEG 1000 and K_2HPO_4/ KH_2PO_4 buffer were tested for their capacity to purify IgY from the egg yolk WSPF. Since the feasibility of these ABS involving PEG 1000 in CPC was carried out using an ABS composed of 12.5 wt% of PEG 1000 + 12.5 wt% of K_2HPO_4, as described above, it was confirmed that the behavior of the ABS constituted by 18 wt% of PEG 1000 + 13 wt% of phosphate buffer when it comes to the stationary phase retention is adequate. A process similar to that previously described was carried out regarding the determination of the S_f value for the CPC assay with this system composition. The first purification trial was performed with the injection of a sample with 8 g of WSPF plus 2 g of mobile phase with an ABS composed of 18 wt% of PEG 1000 + 13 wt% of K_2HPO_4/ KH_2PO_4 buffer at pH 6.0 + 69 wt% of WSPF, using the following operating conditions: ascending mode, 2500 rpm at a 2.05 mL/min flow rate. The ascending mode of operation was chosen since IgY partitioned exclusively to the top phase. In this first assay, it was obtained a remarkably low S_f value, 16.7%, lower than the 20% usually required. However, some IgY purification was achieved. As the purity of the end, IgY sample was of 29.5%, possibly due to the low retention of the stationary phase, further optimization of the process was performed. Since a rotation speed of 2500 rpm lead to an insufficient S_f value, an additional assay was performed in the ascending mode using 2000 rpm and a 3.0 mL/min flow rate. This assay provided better results, presenting a higher S_f than the previous, and a higher IgY purity (50.6%). This IgY purity surpassed all extraction assays using the ABS investigated in this work, disclosing the potential of CPC for the purification of biopharmaceuticals.

4. Conclusions

In this work the WSPF from egg yolk was characterized and PEG 1000 + K_2HPO_4/ KH_2PO_4 ABS were investigated as alternative platforms to purify IgY. Six major proteins were identified in the egg yolk WSPF: the target antibody IgY, serum albumin (α-livetin), ovalbumin, ovotransferrin, vitellogenin 1 and vitellogenin 2. The phase diagrams for PEG 1000 + K_2HPO_4/ KH_2PO_4 + water were determined, and the effect of the pH value on the phase diagrams and proteins partitioning was studied. These systems revealed a high affinity of IgY and remaining proteins to the polymer-rich phase, with the best results obtained with the phosphate buffer at a pH of 6.0. Additional studies on the purification of IgY were carried out by the change of the PEG and salt concentrations. The best results in one-step ABS were obtained with the system formed by 18 wt% PEG 1000 + 13 wt% K_2HPO_4/ KH_2PO_4 buffer at pH 6.0. This ABS was finally applied in CPC, and after optimizing several CPC operating conditions, IgY from the egg yolk WSPF with a purity of 51% was obtained.

Acknowledgements

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Purification of IgY from egg yolk by centrifugal partition chromatography

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Supporting Information

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Experimental Section

ABS phase diagrams and tie-lines

The experimental binodal curves were fitted using equation S1 (J Chromatogr B Biomed Sci Appl 1998;711:285–93; https://doi.org/10.1016/S0378-4347(97)00594-X),

$$[\mathrm{PEG}] = A\,exp[(B[\mathrm{salt}]^{0.5}) - (C[\mathrm{salt}]^{3})] \quad \text{(S1)}$$

where [PEG] and [salt] are the PEG and salt weight fractions percentages, respectively, and *A*, *B* and *C* are fitted constants obtained by least-squares regression.

For the determination of the TLs, the following system of four equations (equations S2 to S5) was used to estimate the concentration of IL and salt at each phase ($[\mathrm{PEG}]_{\mathrm{PEG}}$, $[\mathrm{PEG}]_{\mathrm{salt}}$, $[\mathrm{salt}]_{\mathrm{salt}}$ and $[\mathrm{salt}]_{\mathrm{PEG}}$),

$$[\mathrm{PEG}]_{\mathrm{PEG}} = A\,exp[(B\,[\mathrm{salt}]_{\mathrm{PEG}}^{0.5}) - (C\,[\mathrm{salt}]_{\mathrm{PEG}}^{3})] \quad \text{(S2)}$$

