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Optimized extraction of a single-chain variable fragment of antibody by using aqueous micellar two-phase systems

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ABSTRACT

In this work, the purification of a single-chain variable fragment (scFv) of an antibody by using liquid–liquid extraction in aqueous micellar two-phase systems was optimized by means of central composite design. Protein partitioning assays were performed by using the selected system composition in previous works: Triton X-114 at 4% wt/wt, yeast fermentation supernatant at 60% wt/wt, McIlvaine buffer pH 7.00. The other system component concentrations, Cibacron Blue F3GA (CB), Fabsorbent™ F1P HF (HF) and NaCl, were selected as independent variables. ScFv recovery percentage (%R) and purification factor (PF) were selected as the responses. According to the optimization process both, scFv recovery percentage and purification factor were favored with the addition of HF and NaCl in a range of concentrations around the central point of the second central composite design (HF 0.0120% w/w, CB 0.0200% w/w, NaCl 0.200% w/w). These experimental conditions allowed the concentration and pre-purification of scFv in the micelle-rich bottom phase of the systems with a recovery percentage superior to 88% and a purification factor of approximately 3.5. These results improved the previously presented works and demonstrated the convenience of using aqueous micellar two-phase systems as a first step in the purification of scFv molecules.

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Introduction

Monoclonal antibodies have played an important role in medicine and in different biotechnological fields for several years [1,2]. The most common commercially available monoclonal antibodies (mAbs) are chimeric, humanized, fully human and of murine origin. Other novel therapeutic formats, as for example single-chain variable fragments (scFv) and subtypes, are in development or are undergoing clinical testing [1]. This type of molecules, scFv antibodies, have recently gained importance in the pharmaceutical field because of the several advantages that they present respect to full-sized antibodies, such as, easy manipulation, high permeability and the fact that their immune-complexes can be quickly cleared from the human body [3].

Due to the advantageous features above mentioned, antibody fragment pipeline is now expanding [4,5], thus, its industrial production and purification is highly demanded [6]. Up to date, the production of recombinant antibodies fragments has reached high titers [7–9], which imply that the downstream unit operations delimit the cost of the whole process [7,10–12]. For example, it is known that 30% of the total purification cost corresponds to the Protein A resin, with an estimated value of 4–5 millions dollars in a scale of 10.000 L [11,13]. The reuse of this resin is also expensive (about 120.000 dollars) due to the high buffer volume consumption. This limitation represents the bottleneck for antibody fragment production at industrial levels [6,10] and as a consequence, the use of non conventional methodologies for scFv purification must be evaluated [10].

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The extraction with aqueous two-phase systems (ATPSs[1]) represents an attractive alternative as a bioseparative process [14–16]. Particularly, aqueous micellar two-phase systems (AMTPS) have several desirable features such as high enrichment factor, short-time consumption and low cost [17]. Additionally, it was demonstrated that 87% of the used surfactant can be recovered and re-used, thus reducing notoriously the purification cost [18].

The AMTPS methodology is simple and consists in incubating a mixture of sample and a surfactant solution [19] at certain temperature to allow phase separation and protein partitioning [20]. Furthermore, the partition selectivity can be improved by using mixed micelles, affinity ligands and salts [21–23].

The successful application of liquid–liquid extraction in the purification of recombinant antibodies from different sources (e.g. CHO, *Escherichia coli* cultures, etc.) has already been approached [12,24–28]. Successful purification performances, were obtained with polymer/salt and thermoseparating aqueous two-phase systems [28,29]. Additionally, Fischer and colleagues have used an AMTPS-based methodology to extract an antibody fragment from *E. coli* [18]. Recently, our research group has evaluated the feasibility of using AMTPS of Triton X-114 to extract a scFv antibody directly from *Pichia pastoris* fermentation supernatant [30]. According to our preliminary results, scFv was concentrated and partially purified in a single extractive step with recovery percentages superior to 80% and purification factors of approximately 2 [30]. Even though these results were similar or even better than those reported for other authors [30,31], a further improving of this purification performance would be desirable, thus requiring the application of statistical techniques.

According to a preliminary factorial design (data not shown), the concentration of both ligands (HF and CB) and salt (NaCl), as well as their interactions, showed to be significant factors in the scFv extraction process.

In this context, the aim of this work was to optimize the previously obtained scFv purification parameters, the scFv recovery percentage (%R) and the purification factor (PF), by studying the effect of the mentioned variables.

To identify the independent variables with statistical significance for the process and their significant interactions, the extraction experiments were performed with the aid of a central composite design [32].

Materials and methods

Chemicals

Polyethylene glycol tert-octylphenyl ether (Triton X-114) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Cibacron Blue F3GA (triazine-based dye, used as free ligand) and Fabsorbent™ F1P HF (synthetic compound, cross-linked to an agarose bed, which has been designed to specifically capture antibody fragments) were obtained from Polysciences Inc. and Prometic Biosciences respectively.

