



## Preparation and characterization of plant based lipophilic colorants as water soluble nanoparticle

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### Abstract

Response surface methodology (RSM) was used in this study to obtain empirically significant ( $p < 0.05$ ) models predicting the mean particle size, PDI of the prepared nanodispersions of alizarin (a plant based pigment), extracted from *Rubia tinctorum* L. roots, as a function of organic to aqueous phase addition rate, emulsifier concentration and polymer concentrations. The mentioned processing variables were then optimized to produce the nanodispersions with minimum particle size and maximum homogeneity (minimum PDI). The significant ( $p < 0.05$ ) correlation between the process parameters and physical characteristics of alizarin nanodispersions through second order polynomial models were obtained. It was demonstrated that mixing the organic phase containing alizarin and the aqueous phase containing Tween 20 as emulsifier in concentration of 3.5 ml and PVP as polymer with concentration of 240 mg with the rate of 0.45 ml/min would give the most desired alizarin nanodispersions with minimum particle size (45.15 nm) and PDI (0.1898). No significant differences observed between the experimental and predicted values of response variables, confirmed the adequacy of models.

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## Introduction

Textile coloring industries are known as an unsafe, allergic and environmental polluting process due to uses the synthetic colorants. Therefore, development of natural pigments especially with plant origins has been found great interest and consideration in recent researches (Santis and Moresi, 2007).

Common madder (*Rubia tinctorum L.*), is the most important and plant based colorant had been widely used from 2000 BC. It is a red coloring material extracted from the roots of the Rubiaceae plants, widely grown in all over the world, especially Middle East area (Santis and Moresi, 2007; Fabbri *et al.*, 2000). Madder contains a mixture of hydroxyanthraquinones, free or in the form of glycosides. The principal compound occurring in the dried root is alizarin, 1,2-dihydroxyanthraquinone, (Fig. 1), which have potential uses either as colorant in textiles or medical in the treatment of urinary tract infections and dissolution of bladder stones (Santis and Moresi, 2007; Angelini *et al.*, 1997). However, as most of other plant based pigments, it suffers from poor water solubility and consequently, considerable problems for water based coloring uses (Santis and Moresi, 2007; Angelini *et al.*, 1997). Thus, it is vital to increase the water solubility or dispersibility of these pigments before uses. Since, nanotechnology offers a worthy opportunity to increase the solubility of such colorant compounds, as the dissolution rate of the compound is proportional to the surface area. Moreover, the saturation solubility of a compound also increases with reductions in their particle sizes (Tan and Nakajima, 2005). Nanotechnology provides the development of nano-size delivery tools, known as nanodispersions, used for size reduction of functional lipid compounds, leading to considerably increase in their water solubility and disperibility (Tan and Nakajima, 2005; Anarjan *et al.*, 2010).

Nanodispersions are submicron colloidal systems which can be prepared through either bottom-up or top-down approaches (Saber *et al.*, 2013). The top-down methods which start with larger solid particles are capable of producing fine particles and can be

easily used on an industrial scale. However, these methods are time consuming and involve high energy input to the system leading to a considerable increase in their process costs (Tan and Nakajima, 2005; Anarjan *et al.*, 2010). In contrast, the bottom-up methods fabricate nanosized particles by starting at the atomic level. Therefore, they provide better control over size, morphology and crystallinity of nanodispersion systems in addition to consuming less energy during the process. In these methods, nanoparticles can be produced by either non-solvent addition to the system or solvent elimination through evaporation (Silva *et al.*, 2011; Thorat and Dalvi, 2012). This technique, which also known as nanoprecipitation method, is an easy and reproducible method involving the dispersion of preformed compounds, based on their interfacial deposition of following displacement of the semi-polar solvent miscible with water from the lipophilic solution (Silva *et al.*, 2011; Thorat and Dalvi, 2012). After mixing the solution and antisolvent and generation of super-saturation, the nucleation will be started in the system through solvent an evaporation step. Under uncontrolled conditions the fine particles produced will be grow by coagulation, condensation, or agglomeration. Therefore, to obtain nanoparticles with a narrow size distribution it is necessary to create a high degree of supersaturation, uniform spatial concentration distributions in solutions and restriction of the growth of the particles (Silva *et al.*, 2011; Zhao *et al.*, 2014).

