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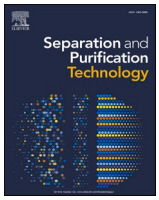

Purification of immunoglobulin Y from egg yolk using thermoresponsive aqueous micellar two-phase systems comprising ionic liquids

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ABSTRACT

Immunoglobulin Y (IgY) represents an important class of antibodies, being present in egg yolk, with relevant medical applications and involving non-invasive methods of extraction. However, due to the complexity of egg yolk, the purification levels required for use in most medical applications demands the application of multi-step and cost-intensive techniques. Therefore, it is of upmost importance to develop a biocompatible and cost-effective downstream process to purify IgY from egg yolk. In this work, IgY was purified from the egg yolk water-soluble protein fraction (WSPF) by the application of thermoresponsive aqueous micellar two-phase systems (AMTPS) composed of the non-ionic surfactant Triton X-114 and surface-active ionic liquids (SAILs) as co-surfactants. The best thermoresponsive systems allowed, in a single step, to recover IgY in the surfactant-poor phase with a purity level of 69%, and up to 73% if consecutive extraction cycles are performed, while maintaining the structural integrity of the antibodies. Considering these results, a downstream process was designed and proposed, consisting of four main steps: (i) recovery of the WSPF; (ii) purification of IgY by applying AMTPS; (iii) isolation of the IgY from the main solvents using an ultrafiltration step; (iv) recycling of the AMTPS phase-forming components by precipitating the contaminant proteins.

1. Introduction

In the last few years, antibody-based therapeutics have attracted significant attention from pharmaceutical industries, leading to considerable research efforts in this field. Several antibody-based therapies have been developed to treat different disorders and/or diseases, such as cancer [1,2], transplant rejection [3], auto-immune disorders [4], asthma [5], infectious diseases [6], among others. However, any new compound within the medical field requires high purity levels and must undergo several steps and clinical trials up to be approved by the regulatory agencies, such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Polyclonal immunoglobulin G (IgG) obtained from human serum is currently used as IgIV (immunoglobulin intravenous) solutions to treat several disorders and immunodeficiency [7,8]. Nevertheless, the extraction of IgG from mammal serum resorts to an invasive technique, while being of more limited access. On the other hand, monoclonal IgG antibodies can be obtained through mammalian cell cultures and recombinant technology, requiring the transfection of cells and culture media [9].

As a substitute of mammalian antibodies, immunoglobulin Y (IgY) that can be obtained from hens' egg yolk emerges as a promising alternative [10]. IgY has a similar structure to IgG and does not require an invasive extraction technique, since it is obtained from the egg yolk. Furthermore, the quantities present in egg yolk are significantly higher (100–150 mg.egg^{-1}) when compared with the amount of IgG present in blood serum (100–150 mg in 20–30 mL of blood) [11]. IgY also displays phylogenetic distance from mammals, allowing the production of antibodies against highly conserved mammalian proteins. Additionally, the use of hens as the host for IgY production has lower costs when compared to the use of mammals [12]. Based on these advantages, IgY has raised considerable attention, reaching a market value of 4 million USD in 2020 (estimation carried before COVID-19 pandemic) and it is expected to reach 9 million USD by 2024 [13]. Nonetheless, it should be

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stressed that this value is also dependent on the IgY costs, which are in turn dependent on its purification steps. The current techniques being applied for the IgY isolation from egg's yolk are quite expensive due to be multistep processes [14], and involve precipitation [15], liquid–liquid extraction with chloroform [16], chromatography [17] and/or filtration [18]. Recently, the use of aqueous biphasic systems (ABS) has been proposed to extract IgY from egg yolk, however with no purity levels being reported [19].

Among liquid–liquid extraction techniques, the use of thermoresponsive aqueous micellar two-phase systems (AMTPS) appears to be an attractive approach since these comprise large amounts of water, higher than in ABS, thus being able to maintain the native conformation and biological activities of biomolecules if properly designed, including proteins [20–22] and antibodies [23]. In addition, these systems only require a surfactant, water and temperature to be formed. Upon phase separation, two distinct environments are created with different polarities, namely a hydrophobic surfactant-rich phase and a hydrophilic surfactant-poor phase [21,24]. The different characteristics of the phases formed are also pointed out as advantageous features, especially when working with real matrices. Some interesting developments were recently carried out by combining AMTPS comprising non-ionic surfactants and surface-active ionic liquids (SAILs) as co-surfactants to improve the extractive performance and selectivity of these processes [21,22], features achieved by the tailoring ability of ILs. Thereby, this work aims to develop a sustainable purification and concentration process, efficient for the recovery of IgY from the water-soluble protein fraction (WSPF) of the egg yolk using thermoresponsive AMTPS involving the non-ionic surfactant Triton X-114 and SAILs belonging to the imidazolium and phosphonium families. Studies were also undertaken to evaluate the structural stability of the antibody before and after extraction using AMTPS through Circular Dichroism (CD) and Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR). Considering the relevant role of the proposed process for the purification of IgY, the final downstream process was considered and is here proposed, consisting of four main steps: (i) recovery of the WSPF; (ii) purification of IgY by applying AMTPS; (iii) isolation of the IgY from the main solvents using an ultrafiltration step; (iv) recycling of the AMTPS phase-forming components by precipitating the contaminant proteins.

2. Experimental section

2.1. Materials

All reagents used in this work, namely the surfactant, the different SAILs (chemical structures given in Fig. 1) and the buffers' components are described in Table 1, in addition to their manufacturer and purity. The electrophoresis gel used in the SDS-PAGE analysis was purchased from Amersham, while the remaining chemicals used are presented also in Table 1.

Table 1
List of compounds employed with their respective manufacturer and purity.

Compound	Manufacturer	Purity (%)
Triton X-114	Acros Organics	–
1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl	Iolitec	>98.0
1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl		
1-tetradecyl-3-methylimidazolium chloride, [C_{14}mim]Cl		
1-hexadecyl-3-methylimidazolium chloride, [C_{16}mim]Cl		
1-octadecyl-3-methylimidazolium chloride, [C_{18}mim]Cl		
trihexyltetradecylphosphonium chloride, [$P_{6,6,6,14}$]Cl	Cytec	>93.0
trihexyltetradecylphosphonium bromide, [$P_{6,6,6,14}$]Br		>96.0
trihexyltetradecylphosphonium decanoate, [$P_{6,6,6,14}$][Dec]		>97.0
trihexyltetradecylphosphonium bis (2,4,4-trimethyl (pentyl)phosphinate, [$P_{6,6,6,14}$][TMPP]		>93.0
Citric acid, $C_6H_8O_7$	Panreac	99.5
Sodium phosphate dibasic, Na_2HPO_4	Merck	99.0
Disodium hydrogen phosphate heptahydrate, $Na_2HPO_4{\cdot}7H_2O$	Panreac	>98
Sodium dihydrogen phosphate, NaH_2PO_4		
NaCl		
Coomassie Blue (G-250)	AMRESCO	–
Methanol	Fisherchem	–
Ethanol		–
Bromophenol Blue	Merck	–
Tris base	Pronolab	–
Glycine	Acros Organics	99
Glycerol	Sigma Aldrich	–
Dithiothreitol	Acros Organics	–
Sodium dodecyl sulphate (SDS)	Acros Organics	99

