Optimization of processing conditions to improve antioxidant activities of apple juice and whey based novel beverage fermented by kefir grains

Nayereh Sabokbar · Faramarz Khodaiyan ·
Marzieh Moosavi-Nasab

Revised: 12 April 2014 / Accepted: 24 April 2014 / Published online: 14 May 2014
© Association of Food Scientists & Technologists (India) 2014

Abstract A central composite design (CCD) was used to evaluate the effects of fermentation temperature (20–30°C) and kefir grains amount (2–8%w/v) on total phenolic content and antioxidant activities of apple juice and whey based novel beverage fermented by kefir grains. The response surface methodology (RSM) showed that the significant second-order polynomial regression equation with high R^2 (>0.86) was successfully fitted for all response as function of independent variable. The overall optimum region was found to be at the combined level of 7.56%w/v kefir grains and temperature of 24.82°C with the highest value for total phenolic content (TPC) and antioxidant activities. At this optimum point TPC, 1, 1-Diphenyl-2-picrylhydrazyl radical scavenging, metal chelating effect, reducing power, inhibition of linoleic acid autoxidation and inhibition of ascorbate autoxidation were 165.02 mgGA/l, 0.38 ml/1 ml, 0.757 (absorbance at 700 nm), 46.12 %, 65.33 % and 21 %, respectively. No significant difference ($p<0.05$) was found between actual values and predicated values.

Keywords Apple juice · Whey · Kefir · Fermentation · Total phenolic content · Antioxidant activities

N. Sabokbar
Department of Food Science and Technology, School of Agriculture,
Shiraz University, Shiraz, Islamic Republic of Iran
e-mail: nayereh.sabokbar@yahoo.com

F. Khodaiyan (✉)
Department of Food Science, Engineering and Technology, Faculty
of Agricultural Engineering and Technology, Campus of Agriculture
and Natural Resources, University of Tehran, Karaj, Islamic
Republic of Iran
e-mail: khodaiyan@ut.ac.ir

M. Moosavi-Nasab
Department of Food Science and Technology and Seafood
Processing Research Group, School of Agriculture, Shiraz
University, Shiraz, Islamic Republic of Iran

Introduction

Several free reactive radicals such as superoxide radical, hydroxyl radical, hydrogen peroxide and peroxide radical, generated by exogenous or endogenous metabolic processes, are known to cause oxidative damages in food systems and human body. These free radicals can result in danger diseases including cancer, atherosclerosis, hypertension, arthritis, emphysema and cirrhosis (Kehrer 1993). However there is an inherently anti oxidative system including superoxide dismutase, glutathione peroxidase and uric acid in our body that can protect us from some damages generated by reactive oxygen species (ROS), but this system is not enough for protecting against all of these damages (Simic 1988). Many kinds of fruits and vegetables are known as natural antioxidant sources which are more desirable than chemical antioxidant. Apple (*Malus domestica*) contains many kinds of polyphenol components such as phenolic acid, flavonol, procyanidin, anthocyanin and catechin. Because of polyphenol existence in fruits and vegetables that have redox properties, fruits and vegetables can act as reducing agent, hydrogen donor and singlet oxygen quencher (Rice-Evans et al. 1996). Therefore, antioxidant activities of fruits, vegetables and also teas, are contributed to the polyphenol components. Also, proteins from several animal sources can act as antioxidant. For instance milk protein hydrolysates and individual peptides released after hydrolysis, expressed antioxidant activity against some oxidative components (Suetsuna et al. 2000; Pena-Ramos and Xiong 2001; Rival et al. 2001). Certain amino acid sequences like histidin and some hydrophobic amino acids are known as natural antioxidant sources (Suetsuna et al. 2000). Whey is a rich source of proteins with high biological value. Whey proteins include α -lactalbumin (α -La), β -lactoglobulin (β -Lg), bovine serum albumin (BSA) and immunoglobulins. β -Lg shows many biological activities such as anti-hypertensive, anti-cancer, hypo-cholesterolemic and anti-

microbial (Chatterton et al. 2006). Use of lactic acid bacteria in food industry, has been utilized for production of functional foods in recent years. Kefir made with kefir grains is a refreshing, naturally carbonated fermented dairy beverage that has a slightly acidic taste, yeasty flavor and creamy consistency (Powell et al. 2007). The traditional kefir is produced by the addition of small kefir grains to fresh milk. Kefir grains are like small cauliflower florets, 1–3 cm in length, lobed, irregularly shaped, white to yellow-white in color, and have a slimy but firm texture (La Rivière et al. 1967). Kefir grains contain proteins, polysaccharides and complex mixture of microorganisms. Lactic acid bacteria (LAB) and yeasts have a complex symbiotic relationship in kefir grains and responsible for alcoholic and lactic acid fermentation, respectively. Some of distinctive features of lactic acid fermentation are growth of lactic acid bacteria, production of different organic acids, degradation of some anti-nutritional factors such as metal chelating agents in raw plant materials like phytate, oxalate and tanins and decrease in pH (Reddy and Pierson 1994).

In the current study, a central composite design (CCD) was applied to assay the effect of two independent variables—temperature (20–30°C) and kefir grains amount (2–8 % w/v)—on total phenolic content and antioxidant activities of apple juice and whey based novel beverage fermented by kefir grains. Therefore, the objective of this work was to formulate and find an optimum quantity of independent variables for accessing the maximum total phenolic content and antioxidant activity of this beverage.

Materials and methods

Kefir grains were collected from a household in Tehran, Iran. The grains were kept in pasteurized milk at room temperature. Milk was exchanged every 2 days to maintain the grains viability. The commercial concentrated apple juice and whey used in this study were supplied from Alifard (Sunich, Iran, Saveh) and Safadasht (Iran, Karaj) cheese making company, respectively.