Stabilizers are necessary during colloid preparation to limit crystal growth and particle agglomeration (McClements, 2004). They play a major role in the formation of nanodispersions in aqueous solutions by decreasing the interfacial tension between the functional lipid compounds and the water phases, reducing the amount of energy required to disrupt the droplets and leading to smaller size droplets. Moreover, they form a protective coating surrounding the droplets thus preventing coalescence (Thiele and von Lavern, 1965). Stabilizers can be either in small molecular or large molecular form such as polymers. Polyvinylpyrrolidone (PVP), is commercially available

surface active polymer, relatively inexpensive, well water-soluble, nontoxic, and very effective stabilizers for colloid preparation (Anarjan and Tan, 2013). It contains a long-carbon-chain backbone with different functional groups, where PVP contains pyrrolidone groups (McClements, 2004). Polyoxyethylene sorbitan mono-laurate (Tween20) is a non-ionic small molecular emulsifier that adsorbs quickly at the oil–water interface, and has shown good results in small particles for various applications (Cheong *et al.*, 2008).

Although considerable works have been conducted to produce the functional lipid food and medicine compounds' nanodispersions (Tan and Nakajima, 2005; Cheong *et al.*, 2008; Anarjan *et al.*, 2010; Anarjan and Tan, 2013), the researches on pigment nanodispersions for textile coloring uses are still limited. Therefore, the main objective of this work were to preparation of alizarin nanodispersions through a low energy solvent displacement technique and to study the effects of organic to aqueous phase addition rate (ml/min), Tween 20 concentration (ml) and polymer concentration (PVP) (mg) on the mean particle size, size distribution (PDI) of produced nanodispersions. The selected independent variables are then optimized to obtain the nanodispersions with the smallest particle size and PDI.

## Materials and methods

### Materials

Accession of *Rubia tinctorum* L. belonging to Rubiaceae family were collected during the autumn of 2012 at almost full growth of roots from wild populations (East Azerbaijan-Tabriz -Sardrood; 2200m). The plant materials were identified at the botany department of the medicinal plants research institute at tabriz university of medical sciences. Polyoxyethylene sorbitan monolaurate (Tween 20) and Ethanol (absolute,  $\geq 99.8\%$ ) were purchased from Merck (Merck Co. Darmstadt, Germany). Polyvinylpyrrolidone (PVP, K 30, Average Mw 40,000) was provided from Sigma–Aldrich.

### Soxhlet extraction

In classical Soxhlet extraction, 10 gr of *Rubia tinctorum* L was placed into a thimble and was extracted with 300 mL of ethanol. Root materials were separated, washed, cut in to small parts, air-dried, and ground to fine powder dust before extraction. Extraction was carried out for 1– 4 h with the approximate cycle of 7 cycles  $h^{-1}$ . The total concentration of Alizarin in final extracted sample was  $37.8 \pm 3.5$  mg/L.

### Preparation of Nanodispersions

Alizarin nanoparticles were formulated according to the solvent displacement technique. PVP and Tween 20 in designed concentrations (Table 1) were completely dissolved in aqueous phase. While magnetically stirring the aqueous phase (900 rpm), the ethanol extracted Alizarin with constant concentration (approximately 37.8 mg/L) was added with different rates to aqueous phase using syringe pump (SP1000, Nmp, Tehran, Iran), according to the experimental design (Table 1). The organic to aqueous volume phase ratio was set at 30:70. The mixture were then homogenized at 13000 rpm using an Ultra-Turrax homogenizer (SilentCrusher M, 26000, Heidolph, Germany), for 10 min. The acetone was then removed from the systems in vacuum rotary evaporator (Heidolph- Laborota 4010 HP digital, Germany) working at 200 kPa, 550 °C and 151 rpm.

### Characterization of Alizarin Nanodispersions

#### Particle Size Analysis

The prepared alizarin nanodispersions were characterized in terms of particle size and size distribution. Particle size and polydispersity index (PDI) measurements were performed using a dynamic light-scattering particle size analyzer (Zetasizer Nano ZS, Malvern Instruments, UK), on undiluted samples one day after the sample preparations. The dynamic light scattering technique measures the Brownian motion of particles in a system optically. According to Stokes–Einstein theory, particle motion is determined by the continuous phase viscosity, the temperature and the size of the particle (Anarjan *et al.*, 2010; Cheong *et al.*, 2008). Therefore, measurement of the particle

motion in a system at a known temperature and viscosity, can lead to the particle size determination of the nanodispersions produced. PDI is a measure of the width of the distribution ranging from 0 (mono dispersed) to 1 (very broad distributed) (Anarjan *et al.*, 2010; Bezerra *et al.*, 2008).