All the other reagents were of analytical grade and used as received. All the solutions were prepared in McIlvaine's buffer, pH 7.00, consisting of 1.38 mM citric acid and 5.3 mM disodium phosphate purified by filtration through a Millipore Milli-Q ion-exchange system (Bedford, MA, USA). All glassware used in the experiments was washed in a 50:50 ethanol/1 M sodium hydroxide bath, washed in a 1 M nitric acid bath, rinsed copiously with Milli-Q water and dried in an oven at 70 °C.

Biologicals

The recombinant *P. pastoris* SMD 1168 (Δpep4::URA3 Δkex::SUC2 his4 ura3, phenotype His- Mut+), an anti-LDL electronegative his-tagged single-chain antibody fragment (scFv) producing yeast (Invitrogem), was kindly provided by Professor Dulcineia Saes Parra Abdalla from the São Paulo University (Brazil) and stored at −70 °C with glycerol 20% wt/wt [33].

Yeast growth and scFv production

Buffered Glycerol Complex medium (BGCM) [30] was used to start *P. pastoris* culture. The yeast growth was initialized for 16 h in a 250-mL orbital shaker (250 rpm) at 20 °C. Afterwards, the inoculum was transferred into a 3-L bioreactor and grown for 96 h at 20 °C in BGCM medium. 1% v/v of inductor (methanol) and 1 mM of the proteases inhibitor phenylmethanesulfonylfluoride (PMSF, from Sigma–Aldrich) were added every 24 h maintaining the pH at a constant value of 6.80. The final fermented broth was then centrifuged for 10 min at 2000 rpm in order to remove yeast cells. The supernatant was storage at −4 °C before use.

Protein partitioning

Protein partitioning assays were performed by using the previously selected system composition [30]: Triton X-114 at 4% wt/wt, yeast fermentation supernatant at 60% wt/wt, McIlvaine buffer pH 7.00. The other components: CB, HF and NaCl, were added according to the experimental design described in section "Experimental design and statistical analysis". The resulting solutions were mixed at 8 °C for 1 h. Subsequently, the solutions were incubated at 17 °C for 3 h to attain partitioning equilibrium. Samples from both upper and lower phases were then taken for the determination of scFv concentration and total protein content.

Recombinant antibody fragment and total proteins assays

The quantity of single-chain antibody fragment (scFv) was determined by immobilized metal affinity chromatography (IMAC). Samples from both, yeast broth and phase systems, were passed through the chromatographic column. Bottom phases were previously diluted at least four times in PBS buffer at pH 7.40 to avoid surfactant interference. The chromatographic procedure was performed according to the manufacturer's instructions (GE Healthcare, Munich, Germany), getting a scFv sample with a final purity (P_{scFv}%) of 95 [33]. After elution, scFv concentration was estimated by using the Bicinchoninic Acid method (BCA; Pierce, Rockford, IL, USA). Bovine Serum Albumin (BSA) was used as standard. Total proteins concentration was determined with the same methodology. All experiments were run in triplicate and the medium effect was discounted.

Extraction performance parameters

The partition coefficient (K) of scFv and total proteins was calculated as follows:

$$K = \frac{C^T}{C^B} \quad (1)$$

[1] *Abbreviation used:* AMTPS, aqueous micellar two-phase systems; ATPS, aqueous two-phase systems; BCA, bicinchoninic acid; BSA, bovine serum albumin; C^0, protein concentration (scFv o TP) (mg/L) in the clarified broth; $C_{(T/B)}$, protein concentration (scFv o TP) (mg/L) in top (T) or bottom (B) phase; CB, cibacron blue F3GA; CP, cloud point; ΔCP, CP at condition A – CP at condition B (°C); HF, Fabsorbent™ F1P HF; IMAC, immobilized metal affinity chromatography; K, partition coefficient; MB, mass balance; P%, total purity; PBS, phosphate buffered saline; PEG, polyethylene glycol; PF, purification factor; scFv, single-chain antibody fragment; TP, total proteins; V^0, volume of added yeast supernatant (mL); $V_{(T/B)}$, top (T) or bottom (B) phase volume (mL); %wt/v, grams of specific compound in 100 mL of total system/solution; %wt/wt, grams of specific compound in 100 grams of total system/solution.

Table 1
Variable values of a central composite design with six repetitions at the central point and $\alpha = 1.68$ used to investigate the effect of NaCl and affinity ligands on scFv purification from *P. pastoris* broth by AMTPS.

Variables	Coded values				
	−1.68	−1	0	+1	+1.68
(1) HF (%wt/wt)	0.0034	0.0050	0.0075	0.0100	0.0117
(2) CB (10^{-3}%wt/wt)	0.0032	0.0100	0.0200	0.0300	0.0368
(3) NaCl (%wt/wt)	0.1160	0.2000	0.2500	0.3000	0.3340

where C^T and C^B represent either scFv or total protein concentrations in the top (T) phase (micelle-poor) and bottom (B) phase (micelle-rich), respectively.

