APPLICATION OF SURFACE RESPONSE ANALYSIS TO THE OPTIMIZATION OF PENICILLIN ACYLASE PURIFICATION IN AQUEOUS TWO-PHASE SYSTEMS

J. C. Marcos[1*], L. P. Fonseca[2], M.T. Ramalho[1] and J. M. S. Cabral[2]

1 – Centro de Química- IBQF (Pólo de Braga), Universidade do Minho, Campus de Gualtar, 4700-320 Braga, Portugal

2 - Centro de Engenharia Biológica e Química, Instituto Superior Técnico, 1049-001 Lisboa, Portugal

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*- Corresponding author

E-mail : jcmarcos@quimica.uminho.pt

Telf: +351 253604386

Fax: +351 253678983

ABSTRACT

Penicillin acylase purification from an *Escherichia coli* crude extract using PEG 3350 – sodium citrate aqueous two phase systems was optimized. An experimental design was used to evaluate the influence of PEG, sodium citrate and sodium chloride on the purification parameters. A central composite design was defined centred on the previously found conditions for highest purification from an osmotic shock extract. Mathematical models for the partition coefficient of protein and enzyme, balance of protein and enzyme, yield and purification were calculated and statistically validated. Analysis of the contours of constant response as a function of PEG and sodium citrate concentrations for three different concentrations of NaCl revealed different effects of the three factors on the studied parameters. A maximum purification factor of 6.5 was predicted for PEG 3350, Sodium Citrate and NaCl concentrations of 15.1%, 11.0% and 8.52% respectively. However under these conditions the predicted yield was 61%. A better compromise between these two parameters can be found by superimposing the contour plots of the purification factor and yield for 10.3% NaCl. A region in the experimental space can be defined where the purification factor is always higher than 5.5 with yields exceeding 80%.

Keywords – penicillin acylase, purification, aqueous two-phase systems, experimental design

1. INTRODUCTION

Aqueous two-phase systems (ATPS) have been used for the purification of proteins [1], nucleic acids [2,3] and antibiotics [4,5]. Their technical simplicity, easy scale-up and suitability for continuous operation makes this method a favoured choice for a large-scale operation [6,7]. Despite their apparent simplicity, the partition of compounds in these systems is very complex due to the several factors involved. In fact the interaction of a compound with each one of the phases, include hydrogen bonds, charge and hydrophobic interactions and steric effects, in a mainly surface dependent process. It should be noted however that the complexity of these systems is even greater because these factors are not completely independent from each other.

Several theoretical approaches had been developed to model both the formation and the partition in ATPS. The Flory-Huggins model [8-11] is the oldest ant the best known. Although it describes phase separation and qualitatively predicts solute partition it is based on a model for polymers in apolar solvents. The osmotic virial expansion model [12-13] also succeeds in describing phase separation and solute partition. However the virial coefficients employed to represent solute interactions between the systems components are thermodynamically defined for diluted solutions. In addition both models consider exclusion the main factor governing partition. A statistical geometrical approach [14-15] has recently been proposed to describe phase separation. Whereas this is a more realistic treatment it application to the partition of solutes is so far unknown.

Empirical models have also been applied to the partition of proteins and peptides in ATPS [16]. Recently the partition in PEG-phosphate systems was modelled based on the model previously suggested by Eitman and Gainier [16] with the aid of molecular

modelling techniques. A good agreement between the model and the experimental values was obtained [17].

The above mentioned models aim at predicting the partition behaviour of an isolated compound. However when separating a compound from a mixture besides the interaction of the compound with the phases there will be interactions between the different components of the mixture. In the case of proteins it was suggested that they may form aggregates, changing the partition behaviour [18,19]. Due to this complexity, purification studies in ATPS are mostly empirical. The best conditions are usually attained by systematic variation of several factors such as polymers molecular weight, salt concentration and pH. In the ideal situation the target and contaminant compounds should accumulate in different phases. Even in this situation further optimisation can be achieved by variation of phase volume ratio. In this case, theoretically, the purification factor increases and the yield decreases with the decrease of phase volume ratio. The best compromise between these two parameters therefore requires the manipulation of the phase volume ratio.

Penicillin G acylase (penicillin amidohydrolase EC 3.5.1.1) is an enzyme that catalyse the penicillin G hydrolysis yielding 6-aminopenicillanic acid (6-APA) and phenylacetic acid [20]. The product 6-APA, is the starting material for the synthesis of semi-synthetic β-lactam antibiotics [21]. The preparation of pure enzyme catalyst is therefore an important step in the production of these drugs. Current purification protocols usually involve several chromatographic steps that increase the cost of the process and reduce yield [22-25]. ATPS seems to be a good alternative to a first step purification as this allow the removal of several contaminants.

In a previous study we described the partial purification of penicillin G acylase from an osmotic shock extract using PEG-sodium citrate ATPS. The purification factor obtained was 5.7 with 85% yield [26]. Variation of phase volume ratio to optimise purification conditions showed that both total protein and enzyme partition coefficients changed with this parameter. Furthermore, in the case of the enzyme, the variation trend depended on the system studied [27].

In this work the previous results were used as a basis to optimise the purification of penicillin acylase from a crude extract. Due to the complex dependence of the factors involved an experimental design was used to better evaluate the interaction among them.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents used were of analytical grade. Polyethylene glycol 3350 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Citric acid, sodium citrate and sodium chloride were obtained from Vaz Pereira Inc, Portugal.