#### Experimental Design and Statistical Analysis

Response surface methodology (RSM) using a central composite design (CCD) with three independent variables, namely, Addition rate (1-5 ml/min), Tween20 concentration (1-5 ml) and polymer concentration (100-500mg), was applied for determination the least mean particle size ( $Y_1$ ) and PDI ( $Y_2$ ) of the alizarin nanodispersions. Due to advantages of RSM over classical one-variable-at-time optimization, such as the generation of large amounts of information from a small number of experiments and the possibility of evaluating the interaction effect between the variables on the response, it is a suitable procedure to assess the relationships between the studied responses and process variables and to optimize them in order to gain the desired characteristics of the product (de Carvalho *et al.*, 2013). Each independent parameter was studied at five different levels, namely, central point ( $X_1$ : 2.55 ml/min,  $X_2$ : 3ml,  $X_3$ : 300 mg), level -1 ( $X_1$ :0.1 ml/min,  $X_2$ :1 ml,  $X_3$ :100 mg), level 1 ( $X_1$ :5 ml/min,  $X_2$ :5 ml,  $X_3$ :500 mg), level  $-\alpha$  ( $X_1$ :1 ml/min,  $x_2$ :1.8 ml,  $X_3$ :4.2 mg) and level  $+\alpha$  ( $X_1$ :4 ml/min,  $X_2$ :4.2 ml,  $X_3$ :422 mg).  $\alpha$  value (1.682) was obtained from the equation  $\pm \sqrt{3}$  for  $k = 3$  (three independent variables). Five replicates were performed at a central point for estimation of the pure error (Montgomery, 2012). Therefore, a total of 20 experiments, including 8 cube points (levels  $\pm 1$ ), 6 axial points (levels  $\pm \alpha$ ), and 6 central points were created using the software Minitab v. 16 statistical package (Minitab Inc., PA, USA) (Table 1). A second order polynomial equation (Eq.1) was used to express the mean particle size ( $Y_1$ ), PDI ( $Y_2$ ) of the nanodispersions as a function of the studied mixing variables.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad \text{Eq. 1}$$

where  $Y$  is the response variable,  $\beta_0$  is a constant,  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  represent the linear terms,  $\beta_{11}$ ,  $\beta_{22}$ ,  $\beta_{33}$  correspond to the quadratic terms and  $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{23}$ , represents the interaction terms. The adequacy of model was examined accounting for the coefficient of determination ( $R^2$ ) and adjusted the coefficient of determination ( $R^2$ -adj). To fit the second order polynomial equation, analysis of variance (ANOVA) was used to analyze the experimental data by multiple linear regressions, and the statistical significance of each regression term was evaluated using the. P value and t value from the pure error obtained from replicates at the central point. The p values lower than 0.05 were considered to be statistically significant. For the graphical analysis of the independent variable interactions, three dimension surface plots and two dimension contour plots were obtained from the fitted polynomial equations and used successfully (Gharibzahedi *et al.*, 2012). The surface plots were used to explain clearly the interactive effects of the independent variables with the response variable (Anarjan *et al.*, 2010). It should be noted that the presented correlation is valid only within the range of variables investigated. Graphical and numerical multiple response optimizations were used to determine the optimum levels of mixing addition rate, Tween20 concentration and polymer concentration to attain the desired response goals (Anarjan *et al.*, 2011). In fact, optimal conditions were determined by evaluating the obtained surface plots with restrictions on the responses of a minimum value for mean particle size and PDI. Subsequently, five additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies (Anarjan *et al.*, 2010; Anarjan *et al.*, 2011).

## Results and discussions

### Fitting the Response Surface Models

The responses obtained from the experimental runs, which are shown in Table 1, were fitted to second order polynomial models by applying multiple regression analysis using RSM for studying selected three process variables. Therefore, the estimated regression coefficients, for initial and reduced

models, as well as the corresponding significance of regressions are shown in Table 2. The reduced models were obtained after removing the insignificant terms. However, the insignificant main effect of parameters should be kept in the reduced model due to their either quadratic or interaction significant ( $p < 0.05$ ) effects (Tables 2, 3) (Anarjan *et al.*, 2010). F ratio and p value of each term in suggested models, which provide their significance determinations, can also be seen in Table 3. It should be considered that in significance determination of terms, lower p value and higher F ratio corresponds to a higher significance of a term on studied response variations'. Moreover, the suggested models might be significant ( $p < 0.05$ ) only in the studied independent variable levels and they may not be extrapolated outside these ranges (Anarjan *et al.*, 2010; Anarjan *et al.*, 2011). Since the coefficients of determinations ( $R^2$  and

adjusted  $R^2$ ) are good measure for overall model performance, their obtained relatively high values confirmed the suitability of suggested models. Furthermore, attained non-significant lack of fits for suggested models ensured their adequate fitness to the independent variable effects (Table 2). As shown in Table 3, all selected independent parameters had significant ( $p < 0.05$ ) effects on both studied characteristics of produced nanodispersions. While the quadratic effect of polymer concentration had the most significant ( $p < 0.05$ ) effect on mean particle size variations, main effect of Tween 20 concentration was affected the PDI changes, the most considerably, as compared to other process variables. As stated before, insignificant main effects of processing conditions were kept in reduced model due to their quadratic or interaction significant ( $p < 0.05$ ) effects.

