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Investigation on Escherichia Coli Inactivation and Some Quality Changes in Carrot Juice by Ultrasound Technique

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ABSTRACT

In this study Response Surface Methodology was used to optimize process conditions and to evaluate the effect of ultrasound on quality attributes (antioxidant activity, pH, total soluble solid, turbidity) and the inactivation of Escherichia coli bacteria in carrot juice. Independent variables in this study were temperature (25-50°C), time (20-40 min) and frequency (0-130 kHz). In this study thermal process (85°C, 10 min) was chosen as control sample. The Browning index (BI) was used to evaluate the color changes of carrot juice. Results showed that linear effect of frequency (X_3) and also interaction effect of frequency-time (X_2-X_3) were significant ($p<0.05$) in the inactivation of E. coli. Moreover about antioxidant activity, it was shown that, linear and quadratic effects of time were significant ($p<0.05$). The pH of samples was changed significantly ($p<0.05$) under the effect of linear (X_2) and quadratic effects of time and linear (X_3) and quadratic (X_2^2) effects of frequency and also interaction effect of temperature-frequency (X_1-X_3). None of parameters had significant (X_3^2) effect on turbidity and total soluble solid ($p>0.05$). Control sample showed higher value for browning index comparing other treatments.

Keyword: Ultrasound; Carrot juice; Antioxidant activity; E. Coli inactivation; Browning index; Optimization.

1. INTRODUCTION

Carrot juice is one of the most high consuming vegetable juice [1] containing high amounts of A provitamin (such as beta carotene). Therefore, it is used for production of ATBC (alpha tocopherol beta carotene) drinks [2, 3]. Carotenoids such as beta carotene act as antioxidants in human immune systems [4]. This product also contains B (B1, B2, B6 and B12) vitamins and minerals [5]. 100 g of

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fresh carrot juice contains 0.08 g Ca, 0.53 g P and 0.001 g Fe. Carbohydrate, fat and proteins are found at the amounts of 2.6, 0.10 and 0.9 g in 100 g of carrot juice respectively. Regarding beta carotene, this value is 1980 μg in100 g of fresh carrot juice [6]. Concerning acidity, carrot juice is considered as a low acid food due to its moderate (pH = 6), due to its pH, a bacterial infection control is required [7]. Heat treatment is a common expensive way of microorganisms' inactivation in fruit juices which reduces the number of most resistant pathogens to 5 logs [8]. Furthermore, this method has some undesirable effects on food quality in terms of flavor. Thus tendency is to propose a new method that can improve shelf-life of the product while decrease these effects [9]. Membrane filtration, osmotic dehydration, electrical pulse, irradiation, high pressure and ultrasound are some non-thermal new methods [10]. The intensity of micro-organism's inactivation by ultrasound treatment depends on the type of microorganism, environmental conditions and process parameters. It has been reported that this non thermal technique didn't have damaging effects on spherical cells as well as on spores [11]. Power ultrasound (high intensity) combined with other methods have been successfully applied for the disinfection of various food products. Other methods consist of heat treatment, chlorination, and the use of hydrogen peroxide and etc. [12, 13]. In a study the use of sonication (50W, 20 kHz) along the concentration and storage at high pressure led to decrease in salmonella count in orange juice [14]. In another study in 2011, it was found that sonication can improve the quality of lemon juice [15]. Sonication is an effective method for reduction in process time and enhancing output due to its low energy consumption [16, 17].

In this study Response Surface Methodology (RSM) was used to optimize ultrasound treatment conditions including temperature, time and frequency and base on some response variables to evaluate the ability of ultrasound in *Escherichia coli* destruction. in *Escherichia coli* is a gram negative rod shaped non sporogenic bacterium with a length of 2 μ, diameter of 0.5 μ and volume of 0.6-0.7 μ and can live on a broad range of substrates [18]. Quality characteristics of carrot juice (such as pH, total soluble solids, turbidity and antioxidant activity) are also investigated in this study. Moreover the browning index of carrot juice samples is studied by the way of Duncan's multiple rang test.