ScFv recovery percentage ($R_{scFv(T/B)}$%) was calculated at the phase it preferentially partitioned (T or B) according to the following equation:

$$R_{scFv(T/B)}\% = \left(\frac{C_{scFv(T/B)} * V_{(T/B)}}{C^0_{scFv} * V^0}\right) * 100\% \quad (2)$$

where $C_{scFv(T/B)}$ represents scFv concentration in the phase and C_{scFv^0} represents scFv concentration in the clarified fermented broth. $V_{(T/B)}$ and V^0 are, respectively, the volumes of the phases and the volume of added yeast supernatant.

ScFv purification factor ($PF_{scFv(T/B)}$) was determined as follows:

$$PF_{scFv(T/B)} = \frac{C_{scFv(T/B)}/C_{TP(T/B)}}{C^0_{scFv}/C^0_{TP}} \quad (3)$$

where C_{TP} and C^0_{TP} represent total protein (TP) concentration in the phase and in the fermented broth, respectively.

ScFv total purity (P_{scFv}%) in either the phase or the broth supernatant was calculated according Eq. (4):

$$P_{scFv}\% = \left(\frac{C_{scFv}}{C_{TP}}\right) * 100\% \quad (4)$$

where C_{scFv} and C_{TP} represent scFv and total protein concentrations in the sample.

To evaluate the possible precipitation of scFv/total proteins at the interface, the mass balance (MB) was calculated in each case according to the following expression:

$$MB = \left(\frac{C_{(T)} * V_{(T)} + C_{(B)} * V_{(B)}}{C^0 * V^0}\right) * 100\% \quad (5)$$

where $C_{(T)}$, $C_{(B)}$ and C^0 are scFv or total protein concentrations in top phase, bottom phase and in the crude extract, respectively. $V_{(T)}$, $V_{(B)}$ and V^0 are the respective volumes in each case.

Experimental design and statistical analysis

Statistical design was carried out for scFv extraction using AMTPS in which the independent variables were Fabsorbent™ F1P (HF) concentration (1), Cibacron Blue F3GA (CB) concentration (2) and NaCl concentration (3) at 2 levels (low and high levels coded as −1 and +1, respectively) with six repetitions at the central point (level 0) (Table 1). The axial points were calculated as follows: $\alpha = (2^n)^{1/4} = 1.68$ (n is the number of studied variables) [34]. The responses were scFv recovery percentage (R%) and scFv purification factor (PF).

The regression and analysis of data were carried out with the aid of Statistica Version 10.0 (Statsoft, Tulsa, OK, USA) software. Different models, e.g. linear and nonlinear adjustments, were evaluated. A given model was accepted when its correlation coefficient (R^2) was higher than 0.80, thus meaning that at least 80% of the data was explained by the model.

The statistical significance of the regression coefficients was evaluated by analysis of variance (ANOVA) at a significance level $p \leqslant 0.05$. Six repetitions were made at the central point in order to estimate the error variability of the experiments.

Optimization of scFv purification

Once the more relevant variables were selected, a second central composite design was performed aiming to obtain the maximum responses (scFv recovery percentage and scFv purification

Table 2
Results of scFv purification by AMTPS on responses recovery percentage (R%) and purification factor (PF), in the bottom phase of the micellar systems, according to the central composite design. R% pure error: 1, PF pure error: 0.01. The best purification conditions are highlighted with green circles.

Runs	Coded values of independent variables			R%	PF
	HF	CB	NaCl		
1	-1	-1	-1	27	0.94
2	+1	-1	-1	89	2.09
3	-1	+1	-1	53	0.99
4	+1	+1	-1	65	2.35
5	-1	-1	+1	64	0.82
6	+1	-1	+1	71	2.24
7	-1	+1	+1	49	2.26
8	+1	+1	+1	55	3.44
9	-1.68	0	0	55	1.55
10	+1.68	0	0	78	2.85
11	0	-1.68	0	69	1.70
12	0	+1.68	0	49	1.92
13	0	0	-1.68	69	1.96
14	0	0	+1.68	50	2.09
15	0	0	0	61	1.94
16	0	0	0	69	2.06
17	0	0	0	60	1.76
18	0	0	0	63	2.05
19	0	0	0	61	1.98
20	0	0	0	62	1.93