Preparation of beverage

Concentrated apple juice was diluted by whey to brix 14°. Mixture was pasteurized at 60°C for 30 min. Kefir grains were removed from milk, washed with distilled water and then inoculated to the prepared beverage in different levels (2–8 % w/v). After that mixtures were incubated at variety temperature (20–30°C) for 48 h. Control samples (unfermented beverage) contained the same proportion of whey and concentrated apple juice, but the fermentation process by kefir grains did not apply for it.

Measurement of total phenolic content (Folin–Ciocalteu assay)

The total phenolic content (TPC) of each sample was determined according to Folin–Ciocalteu method (Singleton and Rossi 1965). Briefly, 0.2 ml of diluted beverage was added to 1 ml of Folin–Ciocalteu reagent (Folin–Ciocalteu reagent prediluted 10-fold with distilled water) and shaken well. Mixture was allowed to stand at room temperature for 8 min. Then, 0.8 ml of sodium carbonate (7.5 %) was added to mixture, shaken and left at room temperature for 30 min. Absorbance was measured at 765 nm. The TPC was assessed by plotting the gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per liter of sample. The equation for the gallic acid calibration curve was $Y = 89.014X - 34.479$ (where X = measured absorbance and Y = concentration of gallic acid equivalents expressed as milligrams of GA per liter of sample), and the correlation coefficient was $R^2 = 0.9751$.

Antioxidant capacity determination

Scavenging effect upon 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radicals

The free radical scavenging activity of beverages was measured by DPPH• using the method of Brand-Williams et al. (1995). Three dilutions of each beverage were prepared. 3.9 ml of a 25 mg/l methanolic solution of DPPH• was added to 0.1 ml of each diluted samples. Mixtures were shaken well. The control sample was prepared with the same volume of methanol instead of beverage. Mixtures were left at room temperature (in a dark place) for 30 min and after that absorbance were measured at 515 nm. The DPPH• concentration in the reaction mixture was calculated using equation $y = 35.919A_{515} - 1.9031$ ($R^2 = 0.9971$) as was obtained by linear regression containing different concentration of DPPH•. The % of remaining DPPH was calculated as follows:

$$\%DPPH^{\bullet}_{rem} = \frac{[DPPH^{\bullet}]_t}{[DPPH^{\bullet}]_{t=0}} \times 100$$

Where $[DPPH^{\bullet}]_t$ and $[DPPH^{\bullet}]_{t=0}$ were the DPPH• concentration of reaction mixture after 30 min (steady state) and DPPH• concentration of control, respectively. The %remaining of DPPH• was plotted against the beverage concentration to obtain EC_{50} . EC_{50} is the concentration of beverage which can decrease the initial DPPH• concentration by 50 %. Lower EC_{50} value shows higher radical scavenging activity.

Reducing power

The reducing power of beverages was measured according to the Oyaizu's method (1986). 2.5 ml of each beverage was

mixed with 2.5 ml of sodium phosphate buffer (0.2 M, pH=6.6) and 2.5 ml of potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of trichloro acetic acid (1 %) was added to the mixture and mixture was centrifuged at 1,400 g for 10 min. The upper layer of solution (5 ml) was mixed with 5 ml of distilled water and 1 ml of ferric chloride (0.1 %). The absorbance of the mixture was measured at 700 nm. Greater absorbance shows greater reducing power.

Ferrous ion chelating ability

The ferrous ion chelating ability of beverages was measured according to the method of Decker and Welch (1990). Briefly, 5 ml of beverage was mixed with 0.1 ml of ferrous chloride (2 mM) and 0.2 ml of ferrozine (5 mM). The mixture was shaken and allowed to stand at room temperature for 10 min. Then, absorbance was measured at 562 nm. Chelating effect of samples was calculated as follow:

$$\text{Chelating effect (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where the A_0 is the absorbance of control and A_1 is the absorbance in the presence of the beverage. The control contains FeCl_2 and ferrozine, complex formation molecules.

Inhibition effect upon Lipid Peroxidation

The inhibition effect of beverage upon lipid peroxidation was determined according to Yen et al. (2000). The linoleic acid emulsion was prepared by mixing equal volumes of linoleic acid, Tween 20, and phosphate buffer (0.02 M at pH 7.0). Samples (0.5 ml) were mixed with 2.5 ml of linoleic acid emulsion (0.002 M) and 2 ml of phosphate buffer (0.2 M at pH 7). The reaction mixture was incubated at 50°C in a dark place for 10 min, and the degree of oxidation was measured according to the thiocyanate method (Yen et al. 2000). 4.7 mL of ethanol (75 %), 0.1 mL of ammonium thiocyanate (30 %), 0.1 mL of sample solution, and 0.1 mL of ferrous chloride (20 mM) were added sequential in HCl (3.5 %). Mixture was stirred for 3 min and peroxide value was determined by reading the absorbance at 500 nm. The relative inhibition of linoleic acid peroxidation was calculated as below:

$$\text{Lipid peroxidation inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where the A_0 is the absorbance of control and A_1 is the absorbance in the presence of the beverage.

Inhibition effect upon ascorbate autoxidation

The method of Mishra and Kovachic (1984) was used to determine the inhibition of ascorbate autoxidation. 0.1 ml of sample or distilled water (control) was mixed with an

ascorbate solution (0.1 ml, 5.0 mM) and phosphate buffer (9.8 ml, 0.2 M, pH 7.0). Mixture was placed at 37°C for 10 min and then the absorbance of this mixture was measured at 265 nm. The ascorbate autoxidation inhibition rate of the sample was calculated according to the following equation:

$$\text{Inhibition effect (\%)} = [(A_1 / A_0) - 1] \times 100$$

A_0 is the absorbance of control and A_1 is the absorbance of sample.