2.2. Production of penicillin acylase

A mutant strain of *Escherichia coli* ATCC 9637 was grown on a 5 litre B.Braun Biostat MD fermenter with 4 litre of medium containing 1% (w/v) yeast extract and 0.3% (w/v) phenylacetic acid. The pH value of the medium was adjusted to pH 7.0 before inoculation and maintained at 7.0±1.0 by automatic addition of 2.0 M NaOH solution or 2.0 M HCl solution. This allowed the initial decrease of pH and the subsequent increase up to 8.0 needed to achieve high levels of penicillin acylase activity. Air flux was settled to 1vvm. Stirring was controlled in cascade with air flux to maintain 5% pO_2. The minimum level of stirring allowed was 400 rpm. Cells were harvested by centrifugation at 12000g, for 10 min, at the end of the exponential phase (20h), washed with 200mM phosphate buffer pH 7.5, and stored at 4ºC until used.

Cell homogenate was obtained by passing concentrated cells (35 g/l) through a Rannie Laboratory Homogenizer model Mini-Lab, type 8.30H at high pressure (500 atm). Release of penicillin acylase was monitored by measuring its activity on the pellet obtained after homogenate centrifugation at 12000g, for 10min. The operation was repeated several times until complete rupture of the cells was achieved. Between each step the cells were cooled to 15ºC in an ice bath.

2.3. Preparation of aqueous two phase systems

Sodium citrate concentrated solution (35.3%) was prepared by mixing appropriate amounts of equimolar solutions of tri-sodium citrate dihydrate and citric acid monohydrate to pH 6.9. The required amount of the previous solution was mixed at 20±1ºC, with 50% PEG 3350 solution, solid NaCl and cell homogenate, in 15ml graduated tubes with conical tips. Water was added to a final amount of 8g. After Vortex shaking for 1 min the two phases were separated by centrifugation and assayed for protein concentration and penicillin acylase activity.

The concentration of sodium citrate in a weight/weight basis was determined using its average molecular mass. This value was calculated by the sum of the molar fraction of each species multiplied by its molecular mass. In the experimental conditions used, only the divalent and trivalent citrate are meaningful and so, by the Henderson-Hasselbalch equation and the K_a value for the last ionization of citrate the molar fraction of each specie at the chosen pH was calculated.

2.4. Analytical methods

Protein concentration was determined by the method of Bradford [28]. To correct for PEG and citrate interference the samples were diluted and read against blank samples with the same composition, but without enzymatic extract.

Penicillin acylase activity was assayed by the method of Kutzbach and Rauenbusch [29]. The hydrolysis of 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) was followed spectrophotometrically by the increase in absorbance at 410nm. The reaction was performed at 37ºC in 100mM phosphate buffer pH 7.5 in stirred cells.

Under these conditions neither PEG nor citrate interfere with the enzymatic activity. One international unit (IU) was defined as the amount of enzyme that catalyse the hydrolysis of 1μmol of NIPAB per minute. The activity was expressed as IU per millilitre and calculated by the following expression:

$$\text{Act. (IU/ml)} = \frac{\Delta Abs}{\Delta t \times v \times 4.49} \qquad (1)$$

where v is the volume of the sample analysed.

2.5. Experimental design

The central composite design used consists in the summation of a star design with 2^k factorial design where k stands for the number of variables. For the case of three variables it means that the data points are the apexes of a cube plus 6 points at a distance $\pm\alpha$ from the centre of the cube [30]. The variables chosen were PEG, sodium citrate and sodium chloride concentration. The design was centred on the variable values where the highest purification factor was previously achieved. A range of values around this point was selected taking into account the conditions needed to achieve aqueous two-phase systems formation. The values were coded according to the chosen design, setting the value of α to 2. In addition six replicates were performed in the centre of the design to estimate experimental error. In Table 1 the real and coded values are presented. In each experiment the following parameters were calculated: the partition coefficient of total protein (K_p) and enzyme (K_e), the yield and purification factor of the enzyme (P.F.), the balance of total protein and enzyme.

2.6. Model fitting and validation

The data obtained for each parameter with the previous experimental design were fitted to full second order models. The models obtained were refined and validated by the following procedure:

1 – The significance of each parameter on the model was evaluated by the t - test. Parameters with less than 95% significance were discarded. Analysis of variance (ANOVA) for the complete and reduced models was performed, and the significance of the discarded parameters was evaluated by F test for a set of parameters according to the following expression:

$$F=[SS_{exp}/(p-g)]/[SS_{re}/(n-p)] \quad (2)$$

where SS_{re} refers to the sum of squares of residuals in the reduced model, SS_{exp} refers to the difference between the previous value and the sum of squares of residuals in the complete model. The letters n, p and g stand for the total number of experiments, the number of parameters in the complete model and the number of parameters in the reduced model respectively. If F significance was lower than 95% the reduced model was accepted. Otherwise the discarded parameters with the highest significance were added until the previous condition was fulfilled.

2- The obtained models were submitted to F-test for the significance of the regression (SOR), and lack of fit (LOF), and the coefficient of multiple determination (R^2) was calculated. The expressions used were:

$$F\ (SOR) = [SS_{fact}/(p-1)]/[SS_r/(n-p)] \quad (3)$$

$$F\ (LOF) = [SS_{lof}/(f-p)]/[SS_{pe}/(n-f)] \quad (4)$$

where SS_{fact} refers to the sum of squares due to factors, SS_r refers to the sum of squares of residuals, SS_{lof} refers to sum of squares due to lack of fit and SS_{pe} refers to the sum of squares due to purely experimental uncertainty. The letters n and p have the same

meaning as above and f stands for the number of distinctly factor combinations at which experiments have been carried out. Models were accepted if the significance of the F(SOR) is higher than 95% and the significance of F(LOF) is lower than that value. If one of these conditions was not satisfied the model was accepted when R^2>0.95 which means that more that 95% of the data is explained by the model.