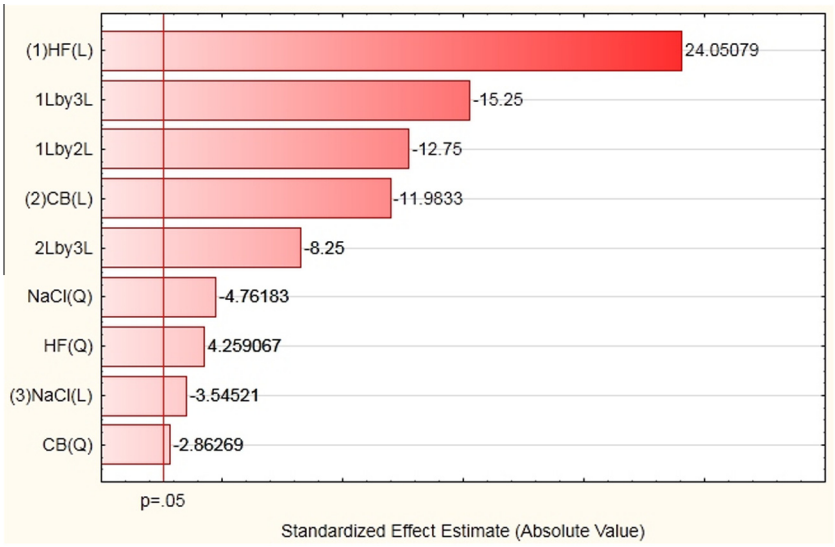

Fig. 1. Pareto chart for the effect of Fabsorbent™ F1P HF (HF) concentration (1), cibacron blue (CB) concentration (2) and NaCl concentration (3) in both, lineal (L) and quadratic (Q) relationship, on scFv recovery percentage (*R*%) in the bottom phase of AMTPS. $R^2 = 0.8427$. Effects significant at a confidence level of 95%.

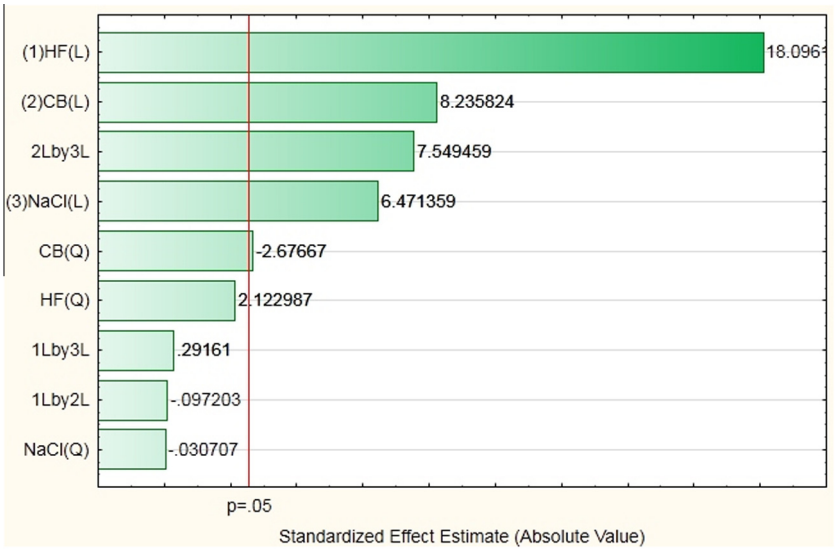

Fig. 2. Pareto chart for the effect of Fabsorbent™ F1P HF (HF) concentration (1), cibacron blue (CB) concentration (2) and NaCl concentration (3) in both, lineal (L) and quadratic (Q) relationship on purification factor (PF) of scFv in the bottom phase of AMTPS. $R^2 = 0.8732$. Effects significant at a confidence level of 95%.

factor). The statistical analysis was carried out as explained in section "Experimental design and statistical analysis". Notice that it was decided to keep constant the alpha value (1.68).

Results and discussion

ScFv purification from P. pastoris broth

Table 2 shows the obtained results for scFv purification based on the central composite design (Table 1). Notice that the purification performances were calculated in the bottom phases because it was the phase where scFv preferentially partitioned in most of the assayed conditions. That behavior has been previously attributable to the presence of affinity ligands in the micellar-rich phase [30].

As shown in Table 2, the highest value for scFv recovery was obtained when the affinity ligand HF was used at the highest concentrations (see runs 2 and 10). These results agreed with our previous work that demonstrated the capacity of HF ligand to produce an uneven antibody fragment partitioning toward the micellar-rich bottom phase [30], in which this ligand is preferentially partitioned.

As far as PF is concerned, it can be appreciated that the best purification (PF of 3.44) was obtained when all the evaluated factors (e.g. HF, CB and NaCl concentration) were present at the highest level (see run 8 in Table 2).

The significance and the magnitude of each variable effect were determined by the analysis of variance (ANOVA). Figs. 1 and 2 show the Pareto charts for scFv recovery percentage and purification factor, respectively. In the Pareto chart, the length of each bar is proportional to the standardized effect of the related variable or interaction, and the bars extending beyond the vertical line correspond to the statistically significant effects at a confidence level of 95% [35].