Experimental design and statistical analysis

The software Design-Expert (trial version 8.0.7.1, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, regression analysis of the experimental data and quadratic model building. RSM was used to determine the effect of two most significant variables namely kefir grains amount (2–8%w/v) and fermentation temperature (20–30°C) on total phenolic content, DPPH radical scavenging, reducing power, metal chelating effect, inhibition effect upon linoleic autoxidation and inhibition effect of ascorbate autoxidation. Thirteen treatments were conducted base on the central composite design (CCD) each at five coded levels –1.41, –1, 0, 1, 1.41 (Table 1). Experiments were randomized in order to minimize the effects of unexplained variability in the observed response due to extraneous factors. The responses functions (y) were related to the coded variables (x_i , $i=1, 2$) by a second-order polynomial using equation below:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$$

The coefficients of the polynomial were represented by b_0 (constant term), b_1 and b_2 (linear effects), b_{11} and b_{22} (quadratic effects), and b_{12} (interaction effects). The quality of the fit of polynomial model was expressed by the coefficient of determination R^2 and R^2_{adj} in equation 1 and 2, respectively. In these equations SS is the sum of squares, DF is the degrees of freedom.

$$R^2 = 1 - \frac{SS_{residual}}{SS_{model} + SS_{residual}} \quad (1)$$

$$R^2_{adj} = 1 - \frac{SS_{residual} / DF_{residual}}{(SS_{model} + SS_{residual}) / (DF_{model} + DF_{residual})} \quad (2)$$

All experiments were carried out in triplicate and each sample was analyzed in duplicate. The results are expressed

Table 1 Matrix of the central composite design (CCD) and experimental data obtained for the response variables (y_i) (mean $\pm$ SD)

Run	Block	Independent variables		Response variables			
		temperature (x_1 , °C)	Kefir grains amount (x_2 , %w/v)	TPC (y_1 , mgGA/l)	EC ₅₀ (y_2 , ml/1 ml)	Reducing power (y_3 , absorbance at 700 nm)	Metal chelating effect (y_4 , %)
1	1	25	5	150.11 $\pm$ 2.02	0.45 $\pm$ 0.02	0.621 $\pm$ 0.03	46.12 $\pm$ 0.8
2	1	25	5	150.03 $\pm$ 2.33	0.49 $\pm$ 0.027	0.728 $\pm$ 0.043	46 $\pm$ 0.8
3	1	25	9.24	180.21 $\pm$ 3	0.29 $\pm$ 0.015	0.9 $\pm$ 0.047	46.05 $\pm$ 0.75
4	1	30	2	95.41 $\pm$ 2.91	0.74 $\pm$ 0.03	0.114 $\pm$ 0.01	45.84 $\pm$ 0.84
5	1	25	5	155.23 $\pm$ 2.63	0.41 $\pm$ 0.02	0.592 $\pm$ 0.028	46.19 $\pm$ 0.74
6	1	20	8	162.8 $\pm$ 3.95	0.44 $\pm$ 0.035	0.456 $\pm$ 0.025	45.99 $\pm$ 0.99
7	1	25	5	165.02 $\pm$ 1.99	0.4 $\pm$ 0.023	0.728 $\pm$ 0.04	46.13 $\pm$ 1
8	1	20	2	139.89 $\pm$ 3.08	0.57 $\pm$ 0.03	0.215 $\pm$ 0.024	45.89 $\pm$ 0.64
9	1	25	0.76	130.04 $\pm$ 1.04	0.6 $\pm$ 0.031	0.202 $\pm$ 0.02	45.95 $\pm$ 0.65
10	1	25	5	157.65 $\pm$ 2.65	0.44 $\pm$ 0.025	0.667 $\pm$ 0.034	46.1 $\pm$ 0.9
11	1	17.93	5	145.65 $\pm$ 2	0.52 $\pm$ 0.035	0.305 $\pm$ 0.025	45.8 $\pm$ 0.73
12	1	32.07	5	99.97 $\pm$ 1.02	0.74 $\pm$ 0.035	0.268 $\pm$ 0.03	45.88 $\pm$ 0.94
13	1	30	8	105.92 $\pm$ 3.89	0.63 $\pm$ 0.022	0.346 $\pm$ 0.025	46.04 $\pm$ 0.55
Run	Block	Independent variables		Response variables			
		temperature (x_1 , °C)	Kefir grains amount (x_2 , %w/v)	Inhibition of linoleic acid autoxidation (y_5 , %)		Inhibition of ascorbate autoxidation (y_6 , %)	
1	1	25	5	54.9 $\pm$ 0.51		21 $\pm$ 0.19	
2	1	25	5	50 $\pm$ 0.42		17.93 $\pm$ 0.13	
3	1	25	9.24	71.32 $\pm$ 0.6		22.5 $\pm$ 0.19	
4	1	30	2	26.12 $\pm$ 0.34		13.41 $\pm$ 0.12	
5	1	25	5	62.77 $\pm$ 0.54		18.83 $\pm$ 0.14	
6	1	20	8	53.2 $\pm$ 0.46		19.34 $\pm$ 0.15	
7	1	25	5	60 $\pm$ 0.45		17.35 $\pm$ 0.2	
8	1	20	2	31 $\pm$ 0.23		13.33 $\pm$ 0.1	
9	1	25	0.76	22 $\pm$ 0.15		11.64 $\pm$ 0.12	
10	1	25	5	62 $\pm$ 0.47		19.01 $\pm$ 0.2	
11	1	17.93	5	42.21 $\pm$ 0.32		17.25 $\pm$ 0.13	
12	1	32.07	5	30.34 $\pm$ 0.24		18.3 $\pm$ 0.12	
13	1	30	8	45.11 $\pm$ 0.2		20.08 $\pm$ 0.21	

as means $\pm$ SD. Also duncan's multiple range tests were used to compare the difference among mean values of beverage's properties at the level of 0.05 and SAS software (version 9.1; statistical analysis system institute Inc., Cary, NC, USA) was used for analysis.