3- Models that were not accepted by the previous tests were improved by addition of third order terms until the determined conditions were fulfilled. In each addition the significance of the added term for the model was F–tested according to equation (2). As before only parameters with significance higher than 95% were accepted.

2.7. Response surface maximum identification

The highest purification factor achievable was determined by the identification of the response surface maximum of its model. The stationary point of the surface, where the three derivatives are simultaneous zero, was calculated. The characteristics of this point was determined by canonical analysis [30] to confirm that it is a maximum.

3. RESULTS AND DISCUSSION

3.1. Model Building

The strategy used to attain the goal of this work was to explore the experimental space around the previous selected conditions for the purification from an osmotic shock extract. An experimental design was implemented in order to better evaluate the interactions between the several variables. The central composite design was chosen as the one that allows the fitting of several mathematical models from the data obtained.

Table 2 presents the results obtained and Table 3 the data resulting from the fitting of each parameter to a model following the methodology described in materials and methods.

The fitting of every parameter was possible according to the defined criteria and with a maximum number of ten factors. The discarded factors from the complete model have a low significance being less than 10% in most of the cases. Only for the partition coefficient and balance of total protein these values are greater, but much lower than the defined limits for accepting the corresponding factors. In the cases where third order factors were needed to improve the fitting of the models the significance of this addition was very high. Therefore the models are statistically valid. However although the defined criteria have been respected, there is an apparent contradiction between the significance of the regression and the significance of the lack of fit. For all models the significance of the regression is 99.99%, but except for the model of the purification factor the significance of the lack of fit is always higher than 96%. This kind of problem has been previously reported, and happens when the model is well fitted to the data but the measurement method is very precise [30]. In the present case the estimate for the experimental error is very low in the centre of the design, being about 3% for the partition coefficient of the protein, yield and balance of both enzyme and protein, and less than 6% for the purification factor and partition coefficient of protein. On the other hand this design takes the experimental space as homocedastic, meaning that the error is assumed to be the same in every point. If this is not the case and the experimental error is higher in the extremes of the design the standard error might have been estimated by defect, being the reason for the high significance of the lack of fit tests.

3.2. Model Analysis

The previous models were used to calculate the contours of constant response for the parameters. The curves of isoresponse as a function of PEG 3350 concentration and sodium citrate concentration, keeping the concentration of sodium chloride constant, were calculated for three different concentrations of sodium chloride: 7.3, 8.8 and 10.3% (w/w). The plots are presented in Figures 1 to 6.

All the curves for the partition coefficient of total protein have a similar shape irrespective of the concentration of NaCl (Fig. 1). However the values of K_p increase with the concentration of the salt as had been previously observed in the experiments with an osmotic shock extract. These results show that although the value of K_p is dependent on the concentration of NaCl there is no interaction between this variable and the other two. This means that the effect of the NaCl concentration does not depend on the concentrations of sodium citrate and PEG 3350. The contours as a function of these two variables present a curved shape contradicting the theoretical expectation that the partition coefficients remained constant along the same tie-line. However they agree with our previous results where it was observed the increase of K_p with the decrease of phase volume ratio [27]. In the present case the behaviour does not seem so linear. However being the tie lines roughly parallel to the oblique line that limits the experimental space, the previous trend is verified for most of it.

The balance of protein does not seem to be significantly influenced by the concentration of NaCl, as the contours are very similar for the three different concentration of this salt (Fig. 2). The increase of PEG and sodium citrate concentrations decreases significantly the balance of protein probably due to its precipitation. In fact in experiments with high concentration of PEG and citrate a

precipitate was observed that accumulates on the interface or sediments depending on the density of the lower phase. The effect of PEG is more pronounced but it seems to be a synergistic effect as the lower recoveries are observed for intermediate concentrations of the two components.

In opposition to the parameters discussed above, K_e shows a marked influence of NaCl on the shape of the contours, increasing their complexity with the increase of salt concentration (Fig. 3). The contour plot shows that the effect of NaCl is very dependent on the concentration of the other two components. It is observed that for low concentrations of PEG and sodium citrate, corresponding to short tie lines, the increase in NaCl concentration results in an increase on the enzyme partition coefficient. However for longer tie lines the effect is variable, depending on the relative concentrations of PEG and sodium citrate. For each different concentration of NaCl the dependence of K_e with the concentrations of the aqueous two-phase forming components is very different.

The balance of enzyme seems to be just slightly increased with the increase of NaCl concentration (Fig. 4). Generally the increase of PEG and sodium citrate concentration results in a decrease of the enzyme balance similar to what happens with the total protein. However in certain conditions at equal PEG concentration, the lowest recovery is obtained for an intermediate value of sodium citrate concentration. This means that there are other factors related with the characteristics of the two phases besides the concentration of the two components that determines the precipitation and/or inactivation of the enzyme.

The contour plots for the purification factor have all similar shapes irrespective of NaCl concentration (Fig.5). In all of them is visible a maximum whose location and

magnitude are dependent on NaCl concentration. From the three different NaCl concentrations the maximum is attained for 8.8% (w/w) NaCl. The absolute maximum of the experimental space was determined as described in materials and methods and is obtained with the following concentrations (w/w): 15.1% PEG 3350, 11.0% sodium citrate and 8.52% sodium chloride. The predicted purification is 6.5 fold.

Being the yield dependent on the partition coefficient of the enzyme, its contour plots also present different shapes for the three NaCl concentrations (Fig. 6). However these are not so marked being the major ones observed for low concentration of salt. For the highest concentration of NaCl it is observed a significant decrease of the yield for the higher concentrations of PEG and sodium citrate. This must be related with the previously observed decrease of the enzyme partition coefficient because in these conditions its balance increases as was referred.