From the analysis of the data concerning to *R*% (Fig. 1), a quadratic model showed to be the most adequate in terms of the

determination coefficient (R^2) value, which demonstrated that 84% of total response variations can be explained by the selected model. Analyzing the variable effects, it can be seen that HF concentration has the strongest effect on the response variable, thus confirming the results observed in Table 2. The positive sign of this effect means that an increase in HF concentration leads to an increase in scFv recovery.

As far as PF is concerned (Fig. 2), a quadratic model showed to be the most adequate in terms of determination coefficient (R^2) value, which demonstrated that 87% of total variations of the studied response can be explained by the selected model. Similar to the results observed for *R*% in Fig. 1, the HF concentration was found to be the most significant effect (also positive) on the response, meaning that an increase in HF concentration results in an increase in scFv purification factor.

The obtained regression models were also used to calculate the response surfaces for each response variable. Due to the fact that HF concentration was the most significant effect on both, *R*% and PF, this variable was always chosen for the plots.

As can be seen from Fig. 3, at low HF concentration, both CB and NaCl slightly favor the scFv recovery in the micellar phase. Similar trends were observed when each variable was analyzed separately [30]. There are reports about the feasibility of using tryazine-based molecules to purify antibodies [36,37]. Particularly, CB is useful to capture antibodies with affinity to negative charged antigens such as DNA molecules [38].Thus, when CB is added into an AMTPS, it is preferentially directed to the micellar-enriched phase, thus driving scFv to this phase. On the other hand, NaCl, like most of salts, is known to partition to

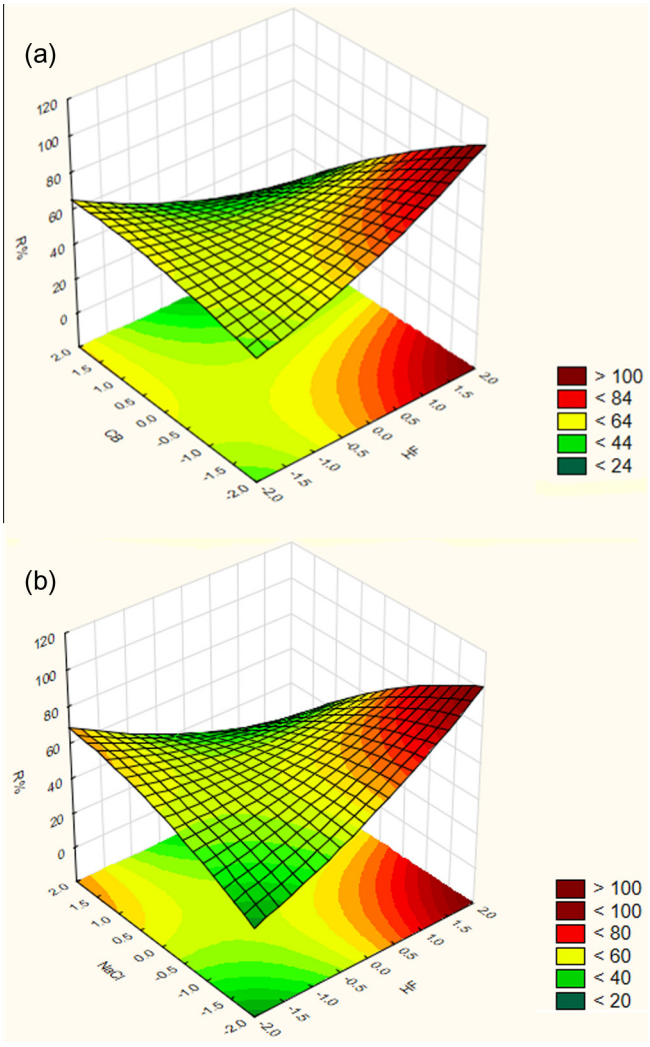

Fig. 3. Fitted surfaces responses for scFv recovery percentage (*R*%) in the bottom phase of AMTPS as a function of HF and (a) NaCl concentration (keeping CB at constant level of 0); (b) CB concentration (keeping NaCl at constant level of 0). For more information, see Table 2.