2.2. Methods

2.2.1. Water soluble protein fraction (WSPF) preparation

The experimental protocol reported by Liu *et al.* [25] was used to obtain the IgY-rich WSPF from the chicken's egg yolk. Briefly, egg yolk was carefully separated from the egg white and thoroughly washed with distilled water to avoid contamination with egg white proteins. Afterwards, the yolk membrane was removed and the yolk contents collected and diluted 1:6 (v:v) with distilled water. The pH of the solution was adjusted to 5.0–5.2 with HCl and mixed for 15 min at 4 °C. After freezing at − 20 °C (8 h) and thawing at 4 °C, the sample was centrifuged at

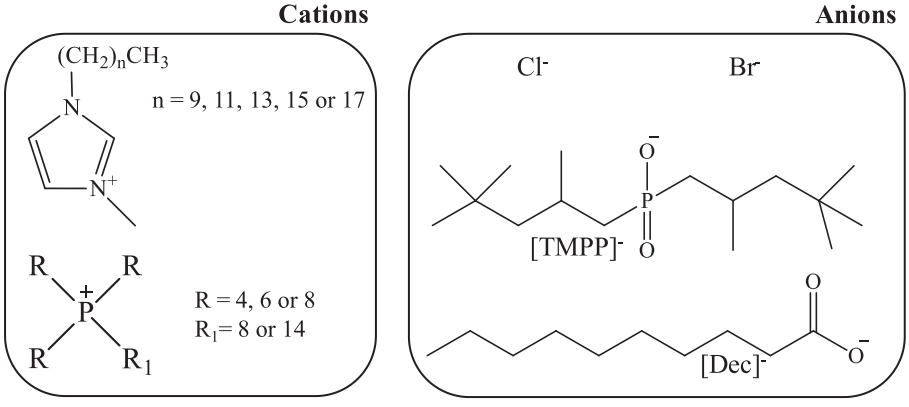

Fig. 1. Chemical structure of the anions and cations composing the SAILs investigated as co-surfactants.

6000g for 20 min (4 °C). The supernatant was collected, being from now on designated as the WSPF. In this process, lipoproteins are mainly removed, leaving only water-soluble proteins, including IgY, in the WSPF.

2.2.2. Preparation of the AMTPS for the IgY purification

Optimization studies of the surfactant and WSPF concentrations were performed using conventional AMTPS (without any SAIL). The surfactant concentration studies were accomplished by weighting all the system components into a falcon tube: 1, 5, 10, 15 or 20 wt% of Triton X-114 + 10 wt% of WSPF + McIlvaine buffer (0.16 M) pH 6.0 to complete a final volume of 10 mL. Then, AMTPS were homogenized at 4 °C in a freezer for at least 2 h, using a rotor apparatus (Stuart SB3) at 35 rpm. Afterwards, the systems were left overnight at 35 °C in a Venticell incubator to reach equilibrium, thus guaranteeing the complete phase separation and IgY partitioning between the coexisting phases. The result was the formation of a surfactant-rich and a surfactant-poor phases, corresponding to the bottom and top phases, respectively. These were carefully separated, and their volumes and weights determined. After the proper optimization of the surfactant concentration, this procedure was repeated for the WSPF concentration study using 20 wt% of Triton X-114 + 10, 17.5 and 25 wt% of WSPF + McIlvaine buffer (pH 6.0) to complete the final volume of 10 mL. Finally, the optimized system was carried out to study the effect of the SAIL addition as co-surfactant (chemical structures of SAILs investigated given in Fig. 1) to the AMTPS: 20 wt% of Triton X-114 + 0.3 or 0.5 wt% of SAIL + 25 wt % of WSPF + McIlvaine buffer pH 6.0 to complete the final volume of 10 mL. All studies were performed in triplicate and the respective average and standard deviations determined.

When working with mixed AMTPS, *i.e.* involving the non-ionic surfactant and the SAIL, an interval of temperatures (35 – 50 °C) was studied due to the cloud point variations imposed by the SAILs addition. For SAILs belonging to the imidazolium family, the phase separation occurred at 37 °C and higher temperatures, whereas the IgY extractions using the phosphonium-based SAILs were performed at 35 °C and 37 °C. These temperatures were chosen considering the phase diagrams previously determined [21].

2.2.3. AMTPS preparation for the consecutive IgY purification cycles

For the three consecutive extraction cycles, only the top/surfactant-poor phase (in which IgY is recovered) of the optimized mixed AMTPS was applied. The new AMTPS composition was reconstituted with 25 wt % of the AMTPS top phase, 20 wt% of Triton X-114, 0.3 wt% of SAIL and the appropriate amount of McIlvaine buffer at pH 6.0 to obtain the same concentration of the original AMTPS components, from where the top phase was recovered. An identical cycle was attempted towards the surfactant-rich phase, in which 20.3 wt% of this phase was added to a new/fresh WSPF to obtain the second AMTPS.

2.2.4. Protein quantification by SE-HPLC analysis

Proteins in both the WSPF and the surfactant-poor phase of each AMTPS were quantified through SE-HPLC with a size exclusion column Shodex Protein KW-802.5 (8 mm × 300 mm). Initially, the surfactant-poor phase was diluted (1:10) in 100 mM of sodium phosphate buffer + NaCl at 0.3 M and pH 7.0 (mobile phase), injected into the HPLC and run isocratically with a flow rate of 0.5 mL.min^{-1} at 40 °C. The injection volume was 25 μL and the wavelength was set at 280 nm using a diode array detector (DAD). The IgY quantification was measured through an external standard calibration method prepared in the range from 0.1 to 1.0 g.L^{-1}. The chromatograms acquired from the HPLC were used for the determination of the IgY purity, purification factor (PF) and yield, using **Eqs. (1)–(3)**:

$$\text{IgY purity } (\%) = \frac{IgY \text{ area}}{(Other\ Proteins + IgY) \text{ area}} \times 100 \quad (1)$$

$$PF_{IgY} = \frac{IgY \quad purity_{surfactant-poor \quad phase} \ (\%)}{IgY \quad purity_{WSPF} \ (\%)} \quad (2)$$

$$\text{IgY yield}(\%) = \frac{IgY \ \ weight_{surfactant-poor \ \ phase}}{IgY \ \ weight_{initial}} \times 100 \quad (3)$$

where the *IgY* and *other proteins areas* correspond to the SE-HPLC peak area of IgY and other proteins, respectively, being determined using Peak fit. *IgY purity$_{surfactant\text{-}poor\ phase}$ (%)* and *IgY purity$_{WSPF}$ (%)* correspond to the IgY purity in the surfactant-poor phase and in the WSPF, respectively. *IgY weight$_{surfactant\text{-}poor\ phase}$* represents the concentration of IgY in the surfactant-poor phase times the volume of the surfactant-poor phase, and the *IgY weight$_{initial}$* corresponds to the IgY concentration in the WSPF times the volume of the WSPF added.