Results and discussion

Model fitting and statistical analysis

The negative or positive effects of two independent variables - Temperature (x_1) and Kefir grain amount (x_2) - on dependent variables (y) were considered using RSM, and their interactive relationship were studied. Analysis of variance (ANOVA) was performed to investigate the adequacy of the suggested models and identify the significant factors.

The independent and dependent variables were fitted by the second-order polynomial equation to the experimental data. Table 2 gives the statistical significance, the linear and quadratic equations and the interaction of effects calculated for each response. As illustrated in Table 2, the insignificant lack-of-fit for all investigated variables shows that the polynomial models were satisfactorily accurate for predicting the relevant responses. Table 2 shows the regression coefficients of the quadratic polynomial model and corresponding coefficients of determination (R^2) for each dependent variable. The higher value of R^2 shows the desirability of the model to elucidate the relationships between variables. The R^2 values were 0.902, 0.922, 0.881, 0.860, 0.921 and 0.915, for TPC, DPPH radical scavenging, reducing power, metal chelating effect, inhibition of linoleic acid autoxidation and inhibition of ascorbate autoxidation, respectively. Moreover, adj- R^2 and coefficient of variation (CV) were estimated to check the model adequacy. A higher adj- R^2 demonstrates that non-significant terms have

Table 2 ANOVA for the experimental variables as a linear, quadratic and interaction terms of each response variable

Source	DF	TPC (mgGA/l)			EC ₅₀ (ml/1 ml)			Reducing power (absorbance at 700 nm)		
		Coefficient	Sum of square	<i>p</i> -value	Coefficient	Sum of square	<i>p</i> -value	Coefficient	Sum of square	<i>p</i> -value
Model	5	155.67	7532.23	0.0022	0.44	0.2	0.0010	0.67	0.65	0.0039
Linear										
b ₁	1	−20.75	3442.89	0.0010	0.084	0.056	0.0019	−0.033	8.668E−003	0.4346
b ₂	1	13.05	1361.67	0.0118	−0.085	0.058	0.0018	0.18	0.27	0.0025
Quadratic										
b ₁₁	1	−19.64	2683.23	0.0021	0.11	0.085	0.0006	−0.22	0.35	0.0012
b ₂₂	1	−3.48	84.35	0.4283	0.018	2.223E−003	0.3694	−0.092	0.059	0.0674
Interaction										
b ₁ b ₂	1	−3.10	38.44	0.5881	5.000E−003	1.000E−004	0.8446	−2.250E−003	2.025E−005	0.9692
Residual	7		835.27			0.017			0.088	
Lack-of-fit	3		681.03	0.0599		0.012	0.1512		0.073	0.0521
Pure error	4		154.24			5.080E−003			0.015	
Total	12		8367.49			0.22			0.74	
R ²		0.9020			0.9216			0.8809		
Adj-R ²		0.8289			0.8656			0.7957		
CV		7.73			9.51			13.78		
Adequate precision		11.02			12.684			7.907		
Source	DF	Inhibition of linoleic acid autoxidation (%)			Inhibition of ascorbate autoxidation (%)					
		Coefficient	Sum of square	<i>p</i> -value	Coefficient	Sum of square	<i>p</i> -value			
Model	5	57.93	2731.31	0.0010	18.12	109.99	0.0003			
Linear										
b ₁	1	−3.72	110.68	0.1115	0.29	0.66	0.5223			
b ₂	1	13.87	1538.43	0.0003	3.50	98.28	<0.0001			
Quadratic										
b ₁₁	1	−11.48	917.12	0.0012	−0.75	3.86	0.1484			
b ₂₂	1	−6.29	275.18	0.0240	−1.10	8.38	0.0480			
Interaction										
b ₁ b ₂	1	−0.8	2.58	0.7892	0.16		0.7930			
Residual	7		232.12			10.25				
Lack-of-fit	3		117.40	0.3783		2.51	0.7417			
Pure error	4		116.34			7.74				
Total	12		2565.05			120.24				
R ²		0.9212		0.9147						
Adj-R ²		0.8649		0.8538						
CV		12.30		6.84						
Adequate precision		10.594		12.058						

not been included in the model. Generally, a low CV shows that variation in the mean value is low and satisfactorily develops an adequate response model (Baş and Boyacı 2007). The low CV values (6.84–13.78 %) for the proposed models indicate the experiments are high precision and reliability. Adequate precision measures the signal-to-noise ratio, with a ratio greater than 4 being desirable. For the proposed

models, these values ranged from 7.907 to 12.684, suggesting good signal-to-noise ratios (Table 2). Comparison between predicted and actual values for the response variables also indicated that the polynomial regression models were suitable to determine optimum formulation for preparing apple juice and whey novel based beverage with maximum TPC and antioxidant activity (Table 4).