2. MATERIALS AND METHODS

2.1. Chemicals

Analytical grade of Methanol (99.9%), hydroxide sodium, phenolphetalein, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchaced from Merck Co. (Darmstadt, Germany). Culture mediums including Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB) were also bought from Merck Co. (Darmstadt, Germany).

2.2. Methods

2.2.1. Carrot juice preparing

Carrot cultivar (Daucus carota L.) in the best quality and value of 50 kg were obtained from local market (IRAN, Boen Zahra region) and kept at ambient temperature until juice extraction. According to the mentioned method in [19] with a little modification, whole of carrots were peeled slightly and washed with potable water and cut into smaller size and immediately converted into carrot juice using juice extractor (Toshiba juicer Jc-17E, Japan). Then prepared carrot juice was filtered by a sterile 3-fold cotton cloth and homogenized using a sterile tool like spoon and kept in PET bottles at 40C. This procedure was done in order to use the same batch of carrot juice during all experiments.

2.2.2. Activation of Escherichia coli and inoculation in carrot juice

The bacterium tested was prepared as lyophilized ampoule from Iranian industrial collection of bacteria and fungi. All contents of the ampoule were transferred to 20 mL culture medium (Becton Dikinson) and incubated at 35°C for 24 hours [20]. Then it was used for preparation of culture inside the sterile micro tube. The inoculation of bacterium

into carrot juice was performed according to method described in [20, 21] with a little modification. Before inoculation of bacterium to carrot juice samples, in order to obtain the same concentration of inoculated volume for all experiments, light density of inoculated suspension was measured at 600 nm by UV-Vis spectrophotometer (CECILE-2, UK) according to Mack Far land standard, and all of the inoculated carrot juice samples were exposed to ultrasound treatment after 10 min of inoculation.

2.2.3. Ultrasound treatment

Glass bottles involved inoculated carrot juice, were put into an ultrasonic bath model (UP200S, Hielscher Ultrasonic GmbH, Teltow, Germany). Temperature was kept constant by re-circulating coolant setting part (ethylene glycol: water, 50:50) during the procedure. Frequency (0-130 kHz) was also performed by adjusting the ultrasonic system in time periods of (20-40 min). Each experiment was done in triplicate.

2.2.4. Thermal treatment

A same sample was prepared as the control sample and it was exposed to thermal pasteurization treatment (85°C, 10 min) and after that it was cooled until 20-25°C. This procedure was done in triplicate.

2.2.5. Survival assay

Immediately after ultrasound process different dilutions (6 dilutions) were prepared by ringer solution under sterile conditions. Two plates of each dilution were incubated on medium (TSA) surface (Becton Dikinson) at 35°C for 48 hours. The survival rate was expressed as cfu/mL [20].

2.2.6. Antioxidant activity

According to method mentioned in [22] with a slight modification, 2,2-Diphenyl-1-picrylhydrazyl methanolic solution (DPPH) was used to measure antioxidant activity of treated carrot juice samples. 1 mL of different diluted of treated carrot juice samples was mixed with 3 mL of DPPH solution in methanol (25 mg/L) which was daily prepared. After mixing (IKA, vortex Genius 3, Germany), samples were kept in a dark place for about 30 min without any movement. Then samples were centrifuged for 10 min at 5000 rpm. Samples absorbance was measured at 515 nm by UV-Vis (CecilCE2502, Cecil Ins., England). Similarly to methods described in [22, 23] antioxidant activity of samples was presented in terms of EC50. Following equation was obtained by standard curve of DPPH methanolic solution, Y= 27.968X+3.8801 (r^2= 0.992). Remained DPPH concentration in samples (Y) was obtained by the way of putting the amount of samples absorbance (X). Furthermore, the control solution was prepared with similar proportions to the major samples using methanol until the remained DPPH percentage is also calculated.

$$\%DPPH_{Rem} = \frac{[DPPH]_t}{[DPPH]\ of\ control\ sample}$$

[DPPH] of control sample is the initial concentration of DPPH and $[DPPH]_t$ is DPPH concentration in treated sample.