3.3. Optimisation of purification conditions

Under the conditions where the maximum of the purification factor is predicted, the yield was 61%. As the predicted balance of enzyme is 62% this means that most of the active enzyme is recovered in the upper phase. However it would be convenient for a first purification step, to achieve a higher yield, even at the cost of a slightly decreased purification factor. The available data allow a first approach for yield improvement. By superimposing the contours of yield and purification factor for 10.3% (w/w) NaCl it is possible to find a region of the experimental space where the yield ranges from 80 to 100% and the purification factor is always higher than 5.5 fold (Fig. 7). These will be the more appropriate conditions for the utilisation of these systems on the purification of penicillin acylase.

4 . CONCLUSIONS

This paper shows the feasibility of using ATPS as a first step in the purification of penicillin acylase from a crude extract. The central composite design used in this work allowed the definition of appropriate models for several purification parameters which in turn led to the definition of the best purification conditions. The obtained results define a large area of the experimental space where a good compromise between purification factor and yield could be attained. A purification factor higher than 5.5 and a yield higher than 80% is undoubtedly a good result for a first step purification from a crude extract. These figures compare very well with the previously obtained results for the purification from an osmotic shock extract [26,27]. The methodology used in this work allowed similar purification factor and yield from a much more crude preparation. This opens promising perspectives for applying ATPS in a large scale process.

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Table 1 – Real and coded values of the variables in the different experiments of central composite design

Experiment	Real Values % (w/w)			Coded Values		
	[PEG 3350] % (p/p)	[NaCitrate] % (p/p)	[NaCl] % (p/p)	X1	X2	X3
1	14	10.76	8.8	0	0	0
2	14	10.76	8.8	0	0	0
3	14	10.76	8.8	0	0	0
4	14	10.76	8.8	0	0	0
5	14	10.76	8.8	0	0	0
6	14	10.76	8.8	0	0	0
7	19	8.71	7.3	1	-1	-1
8	9	8.71	7.3	-1	-1	-1
9	19	12.81	7.3	1	1	-1
10	9	12.81	7.3	-1	1	-1
11	19	8.71	10.3	1	-1	1
12	9	8.71	10.3	-1	-1	1
13	19	12.81	10.3	1	1	1
14	9	12.81	10.3	-1	1	1
15	4	10.76	8.8	-2	0	0
16	24	1076	8.8	2	0	0
17	14	6.66	8.8	0	-2	0
18	14	14.86	8.8	0	2	0
19	14	10.76	5.8	0	0	-2
20	14	10.76	11.8	0	0	2

Table 2 – Results from the experimental design

Experiment	K_e	K_p	F.P.	Yield	Protein balance	Enzyme balance
1	21	0.47	5.9	57	49	62
2	20	0.46	6.6	58	48	64
3	20	0.42	7.0	62	49	68
4	20	0.46	6.4	57	49	63
5	20	0.46	6.2	59	50	64
6	21	0.49	6.5	59	46	65
7	5.6	0.26	5.6	70	53	85
8	0.22	0.17	1.8	11	73	104
9	70	0.64	4.7	70	41	65
10	1.16	0.50	3.0	29	61	93
11	82	0.58	4.1	95	59	103
12	1.05	0.50	2.5	30	68	100
13	106	1.50	3.1	68	42	75
14	57	1.27	5.0	90	56	95
15	0.37	0.61	0.86	4.0	74	100
16	72	1.87	2.5	57	32	58
17	0.56	0.18	3.4	45	67	103
18	217	1.63	5.0	99	41	100
19	0.38	0.14	3.3	18	63	86
20	118	1.22	3.6	96	57	98

Table 3 – Parameters of the fitted models and results from the statistical tests following the methodology described in materials and methods section

	Ln K_p	Ln K_e	F.P.	Yield	Protein Balance	Enzyme Balance
b_0	-0.7862	3.042	6.395	60.00	49.00	65.39
b_1	0.2013	1.335	0.5300	14.06	-9.188	-9.250
b_2	0.5164	1.366	0.3063	11.44	-6.563	-10.42
b_3	0.5013	1.346		17.69		3.125
b_{12}	-0.02515	-0.5458	-0.7000	-16.13		-4.000
b_{13}	-0.04651	-0.4804	-0.7250	-10.13	2.125	3.750
b_{23}			0.1500	4.881		
b_{11}	0.2037	-0.3264	-1.207	-6.500	1.375	4.193
b_{22}		-0.1378	-0.5836	3.875	1.625	9.818
b_{33}		-0.2623	-0.7649		3.125	7.443
b_{123}		-0.7442		-12.02		
b_{222}						2.417
F(SOR) (%)	99.99	99.99	99.99	99.99	99.99	99.99
F(LOF) (%)	99.49	99.99	49.25	99.66	96.64	98.53
R^2	0.9746	0.9913	0.9748	0.9774	0.9598	0.9617
F(CQM)(%)	68.91	2.521	9.922	3.378	40.61	8.396
F(TFA) (%)		99.99		99.94		98.87

SOR – Significance of the regression, R^2 – Coefficient of multiple determination, LOF – Lack of fit, CQM – Complete quadratic model, TFA – Third order factor addition.

FIGURE LEGENDS

Fig. 1 – Total protein partition coefficient constant response contours as a function of [Sodium citrate] and [PEG 3350] for different concentrations of NaCl: a) 7.3%(w/w) b) 8.8% (w/w) c) 10.3 (w/w).

Fig. 2 – Total protein recovery constant response contours as a function of [Sodium citrate] and [PEG 3350] for different concentrations of NaCl: a) 7.3%(w/w) b) 8.8% (w/w) c) 10.3 (w/w).