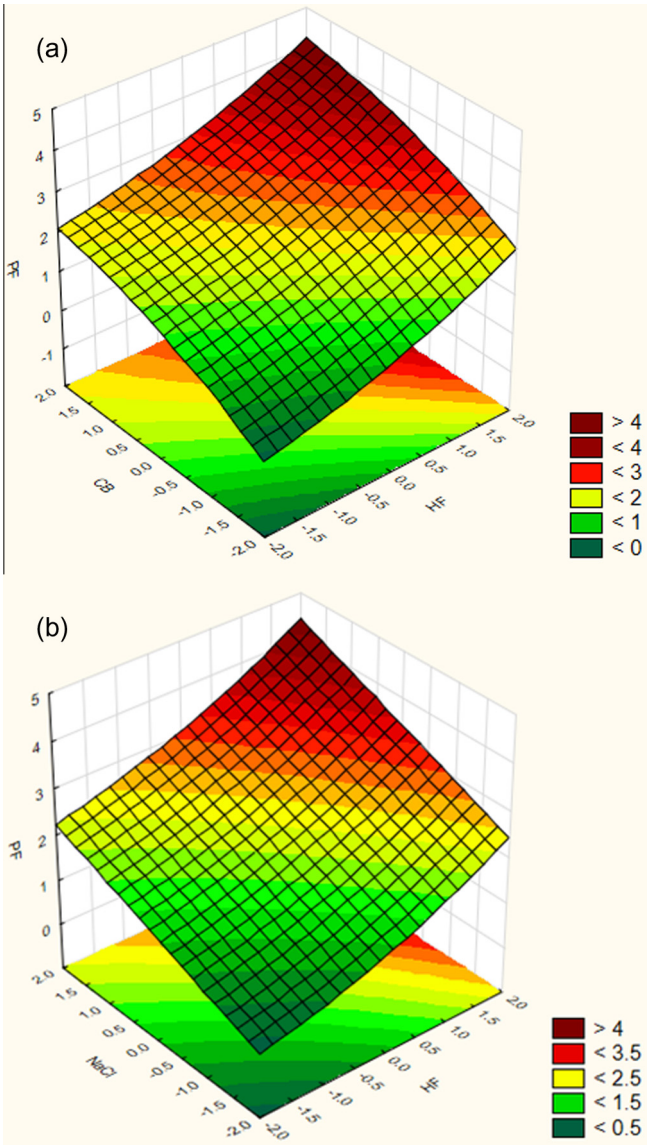

Fig. 4. Fitted surfaces responses for scFv purification factor (PF) in the bottom phase of AMTPS as a function of HF and (a) NaCl concentration (keeping CB at constant level of 0); (b) CB concentration (keeping NaCl at constant level of 0). For more information, see Table 2.

Table 3
Variables values of a central composite design with four repetitions at the central point and $\alpha = 1.68$ used to investigate the effect of NaCl and HF ligand on scFv purification from *P. pastoris* broth by AMTPS.

Variables	Coded values				
	−1.68	−1	0	+1	+1.68
HF (%wt/wt)	0.0053	0.0080	0.0120	0.0160	0.0187
NaCl (%wt/wt)	0.0320	0.1000	0.2000	0.3000	0.3680

the micellar-depleted phase, thus exerting a salting out effect on protein which induces the protein migration to the micellar phase. At high HF concentration, the presence of both CB and NaCl produces the opposite behavior, thus a decrease in the scFv recovery is observed. Other effects, which counteract the affinity interaction with HF, must be taken into account to understand this behavior. It was demonstrated that the addition of CB into an AMTPS produce changes in phase compositions [30] such as an increase in micellar composition in the bottom phase. As a consequence, certain molecules, such as scFv, are driven to the micelle-depleted phase (top phase) due to steric exclusion. The same trend, produced on scFv recovery by NaCl, could

Table 4
Results of scFv purification by AMTPS on responses recovery percentage (*R%*) and purification factor (PF), in the bottom phase, according to the central composite design. *R%* pure error: 2, PF pure error: 0.01. The best purification conditions are highlighted with an orange circle.

Runs	Coded values of independent variables		R %	PF
	HF	NaCl		
1	-1	-1	55	0.45
2	+1	-1	70	1.53
3	-1	+1	38	0.63
4	+1	+1	44	3.01
5	-1.68	0	31	0.50
6	+1.68	0	57	1.10
7	0	-1.68	79	0.59
8	0	+1.68	53	2.85
9	0	0	88	3.45
10	0	0	85	3.68
11	0	0	87	3.49
12	0	0	91	3.54

