Modelling and optimising of physicochemical features of walnut-oil beverage emulsions by implementation of response surface methodology: Effect of preparation conditions on emulsion stability

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Article info

Article history:
Received 30 July 2013
Accepted 21 October 2014
Available online 28 October 2014

Chemical compounds studied in this article:
Tween 80 (PubChem CID: 6364656)
Span 80 (PubChem CID: 6433271)
Isooctane (PubChem CID: 10907)
Isopropanol (PubChem CID: 3776)
Acetic acid (PubChem CID: 176)
Chloroform (PubChem CID: 6212)
Potassium iodide (PubChem CID: 4875)
Sodium thiosulfate (PubChem CID: 24477)

Keywords:
Emulsion stability
Emulsifier
Response surface methodology
Ultrasonic homogenisation
Walnut oil

1. Introduction

Walnut fruit, the seed of Juglans regia L., is a member of Juglandaceae family (Zhang, Liao, Moore, Wu, & Wang, 2009). Walnut trees are widely dispersed throughout the world (Charibzahedi, Mousavi, Hamedi, & Khodaiyan, 2011). Dried ripe walnuts are known to have high nutritious value, due to the presence of abundant phospholipids, proteins, tocopherols and fatty acids. Walnut oil (WO) is mainly composed of linoleic acid followed by oleic, linolenic, palmitic, and stearic acids (Martinez, Mattea, & Maestri, 2008). High concentrations of polyunsaturated fatty acids, such as those found in WO, have been documented to reduce the risk of coronary heart diseases (Pereira et al., 2008). High amounts of natural antioxidants in walnut and its oil have a protective effect against certain types of cancer (Zhang et al., 2009). One possible way to increase consumption of WO is to supply it in the form of walnut juice or other juices and beverages. Emulsion systems are mixtures of two immiscible liquids: one constituting an internal phase in the form of droplets dispersed through the other, which constitutes an external or continuous phase (Tadros, 2005). Emulsions are applied in food, pharmaceutical and personal-care industries and most of them can be placed in one of two broad categories: oil in water (including milk, salad dressing, ice cream mix, etc.) or water in oil (such as butter, margarine, and chocolate) (Coupland & McClements, 2001). Beverage emulsions are unique forms of oil-in-water emulsions. They are prepared in a concentrate form and dilute to prepare a final product. Both concentrate and diluted forms of the beverage must exhibit high stability (Buffo, Reineccius, & Oehlert, 2001). Emulsions do not form spontaneously: they are kinetically stable systems without thermodynamic stability (Borwanker, Lobo, & Wasan, 1992). Emulsion instability...
during storage is a consequence of assorted physicochemical mechanisms including creaming, coalescence, flocculation, and Ostwald ripening (Harmslaiwat, Pongsawatmanit, & McClement, 2006). Emulsion stability can be improved by using a strongly stabilising emulsifier (Sun, Gunasekaran, & Richards, 2007). There are three main groups of emulsifiers applied in beverage emulsion stability including polysaccharides, proteins, and small molecule surfactants. Polysaccharides produce emulsions with high stability against environmental stress during storage but have large particle size. Proteins compared to polysaccharides can produce emulsions with smaller particle size but cannot prevent droplet aggregation during storage than polysaccharides (Qian, Decker, Xiao, & McClements, 2011). Polysorbates and Sorbitanesters are classes of nonionic surface-active materials applied in food industries (Hasenhuettl & Hartel, 2008). They can generate emulsions with small particle size and high stability during storage and are widely used in food industries due to their availability, safety, and acceptable cost (Leong, Wooster, Kentish, & Ashokkumar, 2009).

In emulsions, the particle size of dispersed phase plays an important role in determining stability. A reduction in particle size leads to postpone gravitational separation. Because according to Stocks’ law, the velocity of droplet movements is proportional to the square of the droplet radius. It can be resulted that the tendency of the large fat globules to coalescence is greater than the small ones (McClements, 2005). So by reducing the average particle size to nano dimension, we can increase the stability and therefore shelf life of beverage emulsions. It is demonstrated that nano-emulsions with particle size range between 100 and 500 nm has high stability during storage condition (Constantinides, Chaubal, & Shorr, 2008). Production of beverage emulsions based on walnut oil was studied by Gharibzahedi, Mousavi, Hamedi, and Ghasemlou (2012). They could model and optimise the physicochemical characterisation of walnut oil beverage under defined conditions but their emulsion particles were in micron size. It was exhibit that optimisation of emulsions based on the walnut oil allows the manufacturers to modify the process to produce beverage with high quality. The main objective of the current study is to evaluate, model and optimise the preparation conditions for producing an emulsion of walnut oil in water in nano scale by response surface methodology (RSM). RSM is a valuable group of statistical, mathematical, and empirical techniques to generate models determining optimum conditions in the presence of complex interactions. In this study the effects of ultrasonic time (UT, 5–15 min), walnut-oil content (WO, 4–10% w/w) and Span 80 content (S80, 0.55–0.8) on the particle size, emulsion stability, turbidity loss rate, size index, viscosity and peroxide value changes of prepared walnut-oil were investigated.