2.2.5. SDS-PAGE analysis

The AMTPS surfactant-rich phase was not possible to analyze through SE-HPLC due to the surfactant interference with the column; instead, this phase was analyzed by SDS-PAGE. The SDS-PAGE proteins profile results allow to demonstrate the presence of IgY and other proteins present in the surfactant-rich phase. Firstly, all proteins found in the AMTPS were precipitated to remove the phase-forming components since they interfere with the electrophoresis running. To this end, 1000 μL of acetone/methanol (8:1) were added to 100 μL of sample from each phase [26]. The precipitated proteins were resuspended in 100 μL of McIlvaine buffer (0.16 M) at pH 6.0. Afterwards, all samples were diluted in water so that the amount of protein in each lane was around 0.5 μg. The samples were then diluted in a running buffer followed by a 5 min incubation at 95 °C. The samples and the full-range Amersham rainbow marker (12 to 225 kDa) were loaded into the gel (stacking and resolving gels at 4 and 20%, respectively) and submitted to a run during 90 min at 135 V. Then, the gel was stained with Coomassie Blue G-250 (50 wt% of methanol, 0.1 wt% of coomassie and 7 wt% of acetic acid).

2.2.6. IgY stability studies

To study the effect of the surfactant, as well as the SAIL (as co-surfactant) in the IgY stability after the extraction step, the best AMTPS towards the IgY purification was analyzed by ATR-FTIR and CD. IgY purified from egg yolk by a Pierce Chicken IgY Purification Kit from Thermo Scientific was used. For all techniques, only the top/surfactant-poor phases were analyzed since IgY migrates preferentially towards this phase. Conventional AMTPS and SAIL-based systems were prepared with pure IgY to assure that any variations in the molecular structure were only associated with the antibody and not with the remaining proteins.

The ATR-FTIR analyses were performed in a Perkin Elmer Spectrum Bx spectrophotometer and scanned between 2000 and 1000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans. These conditions were chosen since this is the most important interval when studying proteins owing to the localization of the amides I and II regions for the protein secondary structure determination [27]. Background of McIlvaine buffer (0.16 M, pH 6.0) and each AMTPS blank spectra were subtracted from all samples prior to data analysis to eliminate their interference from the samples containing pure IgY.

Changes in the secondary structure of IgY in presence of AMTPS and SAILs as co-surfactants were addressed by CD spectroscopy (JASCO-1500). Similar to FTIR, the surfactant-poor phase was chosen for CD analysis. For CD analysis, an initial concentration of 1 mg.mL^{-1} of pure IgY in McIlvaine buffer (pH 6.0) was added on the preparation of the AMTPS, and then, the surfactant-poor phase was separated for CD analysis. Pure IgY (1 mg.mL^{-1}) in McIlvaine buffer (pH 6.0) was used as control to compare the secondary structure variations. Spectra were collected in a 1 mm path length quartz cuvette at a scan rate of 100 nm *per* minute at 20 °C. The response time and the bandwidth were 2 s and 0.2 nm, respectively. Three spectra were recorded consecutively to give

single average data. This technique allows to monitor the IgY degradation in presence of the AMTPS components.

3. Results and discussion

The main objective of this work is the development of an efficient downstream process to purify IgY from egg yolk. Aiming at developing an efficient purification strategy, the WSPF was prepared according to the procedure described by Liu *et al.* [25], and different AMTPS comprising Triton X-114 were investigated, considering both the presence and absence of SAILs as co-surfactants, the former forming mixed AMTPS with the WSPF. The main AMTPS process conditions were first optimized, followed by the design of the final process to separate IgY from the remaining proteins present in the WSPF.

3.1. IgY purification from the WSPF using conventional AMTPS

AMTPS offer two distinct environments, namely a hydrophobic surfactant-rich phase and a hydrophilic surfactant-poor phase upon phase separation by temperature increase. The studied conventional AMTPS comprise the non-ionic surfactant Triton X-114, whose phase diagrams were taken from the literature [21]. Based on their lower-critical solution temperature (LCST) behavior, this work took advantage of their thermoresponsive nature to phase separate and purify IgY from the WSPF. Firstly, a pure IgY sample solution obtained from a commercially acquired kit was run through the SE-HPLC alongside the WSPF sample to evaluate their proteins profile, whose results are shown in Fig. 2. The WSPF corresponding chromatogram displays five peaks, where the first peak (peak 1) corresponds to IgY with a retention time of 15 min, being this the protein with a higher molecular weight in solution. Peaks 2 and 3 correspond to other highly abundant water-soluble proteins present in the WSPF, with retention times of 16 and 17 min, respectively, while peaks 4 and 5 correspond to the remaining and less abundant contaminants in the WSPF, displaying retention times of 27 and 37 min, respectively. These results demonstrate the high number and content of other water-soluble proteins present in the WSPF in addition to IgY, and thus the difficulty in purifying this antibody. According to these data, the IgY purity in the WSPF is approximately 35%. The less intense peak appearing before IgY corresponds to IgY and other proteins aggregates, being more evident in the pure IgY obtained with the commercial kit.

After the proper characterization of the WSPF, an optimization study using the conventional AMTPS was performed to select the best conditions of the surfactant Triton X-114 (1, 5, 10, 15 and 20 wt%) and the WSPF (10, 17.5 and 25 wt%) concentrations. The SE-HPLC chromatograms obtained are provided in **Figure S1** in the Supporting Information, while the PF_{IgY} and extraction yield results are depicted in Fig. 3. Overall, IgY preferentially partitions to the surfactant-poor phase, whereas some of the remaining proteins have migrated towards the opposite phase owing to their different sizes and highest hydrophobic nature, hence allowing to improve the IgY purity.