2.2.7. Turbidity

Treated samples were diluted with distilled water (1:10 v/v). Turbidity of treated carrot juices was measured using a turbid meter (Portable TURB 350 IR, TUV) and was presented as Nephelometric Turbidity Units (NTU).

2.2.8. Total soluble solids

Total soluble solids were measured using a refractionmeter (ART.53000C, TR di Turoni & c.snc, Forli, Italy) and expressed as Brix at ambient temperature (approx. 25°C) [24].

2.2.9. pH

pH was evaluated at ambient temperature (approx. 25°C) using a pH meter (IKA, RCT, and Basic Germany) which was calibrated with buffer 7.0.

2.2.10. Browning index

Color of treated carrot juices was determined using a Hunter-Lab Color Flex (A60-1010-615 model

colorimeter, Hunter Lab, Reston, VA). Three color parameters (L, a, b) were used to describe exact 3D situation of color. So samples were poured into the instrument cell and color parameters were read three times. Presence of browning pigments in samples was calculated by browning index (BI), where L, a and b are correlated with (light/dark), (red/green) and (yellow/blue) spectrums respectively [25, 26].

$$BI = \frac{[100(x-0.31)]}{0.17}$$

$$x = \frac{(a+1.75L)}{(5.645L+a-3.012b)}$$

2.3. Experimental design and statistical analysis

In this study Response Surface Methodology was used to evaluate the effect of ultrasound treatment independent variables including temperature X_1 (25-50°C), time X2 (20-40 min) and frequency X_3 (0-130 kHz) on some responses (pH, TSS, turbidity, antioxidant activity, and inactivation of Escherichia coli) in carrot juice samples. Independent variables and their ranges were determined by the way of preliminary experiments and all of experiments were done in triplicate. RSM is a statistical program to optimize the experimental conditions. This method get a pattern called central composite rotatable design (CCRD) to appointment of experiment terms and includes of full factorial design , central and axial points [27, 28]. In this study a table consists of 20 runs (Table 1) with 6 central points obtained by CCRD design (Minitab Version 16 software). The use of RSM allows presenting mathematic models for each of responses as Eq. (1) which showed the significant linear, quadratic and interaction effects of

Table 1: *Matrix of the face central composite design (FCCD) and experimental data obtained for the response variables.*

	Independent variables			**Response variable [a]**				
RUN	Frequency (kHz)	Temprature (°C)	Time (min)	[1]E.C survival	[2]EC50	pH	[3]TSS	[4]Turb
1	0	25	20	450000	0.68	6.82	7.5	3735.7
2	65	37.5	20	350000	0.48	6.92	8	4084.5
3	65	37.5	30	350000	0.64	6.92	7.5	3960.2
4	0	25	40	400000	0.72	6.89	7.5	3833.1
5	130	50	40	18200	0.65	6.85	7.5	4062.2
6	0	50	40	40000	0.73	6.81	7.5	4209.0
7	65	37.5	30	380000	0.61	6.88	7.5	4012.3
8	130	25	40	100000	0.59	6.43	7.5	4137.2
9	0	50	20	203000	0.6	6.9	7.5	4175.7
10	130	37.5	30	350000	0.74	6.51	8	4646.5
11	130	50	20	160000	0.61	6.73	7.5	4339.1
12	65	50	30	32000	0.75	6.79	7	4967.8
13	130	25	20	1350000	0.54	6.4	7	4398.2
14	65	37.5	30	360000	0.62	6.86	8	3927.9
15	65	37.5	30	210000	0.64	6.89	7	3932.2
16	65	37.5	40	250000	0.64	6.89	8.5	3914.5
17	65	37.5	30	350000	0.66	6.9	7	4001.7
18	65	25	30	250000	0.67	6.59	9	2760.4
19	0	37.5	30	400000	0.67	6.6	9	4490.8
20	65	37.5	30	230000	0.65	6.87	7.5	3952.8