Total phenolic content

The data obtained for the TPC indicate that TPC of different beverages increased significantly ($p<0.05$) during fermentation (Table 3). Also, the results in Table 2 show that the changes in TPC during fermentation were related to the linear effect of kefir grains amount and temperature ($p<0.05$) and the quadratic effect of temperature, but the mutual interaction of kefir grains with temperature and the quadratic effect of kefir grains were not significant ($p<0.05$). A possible explanation for increase of TPC during fermentation is related to the fact that the metabolic activities of microorganisms in kefir grains can modify the levels of bioactive components such as different phenolic compounds. During fermentation enzymes such as B-glycosidase derived from the fermentative microorganisms are responsible for hydrolyzing of complex phenolic compounds to simpler types and increase in quantitative amount of TPC (Mousavi et al. 2011). Other enzymes such as protease derived from the microorganisms and or whey can be contributed to the modification of beverage compositions. It has been reported by other researchers that fermentation by lactic acid bacteria or other microorganisms can enhance the level of total phenolic content (Dordević et al. 2010; Young et al. 2006; Coda et al. 2012). On the other hand, it can be seen from Fig. 1a that with increase in kefir grain levels from 2 % w/v to 8 % w/v total phenolic contents increased, too. Also, increase in TPC at temperature which was higher than 25°C was shown to be lower as compared to other temperatures. This might be due to the optimum temperature for enzymes or metabolic activities of microorganisms in kefir grains. Also, difference in the pH of different fermentation is another reason for these results, knowing that optimum pH

influences the liberation of enzymes derived from the microorganisms (Boskov-Hansen et al. 2002). The individual optimization procedure showed that the beverage fermented with 6.5 % (w/v) kefir grains at temperature of 23°C would provide the highest TPC ($y_1=167.036$ mg GA/l).

Antioxidant activity

DPPH radical Scavenging

One of the most important mechanisms for anti-oxidation is proton-radical scavenging. Proton-radical scavenging with DPPH was done in this work. In this method, alcoholic DPPH solution reduces to the yellow-colored non-radical form (diphenyl-picrylhydrazine) of DPPH in the presence of a hydrogen-donating antioxidant. As can be observed in Table 3 fermentation with kefir grains had a positive influence on DPPH radical scavenging and EC_{50} value of beverage decreased as a result of fermentation process. Temperature had significant ($p<0.05$) linear and quadratic effects on DPPH radical scavenging (Table 2). Also kefir grain amount had significant ($p<0.05$) linear effect on DPPH radical scavenging, but quadratic effect of kefir grain amount and interaction effect of independent variables were not significant ($p<0.05$). Fig. 1b shows the variation of DPPH radical scavenging with kefir grain amount and temperature. A rise in kefir grain amount resulted in increase of DPPH radical scavenging activity (as EC_{50} decreased). Also, it can be seen from Fig. 1b between temperature of 20°C and 25°C DPPH radical scavenging was the highest. At higher temperature than 25°C higher EC_{50} value and hence lower DPPH radical scavenging activity was observed. This might be due to optimum

Table 3 Comparison of TPC and antioxidant activities between different fermented beverages and unfermented (control) sample

Independent variables		Response variables					
Temperature (°C)	Kefir grains (%w/v)	TPC (mgGA/l)	EC_{50} (ml/1 ml)	Reducing power (absorbance at 700 nm)	Chelating effect (%)	Inhibition of linoleic autoxidation (%)	Inhibition of ascorbate Autoxidation (%)
20	8	162.8±3.95 ^b	0.44±0.035 ^c	0.456±0.025 ^c	45.99±0.99 ^a	53.2±0.46 ^b	19.34±0.15 ^{bc}
30	2	95.41±2.91 ^f	0.74±0.03 ^a	0.114±0.01 ^f	45.87±0.84 ^a	26.12±0.34 ^{de}	13.41±0.12 ^e
25	9.24	180.21±3 ^a	0.29±0.015 ^f	0.9±0.047 ^a	46.05±0.75 ^a	71.32±0.6 ^a	22.5±0.19 ^a
25	5*	155.94±2.32 ^b	0.44±0.023 ^e	0.667±0.035 ^b	46.11±0.85 ^a	57.93±0.48 ^b	18.83±0.17 ^{bc}
17.93	5	145.65±2 ^c	0.52±0.035 ^d	0.305±0.025 ^{de}	45.8±0.73 ^a	42.21±0.32 ^c	17.25±0.13 ^d
30	8	105.92±3.89 ^e	0.63±0.022 ^b	0.346±0.025 ^d	46.04±0.55 ^a	45.11±0.2 ^c	20.08±0.21 ^b
25	0.76	162.8±3.95 ^b	0.6±0.031 ^{bc}	0.202±0.02 ^e	45.95±0.65 ^a	22±0.15 ^{ef}	11.64±0.12 ^f
20	2	139.89±3.08 ^c	0.57±0.03 ^c	0.215±0.024 ^c	45.89±0.64 ^a	31±0.23 ^d	13.33±0.1 ^e
32.07	5	99.97±1 ^{ef}	0.74±0.035 ^f	0.268±0.03 ^{de}	45.88±0.94 ^a	30.34±0.24 ^d	18.3±0.12 ^{cd}
control		95.43±3.05 ^f	0.73±0.03 ^a	0.072±0.008 ^g	45.95±0.6 ^a	19.53±0.18 ^f	9.07±0.008 ^g

Means ± SD ($n=3$) within each column with same letters are not significantly different ($P<0.05$)

* $n=5$

Fig. 1 3D plots showing the combined effect of temperature and kefir grains on (a) TPC, (b) DPPH radical scavenging, (c) reducing power, (d) inhibition effect upon linoleic acid and (e) inhibition effect upon ascorbate autoxidation

temperature for metabolic activities of microorganisms in kefir grains which are responsible for changing of antioxidant activities. Chelation of transition metals by serum albumin and lactoferrin, an iron-binding glycoprotein, and free radical scavenging activity by amino acids such as tyrosine and cysteine are some of antioxidant activities (Brand-Williams et al. 1995) of milk whey. Also antioxidant activities for some peptide chains in whey such as Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile, has been reported (Hernández-Ledesma et al. 2005).