2. Materials and methods

2.1. Materials

Cold press WO (with the purity of more than 98%, the chemical composition of C16:0: 7.55%, C16:1: 0.22%, C18:0: 2.71%, C18:1: 23.34%, C18:2: 52.91% and C18:3: 13.27%, density of 920.3 kg/m³ and viscosity of 74.2 cP) was purchased from Zeith Co. (Kerman, Iran). Polyoxyethylene (20) sorbitanmonooleate (Tween 80) was purchased from Merck (Darmstadt, Germany). Sorbitanmonooelate (Span 80) was a gift from Jihad Daneshgahi of Tehran University (Tehran, Iran). In this study reverse osmosis is applied for preparing deionised water.

2.2. Preparation of walnut oil emulsion

In current study twenty emulsion samples were prepared. Composition of beverage emulsions were deionised water, WO (4–10% w/w), Tween 80 (1% w/w), S80 (0.5–0.8% w/w), and sodium azide (0.01% w/w). For the preparation of water phase, Tween 80 was added to deionised water and mixed for 5 min (450 rpm) by a blender (IKA-WERK, RK 20 DZM, Staufen, Germany). Mixing process is continued until Tween 80 is completely dissolved in deionised water and then sodium azide (0.01% w/w) was added to emulsifier solution as an antimicrobial agent. Dispersed phase was prepared by adding S80 to cold press WO. The pre-emulsion was prepared in two stages: at first the dispersed phase was added gradually into aqueous phase and mixed for 10 min using the same blender. In the second stage, to achieve fine emulsification, pre-homogenisation was performed using a high shear blender (IKA_WERK, ultra Terrax, T25 basic, Staufen, Germany) for 5 min at a low shear rate (6500 rpm) to avoid excessive foaming. Subsequently, the final pre-emulsion was subjected to ultrasonic homogeniser (UP200S, Hielscher Ultrasonic GmbH, Teltow, Germany) equipped with an 8-mm-diameter ultrasonic probe made of titanium which provided continuous 24 kHz ultrasound waves. The sonotrode probe was symmetrically placed in the coarse emulsion and emulsification time was different for each sample based on the design of the experiments. Eventually, three separate samples were prepared for each experiment.

2.3. Analytical methods

2.3.1. Particle size

Mean particle diameters of prepared emulsions were evaluated by a laser light scattering using a Mastersizer 2000s (Malvern Instrument Ltd., Worcestershire, UK). The samples were diluted with deionised water (1:100) to prevent multiple scattering effects. Experimental data related to the emulsion particle size was achieved with the best fit between light scatterings (Mie) theory and the measured particle size distribution. A refractive index of 1.45 was applied for WO in Mie theory calculations. The size distribution was characterised by surface-weighted mean diameter ($D_{32}$) of emulsion droplets based on the following equation:

$$D_{32} = \frac{\sum n_i d_i^2}{\sum n_i d_i^3}$$  \hspace{1cm} (1)

where $n_i$ is the number of droplets that have the same radius ($d_i$).

2.3.2. Emulsion stability (creaming test)

In order to evaluate emulsion stability, the extent of emulsion to gravitational separation was assessed by calculating the level of cream layer or sediment phase. For this test, 15 ml of freshly prepared emulsion was transferred into a 25 ml test tube and capped to prevent evaporation; tubes were stored at room temperature (23 ± 2) for 35 days. Then emulsion stability index was measured in percentage according to the following equation:

$$ESI (%) = \frac{H_E - (H_C + H_S)}{H_E} \times 100$$  \hspace{1cm} (2)

where $H_E$ is the initial emulsion height, $H_C$ the height of cream layer, and $H_S$ is the sedimentation phase height (Mirmosayie, Tan, Hamid, Yusof, & Chern, 2009). Larger ESI shows that the emulsion has higher stability. Monitoring tests were duplicated and the mean of the two individual trials was taken for data analysis.

2.3.3. Turbidity loss rate

Determination of turbidity loss rate was done based on the method of Buffo et al. (2001). Loss of turbidity is an index for estimating cloud stability or emulsion breakage under accelerated situation. In general, beverage emulsions are prepared in concentrate form and they are to be consumed in a high diluted form. In order to simulate the beverage as a final product that consumes, the effects of ultrasonic time (UT, 5–15 min), walnut-oil content (WO, 4–10% w/w) and Span 80 content (S80, 0.55–0.8) on the cloud stability or emulsion breakage under accelerated situation. In general, beverage emulsions are prepared in concentrate form and they are to be consumed in a high diluted form. In order to simulate the beverage as a final product that consumes,
concentrated emulsion samples were diluted about 2.5/1000 in a sugar solution (10%) to make preparation of specimen for turbidity loss rate studies. Samples were retained in a 300 ml plastic bottle at room temperature (23 ± 2) and each time for testing, samples were taken from the middle of the bottle containing diluted form of WO beverage emulsion and put in a 1 cm path cell. Measuring absorbance was conducted at 500 nm by applying a UV–visible spectrophotometer (DR/4000U-HACH, USA). For absorbance measurement, sugar solution (10% w/w) was employed as a blank. Data analysis is an average of three sequential absorbance readings. For each sample, absorbance vs. time storage data was fitted to the following first-order equation:

\[
\ln A = \ln A_0 - k_1 t
\]  
(3)

where \( t \) is the time storage, \( A \) the absorbance at time \( t \), \( A_0 \) the absorbance at time 0, and \( k_1 \) is the first-order rate constant. According to the above model (Eq. (2)), \( k_1 \) (slope of a first order model) was considered as loss rate of turbidity to evaluate shelf life. According to the above model (Eq.(2)), or more consecutive readings in two different emulsion samples.