$$[\mathrm{PEG}]_{\mathrm{salt}} = A\,exp[(B\,[\mathrm{salt}]_{\mathrm{salt}}^{0.5}) - (C\,[\mathrm{salt}]_{\mathrm{salt}}^{3})] \quad \text{(S3)}$$

$$[\mathrm{PEG}]_{\mathrm{PEG}} = \frac{[\mathrm{PEG}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{PEG}]_{\mathrm{salt}} \quad \text{(S4)}$$

$$[\mathrm{salt}]_{\mathrm{PEG}} = \frac{[\mathrm{salt}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{salt}]_{\mathrm{salt}} \quad \text{(S5)}$$

where the subscripts salt and PEG designate the salt- and PEG-rich phases, respectively, and M is the initial mixture composition. The parameter α is the ratio between the weight of the top phase and the total weight of the mixture. The solution of the described system provides the concentration of the PEG and salt in the top and bottom phases.

In order to calculate each the tie-line length (TLL), equation S6 was applied,

$$\mathrm{TLL} = \sqrt{([\mathrm{salt}]_{\mathrm{PEG}} - [\mathrm{salt}]_{\mathrm{salt}})^2 + ([\mathrm{PEG}]_{\mathrm{PEG}} - [\mathrm{PEG}]_{\mathrm{salt}})^2} \quad \text{(S6)}$$

The correlation parameters of equation 15 and the compositions of the top and bottom phases, were determined using the software Matlab R2015a.

Table S1. Composition of K_2HPO_4/ KH_2PO_4 buffers used at different pH values.

pH	KH_2PO_4(g)	$K_2HPO_4 \cdot 3H_2O$(g)	H_2O (g)
5.5	6.50	3.51	25.04
6.0	5.01	5.01	15.00
6.5	4.01	6.00	15.31
7.5	2.01	8.02	15.00
8.0	1.02	8.99	15.00

Table S2. Ternary mixture composition of ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer for the purification of IgY from the WSPF, and respective TLL.

pH	[PEG]/ wt%	[K_2HPO_4/ KH_2PO_4 buffer]/ wt%	[WSPF]/ wt%	TLL≈
5.5	18.0	15.0	67.0	28.9
6.0	18.0	15.0	67.0	38.3
6.5	18.0	15.0	67.0	38.6
7.5	18.0	15.0	67.0	41.4
8.0	18.0	15.0	67.0	42.3

Table S3. Ternary mixture composition of ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer pH 6.0 for the purification of IgY from the WSPF, and respective TLL.

	[PEG 1000]/ wt%	[K_2HPO_4/ KH_2PO_4 buffer]/ wt%	[WSPF]/ wt%	TLL≈
pH 6.0	18.0	15.0	67.0	42.3
	18.0	13.0	69.0	29.9
	21.0	16.0	63.0	47.6
	21.0	14.0	65.0	42.5

Table S4. Experimental weight fraction data for the systems composed of PEG 1000 (1) + K_2HPO_4/ KH_2PO_4 buffer (2) + H_2O (3) at (25 ± 1) °C.

pH 5.5		**pH 6.0**					
100 w_1	***100 w_2***	**100 w_1**	***100 w_2***	**100 w_1**	***100 w_2***	**100 w_1**	***100 w_2***
55.68	1.68	46.81	2.21	20.49	9.67	7.42	18.28
50.29	2.24	41.98	2.45	20.06	9.95	6.98	18.60
47.0	2.75	40.27	2.82	19.77	10.07	6.28	19.08
42.90	3.17	38.43	3.12	19.50	10.17	6.05	19.40
40.42	3.56	37.50	3.44	19.02	10.34	5.33	19.91
38.15	3.94	36.08	3.84	18.68	10.57	5.01	20.26
36.34	4.21	34.84	4.11	18.36	10.7	4.33	20.94
35.14	4.50	33.56	4.41	18.06	10.94	3.96	21.32
34.02	4.81	32.42	4.73	17.86	11.02	3.51	21.73
32.76	5.21	31.34	4.97	17.57	11.23	3.08	22.12
31.70	5.43	30.68	5.30	17.28	11.41	2.67	22.78
30.77	5.69	29.80	5.48	16.79	11.90	2.23	23.27
29.35	6.29	29.27	5.71	16.00	12.40	1.84	24.28
27.91	7.00	28.71	5.93	15.63	12.72	1.46	25.63
27.23	7.19	28.19	6.17	15.16	12.95		
26.20	7.57	27.43	6.34	14.72	13.28		
25.29	7.98	26.45	6.76	14.48	13.50		
24.34	8.34	25.95	6.96	14.01	13.75		
23.26	8.91	25.52	7.14	13.70	13.94		
22.20	9.42	25.04	7.34	12.94	14.43		
21.13	10.03	24.63	7.52	12.58	14.67		
20.37	10.39	23.82	8.15	12.127	15.00		
19.20	11.08	23.44	8.31	11.78	15.20		
18.14	12.20	23.05	8.48	11.04	15.71		
16.92	12.95	22.70	8.60	10.824	15.87		
14.02	15.08	22.333	8.76	10.04	16.37		
12.61	16.05	21.85	9.07	9.43	16.78		
10.46	17.52	21.52	9.20	8.87	17.17		
		21.18	9.32	8.51	17.48		
		20.79	9.56	7.80	17.86		