[a]Escherichia coli[1] (cfu/mL), Antioxidant activity[2] (%), Total soluble solids[3] (%), Turbidity [4](NTU).

independent variables on each response with their coefficients, respectively.

$$Yk = \beta k0 + \sum_{i=1}^{5} \beta kiXi + \sum_{i=1}^{5} \beta kiiXi2 + \sum_{i=1}^{4} \sum_{j=i=1}^{5} \beta kijXiXj$$

$$(K = 1,2,3,...,5) \qquad (1)$$

Where Y is the predicted response and with different subscripts is explanatory of constant regression coefficients and is correlated with linear, quadratic and interaction terms of independent variables respectively. Analysis of variance table gotten by RSM presents the effect in surface lower than 5% that are explanatory as

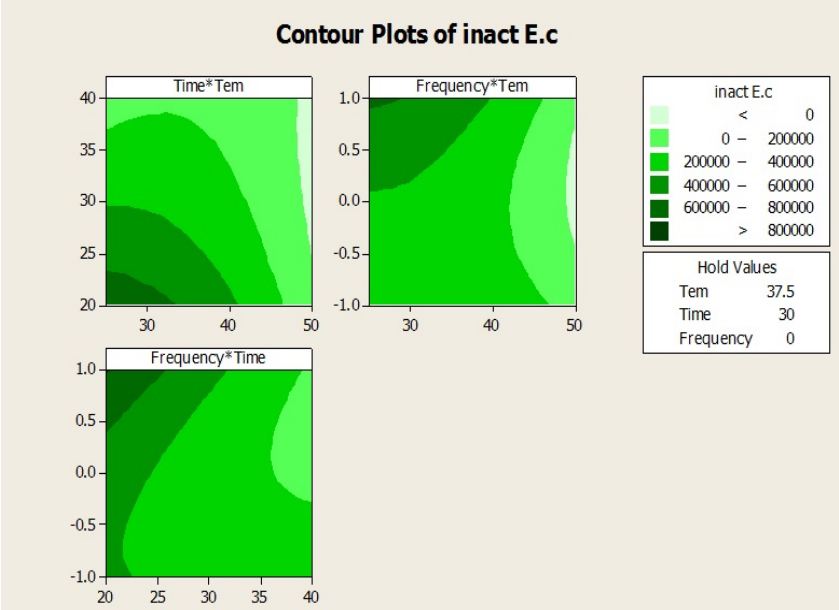

Figure 1-A: *Effects of temperature and frequency of ultrasound on E. coli inactivation.*

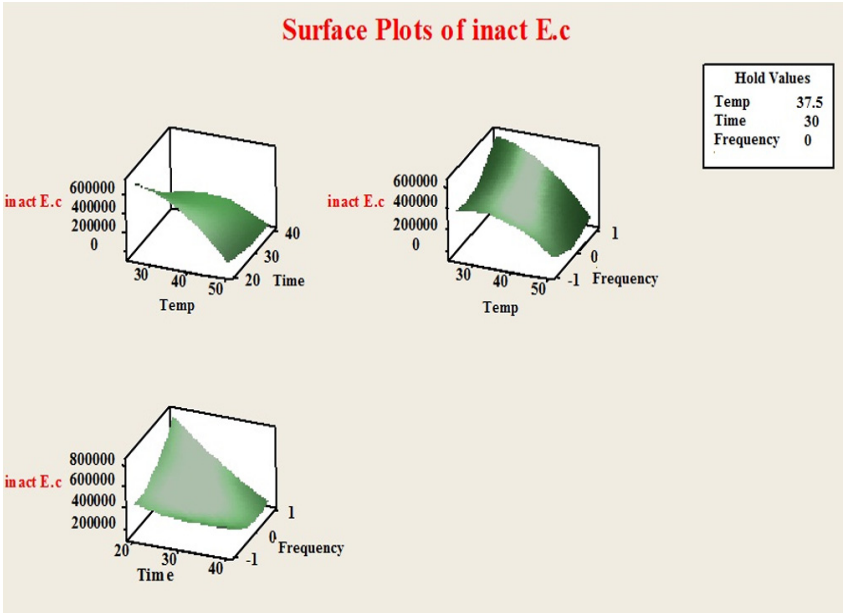

Figure 1-B: *Surface plots of effects of time temperature and frequency of ultrasound on E. coli inactivation.*