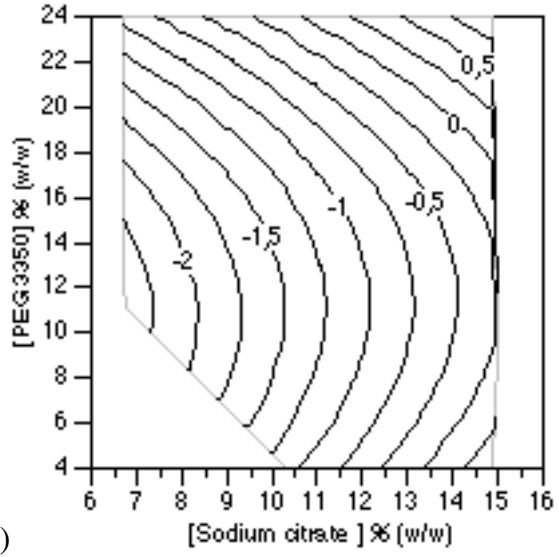
Fig. 3 – Penicillin acylase partition coefficient constant response contours as a function of [Sodium citrate] and [PEG 3350] for different concentrations of NaCl: a) 7.3%(w/w) b) 8.8% (w/w) c) 10.3 (w/w).

Fig. 4 –Penicillin acylase recovery constant response contours as a function of [Sodium citrate] and [PEG 3350] for different concentrations of NaCl: a) 7.3%(w/w) b) 8.8% (w/w) c) 10.3 (w/w).

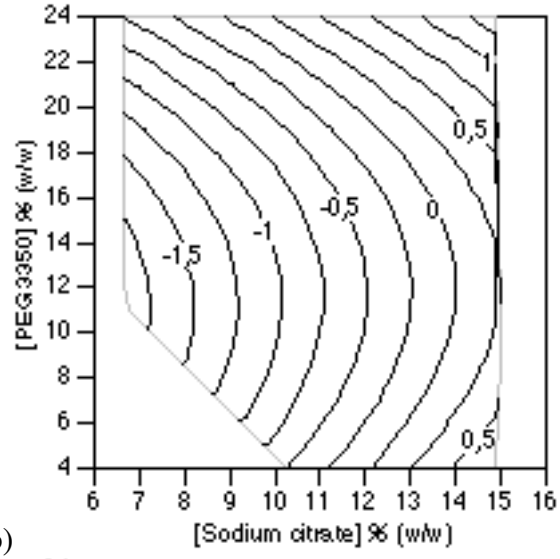
Fig. 5 – Purification factor constant response contours as a function of [Sodium citrate] and [PEG 3350] for different concentrations of NaCl: a) 7.3%(w/w) b) 8.8% (w/w) c) 10.3 (w/w).

Fig. 6 – Yield constant response contours as a function of [Sodium citrate] and [PEG 3350] for different concentrations of NaCl: a) 7.3%(w/w) b) 8.8% (w/w) c) 10.3 (w/w).

Fig. 7 – Constant response contours of purification factor (a) and yield (b) as a function of [Sodium citrate] and [PEG 3350] for [NaCl]=10.3% (p/p) showing the region where is possible to attain purification factor higher than 5.5 and yield higher than 80%.



a)



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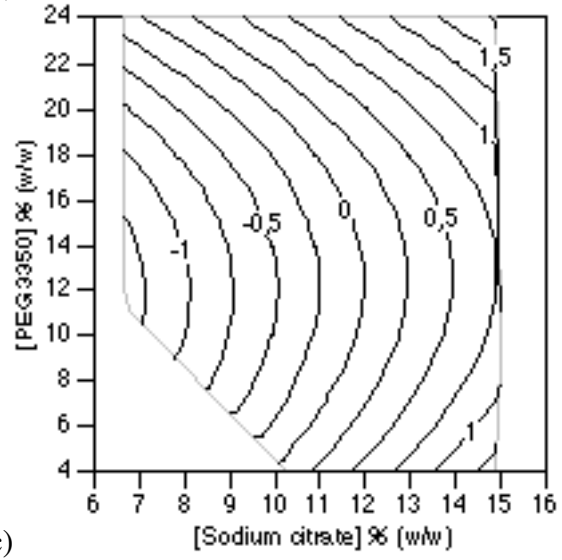
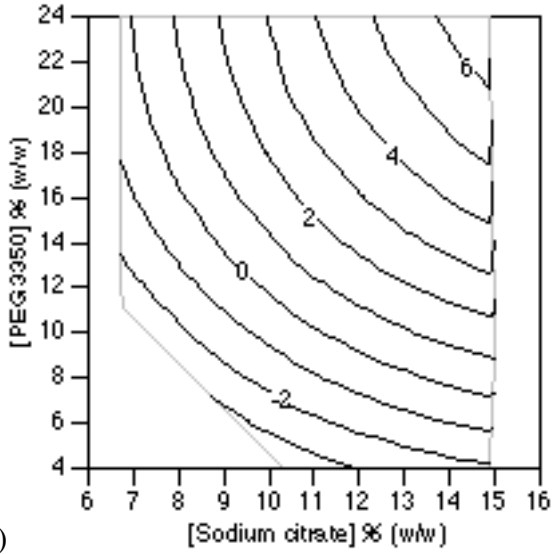


c)