Table S5. Experimental weight fraction data for the systems composed of PEG 1000 (1) + K_2HPO_4/ KH_2PO_4 buffer (2) + H_2O (3) at (25 ± 1) °C.

pH 6.5				pH 7.5					
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
57.13	1.58	16.38	11.33	56.14	2.01	22.18	7.648	6.771	15.779
50.93	2.16	15.83	11.63	47.47	2.11	21.88	7.738	6.047	16.258
45.56	2.84	15.47	11.87	44.64	2.32	21.17	7.821	5.648	16.665
40.65	3.24	15.08	12.12	42.74	2.63	20.88	7.98	4.84	17.15
37.55	3.94	14.65	12.36	40.72	3.246	20.41	8.17	4.36	17.62
34.46	4.49	14.24	12.62	37.78	3.35	20.09	8.34	3.92	18.10
32.45	4.98	13.85	12.90	36.05	3.76	19.55	8.56	3.45	18.58
30.59	5.40	13.43	13.20	34.30	3.87	19.25	8.751	2.97	19.11
29.43	5.92	12.97	13.51	33.63	4.09	18.91	8.93	2.44	19.67
27.91	6.29	12.48	13.83	32.66	4.23	18.56	9.11	2.04	21.04
26.55	6.63	12.00	14.15	32.02	4.44	18.14	9.32		
25.70	7.02	11.44	14.50	31.44	4.64	17.73	9.54		
24.88	7.39	10.88	14.88	30.56	4.79	17.34	9.74		
23.87	7.55	10.27	15.29	29.65	5.31	16.94	9.96		
23.15	7.88	9.67	15.70	28.83	5.42	16.49	10.20		
22.45	8.21	9.29	16.00	28.02	5.63	15.95	10.47		
21.80	8.48	8.57	16.41	27.58	5.80	15.50	10.71		
21.18	8.81	7.83	16.88	27.12	5.95	15.16	10.94		
20.59	9.09	7.45	17.22	26.64	6.16	14.62	11.22		
20.00	9.23	6.66	17.77	26.193	6.29	14.03	11.53		
19.99	9.40	6.26	18.13	25.76	6.41	13.38	11.85		
19.63	9.43	5.90	18.48	25.36	6.54	13.01	12.12		
19.21	9.65	4.95	19.09	24.98	6.65	12.57	12.41		
18.95	9.79	4.487	19.50	24.61	6.76	11.78	12.83		
18.55	10.01	4.03	19.94	24.29	6.86	11.00	13.25		
18.29	10.20	3.58	20.68	23.89	6.97	10.47	13.60		
18.01	10.38	3.13	21.12	23.40	7.25	9.85	13.96		
17.49	10.64	2.59	21.94	23.09	7.36	9.23	14.33		
17.07	10.90	2.01	23.27	22.77	7.47	8.28	14.86		
16.77	11.111	1.52	25.18	22.46	7.56	7.56	15.29		

Table S6. Experimental weight fraction data for the systems composed of PEG 1000 (1) + K_2HPO_4/ KH_2PO_4 buffer (2) + H_2O (3) at (25 ± 1) °C.