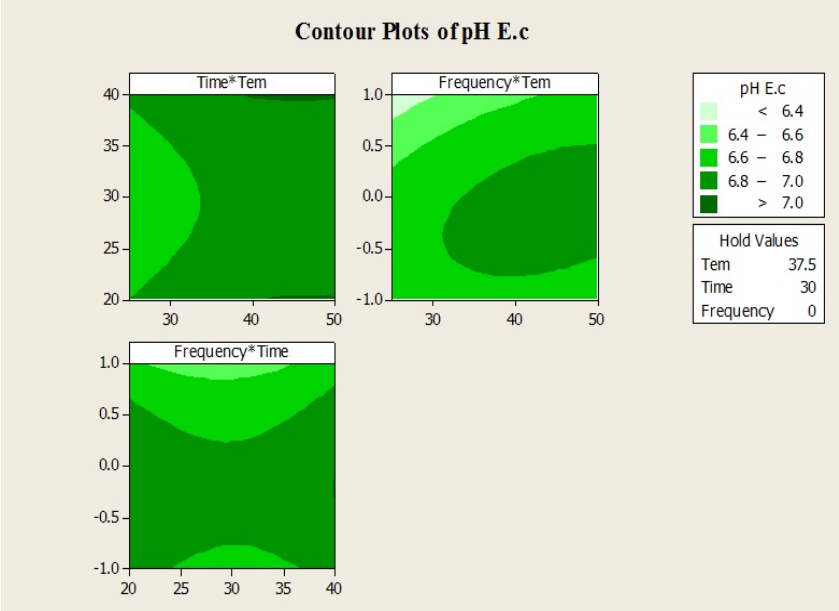

Figure 2-A: *Effects of time, temprature and frequency of ultrasound on pH.*

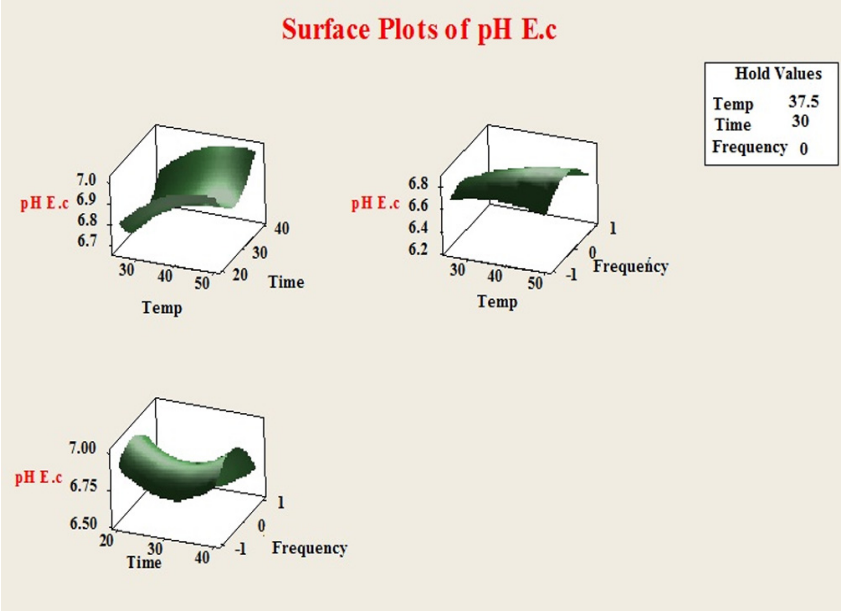

Figure 2-B: *Surface plots of effects of time temperature and frequency of ultrasound on pH.*

significant effects (Table 2) [29]. RSM also shows the interaction effects of independent variables on each of responses using contour and 3D surface plots [30]. Color assay results were not entered into response surface. Duncan's new multiple range test was used to explain the color changes.