From the analysis of the SE-HPLC chromatograms, it can be seen that

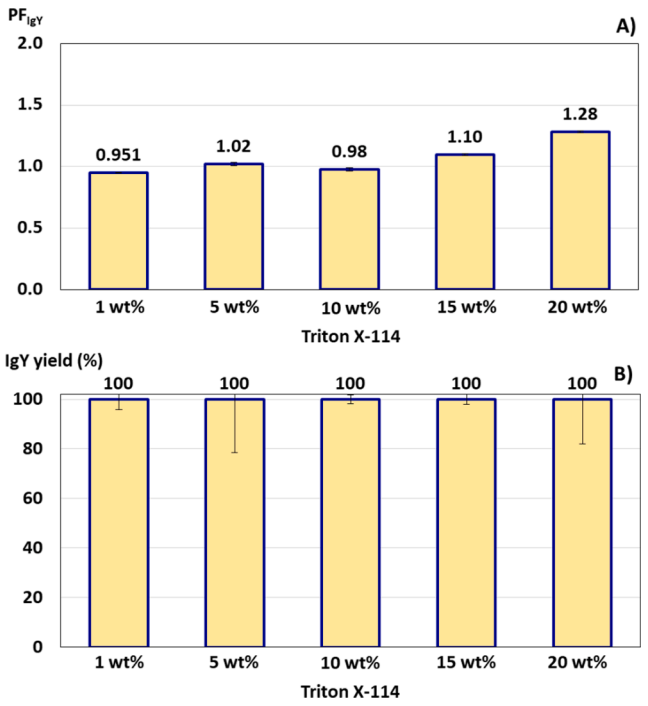

Fig. 3. PF_{IgY} (**A**) and yield (**B**, %) obtained for each AMTPS with different concentrations of Triton X-114 at the surfactant-poor phase upon phase separation at 35.0 ± 0.1 °C.

at 20 wt% of Triton X-114, an inversion in the peaks intensity is observed (**Figure S1** in the Supporting Information), leading to an increase in IgY purity. These chromatograms also show the appearance of another peak at 23 min not visible before in the WSPF, corresponding to the residual presence of the phase-forming components and as proved with control systems with no proteins added. The results provided in Fig. 3**.A)** demonstrate that, when the surfactant concentration is increased, the PF_{IgY} is improved from 0.951 ± 0.002 to 1.28 ± 0.01, a profile which is followed by the complete recovery of IgY into the surfactant-poor (top) phase. This effect seems to be related with the increase of the micelles size, *i.e.* with larger diameters and different shapes [28], as it well-known that nonionic surfactants form spherical aggregates in aqueous solutions above the critical micelle concentration (CMC) but, under certain conditions, such as the surfactant concentration and/or temperature, these spherical micelles grow in size and/or change their shape resulting in the formation of rodlike structures or even long flexible micelles [29]. Thus, when the surfactant concentration is increased, the micelles have a higher capacity to concentrate other proteins inside them. Contrarily to what happens with the remaining proteins in the WSPF, the IgY does not prefer the surfactant-rich phase, which may be due to its higher molecular weight and higher hydrophilicity. Both phenomena, *i.e.* micelles size and IgY *versus* other proteins' hydrophobicity, may contribute to the IgY purification factor

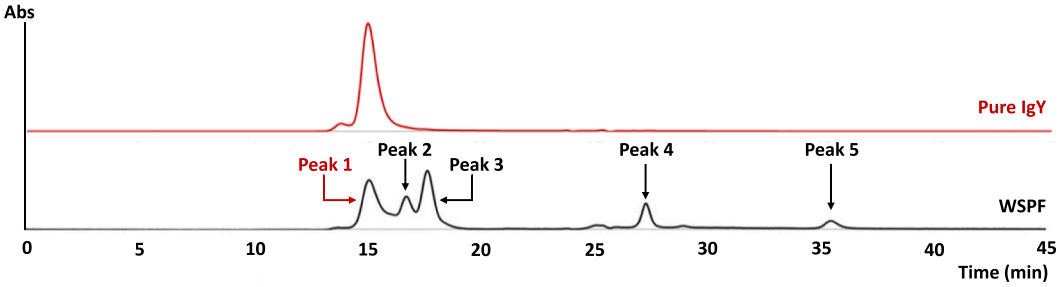

Fig. 2. Chromatograms of pure IgY and the WSPF used in this work.

increase in the surfactant-poor phase.

As a second optimization step, the WSPF concentration was studied, keeping constant the surfactant concentration at 20 wt% and varying the WSPF content (10.0, 17.5 and 25.0 wt%) in the AMTPS. The respective PF_{IgY} and extraction yield results are presented in Fig. 4. Overall, the increment in the WSPF concentration leads to an increase in the IgY purification factor (Fig. 4.**A**), meaning that more IgY is being retained in the surfactant-poor phase when compared to the remaining proteins. These results also suggest that none of the AMTPS phase has reached saturation. This set of studies allowed a PF_{IgY} increase from 1.28 ± 0.01 to 1.48 ± 0.02, with 25 wt% of WSPF, without any IgY loss as can be seen through the IgY yield given in Fig. 4.**B)**.

3.2. IgY purification from the WSPF using SAIL-based thermoresponsive AMTPS

After the optimization of both the amount of surfactant and WSPF, SAILs were investigated as co-surfactants in mixed AMTPS with the goal of designing a more efficient AMTPS for the IgY purification from the WSPF, while taking advantage of their thermoresponsive nature. Several imidazolium- and phosphonium-based ILs were investigated. They were selected due to their well-recognized enhanced extractive performance and selectivity provided by their tailoring ability [21,22]. Both classes of cations with different alkyl side chain length paired with distinct anions, as well as the SAILs' concentration, were studied. In particular, the alkyl side chain length effect was evaluated first with the imidazolium family of ILs, [$C_n$mim]Cl, with *n* varying between 10 and 18. On the other hand, the anion influence was studied using the phosphonium family, with the fixed cation $[P_{6,6,6,14}]^+$, while using Cl^-, Br^-, $[Dec]^-$ and $[TMPP]^-$ as counterions. This family was also used to investigate the cations' symmetry and the alkyl side chain by comparing $[P_{6,6,6,14}]Br$ with $[P_{8,8,8,8}]Br$ and $[P_{6,6,6,14}]Cl$ with $[P_{4,4,4,14}]Cl$. Overall, all the selected ILs have a surface-active behavior with capability to form micelles in aqueous solutions, and with previously determined CMCs [30]. The results corresponding to the imidazolium-based ILs are given in Fig. 5. As previously mentioned, the extraction temperatures were carefully selected according to the cloud points determined elsewhere [21].

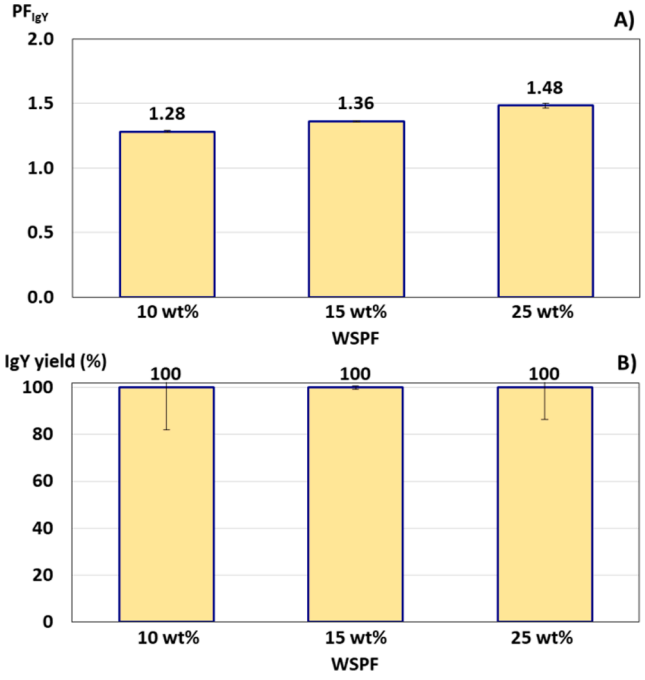

Fig. 4. PF_{IgY} (**A**) and yield (**B**, %) obtained for each system with different amounts of WSPF and 20 wt% of Triton X-114, upon phase separation at 35.0 ± 0.1 °C.