**Table 1.** Central composite design (CCD) and response variables for processing of alizarin nanodispersions.

Sample number	Addition rate (ml/min)	Tween20 concentration (ml)	Polymer concentration (mg)	Particle size (nm)		PDI	
				Exp <sup>a</sup>	Pre <sup>b</sup>	Exp <sup>a</sup>	Pre <sup>b</sup>
1	4.0	4.2	422	70.00	96.928	0.322	0.272
2	1.0	4.2	178	31.16	62.324	0.258	0.216
3	1.0	1.8	422	168.00	178.377	0.250	0.253
4	2.55	3.0	300	66.36	62.845	0.180	0.182
5	2.55	3.0	300	66.00	62.845	0.177	0.182
6	4.0	1.8	178	31.69	28.403	0.628	0.583
7	4.0	1.8	422	137.50	133.022	0.266	0.293
8	4.0	4.2	178	118.40	94.244	0.241	0.223
9	2.55	3.0	300	66.50	62.845	0.185	0.182
10	1.0	4.2	422	75.50	65.008	0.420	0.450
11	1.0	1.8	178	74.00	73.758	0.323	0.358
12	2.55	3.0	300	66.40	62.845	0.184	0.182
13	0.1	3.0	300	92.71	68.330	0.194	0.163
14	2.55	1.0	300	59.90	82.248	0.537	0.517
15	5.0	3.0	300	59.49	57.132	0.167	0.203
16	2.55	3.0	300	67.00	62.845	0.173	0.182
17	2.55	3.0	300	66.00	62.845	0.185	0.182
18	2.55	5.0	300	34.00	43.442	0.342	0.384
19	2.55	3.0	500	193.70	183.086	0.302	0.288
20	2.55	3.0	100	94.54	95.473	0.299	0.334

<sup>a</sup> Experimental values of studied responses

<sup>b</sup> Predicted values of studied responses.

### Mean Particle Size

Mean particle sizes of the obtained alizarin nanodispersions ranged from 31.16 to 193.70 nm as shown in Table 1. In all cases, a mono-modal size distribution was obtained. Therefore, it is possible to prepare alizarin nanodispersions in moderate mixing conditions without applying high energy to the system via any high shear or high pressure homogenization. The high influence of organic to aqueous phase addition rate on particle sizes produced nanodispersions have been reported in various researches, since it affects the rate and supersaturation, and consequently the nucleation rate and particle growth kinetics of these systems (Joye and McClements, 2013; Chu *et al.*, 2007). The negative single effect of addition rate and its insignificant quadratic effect confirmed the contrary changes of particle size with addition rate in studied system. Therefore, the fast addition of organic to aqueous phase led to production of smaller particles.

Decreasing the particle size with increasing the flow rate of solvent into anti-solvent have also been reported by some previous studies. It can be related to occurrence of more efficient mixing; By increasing the addition rate, the jet velocity and shear force will be increased, which increases the mixing efficiency and leads to a decline of particle size (Joye and McClements, 2013). The size decrease in slow phase addition rate at high levels of emulsifier concentrations (Fig 2a) can be related to enough mixing time providing for system to complete coverage of freshly produced nanoparticles and preventing their re-attachments to each other due to their electrostatic repulsive forces. At less addition rate, the phases underwent longer mixing time and consequently greater tendency to aggregate, due to their higher susceptibility to Brownian motion and more chance of collision, coagulation and aggregation, especially in lack of emulsifier conditions (Mora-Huertas *et al.*, 2011).

**Table 2.** Regression coefficients, R<sup>2</sup>, adjusted R<sup>2</sup> (R<sup>2</sup>-adj) and probability values for the final reduced models.