2.3.4. Size index

Measurement of size index was done based on the method of Mirhosseini, Tan, Hamid, and Yusof (2008). As general, light scattering depends on particle size, wavelength and refractive index. Droplets with various sizes scatter light at various wavelengths and with increasing droplet size the light scattering increases. By measuring spectral absorption at two wavelengths, size index is determined and it can predict emulsion stability. In fact size index is determined by ratio of the absorbance at two wavelengths (Kaufman & Garti, 1981). Based on the method of Mirhosseini, Tan, Hamid, et al. (2008), size index is defined as the ratio of absorbance at 800 nm over 400 nm. These wavelengths were selected because they are more sensible to variation of size index than others (Kaufman & Garti, 1981). Size index of the walnut oil in water emulsion was measured by employing a UV–visible spectrophotometer (DR/4000U-HACH, USA). In this test, 1 ml of the prepared emulsions stored in room temperature (23 ± 2) were taken from the middle of the bottle containing sample and diluted by 100 ml distilled water and contained in a 1 cm path cell. Readings of absorbance were repeated at two wavelengths of 800 and 400 nm in triplicate. For absorbance measurement, Distilled water was employed as a blank.

2.3.5. Viscosity

The apparent viscosity of WO beverage emulsion was determined by means of a steady stress Brookfield viscometer (Brookfield DV-II+ programmable Viscometer, Middlboro, MA, USA) equipped with ULA spindles based on the method of Gharibzahedi, Mousavi, Khodaiyan, and Hamedi (2012). The unit of viscosity measured by a Brookfield programmable viscometer DV-II+ is in cP or mPa S. After sample preparation, viscosity of specimen was determined in 25°C for a sample size of about 16 ml in various rotational speeds depending on their torque value. Based on the primary experiments, the best result of viscosity was observed in a torque value between 10% and 100%. Three replicates of each sample were analysed and the mean was reported. Changes of shear stress as a function of shear rate was plotted and a power-law model was applied to study the flow behaviour of emulsion samples:

\[
\sigma = K\gamma^n
\]  
(4)

where \( \sigma \) is the shear stress (Pa), \( K \) the consistency coefficient (Pa s), \( \gamma \) the shear rate (s⁻¹) and \( n \) is the flow behaviour index.

2.3.6. Oxidative stability

In order to evaluate the oxidative stability of prepared emulsion, changes of peroxide value of them were determined during the 35-day storage. Mill equivalent of active oxygen per kilogram of oil (meq/kg) was defined as the peroxide value.

Oil was extracted from the oil-in-water emulsions according to the method of Osborn and Akoh (2004): a mixture of isooctane-isopropanol (3:2) was added to emulsion samples and the mixture obtained was stirred 5 times for 15 s and finally centrifuged for 5 min at 1000 rpm. At the end of centrifuge procedure, upper phase in centrifuge tube was collected and solvent mixture was evaporated under nitrogen. Peroxide value of the extracted oil was determined by the method of AOAC Official Method 965.33 (AOAC: Official Methods of AOAC International, 2000): 30 ml glacial acetic acid and chloroform mixture (3:2) was added to 5 g of the oil and stirred to dissolve the oil. Then 0.5 ml saturated potassium iodide solution was added and shook for 1 min, after which 30 ml of water was added. Finally the obtained mixture was titrated by sodium thiosulfate until its yellow colour disappeared. In this state, 0.5 ml starch solution (1% w/w) was added and titration was continued until the blue disappeared. The peroxide value is expressed in mill equivalents of peroxide oxygen per kilogram of oil and calculated by the following equation:

\[
\text{Peroxide value} = \frac{S \cdot N \cdot 1000}{W}
\]  
(5)

where \( S \) is the volume of applied sodium thiosulfate, \( N \) the normality of thiosulfate and \( W \) is the oil weight.

And changes of peroxide value were calculated according to the following equation:

\[
\text{Changes of peroxide value } \Delta P = \frac{P_1 - P_0}{P_0} \times 100
\]  
(6)

where \( P_0 \) is the content of peroxide value in the first day and \( P_1 \) is the content of peroxide value in the 35th day.

2.4. Experimental design and statistical analysis

Response surface methodology (RSM) is a set of mathematical and statistical approaches that is used to find out a suitable relationship between collections of controllable experimental variables and the obtained data (Myers & Montgomery, 2002). This method has several advantages: it is less expensive and less time consuming, several numbers of factors can be investigated simultaneously with a minimum number of experiments and it can calculate the interaction between considered variables (Bitaraf, Khodaiyan, Mohammadi Far, & Mousavi, 2012). RSM is an effective statistical system applied for optimising and modelling an account of responses. The most popular design of experiment applied in RSM technique is central composite design (CCD). Based on this method a three-factor design is considered for preparation of walnut oil beverage emulsions. These factors include ultrasonic time (UT, 5–15 min, \( x_1 \)), walnut oil content (WO, 4–10% (w/w), \( x_2 \)) and Span 80 content (S80, 0.55–0.8, \( x_2 \)) in five coded levels (−1.68, −1, 0, 1 and 1.68). Preliminary experiments determined the range and the centre point value of the defined independent variables. A total number of 20 runs of experiment including 2⁵ orthogonal factorial, six star point (x = 1.68) and six replicate at the centre point were employed to evaluate and optimise the effect of preparation conditions on response variables namely particle size (nm) (\( Y_1 \)), emulsion stability (%) (\( Y_2 \)), turbidity loss rate (A/day) (\( Y_3 \)), size index value (\( Y_4 \)), viscosity (\( Y_5 \)) and changes of peroxide value (\( Y_6 \)) of the walnut oil beverage emulsions. The design
matrix of independent variables (in the form of actual unit), observed responses and predicted values are presented in Table 1. All experiments were carried out three times and response data are mean of them. In order to minimise influences of unexpected variability in the responses obtained, experimental runs were conducted in random. For the statistical treatment, the test or real factors were coded according to the following equation:

\[ x_i = \frac{X_i - X_0}{\Delta X} \quad i = 1, 2 \text{ and } 3 \]  

where \( x_i \) is a dimensionless coded value of an independent factor, \( X_i \) is its corresponding actual value, \( X_0 \) is the real value of \( X_i \) at the centre of the domain and \( \Delta X \) is the step-change content of uncoded variable \( i \).

In RSM problems, the first and main step is finding an appropriate relationship between defined independent factors and responses. If the data obtained is well fitted by a linear equation of considered variables, the approximation model is the first-order function:

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \varepsilon \]  

where \( Y \) is the response, \( \beta_0 \) the constant coefficient or content of response at the centre point, \( \beta_i \) the linear coefficient of \( r \)th factor and \( \varepsilon \) is the obtained error in the experimental observations.

If any curvature is existed in the approximation function, a polynomial equation with a high degree as second-order function must be applied:

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \]  

where \( \beta_0 \) shows the quadratic term of \( i \)th factor and \( \beta_{ij} \) is the mutual interaction between \( i \)th and \( j \)th variables. In order to achieve terms of mathematical model, the data obtained from central composite design was subjected to regression analysis by utilising least square regression technique. The adequacy and general ability of the proposed regression models could be evaluated based on the coefficient of determination \( (R^2) \) and adjusted coefficient of determination \( (R_{adj}^2) \). For each response, the statistical significant of regression coefficients were checked by analysis of variance (ANOVA). Based on the p-value, significance of coefficients of mathematical model was determined. A software Design-Expert (trial version 8.0.1, Stat-Ease Inc., Minneapolis, USA) was employed for design of experiments, analysis of observed data, calculation of empirical model and depiction of 3D plot by keeping one variable at its centre point in order to better interpret of interaction effect of two other variables in regression models and optimisation of multiple responses. Afterwards four confirmation experiments were carried out to verify validity and accuracy of response surface model.

### 2.5. Optimisation and validation procedures

The individual and overall optimisation procedures were conducted by obtaining optimum level of three independent variables including UT, WO and S80 contents resulting in the desirable response variables purpose. A WO emulsion would be considered an optimum product with the overall desirable aim. In the graphical optimisation, the response models were visualised as three dimension surface plots to better understand the relation and interaction effects of independent variables on the studied physicochemical characteristic. In the numerical optimisation, the exact optimum level of independent variables which resulted in the desirable response variables was achieved by applying response optimiser (Design Expert software). With the numerical response optimisation, we can modify independent variables to get a final product with high quality. The adequacy and efficiency of predicted response surface models were verified by comparing experimental data and predicted ones. On the other hand, in order to check the validity of the models obtained, optimum sample was predicted by models and three additional samples were prepared and their response variables were compared with their predicted value.

### 3. Results and discussion

#### 3.1. Model fitting and statistical analysis

RSM was applied to analyse the influence of independent parameters – UT \((X_1)\), WO \((X_2)\) and S80 \((X_3)\) – on particle size.
(\(Y_1\)), emulsion stability (\(Y_2\)), turbidity loss rate (\(Y_3\)), size index value (\(Y_4\)), viscosity (\(Y_5\)) and peroxide value change (\(Y_6\)) of prepared emulsions. Variance and regression analysis was employed to fit the suggested regression models and investigate the statistical significance of model factors. The regression coefficients of the empirical models and the corresponding \(R^2\), adjusted \(R^2\) values, CV and adequate precision are shown in Table 2. Based on the \(p\)-value index, statistically significant models and equation terms were investigated, and insignificant factors (\(p > 0.05\)) were identified. As shown in Table 2, all of the predicted response surface models were significantly fitted (\(p < 0.05\)) to observed data. In this study, \(R^2\) values for particle size, emulsion stability, turbidity loss rate, size index value, viscosity and peroxide value were 0.9509, 0.9591, 0.9319, 0.9653, 0.9645 and 0.9801, respectively. For an appropriately fitted model, \(R^2\) should be greater than 0.8. When this parameter is close to unit, variations in the response data can be explained well by regression models. In this study, empirical models explained a high percentage (>0.93) of response variations. As is known, addition of variables to model will always increase \(R^2\), whether they are statistically significant or not. In this study, the difference between \(R^2\) and \(R_{adj}^2\) values for all responses was less than 0.2 (Table 2) implying that models did not include insignificant terms. Moreover, the quality of fitness models was assessed by a lack-of-fit test (\(p > 0.05\) for all responses); this test determines model accuracy to predict variation. The high reliability and precision of the response data were confirmed by values of CV that varied between 0.48 and 8.63. Adequate precision is a statistical index that indicates signal to noise ratio; values higher than 4 are acceptable. For all responses studied, this indicator is greater than 4. Fig. 1 indicates that observed responses are in acceptable agreement with the empirical equations. The results obtained from experimental observation were compared with predicted values from the regression equations. The results showed that the models can establish optimum preparation conditions for walnut-oil beverage emulsions.