pH 8.0					
100 w_1	***100 w_2***	**100 w_1**	***100 w_2***	**100 w_1**	***100 w_2***
56.88	1.65	19.41	8.51	11.47	12.90
48.42	2.20	19.11	8.65	11.29	13.02
43.18	2.60	18.64	8.94	11.17	13.06
39.84	3.03	18.36	9.03	11.00	13.18
37.72	3.44	18.08	9.11	10.87	13.20
35.36	3.82	17.82	9.19	10.71	13.30
33.29	4.046	17.42	9.46	10.70	13.41
32.35	4.40	17.15	9.546	10.37	13.61
31.37	4.76	16.90	9.66	9.50	13.97
30.22	4.92	16.57	9.87	9.05	14.206
29.43	5.20	16.32	9.96	8.70	14.55
28.705	5.47	16.05	10.08	8.27	14.80
27.68	5.65	15.72	10.27	7.78	15.08
26.99	5.91	15.50	10.34	7.32	15.39
26.32	6.11	15.19	10.52	6.81	15.68
25.71	6.33	14.98	10.59	6.33	16.00
24.91	6.41	14.67	10.78	5.79	16.33
24.37	6.55	14.48	10.82	5.40	16.77
23.92	6.725	14.204	11.01	4.79	17.19
23.46	6.90	13.79	11.41	4.28	17.55
22.97	7.08	13.53	11.58	3.38	18.29
22.57	7.23	13.37	11.63	2.77	18.823
22.15	7.42	13.15	11.76	2.12	19.80
21.75	7.59	12.92	11.89		
21.37	7.74	12.75	11.94		
21.03	7.90	12.61	12.00		
20.65	8.04	12.38	12.17		
20.35	8.13	12.25	12.20		
20.00	8.27	12.06	12.32		
19.69	8.41	11.60	12.88		

Table S7. Correlation parameters and respective standard deviations (σ) of equation 15 used to describe the experimental binodal data of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer.

pH	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
5.5	99.2 ± 4.0	-0.49 ± 0.02	2.7 ± 2.0	0.993
6.0	81.3 ± 2.4	-0.40 ± 0.01	10.2 ± 0.8	0.998
6.5	110.4 ± 2.7	-0.54 ± 0.01	9.01 ± 0.7	0.998
7.5	109.5 ± 6.0	-0.56 ± 0.03	12.5 ± 2.5	0.992
8.0	113.5 ± 3.8	-0.58 ± 0.02	11.8 ± 1.5	0.996

Table S8. Experimental TLs and TLLs of the ABS composed of PEG 1000 + K_2HPO_4/ KH_2PO_4 buffer + H_2O at (25 ± 1) °C.

pH	Weight fraction composition / wt%						
	$[PEG]_{PEG}$	$[buffer]_{PEG}$	$[PEG]_M$	$[buffer]_M$	$[PEG]_{buffer}$	$[buffer]_{buffer}$	TLL
5.5	27.50	6.89	17.92	14.98	5.39	25.57	28.94
	38.46	3.80	24.63	15.06	2.18	33.35	46.80
6.0	33.74	4.43	18.10	14.79	1.82	25.56	38.29
	39.99	2.92	24.82	15.00	0.10	34.69	50.99
6.5	34.27	4.70	18.01	15.03	1.70	25.40	38.59
	42.25	3.20	24.86	14.91	0.34	31.42	50.52
7.5	36.12	3.85	17.97	14.96	0.82	25.47	41.39
	43.75	2.66	25.05	14.98	0.10	21.42	52.28
8.0	37.44	3.60	18.32	14.85	0.95	25.07	42.33
	43.29	2.74	24.98	15.00	0.10	31.69	51.99

Table S9. Purity and yield of IgY and partition coefficients of each contaminant protein, $K_{impurity1}$ and $K_{impurity2}$, in the 18 wt% PEG 1000 + 15 wt% K_2HPO_4/ KH_2PO_4 buffer + 69 wt% WSPF systems at (25 ± 1) °C.

pH	IgY		Impurities	
	Purity%	Yield%	$K_{impurity1}$	$K_{impurityt2}$
5.5	41.8 ± 0.1	89.1 ± 3.4	1.6 ± 0.1	1.4 ± 0.1
6.0	41.1 ± 0.5	79.8 ± 3.1	2.0 ± 0.1	1.7 ± 0.1
6.5	33.9 ± 3.6	79.9 ± 0.7	12.7 ± 3.8	2.5 ± 0.9
7.5	27.2 ± 4.0	74.9 ± 3.4	10.8 ± 3.1	3.0 ± 0.7
8.0	28.1 ± 1.6	79.9 ± 5.6	25.5 ± 2.4	3.5 ± 0.6