Regression Coefficient	Particle Size (nm)		PDI	
	Initial Model	Reduced Model	Initial Model	Reduced Model
β <sub>0</sub> (constant)	50.2992	131.523	1.28726	1.27871
β <sub>1</sub> (main effect)	-23.7524	-34.484	0.16968	0.17599
β <sub>2</sub> (main effect)	46.8891	14.435	-0.53011	-0.52921
β <sub>3</sub> (main effect)	-0.2638	-0.418	-0.00311	-0.00310
B <sub>11</sub> (quadratic effect)	1.2084	-	0.00125	-
β <sub>22</sub> (quadratic effect)	-5.4091	-	0.06716	0.06701
β <sub>33</sub> (quadratic effect)	0.0019	0.002	0.00000	0.00000
β <sub>12</sub> (interaction effect)	10.7327	10.733	-0.03028	-0.03028
β <sub>13</sub> (interaction effect)	-0.0562	-	-0.00026	-0.00026
β <sub>23</sub> (interaction effect)	-0.1699	-0.170	0.00057	0.00057
R <sup>2</sup> (%)	94.03	88.74	94.87	94.84
R <sup>2</sup> -adj (%)	88.66	83.54	90.26	91.08
p value (regression)	0.000	0.000	0.000	0.000

β<sub>0</sub> is a constant, β<sub>i</sub>, β<sub>ii</sub> and β<sub>ij</sub> are the linear, quadratic and interaction coefficients of the quadratic polynomial equation, respectively; 1: Addition rate 2: Tween20 concentration 3: Polymer concentration.

It was also shown that neither single nor quadratic effects of Tween 20 concentration in studied ranges were not significant (p>0.05) on particle size variation. But this response was influenced affectively by interaction of emulsifier concentration with addition rate and PVP concentrations (Fig. 2a and

Fig. 2b). Depends on the role of surface active compounds in nanoparticle formation and growth, these agents are found as critical factors for ensuring the nanodispersions' stabilities. Therefore, finding their best concentrations have been considered in related studies. The insignificant effect of emulsifier

concentrations have already seen in prepared nanodispersions via solvent displacement technique (Joye and McClements, 2013; Sinha *et al.*, 2013).

Particle size reduction can be seen by increasing the emulsifier concentration in low concentrations of used polymer. However, at very higher Tween 20 concentrations (especially in upper levels of polymer

concentration) this effect was inverted, as seen in Fig. 2b, because the exceeded polymer or emulsifier concentrations from critical flocculation concentration can promote the particle aggregation through a depletion flocculation mechanism (Joye and McClements, 2013; Chu *et al.*, 2007; Kanafusa *et al.*, 2007).

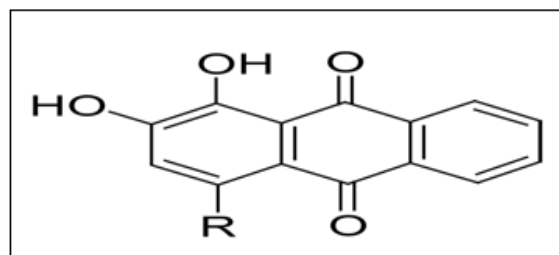
**Table 3.** The significance probability (p value, F ratio) of regression coefficients in final reduced second order polynomial models.

Main effects		Main effects			Quadratic effects			Interacted effects		
		$x_1$	$x_2$	$x_3$	$x_1^2$	$x_2^2$	$x_3^2$	$x_1x_2$	$x_1x_3$	$x_2x_3$
Mean particle size ( $Y_1$ , nm)	p value (initial model)	0.149	0.044	0.224	0.511	0.068	0.000	0.003	0.075	0.001
	F ratio (initial model)	2.44	5.30	1.68	0.45	4.19	51.11	14.42	3.96	25.10
	p value (reduced model)	0.007	0.366 <sup>ns</sup>	0.092 <sup>ns</sup>	ns	ns	0.000	0.008	ns	0.001
	F ratio (reduced model)	10.38	0.88	3.32	ns	ns	36.46	9.94	ns	17.29
PDI ( $Y_2$ )	p value (initial model)	0.002	0.000	0.000	0.801	0.000	0.001	0.003	0.007	0.000
	F ratio (initial model)	16.89	91.88	31.66	0.07	87.71	20.43	15.57	11.21	37.66
	p value (reduced model)	0.000	0.000	0.000	ns	0.000	0.000	0.002	0.005	0.000
	F ratio (reduced model)	30.44	100.45	34.53	ns	56.31	96.03	17.02	12.25	41.15

ns: Not significant.

It was also observed that the PVP concentration affected the particle size positively in quadratic form. Thus increasing the PVP concentrations, especially in high levels, resulted the production of nanodispersion systems with considerable large particles. It is apparent that the nanoparticle formation via solvent displacement process is extremely sensitive to used polymer concentration. In concentrated aqueous solutions due to high levels of PVP, the mixing efficiency can be decreased, Ostwald ripening will be increased due to higher viscosity of systems and the particle growth rate will be increased. The increasing the viscosity of system by polymer concentrations might be explained by the increase in polymer chain association as the polymer concentration increases.