3. RESULTS

3.1. Escherichia coli inactivation

According to results of analysis of variance (ANOVA) shown at Table 2, among of linear effects only frequency (X_3) has significant linear effect on inactivation of the bacterium and among of

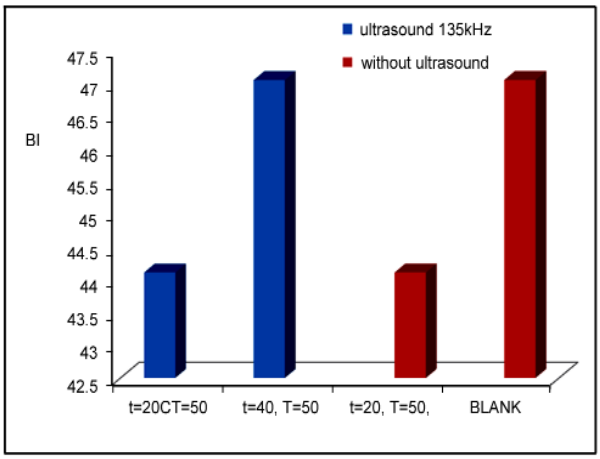

Figure 3: *Effect of time and ultrasound on browning index at constant temperature.*

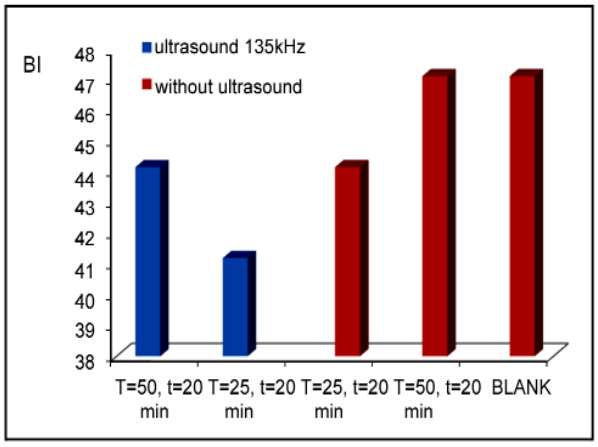

Figure 4: *Effect of temperature and ultrasound on browning index at constant time.*

Table 2: *ANOVA and regression coefficients of the second-order polynomial models for the response variables.*

Turb[4] (NTU)		TSS[3] (%)		PH		EC50[2] (%)		EC survival[1](cfu/mL)		DF	Source
PV	Coefficient	PV	Coefficient	PV	Coefficient	PV	Coefficient	PV	Coefficient		
0.977	83.590	0.467	3.88750	<0.001	7.14125	0.669	0.134	0.147	1867640	9	Model
0.286	142.125	0.507	0.15573	0.138	0.04265	0.084	-0.025716	0.856	9765	1	X_1
0.704	61.753	0.810	0.06966	0.029	-0.08419	0.005	0.060655	0.253	-79273	1	X_2
0.286	720.865	0.657	-0.52500	0.007	-0.45500	0.313	-0.072000	0.019	73987	1	X_3
0.357	-1.555	0.496	-0.00204	0.222	-0.00044	0.085	0.0003226	0.286	-752	1	X_1^2
0.678	-1.075	0.883	-0.00068	0.020	0.00146	0.004	-0.000991	0.699	415	1	X_2^2
0.096	461.609	0.695	0.18182	0.003	-0.20364	0.116	0.045909	0.290	116509	1	X_3^2
0.947	-0.080	0.818	-0.0005	0.783	-0.00007	0.537	0.00008	0.069	995	1	X_1X_2
0.439	-9.5	0.818	0.005	0.013	0.00750	0.414	0.002	0.204	-6648	1	X_1X_3
0.583	-8.357	0.818	0.00625	0.507	0.00212	0.537	-0.001	0.037	-14735	1	X_2X_3
<0.001	-	0.027	-	0.001	-	0.012	-	0.012	-	5	Lack-of-fit
-	0.5047	-	0.1578	-	0.86	-	0.7618	-	0.7967	-	R^2