This peptide had higher radical scavenging activity as compared to butylated hydroxyanisole (BHA). Another compound of beverage prepared in this study which can have antioxidant activity, is phenolic compounds since antioxidant activities (DPPH radical scavenging) of phenolic compounds in fruits have been reported (Lu and Foo 2000). It can be seen direct correlation exists between TPC and DPPH radical scavenging (Table 1). Young et al. (2006) also have reported there is direct relation between TPC and increase of radical scavenging activity after fermentation. Marazza et al. (2012)

have reported that fermentation of soymilk by *L. rhamnosus* can increase DPPH radical scavenging. Similar results of DPPH radical scavenging increase after fermentation have been reported by other researchers (Liu et al. 2005; Vong et al. 2006; Dordević et al. 2010).

During fermentation antioxidant compounds in kefir grains are transferred to beverage and lead to increase in DPPH radical scavenging activity (Lin and Yen 1999). Also, both intact cells and intracellular cell free extracts of *L. acidophilus* have ability of DPPH radical scavenging (Lin and Chang 2000) and *L. acidophilus* is one of the bacteria found in kefir grains. Fermentation can release amino acids such as cysteine in peptide chains of whey protein. Cysteine is able to donate hydrogen atom to DPPH radicals and therefore can neutralize this radical from the purple color to yellow color of non-radical form (diphenyl-picrylhydrazine). Besides, synergistic effect of phenolic compounds with each other or other compounds can result in enhancement of antioxidant activities such as DPPH radical scavenging (Shahidi et al. 1994). Polyphenols in apple and other fruits showed good antioxidant activities. As regards to correlation between TPC and DPPH radical scavenging observed, it can be said increase in TPC after fermentation with kefir grains can affect DPPH radical scavenging activity. The optimum amount of kefir grains and temperature for the minimum EC₅₀ value was around 6.72 % w/v and 23.5°C, respectively ($y_2=0.39$ ml/1 ml).

Reducing power

Fermented beverage reducing powers were significantly higher ($p<0.05$) than those of the control as can be observed in Table 3. Also Table 2 shows that the quadratic effect of temperature and the linear effect of kefir grain amount on reducing power were significant ($p<0.05$), but the linear effect of temperature, the quadratic effect of kefir grain amount and the mutual interaction effect between kefir grain amount and temperature on reducing power were not significant ($p<0.05$). Fig. 1c shows that the higher reducing power was observed in higher level of kefir grain amount and medium temperature in the range, around 25°C. It has been reported by Liu et al. (2005) the reducing power of both milk and soymilk increases significantly after fermentation by kefir grains. Also, Marazza et al. (2012) have observed reducing power of soymilk increases (1.5-fold) after fermentation by *Lactobacillus rhamnosus*. Wong and Kitts (2003) attributed reducing activity of certain butter milk solids to the sulfhydryl and free hydroxyl groups. Whey is a rich source of sulfhydryl amino acids such as cysteine and liberation of cysteine during fermentation can increase reducing power. In addition, formation of reductants during fermentation may be another reason for the increase of reducing ability. These reductants can react with free radicals, stabilize them and suppress radical chain reactions. It has been reported reducing power of some lactic

acid bacteria is excellent (Lin and Yen 1999). Also, increase in reducing power may be contributed to the intracellular antioxidants, peptides of the starter organisms and their hydrogen-donating ability (Yang et al. 2000). Beside of these, apple is a rich source of phenolic components and Khanizadeh et al. (2008) showed direct relation between phenolic components and reducing activity. It has been observed by other researchers that the reducing power is closely related with the antioxidant activity of polyphenolic compounds (Yen et al. 2000). After fermentation TPC increases and this can lead to increase in reducing power. On the other hand, the composition, structure and polarity of antioxidant biofactors in the fermented sample may be altered by fermentation and may thus results in the variation of antioxidant activities observed in the sample with and without fermentation (Sun et al. 2009). From the individual optimization data, a combination of 7.85 % (w/v) kefir grains and 25.25(°C) temperature was predicted for achieving the desirable reducing power (the highest value, $y_3=0.755$ (absorbance at 700 nm)).

Metal chelating effect

No significant ($p<0.05$) change was observed for metal chelating effect of beverages after fermentation (Table 3). This result is similar to findings of Liu et al. (2005) who have reported ferrous chelating activity of milk and soy milk do not change by fermentation with kefir grains. Whey proteins have ability of binding ferric or ferrous ions. The ability of lactoferrin, serum albumin, casein, and a high molecular-weight fraction of whey to chelate ferrous ion have investigated by several researchers (Tong et al. 2000). Milk fractions which have greater number of phosphoserine groups show greater affinity for iron. Although carboxyl group of the amino acids asparagine and glutamine have good ability for binding iron (Wong and Kitts, 2003). Phenolic compounds are another factors that chelating effect of fermented beverage may be attributed to them and apple contains different phenolic compounds. Phenolic compounds derived from soybean, were able to chelate ferrous ion make a safe and catalytically inactive form (Moran et al. 1997).

Inhibition effect upon lipid peroxidation

Inhibition of lipid peroxidation has great importance for prevention of the food quality deterioration and human diseases relating to free radicals. As shown in Table 3 fermented beverages demonstrated a more substantial inhibitory effect upon linoleic acid peroxidation than unfermented sample (control). Table 2 shows that inhibitory effect upon linoleic acid (y_5) depended on kefir grain amount, as its linear and quadratic effects were positive and negative at $p<0.05$, respectively. However temperature only had a significant quadratic effect ($p<0.05$). Also, the interaction effect between

Fig. 2 Schematic representation of the optimum values of factors, response, and the corresponding level

temperature and kefir grain amount was not significant at $p < 0.05$. As can be observed in Fig. 1d increase in kefir grains amount enhanced the inhibitory effect upon linoleic acid peroxidation. Also, the highest inhibitory effect was observed around temperature of 25°C (Fig. 1d).