Fig. 1 Marcos *et. al.*



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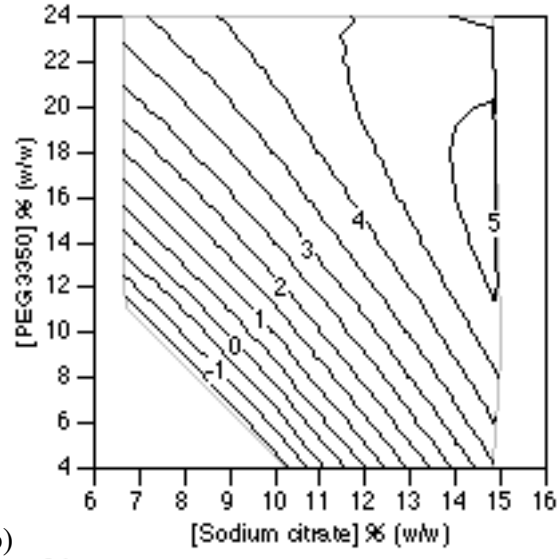

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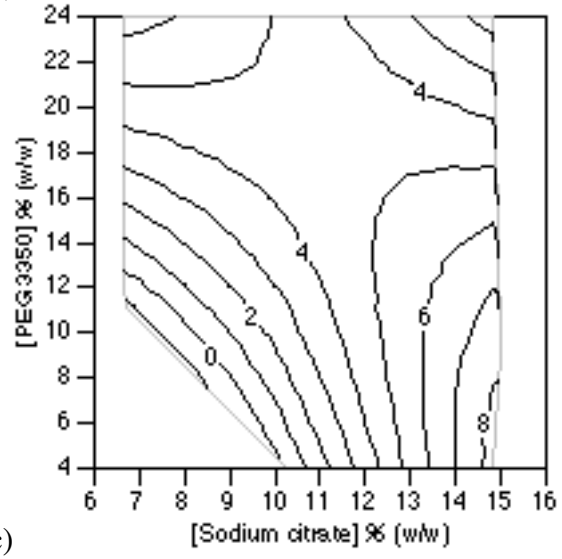
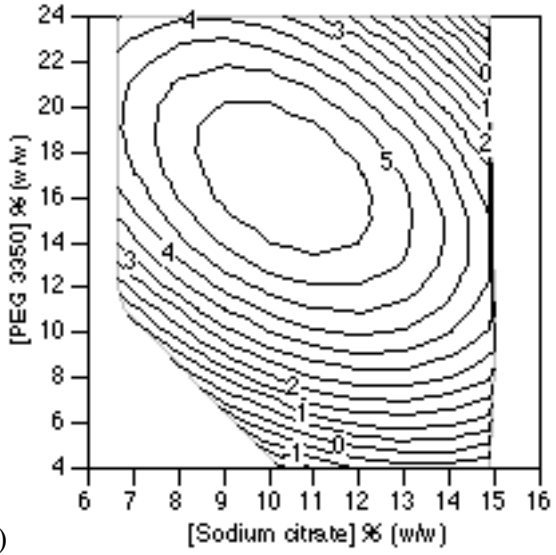


c)

Fig. 3 Marcos *et. al.*



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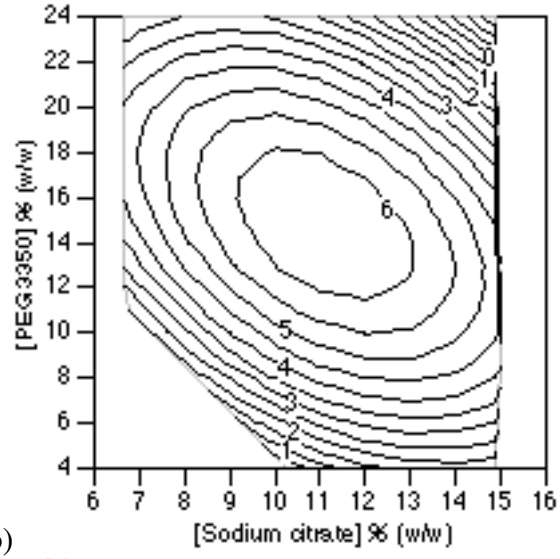

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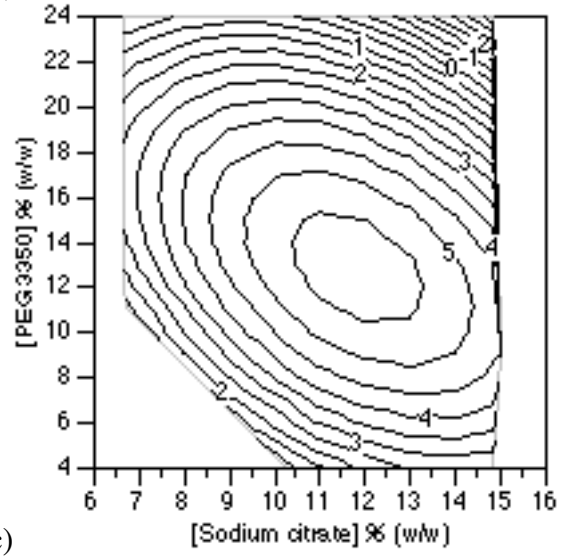
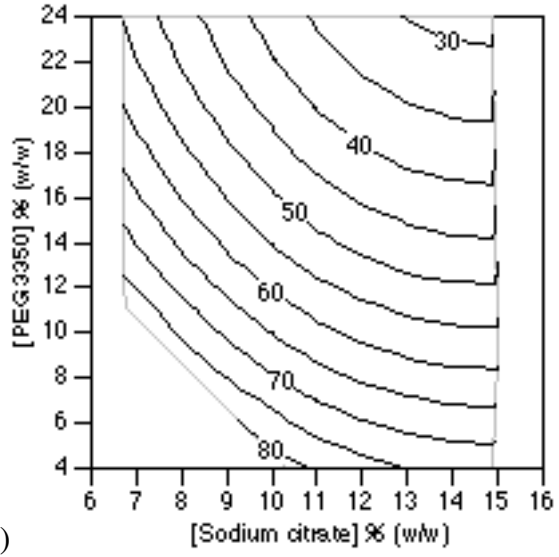


c)