### 3.2. Particle size

Table 2 shows that linear and quadratic effects of UT and WO content on the particle size of beverage emulsion were significant. Also the main effect of S80 was significant. The results demonstrated that emulsion droplet size was adversely affected by UT and S80 and positively related to oil content. Fig. 2a shows positive effect of homogenisation time and negative effect of oil content on the particle size. As shown in Table 2, quadratic term of all variables positively associated with the particle size that reveal high level of them lead to increasing droplet size. Emulsification is an important factor that influence on the rate and volume of surfactant

![Fig. 1](image-url)
absorption on the surface of recently developed particles. Intensity of Shear force, turbulence and cavitation created by homogeniser determined average particle size. Increasing homogenising time leads to temperature rising (Anarjan, Mirhosseini, Baharin, & Tan, 2010). Interfacial tension and viscosity were reduced as a result of temperature increasing. Decrease of interfacial tension causes increase of interfacial instability. On the other hand, vapour pressure of cavitation region increases as a consequence of temperature raising that leads to development of cavitation intensity. As the cavitation intensity is increased, breakage of droplet oil and form newly fine droplet is accelerated (Gaikwad & Pandit, 2008).

As previously mentioned, high level of ultrasonic time was lead to increase particle size. These results are because of lack of sufficient emulsifier in the emulsifying chamber. In fact, in these situations, small particle size is formed by homogeniser but due to the emulsifier shortage, particle join to each other and lead to droplet coalescence (Anarjan et al., 2010).

As shown in Table 2 oil content had a positive effect on the particle size. During homogenisation process, oil droplets break to smaller ones that cause increasing interface area. In this situation there has not been sufficient emulsifier to cover oil droplet (Gaikwad & Pandit, 2008). As previously mentioned, high level of ultrasonic time was lead to increase particle size. These results are because of lack of sufficient emulsifier in the emulsifying chamber. In fact, in these situations, small particle size is formed by homogeniser but due to the emulsifier shortage, particle join to each other and lead to droplet coalescence (Anarjan et al., 2010).

As shown in Table 2 S80 content had a positive effect on the particle size. Magnitude of Laplace pressure determines disperse phase resistance to deformation which is governed by interfacial tension of surfactant. Leong et al. (2009) in their study indicated mixture of surfactant and co-surfactant can modify interfacial tension of oil and water as a result of surfactant synergy. In this study, in all emulsion samples Tween 80 content was constant (1% w/w) and S80 content was vary between (0.5 and 0.8% w/w).

As revealed in Table 2, low amount of S80 caused a reduction in average particle size and high level of it had reverse trend on the oil droplet size. The reduction effect on droplet size is due to surfactant synergy of Tween 80 and Span 80 with opposing HLB/packing geometries. Tween 80 as a hydrophilic surfactant has an interfacial molecular area of 2.48 nm$^2$ and Span 80 as a hydrophobic surfactant has an interfacial molecular area are 0.46 (Liu, Sun, Li, & Xu, 2006). Application of these emulsifiers in emulsion system leads to decrease interfacial tension as a result of increasing surfactant packaging and improves preferential interfacial curvature. However, high contents of Span 80 cause the surfactant system too hydrophobic which lead to increasing average particle size and emulsion instability (Leong et al., 2009).

From the optimisation results, a combination of 11.427 min UT, 4.03% (w/w) WO and 0.772% (w/w) S80 was predicted to provide the desirable emulsion stability (the highest value, $Y_1 = 197.211$).

3.3. Emulsion stability

In general, the emulsions with higher stability values would be regarded as more acceptable, as they would result in higher consumer satisfaction with the final beverage emulsions. The results in Table 2 show that the linear terms of UT, WO content and S80 had significant influences on emulsion stability ($p < 0.01$ and $p < 0.05$). The quadratic terms of all independent variables had
significant and similar effects on emulsion stability at the levels of less than 0.01% and 5%. The mutual interaction between UT and WO (p < 0.01), WO and S80 (p < 0.01) were also found to be significant. The results in Table 2 indicate that the variables with the largest effects were the quadratic terms of S80 and UT and linear effects of UT. The combined effect of WO content and S80 was observed from the curvature of the 3D emulsion stability. UT had an inverse effect on droplet size. Smaller droplet size prevented flocculation and coalescence, as the droplets were non-deformable and led to higher stability of the emulsion.