[a]Escherichia coli1 (cfu/mL), Antioxidant activity2 (%), Total soluble solids3 (%), Turbidity 4(NTU).

interactive effects between independent variables only frequency time interaction (X_2-X_3) was significant (P<0.05). Model for inactivation is obtained as followings:

Inactivation of *Escherichia coli*= 1867640 + 739870X_3 - 14735X_2 X_3

As it can be seen from Figure 1-A, the survival rate depends on time and time had more effect on survival rate than frequency. Figure 1-B shows the survival rate decreased with time which is more pronounced at higher frequencies.

3.2. Antioxidant activity

Results of ANOVA presented at Table 2 showed that only linear effect of time (X_3) and quadratic effect of time (X_2) on antioxidant activity were significant (P<0.05). Contour and surface plots of samples showed that antioxidant activity was decreased with time and the highest antioxidant activity was observed at times less than 25 min. Model for antioxidant activity of samples is obtained as followings:

Antioxidant activity= 0.134000 + 0.060655X_2 - 0.000991

3.3. pH

Based on results of analysis of variance X_3 (frequency's linear effect), X_2 (time's linear effect), $X_2$2 (time's quadratic effect) and $X_3$2 (frequency's quadratic effect) had negative effects on pH (P<0.05). Among interactive effects, only X_1-X_3 interaction (temperature - frequency) had a significant effect on pH. Since pH had a limited variation range, the only factor exerting the highest effects on pH was frequency so that an increase in frequency led to pH reduction (Figures 2-A and B).

pH= 7.14125 - 0.08419X_2 - 0.45500X_3 + 0.00146 X_2^2 - 0.20364X_3^2 + 0.00750X_1X_3

3.4. Total soluble solids and turbidity

Results of AVOVA presented at Table 2 showed that none of linear, non- linear and interactive effects on total soluble solids were significant (P> 0.05). This was the same for turbidity.

Turbidity= 83.590 + 142.125X1 + 61.753X_2 + 720.865X_3 - 1.555X_1^2 - 1.075X_2^2 + 461.609X_3^2 - 0.080X_1X_2 - 9.500X_1X_3 - 8.357X_2X_3

Total soluble solids= 3.88750 + 0.15573X_1 + 0.06966X_2 - 0.52500X_3 - 0.00204X_1^2 - 0.00068 X_2^2 + 0.18182X_3^2 - 0.00050X_1X_2 + 0.005 X_1X_3 + 0.00625X_2X_3

3.5. Browning index

Figures 3 and 4 show that the highest value for browning index belonged to control sample. At a constant temperature (Figure 3) browning index was increased with time and the use of ultrasound had no effect on this index. Furthermore, at a constant time (Figure 4) browning index of control sample was higher than that of ultrasound treated samples. It can be concluded that browning index was increased with temperature.

4. DISCUSSION

4.1. Escherichia coli inactivation

As Figures 1 A and B show survival rate was decreased with time especially at higher requencies. Another study in 2011 showed that microbial load reduced by sonication depended on time. Considering the effect of sonication on total plate count (TPC), they found that reduction in microbial load occurred only after 60 minutes and microorganism cellular wall was destructed only when sonication time was increased to longer periods. They also attributed microorganism killing during sonication process to the series of physical and chemical mechanisms occurred during cavitation [15]. Ahmad and Russell (1975) obtained the same result during inactivation of Bacillus cereus and Candida albicans spores by ultrasonic bath technique. They found that applying of ultrasound was useful for time periods upper than30 min [31]. Another group of researchers (2008) stated that there were several targets for killing cells

by ultrasound waves including cellular wall, cytoplasmic membrane, DNA, intracellular structure and external membrane [32]. In accordance with this, another research (1989) showed that ultrasound treatment could have crucial effect on cytoplasmic membrane and that destruction rate of microbial cells by ultrasound depended on experimental conditions and microorganism species. Based on this study ultrasound by itself can't kill spores [33]. Oyane et al., (2009) attributed microorganism death to the formation of free radicals and hydrogen peroxide [34].