It has been previously reported that fermentation by kefir grains can increase inhibitory effect of milk and soy milk upon linoleic acid peroxidation as compared with unfermented sample (Liu et al. 2005). Pena-Ramos and Xiong (2001) showed that peptides deriving from milk protein hydrolysates inhibited oxidation and they related this effect to the specific amino acid residue side-chain groups or the specific peptide structure of the antioxidative peptides. Thus, we suggest that the increase in inhibitory effect upon lipid peroxidation may be contributed to the peptides deriving from whey proteins. The highest response for inhibitory effect upon linoleic acid peroxidation in the range studied was observed when the

beverage was prepared with 6.85%w/v kefir grains at temperature of 23.88°C (63.83 %).

Inhibition effect upon ascorbate autoxidation

Table 3 shows that fermentation with kefir grains significantly ($p < 0.05$) increased the inhibition rate of ascorbate autoxidation. The inhibition of ascorbate autoxidation observed with beverage may be attributed to the phenols found in apple juice. Wang et al. (2006) found soymilk exhibited inhibition of ascorbate autoxidation and they attributed this to soybean phenol components. Liberation of some phenol content (Chien 2004) through the some enzymatic catalytic actions and intracellular antioxidants of starter organisms (Lin and Yen 1999) may be accounted for the rise of inhibition upon ascorbate autoxidation. As shown in Table 2 the linear and quadratic effect of kefir grain amount showed significant ($p < 0.05$) effect on the inhibition of ascorbate autoxidation. The linear and quadratic effect of temperature and also mutual interaction of temperature and kefir grains were not significant ($p < 0.05$). Fig. 1e shows that the inhibition rate upon ascorbate

Fig. 3 Optimum region, obtained by overlaying contour plots of the responses evaluated as a function of kefir grain amount and fermentation temperature

Table 4 Predicted and experimental values of the responses at optimum conditions

Optimum condition		Response variable
Predicted	Experimental ^a	
165.02	163.99±2.05	TPC (mgGA/l)
0.38	0.39±0.021	EC ₅₀ (ml/1 ml)
0.757	0.749±0.03	Reducing power (absorbance at 700 nm)
46.12	47.01±0.54	Metal chelating effect (%)
65.33	66.23±0.79	Inhibition of linoleic acid autoxidation (%)
21	20.86±0.17	Inhibition of ascorbate autoxidation (%)

^a Means ± SD ($n=3$)

autoxidation increased as the amount of kefir grains went up. The results suggested that a beverage prepared with 7.92%w/v kefir grains at temperature of 28.56°C would result in the maximum inhibition rate upon ascorbate autoxidation ($y_6=21.14\%$).

Optimization

Numerical and graphical optimization procedures were carried out for predicting the optimum level of independent variables to obtain the maximum value of TPC and antioxidant activities. The RSM package's response optimizer determined the overall optimum region to be at temperature of 24.82°C and 7.56%w/v kefir grains. Fig. 2 shows the results of the optimization. According to this Fig, the corresponding predicted response values under the optimum conditions for TPC, DPPH radical scavenging, reducing power, metal chelating effect, inhibition of linoleic acid autoxidation and inhibition of ascorbate autoxidation were 165.02 mgGA/l, 0.38 ml/1 ml, 0.757 (absorbance at 700 nm), 46.12 %, 65.33% and 21 %, respectively. The use of an overlay plot of the regression model has been highly recommended for the graphical interpretation of independent variables interactions, (Mason et al. 2003). The range of optimum conditions can be visualized by superimposing the contours for the various response surfaces in an overlay plot, which defines a region in which optimum values for all responses can be obtained. Fig. 3 presents the overlaying contour plot for the five responses, which were evaluated as a function of kefir grains and fermentation temperature. The highlight area indicates the optimum conditions in the preparation condition.

Verification of the models

A confirmation of the results using the optimum point (7.56 % kefir grain amount and temperature of 24.82°C) was accomplished by repeating six additional experiments. Results are shown in Table 4. The TPC and antioxidant activities of beverage were evaluated. The results showed that the corresponding experimental values for TPC, DPPH radical scavenging, reducing power, metal chelating effect, inhibition of linoleic acid autoxidation and inhibition of ascorbate autoxidation with the optimum condition were 163.99 mgGA/l, 0.39 ml/1 ml, 0.749 (absorbance at 700 nm), 47.01 %, 66.23% and 20.86 %, respectively. No significant ($p<0.05$) difference was found between the experimental and predicted values, which indicates the high accuracy of the present model.

Conclusion

This study showed that central composite design and response surface methodology could be successfully used in optimizing

formulation variables for apple juice and whey based novel beverage fermented by kefir grains. RSM predicted that a set level of 7.56%w/v kefir grains and temperature of 24.82°C would provide the overall optimum region for preparing beverage with the highest value of TPC and antioxidant activity. The other results obtained in this work showed that fermentation by kefir grains could increase TPC and antioxidant activities.

Acknowledgment The authors would like to thank the food science department microbiology and chemistry laboratory of University of Tehran for providing the laboratory facilities for this project.