As shown in Table 2, S80 had direct effect on the emulsion stability. Previous studies also demonstrated that emulsifiers had a positive impact on stability of beverages emulsions (Gharibzahedi, Mousavi, Hamedi, & Khodaiyan, 2013; Mirhosseini, Tan, Hamid, et al., 2009). According to Stocks low, at higher proportions of emulsifier, viscosity of emulsion system increased which drastically decreased the mobility and upward movement of dispersed phase. In fact in low emulsifier content because of weak viscose forces in the system free the mobility movement of dispersed phase. In fact in low emulsifier content and degree of oil droplet interaction with each other increase increased which drastically decreased the mobility and upward movement of dispersed phase. In fact in low emulsifier content and degree of oil droplet interaction with each other increase and leads to emulsion instability. In the other hand presence of emulsifier can decrease the interfacial tension between oil and aqueous and oil phases and create a cohesive interfacial film around the oil particle therefore improve emulsion stability. It is
believed that emulsifiers could form a film around droplets and thus prevent their coalescence. At high levels of surface active material and low oil concentration, emulsion droplets were suitably covered by emulsifier, giving high emulsion stability (Gharibzahedi, Mousavi, Khodaiyan, et al., 2012). But when oil content increased, oil-droplet coverage and as a result emulsion stability decreased (Bufo et al., 2001) Jacome-Guth, Aserin, and Garti (1991) stated that the emulsifier films act as a mechanical barrier that prevents coalescence. On the other hand, the use of emulsifiers increases droplets’ specific gravity, which reduces the specific-gravity difference between water and oil phase, thereby reducing the creaming rate (Dłuzewska, Stobiecka, & Maszewska, 2006). From the optimisation results, a combination of 15.00 min UT, 4.44% (w/w) walnut oil and 0.8% (w/w) S80 was predicted to provide the desirable emulsion stability (the highest value, $Y_2 = 100.001$).

### 3.4. Turbidity loss rate

In general, it is assumed that beverage emulsions with lower rates of turbidity loss have higher potential to maintain cloudiness, and be more desirable for consumers (Mirhosseini, Tan, & Taherian, 2008). Based on data of Table 1, the positive sign of turbidity loss rate data shows turbidity of all emulsion samples reduced during storage time (35 days). These observations are in a good agreement with previous studies (Mirhosseini, Tan, & Boo, 2009; Mohagheghi, Rezaei, Labbafi, & Ebrahimzadeh Mousavi, 2011; Ray, Johnson, & Sollivan, 1983). The results demonstrated that the main effect of WO was positively proportional to the variations in turbidity loss rate ($p < 0.05$). Main and quadratic effects of UT, the quadratic term of S80, its interaction with WO content and the combined effect of UT and S80 were negatively associated with turbidity loss rate. Table 2 indicates that the linear term of UT, the quadratic term of S80 and its interaction with WO content were among the significant ($p < 0.01$) terms of the model. Increased oil content in low ratios of S80 resulted in an increased turbidity loss rate; however, at high values of S80, WO had the opposite influence on this response. At all levels of oil, S80 elevation leads to decrease in turbidity loss rate (Fig. 2d). Fig. 2e indicates that increase in UT resulted in decreased turbidity loss rate, while WO had the reverse effect. Quadratic scales of these variables are presented in this figure as curves. Kaufman and Garti (1984) illustrated that incorporating two different emulsifiers could be responsible for reduced turbidity. When a proportion of surface active materials cover oil droplets, the remainders stay in the system and lead to depletion flocculation. The higher emulsion stability is due to the lower turbidity loss rate (Mirhosseini, Tan, Hamid, et al. (2008)). Reduction of emulsion turbidity could be as a result of the external layer thinning of emulsifier film located on the interfacial surface of the oil droplets (Dłuzewska et al., 2006). The positive effect of oil concentration on emulsion turbidity is due to the aggregation of oil droplets and changes in the reflective index of disperse and continuous phases. As mentioned by Buffo et al. (2001), the intensity of repulsive interactions and forces (as barrier energy) between emulsion droplets has a direct effect on emulsion stability. Phenomena such as flocculation, coalescence and aggregation lead to increased average droplet size and polydispersity index of samples, and could eventually be responsible for turbidity loss during storage. The opposite effect of UT on turbidity loss can be explained by reduced particle size from increased ultrasonic time. Based on the Stocks Law, particle diameter has a direct relation with the velocity of phase separation; therefore smaller particle leads to reduction of turbidity loss rate because of low separation velocity. The optimum turbidity loss rate (with a lowest value of $Y_3 = 0.0064$) was predicted to occur at 10 min UT, 4% (w/w) WO and 0.55% (w/w) S80.

### 3.5. Size index

Table 2 shows that the linear effects of UT and WO were significant ($p < 0.0001$) on size index ($Y_4$), although S80 was not significant ($p > 0.05$). The quadratic effects of UT and WO content were found to be significant ($p < 0.01$ and $p < 0.05$). The mutual interaction between UT and WO was also significant at the 0.05% level. The most remarkable effects on size index ($p < 0.0001$) were the linear component of UT and WO. Fig. 2f shows the mutual effect of UT and WO content on the size index. It can be seen that at all WO concentrations, increases in UT caused the size index to decrease, and the increasing of WO content also resulted in size index development at all ultrasonic times. The influence of these variables’ quadratic effect is evident in the curvature in the 3D plot of the size index. As general, at the all spectra the reflectance is sensitive to wavelength. The spectral reflectance increases with diameter. The maximum of scattering efficiency is when diameter of droplet is approximately equal to the light wavelength. Above this point, with increasing droplet diameter reflectance decreases because the scattering of dispersed phase decreases therefore the penetration of light beam to emulsion develops (Chantraponchai, Clydesdale, & McClemen, 1999). Hence it can be resulted that increasing in UT and reducing in particle size leads to increase in size index. As shown in Table 2, WO content had direct effect on size index. Increasing in dispersed phase led to elevate in emulsion droplet number. Therefore presence of large number of oil phase caused to increase in refractive index and increase in light scattering efficiency and led to size index development. Gharibzahedi, Mousavi, Hamedi, et al. (2012) reported that oil content has a direct effect on the size index of walnut emulsion. Mirhosseini, Tan, Hamid, et al. (2008) also revealed that oil content had inverse influence on size index. As the content of the dispersed phase increased, so did the size index of emulsion, resulting in low emulsion stability (Dłuzewska et al., 2006). The larger size index revealed low stability; nevertheless, our emulsions did not undergo phase separation due to the presence of emulsifiers that decreased the specific-gravity difference between the oil and water phases. Dłuzewska, Stobiecka, and Maszewska (2006) indicated that oil concentration (dispersed phase) has a direct effect on the size index, independent of the kind of applied emulsifier. But at a constant level of dispersed phase, the growth of size index is a consequence of increased droplet size. From the optimisation results, a WO emulsion composed of a 13.62-min UT, 5.12% (w/w) WO and 0.78% (w/w) S80, estimated to be the individual optimum region leading to the lowest size index ($Y_4 = 0.00199$).