The results displayed in Fig. 5 suggest that the IgY purification can be enhanced by the proper choice of the imidazolium-based SAIL added to the AMTPS and respective concentration, being [C_{18}mim]Cl and [C_{12}mim]Cl, respectively, the best and the worst SAILs, both compared at 0.5 wt%. The SAILs addition as co-surfactants confers the mixed micelles different properties regarding charge (when compared with the common AMTPS), diameter and shape [30–34]. The obtained results can be explained by the formation of mixed micelles, with the SAIL monomers being incorporated in the micelle alongside the nonionic surfactant ones. Here, the best results were achieved with SAILs with longer alkyl side chain length and lower CMC. It is important to notice that the results for the imidazolium-based SAILs were not studied at the same temperature due to their cloud point variation. When the SAIL's alkyl side chain increases, so does the system hydrophobicity and thus, the temperature required to promote the phase separation is lower. It is then required to have this is mind when comparing these results, since these are thermoresponsive systems. For this reason, it was also impossible to have phase separation with 0.5 wt% of [C_{10}mim]Cl considering the absence of a phase separation until 50 °C.

It should be stressed that variations in the temperature can lead to changes in the CMC. As the temperature increases, the hydration shell of the imidazolium head decreases, leading to an increased hydrophobicity of the overall system and favoring solute–solute interactions [35,36]. This hydrophobicity increment can affect the proteins partition and incorporation into the micelle core and, as a consequence, the purification factor results. In this case, a temperature increment leads to a decrease in the IgY purification factor (Fig. 5), which is not associated to the potential denaturation of the antibody since this is stable up to 70 °C [37]. This can be further corroborated by the IgY yield results, as these show that the best yields (100% for [C_{10}mim]Cl and [C_{12}mim]Cl) are obtained at the highest temperature tested, namely 50 °C. This set of results suggests that the characteristics of the mixed micelles might play an important role upon the IgY purification.

By comparing the data obtained for [C_{16}mim]Cl and [C_{18}mim]Cl, accomplished at the same temperature (Fig. 5), it is clear that the elongation of the SAIL's alkyl side chain increases the partition of the other water-soluble proteins towards the surfactant-rich phase, representing a boost in the antibody purity in the opposite phase. This effect is even more pronounced when the SAIL concentration increases from 0.3 to 0.5 wt%, reaching a maximum PF_{IgY} of 2.09 ± 0.03. In summary, the best scenario corresponds to a PF_{IgY} increase from 1.48 ± 0.04 (AMTPS without SAIL at 37 °C, which corresponds to an IgY purity of 51 ± 1%) to 2.09 ± 0.03 (mixed AMTPS with 0.5 wt% of [C_{18}mim]Cl at 37 °C, corresponding to an IgY purity of 69 ± 1%). However, despite the highest purity found in the latest system, a considerably IgY loss (IgY yield of 31 ± 3%) was observed. In this sense, a compromise should be taken and 0.3 wt% of [C_{14}mim]Cl should be considered as a more promising system than 0.5 wt% of [C_{18}mim]Cl, since the first still led to a considerable purification (PF_{IgY} = 1.78 ± 0.03, IgY purity = 62 ± 1 %) while displaying a higher IgY yield (~57 %).

The results corresponding to the addition of phosphonium-based ILs as SAILs are given in Fig. 6. With the exception of $[P_{4,4,4,14}]Cl$ (at 37 °C) and $[P_{6,6,6,14}][TMPP]$ (at 35 °C), the addition of these ILs to AMTPS as co-surfactants reduces the IgY purification factor when compared with the results obtained for the system without SAIL and with the imidazolium-based SAILs. This effect could be related with the high hydrophobic nature of these SAILs, which may create difficulties in the extraction of the remaining water-soluble contaminant proteins. As aforementioned, these systems allow the purification of biomolecules through the different size and hydrophilic/hydrophobic character of the target compound, *i.e.* smaller and hydrophobic molecules tend to stay in the micelle core, whereas bigger and hydrophilic molecules prefer the water-rich pool. These results show that the extractive performance of the SAIL-based thermoresponsive systems depends on the target biomolecule to be extracted and properties of the micelles. For example, the phosphonium-based AMTPS have proven to be considerably more

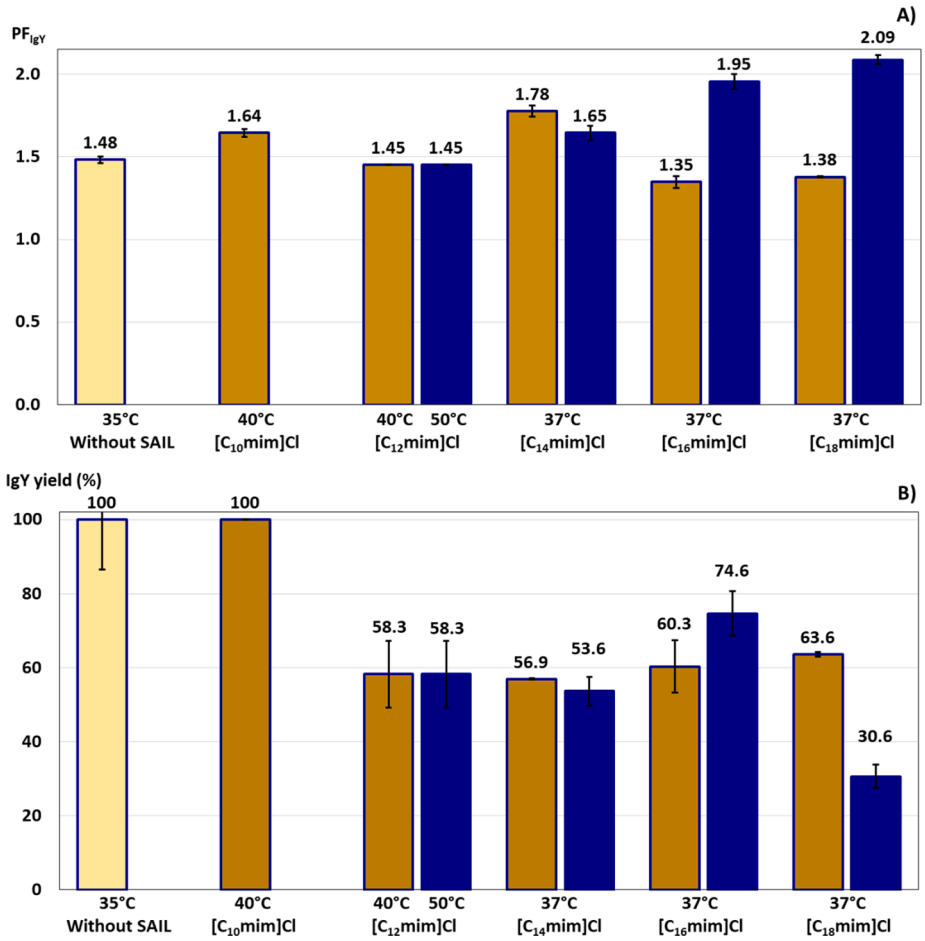

Fig. 5. PF_{IgY} (**A**) and yield (**B**, %) of IgY in the distinct imidazolium-based AMTPS in comparison with the AMTPS without SAIL. The SAIL concentration (wt%) effect is also shown for two concentrations, namely 0.3 (bar without pattern) and 0.5 wt% (bar with pattern).