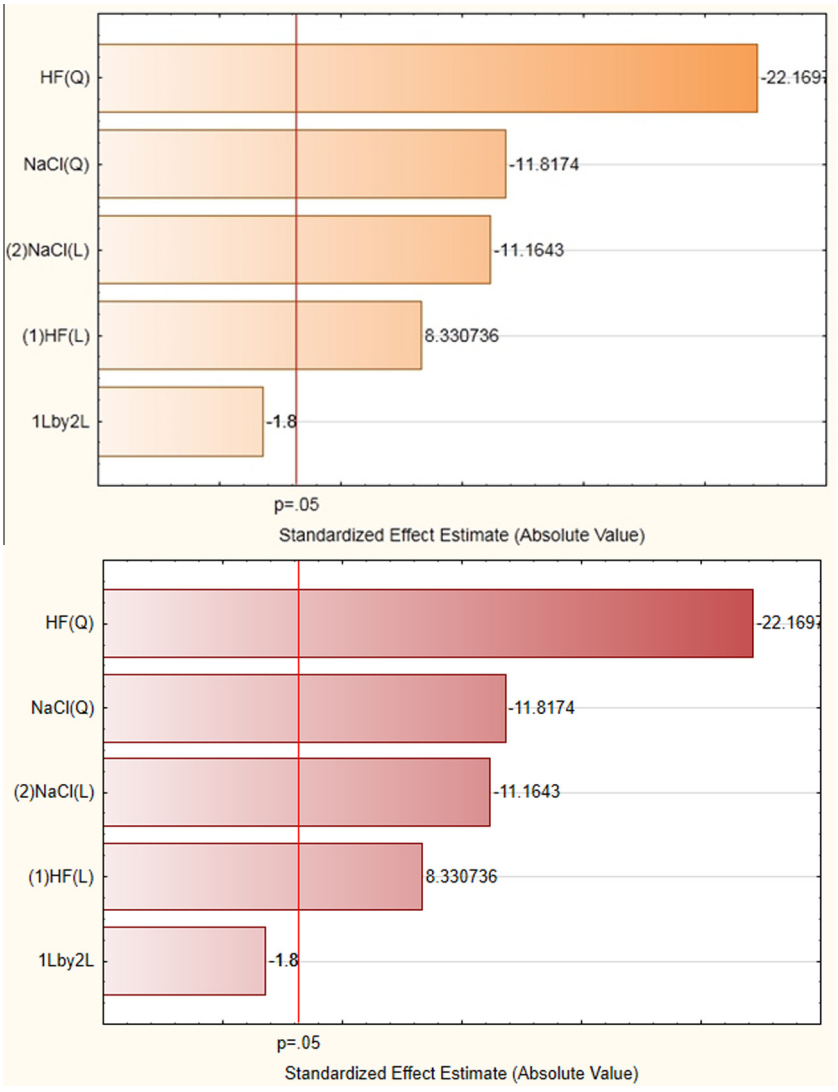

Fig. 5. Pareto charts for the effect of HF concentration (1) and NaCl concentration (2) in both lineal (L) and quadratic (Q) relationship on a) scFv recovery percentage (R^2 = 0.9055) and b) scFv purification factor (R^2 = 0.9051) in the bottom phase of AMTPS. Effects significant at a confidence level of 95%.

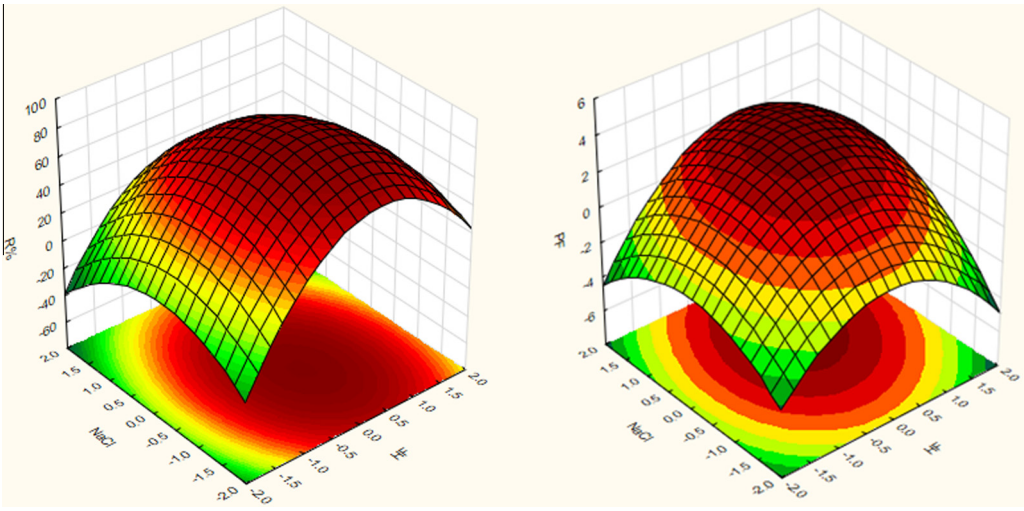

Fig. 6. Fitted surfaces responses for scFv recovery percentage (*R*%) and scFv purification factor (PF) as a function of HF concentration and NaCl concentration. For more information, see Table 4.

Table 5
Reported purification performances for scFv separation in the bottom phase of AMTPS according to a) our preliminary results and b) the process optimization.

	scFv concentration (mg/mL)	$V_{(T)}/V_{(B)}$	MB%	PF	*R*%
(a)	271	1.5	92	2.0	88
(b)*	385	2.2	90	3.5	88

* Calculated as an average of the four central point experiments.

be attributable to the ability of this salt of weakening the affinity interaction HF–scFv and favoring the transfer of free scFv toward the top – poor in micelles – phase. This pattern agrees with results of Table 2 that shows that the highest recombinant antibody recovery (89%) is obtained when the experimental condition of run 2 (HF at level +1, CB and NaCl at level −1) are used.