Fig. 5 Marcos *et. al.*



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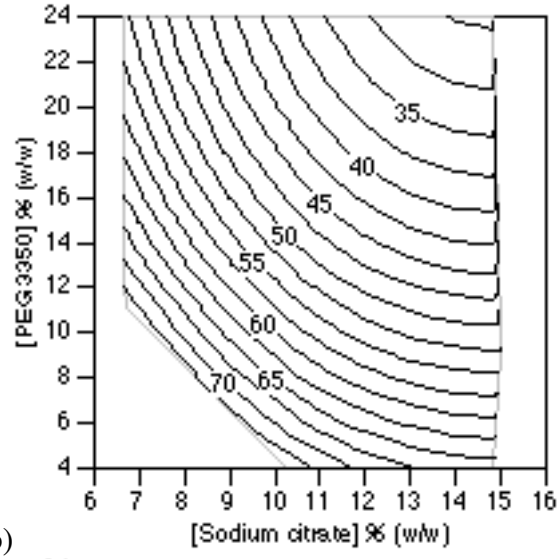

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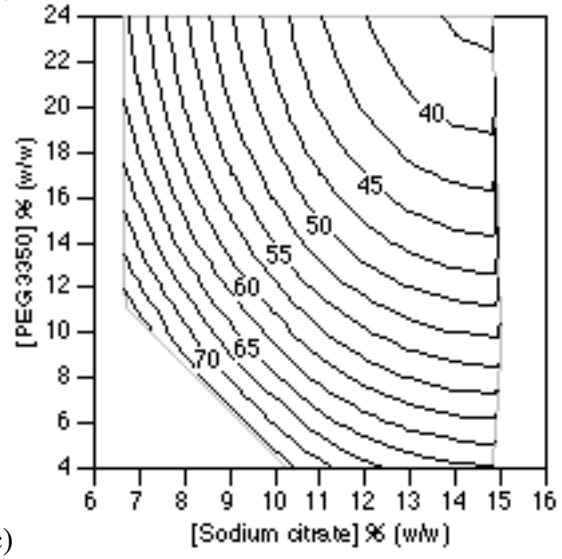
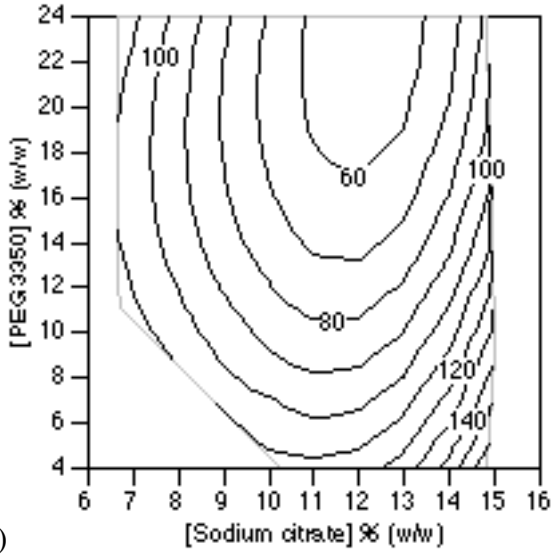


c)

Fig. 2 Marcos *et. al.*



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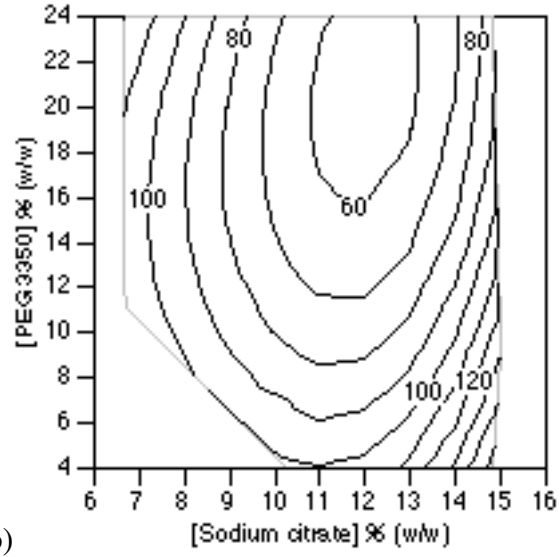

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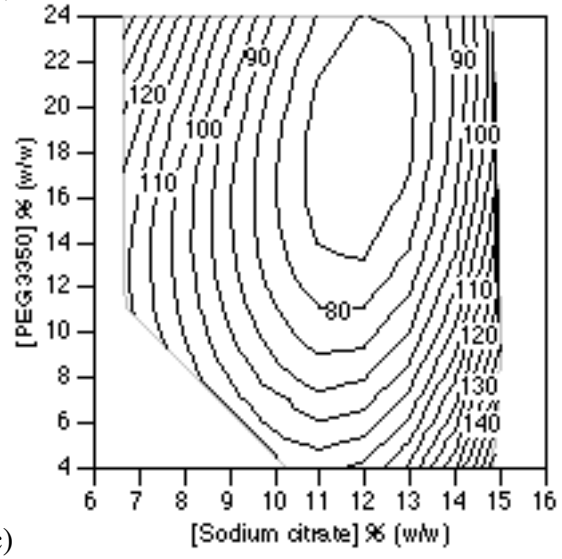
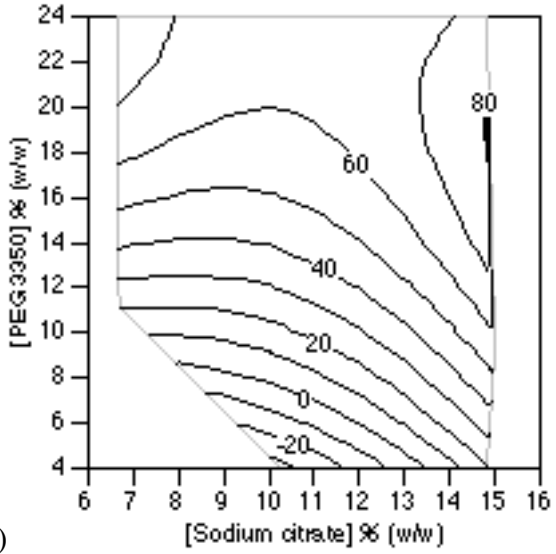


c)