selective than the imidazolium-based counterparts in the purification of bromelain from pineapple stem [22].

Overall, when the cation effect is concerned, the achieved results at 37 °C show that the IgY purification factor follows the increasing tendency: $[P_{8,8,8,8}]^+ < [P_{6,6,6,14}]^+ < [P_{4,4,4,14}]^+$, whereas the IgY yield presents a decreasing trend in the following order: $[P_{6,6,6,14}]^+ < [P_{8,8,8,8}]^+ < [P_{4,4,4,14}]^+$. With this family it is also proven that the temperature increase leads to a decrease in the IgY purification. On the other hand, when considering the counterion effect with the fixed $[P_{6,6,6,14}]^+$, at both concentrations (0.3 and 0.5 wt%), it is shown that the anion effect is not significant for the IgY purification and neither is the SAIL concentration. In summary, phosphonium-based SAILs are less effective for the IgY purification from the WSPF when compared with AMTPS in absence of SAILs or those composed of ILs from the imidazolium family.

The discussed results are corroborated by the SDS-PAGE results provided in **Figure S2** in the Supporting Information. It should be highlighted that in the pure IgY lane, the antibody heavy chain appears in the 65–68 kDa and its light chain is presented around 25 kDa. When the WSPF is considered, the same bands are visible in addition to several other bands, corresponding to other water-soluble proteins. Finally, the SDS-PAGE results provide clearly evidences that the [C_{14}mim]Cl-based AMTPS is the best identified system to purify IgY, being able to retrieve most of the remaining proteins in the surfactant-rich phase while leading to the highest IgY purity of the considered systems. In other words, these results are a proof of the IgY purification, showing an increasing trend in the IgY purity from the WSPF to the conventional AMTPS and finally to the most promising mixed AMTPS (35% < 53% < 62% IgY purity).

3.3. IgY stability studies through ATR-FTIR and CD

Stability studies were conducted to determine the structural integrity of IgY after the extraction step with AMTPS. Conventional AMTPS and SAIL-based systems were prepared with pure IgY to assure that variations identified are only associated with the antibody and not with the remaining proteins. The obtained surfactant-poor phases after extraction with pure IgY were analyzed by ATR-FTIR, whose results are given in Fig. 7**.** From the ATR-FTIR spectra of IgY (Fig. 7**.A**), two different regions were identified as important for the structural integrity analysis of the secondary structure, one is amide I (~1650 cm^{-1}), which corresponds to stretching vibration of the group C = O, and the other is amide II (~1550 cm^{-1}) that corresponds to stretching on C = N group. Amide I region is attributed to β-sheet structures (≈1635 cm^{-1}), as well as α-helices (≈1660 cm^{-1}), random coil (≈1645 cm^{-1}) and β-turn (≈1670 cm^{-1}). These regions are usually used to identify several elements from secondary structures of proteins [27]. By comparing this set of results with the pure IgY in buffer solution, there is no significant difference in the amide I and amide II peaks position in conventional AMTPS and in [C_{14}mim]Cl-based AMTPS, meaning that IgY maintains its secondary structure intact. The same conclusion was achieved from the CD spectra analysis, where the presence of the same negative band around ~ 218 nm was found for both the conventional- and [C_{14}mim]Cl-based AMTPS (Fig. 7**.B**), further confirming the structural integrity of the antibody after the extraction with AMTPS.

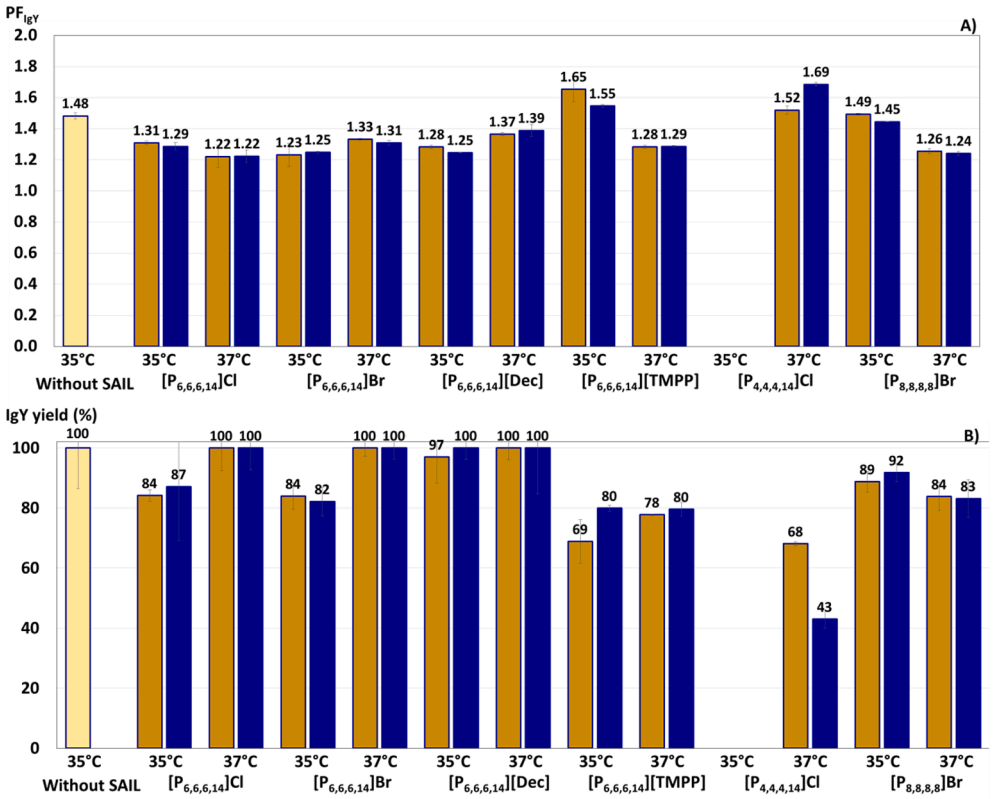

Fig. 6. PF$_{IgY}$ (**A**) and yield (**B**, %) of IgY in the distinct phosphonium-based AMTPS in comparison with the AMTPS without SAIL. The SAIL concentration (wt%) effect is also shown for two concentrations, namely 0.3 (without pattern) and 0.5 wt% (with pattern).