References

- Baş D, Boyaci IH (2007) Modeling and optimization I: usability of response surface methodology. *J Food Eng* 78:836–845
- Boskov-Hansen H, Andersen MF, Nielsen L, Back-Knudsen KE, Meyer AS, Christens LP et al (2002) Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread making. *Eur Food Res Technol* 214:33–42
- Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *Food Sci Technol* 28:25–30
- Chatterton DEW, Smithers G, Roupas P, Brodtkorb A (2006) Bioactivity of β -lactoglobulin and α -lactalbumin-Technological implications for processing. *Int Dairy J* 16:1229–1240
- Chien HL (2004) Change of isoflavones contents in cultured soymilk fermented with lactic acid bacteria and bifidobacteria. M.S. Thesis, National Taiwan University, Taipei, Taiwan
- Coda R, Larena A, Trani A, Gobetti M, Cagno RD (2012) Yogurt-like beverages made of a mixture of cereals, soy and grape must: microbiology, texture, nutritional and sensory properties. *Int J Food Microbiol* 155:120–127
- Decker EA, Welch B (1990) Role of ferritin as a lipid oxidation catalyst in muscle food. *J Agric Food Chem* 38:674–677
- Dordević TM, Šiler-Marinković SS, Dimitrijević-Branković SI (2010) Effect of fermentation on antioxidant properties of some cereals and pseudo cereals. *Food Chem* 119:957–963
- Hernández-Ledesma B, Miralles B, Amigo L, Ramos M, Recio I (2005) Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *J Sci Food Agric* 85:1041–1048
- Kehrer JP (1993) Free radicals as mediators of tissue injury and disease. *CRC Crit Rev Toxicol* 23:21–48
- Khanizadeh S, Tsao R, Rekika D, Yang R, Charles MT, Rupasinghe HPV (2008) Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. *J Food Compos Anal* 21: 396–401
- La Rivière JWM, Kooiman P, Schmidt K (1967) Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis*. *Arch Mikrobiol* 59:269–278
- Lin MY, Chang FJ (2000) Antioxidative effects of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ACTT 4356. *Dig Dis Sci* 45:1617–1622
- Lin MY, Yen CL (1999) Antioxidative ability of lactic acid bacteria. *J Agric Food Chem* 47:1460–1466
- Liu JR, Chen MJ, Lin CW (2005) Antimutagenic and antioxidant properties of milk kefir and soy-milk kefir. *J Agric Food Chem* 53:2467–2474
- Lu Y, Foo LY (2000) Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chem* 68:81–85

- Marazza JA, Nazareno MA, Giori GSD, Garro MS (2012) Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. *J Funct Food* 4:594–601
- Mason RL, Richard F, Gunst RF, Hess JL (2003) Statistical design and analysis of experiments with applications to engineering and science. Wiley, Hoboken
- Mishra OP, Kovachic GB (1984) Inhibition of the autoxidation of ascorbate and norepinephrine by extracts of *Clostridium butyricum*, *Megasphaera elsdenii* and *Escherichia coli*. *Life Sci* 35:849–854
- Moran JF, Klucas RV, Grayer RJ, Abian J, Becana M (1997) Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: prooxidant and antioxidant properties. *Free Radic Biol Med* 22:861–870
- Mousavi ZE, Mousavi SM, Razavi SH, Emam-Djomeh Z, Kiani H (2011) Fermentation of pomegranate juice by probiotic lactic acid bacteria. *World J Microbiol Biotechnol* 27:123–128
- Oyaizu M (1986) Studies on product of browning reaction prepared from glucose amine. *Jpn J Nutr* 44:307–315
- Pena-Ramos EA, Xiong YL (2001) Antioxidative activity of whey protein hydrolysates in a liposomal system. *J Dairy Sci* 84:2577–2583
- Powell JE, Witthuhn RC, Todorov SD, Dicks LMT (2007) Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. *Int Dairy J* 17:190–198
- Reddy NR, Pierson MD (1994) Reduction in anti-nutritional and toxic components in plant foods by fermentation. *Food Res Int* 27:281–290
- Rice-Evans CA, Miller NJ, Paganga G (1996) Structure antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 20:933–956
- Rival SG, Fornaroli S, Boeriu CG, Wichers HJ (2001) Caseins casein hydrolysates. 1. Lipoygenase inhibitory properties. *J Agric Food Chem* 49:287–294
- Shahidi F, Wanasundara UN, Amarowicz R (1994) Natural antioxidants from low-pungency mustard flour. *Food Res Int* 27:489–493
- Simic MG (1988) Mechanisms of inhibition of free-radical processed in mutagenesis and carcinogenesis. *Mutat Res* 202:377–386
- Singleton VL, Rossi JAJ (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16:144–158
- Suetsuna K, Ukeda H, Ochi H (2000) Isolation and characterization of free radical scavenging activities peptides derived from casein. *J Nutr Biochem* 11:128–131
- Sun YP, Chou Ch, Yu RCh (2009) Antioxidant activity of lactic-fermented Chinese cabbage. *Food Chem* 115:912–917
- Tong LM, Sasaki S, Julian McClements D, Decker EA (2000) Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *J Agric Food Chem* 48:1473–1478
- Voung T, Martin L, Matar C (2006) Antioxidant activity of fermented berry juices and their effects on nitric oxide and tumor necrosis factor-alpha production in macrophages 264.7 gamma NO (-) cell line. *J Food Biochem* 30:249–268
- Wang YC, Yu RC, Chou CC (2006) Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. *Food Microbiol* 23:128–135
- Wong PYY, Kitts DD (2003) Chemistry of buttermilk solid antioxidant activity. *J Dairy Sci* 86:1541–1547
- Yang JH, Mau JL, Ko PT, Huang LC (2000) Antioxidant properties of fermented soybean broth. *Food Chem* 71:249–254
- Yen GC, Duh PD, Chuang DY (2000) Antioxidant activity of anthraquinones and anthrone. *Food Chem* 70(4):437–441