Polymer chain association could govern nucleation and growth rates in solvent displacement processes. Moreover, rapid solvent diffusion into the aqueous phase could be delayed in high viscose systems (Anarjan *et al.*, 2011; Mora-Huertas *et al.*, 2011).



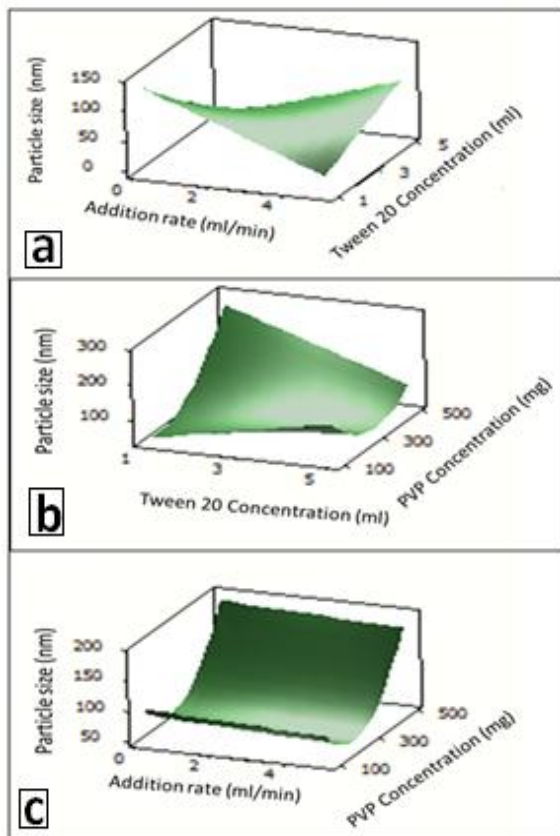
**Fig. 1.** Molecular structures of alizarin (R = H) [3].

No seen significant interacted effect in Fig 2c, were also confirmed by data in Table 3 (higher p value and



low F ratio of interaction term of addition rate and PVP concentrations).

The individual optimum optimization procedure predicted that the minimum average particle size ( $Y_1 = 29.5$  nm) would be obtained at addition rate and concentrations of 3 ml/min, 1 ml and 150 mg for rate of organic phase addition, Tween 20 and polymer concentrations, respectively.



**Fig. 2.** Response surface plots for mean particle size of alizarin nanodispersions as function of significant ( $p < 0.05$ ) interaction effects between component.

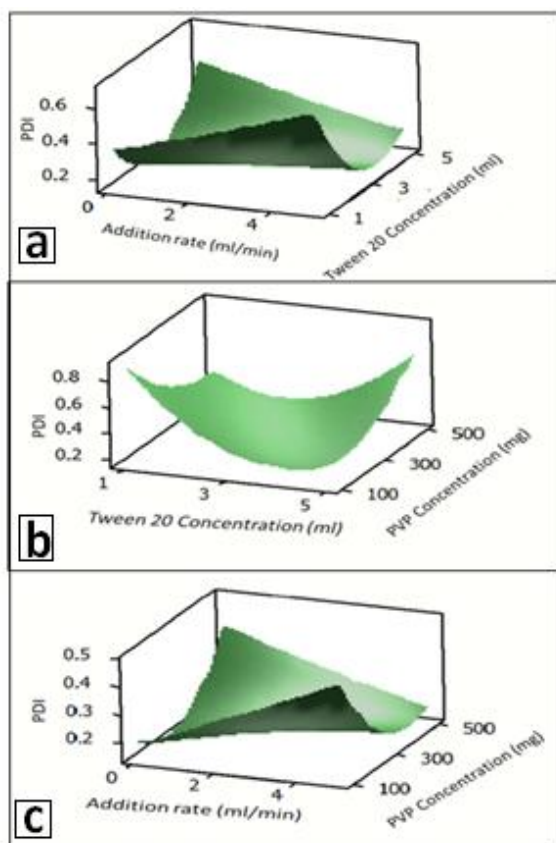
#### *Polydispersity index (PDI)*

The results also confirmed the significant effects ( $p < 0.05$ ) of selected independent parameters on PDI ( $Y_2$ ) variations of produced nanodispersions (Tables 2, 3). Thus, the PDI changes could be explained as a function of addition rate, Tween 20 concentration and used polymer concentration with acceptable determination of coefficients ( $> 90\%$ ) either in initial or reduced models. Therefore, more than 90% of PDI variations could be predicted by suggested initial or reduced models in this study. The positive main effect