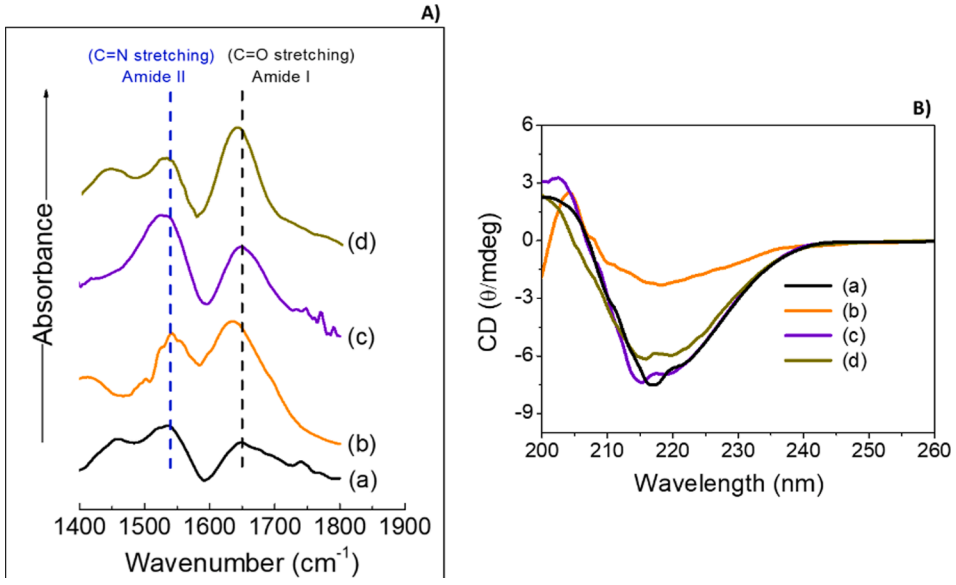

Fig. 7. Infrared (**A**) and CD (**B**) spectra of pure IgY in conventional and mixed AMTPS. **(a)** Pure IgY in McIlvaine buffer (pH 6.0); **(b)** IgY in AMTPS with 1 wt% of Triton X-114; **(c)** IgY in 20 wt% of Triton X-114; **(d)** IgY in the surfactant-poor phase with 0.3 wt% of [C$_{14}$mim]Cl. Concentration of IgY taken initially was 1 mg.mL^{-1} in all cases.

3.4. Conceptual process for the IgY purification

As previously described, the purpose of this work is to purify IgY from the WSPF, while applying a biocompatible and efficient downstream process. The reuse of each phase-forming component (Fig. 8) as well as the IgY isolation were also contemplated in the integrated downstream process envisaged in this work, whose process is summarized in Fig. 9.

Considering the temperature required and the purification and yield of IgY obtained, the mixed AMTPS composed of 0.3 wt% of [C$_{14}$mim]Cl was selected as the most relevant system to purify IgY from the WSPF. Therefore, the surfactant-poor phase of this mixed system was collected and used in three consecutive extraction cycles, being the systems completed with fresh 20 wt% of Triton X-114, 0.3 wt% of [C$_{14}$mim]Cl

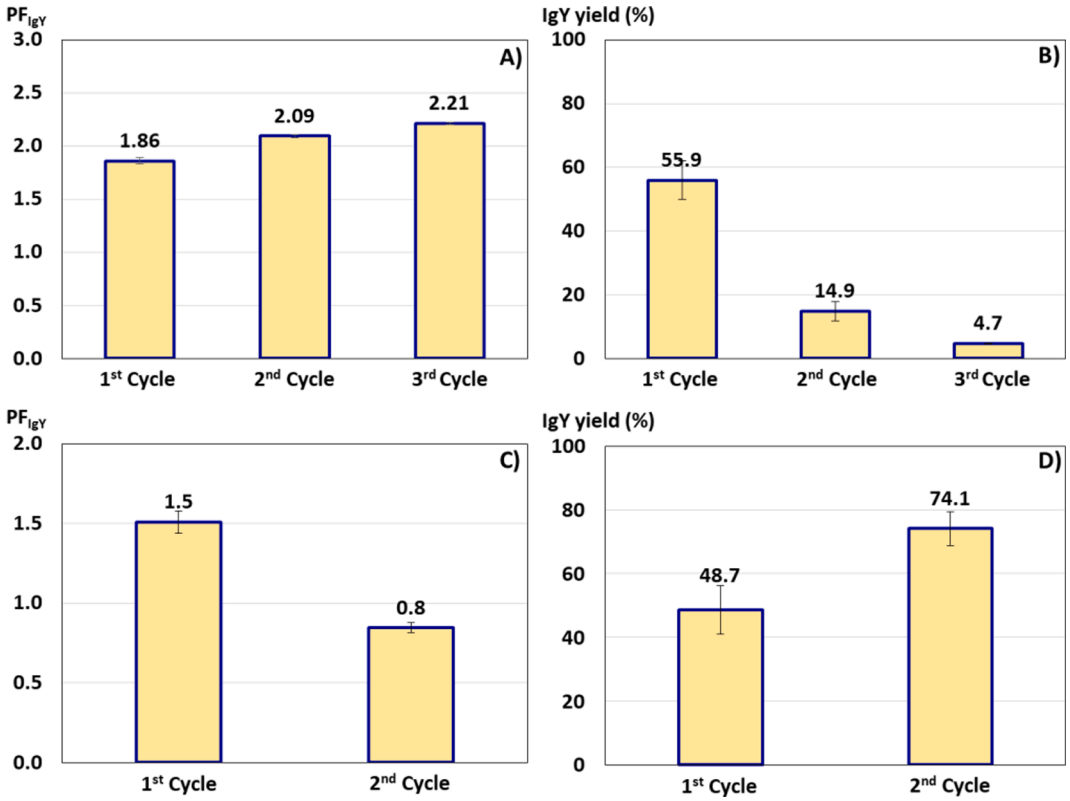

Fig. 8. PF$_{IgY}$ (**A** and **C**) and yield (**B** and **D**, %) of IgY obtained for each extraction cycle with the mixed AMTPS composed of 0.3 wt% of [C_{14}mim]Cl, upon the reuse of the surfactant-poor phase (**A** and **B**) and the surfactant-rich phase (**C** and **D**) with phase separation at 37 °C.