### 3.6. Viscosity

The analysis indicated that all of the twenty walnut beverage samples showed a rheological behaviour as Newtonian fluid. As shown in Table 2 the viscosity of emulsions was positively proportional to the main effect of WO and S80 and negatively related to the main effect of UT (Fig. 2g) and all interaction and quadratic effects of the three variables were not significant ($p > .05$). Based on the Stocks Law, the emulsion instability resulting from creaming or sedimentation was related to viscosity, because velocity of the phase separation is adversely affected by viscosity and high viscosity value is thus desirable in the emulsions. As shown in Fig. 2g the viscosity of WO beverage emulsions increased by increasing WO concentration. The results also demonstrated that as the S80 content was increased, so did significantly the emulsion viscosity. This result may be due to the elevation of flow resistance as a result of increasing particle numbers in emulsion composition that finally led to an increase in the apparent viscosity. As observed in Table 2, UT had negative effect on viscosity, this relation could be as influence of UT on particle size. As previously mentioned
higher homogenising time led to lower particle size. As we know viscosity is defined as resistance against flowing and emulsions with smaller particle size because of lower internal force than larger ones has low resistance to flow therefore show lower viscosity (Sato & Cunha, 2009). The optimum viscosity (with a highest value of Y3 = 3.03) was predicted to occur at 5 min UT, 10% (w/w) WO and 0.8% (w/w) S80.

3.7. Peroxide value changes

In order to evaluate chemical stability of prepared emulsion based on WO, variation of particle size of samples in the form of the percentage of their changes was determined after storage for 35 days. As shown in Table 1, based on Eq. (5), a positive sign of peroxide value change represents an increase of the peroxide value of a WO-in-water emulsion during storage. The results demonstrate that ΔP is negatively correlated with the main effects of WO, while linear terms of oil content and UT indicate a significant positive effect on the ΔP variations. Thus in order to produce emulsion with a desirable ΔP value (minimum level), the parameter must be optimised. Combination effects of ultrasonic time and S80 are shown in Fig. 2h. As observed, the peroxide value increases as UT increases at every level of S80. In addition, S80 had an inverse relation with peroxide value at all levels of UT.

Hydro peroxides as primary oxidation ingredients have lower half-life compared to the secondary ones. Production of peroxides is increased in the presence of light, oxygen, metal ions and heat. It is accepted that interaction between hydro peroxides in oil droplet surfaces and metal ions in aqueous phase is a major reason for oxidation (Paraskevopoulou et al., 2007). Direct effect of homogenisation time on this parameter is possibility due to the increase of surface area of oil droplet after homogenisation. As mentioned previously, ultrasonic time had an inverse effect on droplet diameter. Reduction of particle diameter as a function of homogenisation time leads to development of surface area of disperse phase than unprocessed ones. Larger surface area leads to facility surface contacts between oil particles and surrounding aqueous phase and results in increasing peroxide value. On the other hand, cavitation in homogenisation process generates free radicals and leads to faster creation of oxidant component (Tan & Nakajima, 2005). As stated, emulsions with high oil contents had high peroxide value changes in comparison with low oil content emulsions. As mentioned previously, 89.74% of used WO is composed of mono (oleic, 23.34%) and poly (linoleic, 52.91% and linolenic, 13.27%) unsaturated fatty acid. The pro-oxidant components cause lipid hydroperoxides (ROOH) decomposes into highly reactive peroxyl (ROO') and alkoxyl (RO') radicals. Theses radicals react with unsaturated fatty acid within the oil droplets or at the oil–water interface, resulted in the formation of lipid radicals (Paraskevopoulou et al., 2007). Therefore it can be resulted that in high oil content emulsions, the concentration of unsaturated fatty acids is more than low oil content ones and this type of emulsions are more susceptible to oxidation.

The presence of Tween 80 and Span 80 at the surface of oil droplet because of their surface active functions, changes the amount of oxidation in emulsions.

Lipid hydroperoxides are surface active, tend to move to the oil droplet surface and interact with the aqueous phase oxidation catalysts. The Reverse effect of S80 on the oil oxidation might be related to membrane formed around the droplets by the emulsifiers. In fact, the surfactant layer around the oil droplet acts as a barrier, reducing the possibility of collision between oil droplet and oxidants and, in turn, leads to a decrease in the rate of oil oxidation. The optimum ΔP (the least value, Y3 = 12.654) was predicted to be obtained at 15.00 min of UT, 4.00% (w/w) WO concentration and 0.74% (w/w) S80.