and insignificant quadratic effect of addition rate on PDI revealed that the heterogeneity of system was increased by rapid addition of solvent to aqueous phase. It was concluded that an increase in the flow rate of solvent to non-solvent, reduces their mixing time, resulting rapid and uneven supersaturation and causing the PDI values of system (Sinha *et al.*, 2013). The results also showed the negative main and positive quadratic effects of emulsifier and polymer concentrations on PDI variations of produced nanodispersions (Tables 2, 3). The comparison of p value and F ratio of these terms indicated that the main effect of Tween 20 and the quadratic effect of PVP concentrations were the most significant on PDI changes. It can be observed that at low stabilizer concentrations, increasing the Tween 20 led to production of uniform nanodispersions with less PDI values. However, at high stabilizer concentrations, further increases of either emulsifier or polymer (especially polymer) increased the heterogeneity of system considerably. The probability of micelle production is considerably increased in multiple stabilizer systems; in these systems, the transition from a surfactant-poor regime to a surfactant-rich regime could occur at low Tween20 concentrations. Therefore, further increases in Tween20 concentration caused heterogeneity in the particle size and so increased the PDI. Several previous studies have reported different results for the effect of Tween 20 concentrations on PDI; Chu *et al.*, (2007) and Mainardes and Evangelista, (2005) reported that the PDI was reduced by increasing the Tween 20 concentration. As mentioned before, these differences in results may be related to different nature of the emulsifier systems used to stabilize the emulsion systems in the different studies. As clearly can be observed in (Fig 3a), since the middle concentrations of Tween 20 at all studied addition rates resulted acceptable uniform nanodispersions ( $PDI < 0.20$ ), using either low concentrations of emulsifier at rapid rates of solvent to non-solvent additions, or high emulsion concentrations in slow phases addition rates led to considerable increase in PDI. The positive interaction of polymer and Tween 20 concentrations showed that simultaneously increasing of polymer



and Tween 20 could increase the heterogeneity of system (Fig 3b). Therefore, the most even and consequently physically stable nanodispersions would be attained at middle levels of emulsifier and polymer concentrations. Fig 3b illustrated that using polymer in middle concentrations also caused the uniform system at all addition rates which was previously found for interaction of Tween concentration with addition rate. Therefore, it can be predicted that minimum values for PDI of produced nanodispersions fell in slow addition rates and medium stabilizer concentrations. The individual optimum conditions indicated that the minimum PDI ( $Y_2 = 0.150$ ) was predicted as being obtained by addition rate and tween 20 concentration and polymer concentration 0.1 ml/min, 3 ml and 220 mg, respectively.

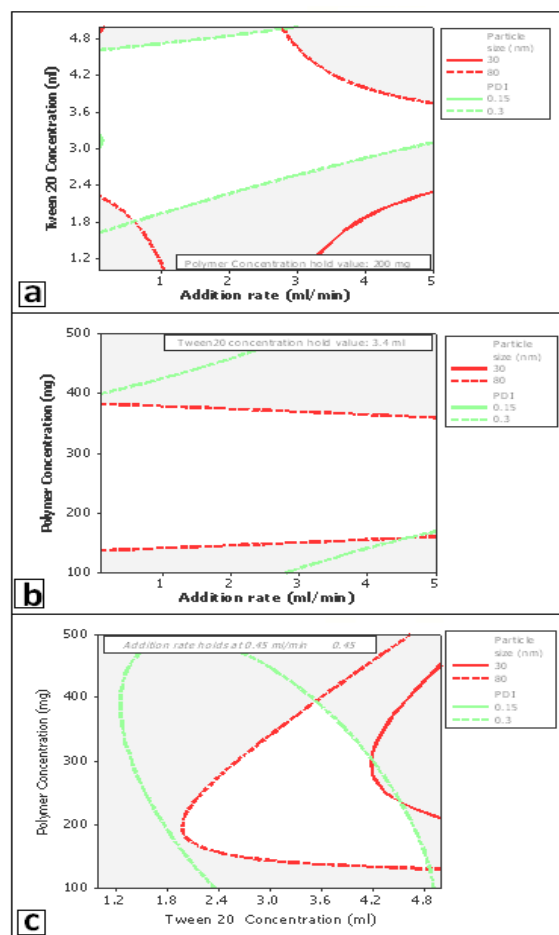


**Fig. 3.** Contour plots for mean particle size of alizarin nanodispersions as function of significant ( $p < 0.05$ ) interaction effects between component.