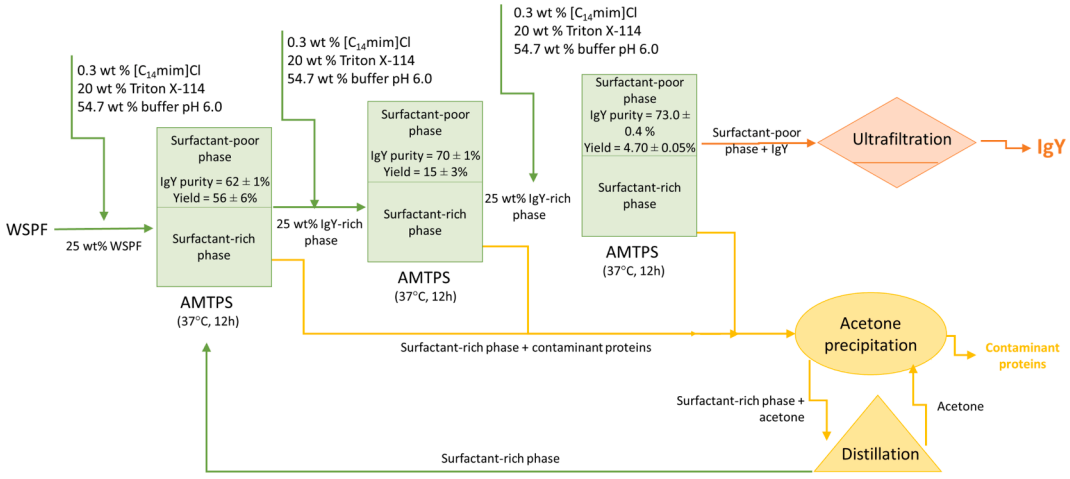

Fig. 9. Integrated downstream process envisioned for IgY purification from egg yolk.

and 54.7 wt% of McIlvaine buffer pH 6.0 at each new cycle. Through the analysis of Fig. 8.**A)** and **B)**, it is verified that the reuse of the surfactant-poor phase slightly enhances the IgY purification from the first to the second cycles of extraction. However, this is accompanied by the loss of IgY between cycles. When the second and third cycles are compared, the same tendency is observed; however, the IgY purification only increases around 3% compared to its losses of around 10%. These results were confirmed through SDS-PAGE (**Figure S3** in the Supporting Information). With these results, it can be concluded that new extraction cycles with the reuse of the surfactant-poor phase from the 0.3 wt% of [C_{14}mim]Cl-based AMTPS are indeed able to extract more protein impurities from the WSPF, while simultaneously concentrating IgY in this phase. On the other hand, the presence of IgY heavy chain bands was also observed in the surfactant-rich phase of each new cycle, though with less intensity and being in accordance with the decreasing values of IgY yield in each new AMTPS. Overall, new extraction and consecutive cycles have the capacity to purify and concentrate IgY, with a maximum PF$_{IgY}$ of 2.21 ± 0.01 (IgY purity = 73.0 ± 0.4%). Moreover, IgY can be further purified by the incorporation of an ultrafiltration step, as demonstrated in the literature [38], and the surfactant-rich phase reused after removing the remaining proteins as demonstrated elsewhere [39].

A similar approach was also applied for the reuse of the surfactant-rich phase, being the system completed with fresh WSPF and McIlvaine buffer pH 6.0 in the same compositions as the initial ones (*cf.* methods section). However, the results were not as good as the ones attained for the surfactant-poor phase. By analyzing the PF$_{IgY}$ and yields

(Fig. 8.**C** and **D**), it is clear that the surfactant-rich phase is saturated, since the PF_{IgY} in the second cycle decreases almost to half, showing that this phase cannot recover more proteins.

Summing up, and considering the low cost of chicken eggs as raw material and the high commercial value of IgY (50 μg of IgY cost 385€ [40]), the final conceptual integrated purification process proposed is depicted in Fig. 9. It should be here highlighted that this is the first time that an AMTPS has been applied in the IgY purification, showing outstanding results for a low-resolution downstream process. Our data show an impressive purification of IgY from the WSPF in one-step, namely from the initial 35% purity to 54% purity of the conventional AMTPS. This was further enhanced by the addition of small amounts as SAILs, as co-surfactants, leading to an IgY purity of 69% with 0.5 wt% of [C_{18}mim]Cl-based AMTPS in a single step or even 73% purity with 0.3 wt% of [C_{14}mim]Cl-based AMTPS in three consecutive purification cycles (PF_{IgY} = 2.21 ± 0.01%). These results evidence the remarkable tailoring ability of ILs, especially considering their low amounts in AMTPS. This is a considerable improvement of the previously reported IgY purification of 60%, achieved by sequential steps of lipids removal and precipitation methods [15]. Apart from this study, our group has also shown the ability to extract IgY using ABS. However, the IgY purity was not determined, being demonstrated these systems ability to achieve IgY extraction efficiencies higher than 79% in one-step [19]. More recently, Balaraman and Rathnasamy [41] have shown the IgY purification from quail eggs using a deep eutectic solvent-based ABS coupled with an ultrasound-assisted liquid–liquid microextraction. The authors reported an increase in the IgY purity (up to 89-fold), but after applying subsequent steps of preparative size-exclusion liquid chromatography and anion exchange chromatography. Furthermore, there are other studies attempting the IgY purification, though they report more complex protocols while using high-resolution techniques, such as chromatography (*cf.* **Table S1** in the Supporting Information), these recognized as time-consuming, expensive and not always easy to scale-up. Overall, AMTPS comprising ILs seem to be a promising and cost-effective downstream process for the IgY purification from the WSPF.

4. Conclusions

This work reports, for the first time, the successful application of thermoresponsive systems (commonly defined as mixed AMTPS) in presence of very small amounts of SAILs as co-surfactants for the IgY purification from the egg yolk. After a careful optimization of the most relevant conditions, namely surfactant, WSPF and SAILs concentrations as well as the influence of the IL cation, anion, and symmetry and length of the alkyl side chain of the cation, an integrated process was envisioned. In a single step, it was possible to enhance the IgY purity from 35% in the WSPF to 54% and 69% when the conventional AMTPS and 0.5 wt% of [C_{18}mim]Cl-based AMTPS were applied, respectively. It was also shown that by applying three consecutive purification cycles with the surfactant-poor phase of the 0.3 wt% of [C_{14}mim]Cl-based AMTPS, IgY purity could be further boosted to 73%. Finally, SDS-PAGE corroborated these purification values while ATR-FTIR and CD showed that IgY maintained its structural integrity intact.

Overall, a cost-effective downstream process for the IgY purification from the WSPF with simple and scalable techniques was here proposed.

CRediT authorship contribution statement

Filipa A. Vicente: Investigation, Methodology, Formal analysis, Writing – original draft. **Leonor S. Castro:** Methodology, Investigation. **Dibyendu Mondal:** Methodology, Formal analysis, Writing – original draft. **João A.P. Coutinho:** Conceptualization, Resources, Funding acquisition, Writing – review & editing. **Ana P.M. Tavares:** Formal analysis, Writing – review & editing. **Sónia P.M. Ventura:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Mara G. Freire:** Resources, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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