3.8. Optimisation

Numerical optimisation was performed to obtain optimum values for independent variables, thus determining desirable response parameters include maximum emulsion stability and viscosity, minimum particle size turbidity loss rate, size index and peroxide value. The optimum conditions for prepared emulsions are defined as 14.63 min for UT, 8.23% for WO content and 0.79 for S80. At this point, particle size, emulsion stability, turbidity loss rate, size index, viscosity and changes of peroxide value of the WO emulsion were calculated as 219.198, 99.184, 0.008, 0.008, 2.438 and 16.65 respectively.

3.9. Model verification

To check the adequacy and validity of response surface models, experimental data and predicted values related to each response were compared (Table 1). Results revealed no statistical significant difference (p > 0.05) between observed and predicted responses. Fig. 1 illustrates that regression models were in a good agreement with observed values, demonstrating the precision and reliability of the empirical models. Moreover, three additional and optimum point experiments were performed to assess the model validity. Defined conditions, experimental data and percentage errors between them are presented in Table 3. These conditions were not among the experimental conditions presented in Table 3, but...
were in defined ranges for the independent variables. According to obtained regression model by software in actual level, the predicted values were obtained for each response. These equations for each response are:

Regression model for particle size:

\[ Y_1 = 464.61 - 19.59x_1 - 11.153x_2 - 247.23x_3 + 0.016x_1x_2 + 9.2x_1x_3 + 3.3x_2x_3 + 0.5x_1^2 + 1.04x_2^2 + 25.85x_3^2 \]

Regression model for emulsion stability:

\[ Y_2 = 134.64 - 0.6x_1 - 1.93x_2 - 90.43x_3 - 0.042x_1x_2 + 0.54x_1x_3 + 2.5x_2x_3 + 0.03x_1^2 + 0.03x_2^2 + 54.58x_3^2 \]

Regression model for turbidity loss rate:

\[ Y_3 = -0.06 + 3.86x_1 + 0.01x_2 + 0.08x_3 - 1.26E - 004x_1x_2 + 1.8 \]
\[ - 003x_1x_3 - 0.01x_1x_3 - 7.96E - 005x_2^2 - 3.48E - 004x_2^2 \]
\[ - 0.03x_3^2 \]

Regression model for size index:

\[ Y_4 = 0.017 - 4.72x_1 - 1.84x_2 + 0.08x_3 - 2.25E - 004x_1x_2 + 2E \]
\[ - 004x_1x_3 + 3.66E - 003x_2x_3 + 1.91E - 004x_2^2 + 3.14E \]
\[ - 004x_2^2 - 0.08x_3^2 \]

Regression model for viscosity:

\[ Y_5 = -1.10 + 0.04x_1 + 0.27x_2 + 4.89x_3 - 2.66E - 003x_1x_3 \]
\[ - 0.02x_1x_3 - 0.18x_1x_3 - 2.23E - 003x_2^2 - 7.06E - 004x_2^2 \]
\[ - 1.54x_3^2 \]

Regression model for peroxide value changes:

\[ Y_6 = 62.50 - 1.78x_1 + 4.82x_1 - 131.30x_3 - 0.02x_1x_2 + 1.58x_1x_3 \]
\[ - 4.8x_2x_3 + 0.02x_1^2 - 0.03x_3^2 + 84.12x_3^2 \]

It should be noted that there are two kinds of regression model: regression model with coded equation useful for identifying the relative influence of the factors on response variable by comparing the factor coefficients which is presented in Table 2 and regression models with the equation in the form of actual factors can be applied for make predicted value about the response for given levels of each factor which is presented in this section.

Maximum percentage error for particle size, emulsion stability, turbidity loss rate, size index, viscosity and changes of peroxide value are –2.4, –5.58, –7.6, 9.9, 5 and –6.67 respectively. It can be concluded that the proposed regression models are valid.

4. Conclusion

Response surface methodology in conjunction with central composite rotatable design was developed to investigate the effects of emulsion-preparation conditions on particle size, emulsion stability, turbidity loss rate, size index, viscosity and peroxide value changes. This study applied this technique to walnut-oil beverage emulsions. Results indicate that RSM with CCRD is a practical technique to model variations of response variables as a function of independent variables. All mathematical equations developed in this process demonstrated high determination coefficients (greater than 0.93) and insignificant lack of fit (p > 0.05). While increasing ultrasonic time increases emulsion stability and peroxide value, it decreases particle size, turbidity loss rate, size index, viscosity and peroxide value. Increases in walnut-oil concentration reduce emulsion stability but increase particle size, turbidity loss rate, size index, viscosity and peroxide value. Turbidity loss rate and size index are not affected by S80, although it does had a direct influence on emulsion stability and viscosity and a reverse effect on changes of peroxide value.

Acknowledgements

This work has been partially funded by a grant provided by “the Council for Research at the School of Agriculture and Natural Resources of the University of Tehran” and “Research Council of the University of Tehran”. Gratitude is expressed to the ZamZam Iran Co. (Tehran, Iran) for providing the laboratory facilities, the equipment, and also for the financial support for the project.

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