Fig. 4 Marcos *et. al.*



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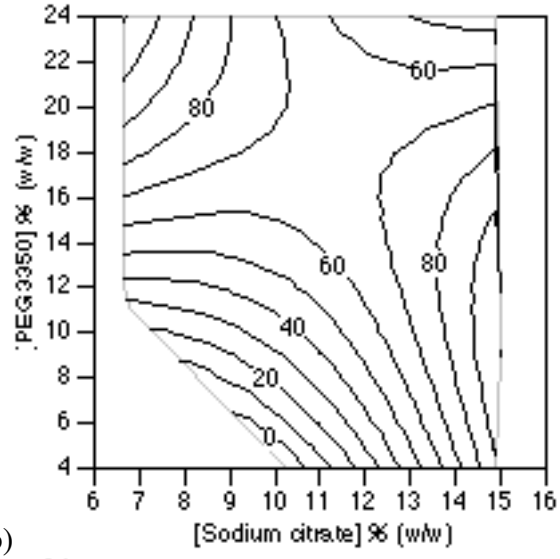

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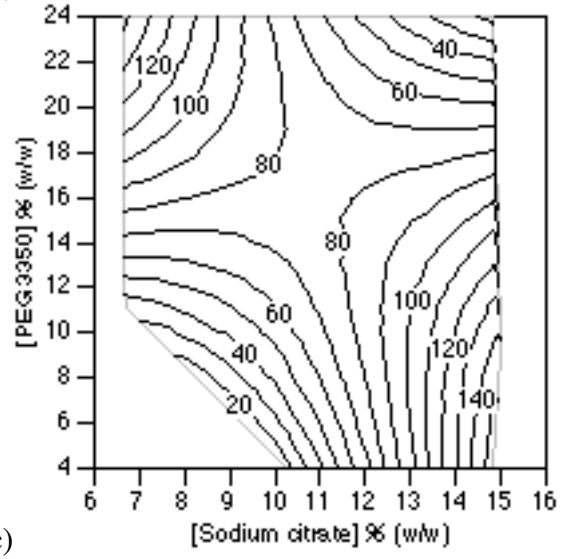
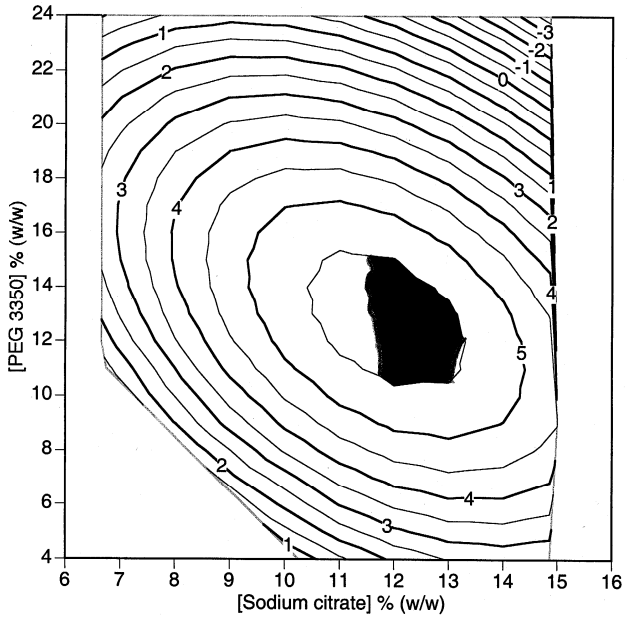


c)

Fig. 6 Marcos *et. al.*



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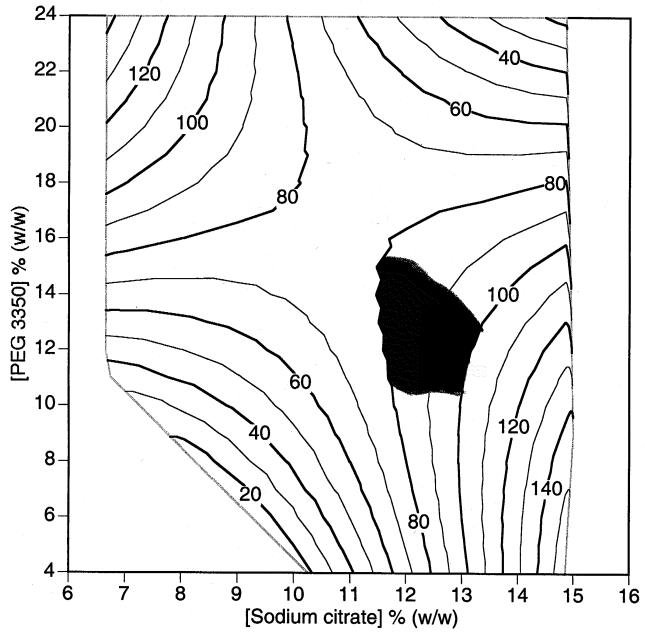

b)

Fig. 7 Marcos *et. al.*