The response surface plots for scFv purification (Fig. 4) show a notorious increase in scFv PF when the concentrations of all independent factors (HF, CB and NaCl) are increased.

The increase of the antibody fragment purity in presence of HF is a predictable behavior because of the high specificity of this ligand for scFv molecules [39]. However, the favorable effect of NaCl and CB -in presence of HF- on the purification performance was unexpected.

Several reports showed that the magnitude of CB-protein interaction is not the same for diverse proteins [40,41]. In this way, the improvement in scFv PF, produced by CB, can be explained by different affinity interaction between CB and scFv/total proteins which results in a more preferential partitioning of scFv toward the bottom phase than total proteins.

The favoring effect of NaCl on scFv purification – at high HF concentration – is more difficult to understand since NaCl affects protein (scFv and total protein) partitioning by two opposite mechanisms. On the one hand, the weakening effect of affinity interaction which drives scFv to top phase and on the other hand, the salting out effect which drives scFv and total proteins to the bottom phase. The observed enhancement of scFv PF shows the prevalence of those mechanisms that induce a differential displacement of scFv to the micellar phase respect to total proteins. This behavior is reflected in Table 2, since run 8 shows the best purification factor (3.44) when level of all variables is the highest (+1).

Taking into account the results presented in Figs. 3 and 4, it can also be observed that none of the response surfaces exhibits a maximum value, which means that an extra optimization step should be developed in order to improve the response variables.

In this context, a second central composite design was carried out by extending HF and NaCl concentrations toward a response surface region of highest values of *R*% and PF (Table 3). CB concentration was kept constant at level 0 (Table 1) since higher CB concentration makes the bottom-phases too dark, thus interfering the protein quantification method.

The obtained results for scFv purification according to the second central composite design are presented in Table 4. As can be seen from this table, the highest recovery percentage (*R*% > 85) and purification factor (PF > 3.4) for scFv were obtained at the central points (runs 9–12). These results demonstrate that in the second composite design it is possible to simultaneously optimize both parameters (*R*% and PF).

The analysis of significance of the assayed factors (HF and NaCl concentration) on the responses R% and PF are shown in Fig. 5.

For both responses, a second-order model showed to be the most adequate in terms of determination coefficient (R^2) value, which demonstrated that 90% of total variations for *R*% and PF can be explained by the selected model. Additionally, it can be observed that HF and NaCl concentrations, in a quadratic relationship, exert the most significant effect (with negative sign) on *R*% and PF. Notice that the quadratic significant effects are negative, therefore, it is expected that both factors causes an increase in scFv recovery and purification until a limit concentration is reached, and then, a further increase in their composition causes the opposite behavior. From the analysis of the surface responses presented in Fig. 6 it can be appreciated that in both plots, the maximum responses correspond to central region. These results suggest that the limit concentrations of NaCl and HF, at which the responses optimize, are those belonging to the central point or around it (Table 5).

Even though the scFv recovery reduction by increasing the affinity ligand concentration seems to be an anomalous behavior, this is an indirect consequence of the effect of HF on the phase compositions (bottom phase becomes more enriched in micelles) which leads to an increase in excluded-volume phenomena in the micelle-rich phase [30].

Analyzing the maximum responses values, it can be appreciated that the obtained parameters (*R*% 88 and PF 3.5, approximately) resulted to be superior to those presented in our previous work [30] in terms of purification factor and final scFv concentration (see Table 5) and even better than those results obtained from other single-step purification methodologies such as packed bed chromatographies and polymer-based precipitation [10,30].

Conclusion

In this work, the purification of a single-chain variable fragment (scFv) of antibody by using liquid–liquid extraction in aqueous micellar two-phase systems was optimized by means of central composite designs. The best scFv purification performance was found in the central points of the second central composite design (HF 0.0120% w/w, CB 0.0200% w/w, NaCl 0.200% w/w) with an averaged recovery of 88 and a purification factor of approximately 3.5.

An apparent disadvantage of this methodology is that the target protein, scFv, is recovered in a surfactant (Triton X-114)-enriched phase. However, it was already demonstrated that working at temperatures of approximately 17 °C, the used surfactant does not significantly modify the protein structure and stability [31]. Besides, when a further purification grade of scFv is required, the obtained sample is able to be applied in other additional separation processes (i.e. chromatography affinity columns), after an appropriate dilution without requiring an extra step for surfactant removal.

Finally, taking into account the obtained purification performances as well as the simplicity and the low cost of the studied separative methodology, it can be concluded that the assayed AMTPS present a favorable benefit/cost balance among other methodologies and can be considered for an early step in scFv purification protocols.

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