#### Optimization of Processing Parameters for the Production of Alizarin Nanodispersions

The alizarin nanodispersions would be considered as optimum product if the process resulted in the

smallest mean particle size and PDI. Therefore, an overlaid contour plot as a graphical optimization approach was used to find the optimum region for mixing variables in order to produce alizarin nanodispersions with the minimum particle size and PDI (Fig. 4). In overlaid contour plots minimum and mean values of responses found from experiments were considered as their goal and maximum acceptable values, respectively.

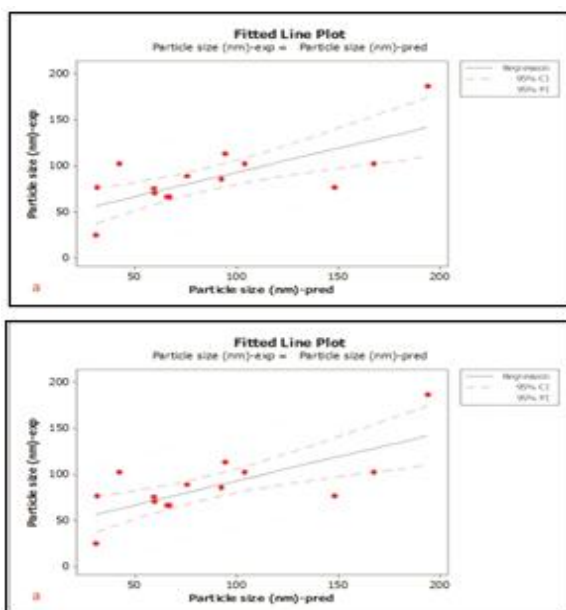


**Fig. 4.** Overlaid contour plot of particle size, PDI with acceptable levels as function of solvent to nonsolvent addition rate, emulsifier and polymer concentrations.

The white area in Fig. 4a-c, indicated the desired addition rate, Tween 20 and PVP concentration levels to get the optimum nanodispersion products. The most desirable products were obtained in the addition rate of 0.1-3 ml/min, Tween 20 concentration of 2 - 3.6 ml and polymer concentration of 150-350 mg.

Numerical multiple optimization was also performed

to find the exact optimum levels of studied processing variables. It was shown that addition of solvent to nonsolvent phases with the rate of 0.45ml/min, and using 3.5 ml Tween 20 as small molecular emulsifier and 240 mg PVP as surface active protein can produce the optimum alizarin nanodispersions with mean particle size and PDI of 45.15 nm and 0.1898 respectively, with composite desirability of 0.73.



**Fig. 5.** Fitted line plots between the experimental and predicted values of mean particle size (a) and PDI (b).

The adequacy of the regression equations obtained was checked by either plotting the experimental values versus predicted ones using final reduced models, or preparation the final optimum suggested alizarin nanodispersions in triplicate and comparing their experimental mean particle size and PDI to predicted ones using one sample t-test. The measured mean particle size and PDI were  $50.63 \pm 5.55$  and  $0.1943 \pm 0.0261$ . The attained p value of performed t-test ( $p \text{ value} = 0.492 > 0.05$ ) confirmed the suitability of obtained polynomial models connected the studied independent and response variables. Moreover, as shown in Fig. 5, the obtained linear plots with intercepts of zero and slope of one holding relatively high  $R^2$  values ( $> 0.78$ ), re-confirmed the adequacy of reduced models.

## Conclusion

The effects of the most important processing

parameters, namely, the organic to aqueous addition rate, emulsifier concentration and polymer concentration on the mean particle size and PDI of alizarin nanodispersions prepared via solvent displacement technique was considered, studied and described in this work. Alizarin was extracted in classical Soxhlet extraction from (*Rubia tinctorum .L*) using acetone in final concentration of 37.8 mg/L. Analysis of variance (ANOVA) presented high coefficient of determinations  $R^2 > 90\%$  for suggested reduced second order polynomial regression models. The results showed that since all selected process parameters influenced the characterizations of nanodispersions considerably ( $p < 0.05$ ), the quadratic effect of polymer concentration and main effect of emulsifier concentration were the most significant (with highest F ratio) on mean particle size and PDI variations, respectively. The multiple optimization procedure designated that the overall optimum processing region for producing the most desired alizarin nanodispersions was obtained by organic to aqueous phase addition rate of 0.45 ml/min and using Tween 20 and PVP in concentrations of 3.5 ml and 240 mg, correspondingly. The experimental values were obtained in good agreement with the predicted values by achieved regression models.

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