4.2. Antioxidant activity

It should be noted that antioxidant activity in the diet is related to presence of bioactive phenolic compounds, ascorbic acid, tocopherol and carotenoides in plants which increases body resistance to oxidative stress [35]. The same result was obtained on ascorbic acid content of melon juice under thermo-sonication treatment; in mentioned study when process time was increased from 0 to 10 minutes, ascorbic acid content was decreased and at extreme conditions (the highest amplitude, frequency and time) ascorbic acid percent was reduced to 50% significantly ($P<0.05$). Furthermore significant decrease was observed on phenolic compounds content of melon juice when temperature increased up to 45°C at higher frequencies and times [36]. Ascorbic acid decomposition can attribute to the intensified physical conditions occurred in bubbles during cavitation [37, 38] and to simultaneous or separate disintegration of these bubbles. In other words because these bubble are full of vapor and soluble gases such as O_2 and N_2 they bring about consequent sonochemical reactions [39]. Ascorbic acid decomposition in higher frequencies and times has also been attributed to oxidation by free radicals [40]. Another same result was observed by Zhou et al., (2006) on destruction of Astaxanthin (one kind of carotenoid pigments) under ultrasound treatment. They stated that these changes are more severe at higher times and powers of ultrasound [41].

4.3. pH

The only factor influencing pH significantly was frequency which was probably due to the partial decomposition of some compounds as a result of ultrasound which leads to the formation of H^+ ions, higher solubility and enhancement of acidity. In a study (2010) done on the use of ultrasound for grape puree, it was found that sonication treatment increased total acidity by 13.6% compared to control treatment (traditional enzymatic treatment). Their result was attributed to better derivation of acidic compounds by ultrasound [42]. It should be noted that effect of ultrasound on pH, depends on intensity of frequency, treatment time, temperature and type of juice. Thus Tiwari et al., (2009a) found any significant effect on pH in treated orange juice. They attributed this observation to extents of applying frequency, temperature and time during sonication [43]. Another study was done in 2006 on apple cider and showed insignificant effect on pH [44]. Dizadji et al., (2012) studied on the effect of ultrasound in kiwi juice and found no significant effect on pH due to buffer effect of kiwi juice [45].

4.4. Browning index

As Figures 3 and 4 show, the highest amounts of browning substances were produced in control sample. At higher intensities of ultrasound and temperature beta carotene decomposition rate was decreased. The reason was that bubbles formed due to the cavitation process inhibited emission of ultrasound waves under these conditions as a result of enhanced size. Also disintegration of these large bubbles led to decrease in cavitation effects [46]. Therefore the factor causing the highest changes in samples color was temperature. This can be attributed to the formation of dark compounds at higher temperatures. In order to study [47] they showed a relationship between the formation of insoluble brown compounds and mechanisms of hydrolysis or decomposition of anthocyanin caused by heat. Formation of browning compounds requires sugar. Presence of bacterium in samples was not ineffective in color changes. Therefore, it can be concluded that at higher temperatures higher number of bacterium was killed and

consequently the consumption of sugar matters was decreased. This can lead to the presence of higher amounts of sugar substances for involvement in browning reactions.

5. OPTIMIZATION

In this study two responses; Escherichia coli inactivation and antioxidant activity; were optimized by RSM. The main purpose of our study was achieving to the highest level of Escherichia coli inactivation and the least level of antioxidant activity destruction. These two responses vary inversely. It means that each factor causes further Escherichia coli inactivation, it can also cause a decrease in antioxidant activity which is not desirable. The best conditions to obtain maximum E. coli inactivation and minimum antioxidant activity destruction were determined as temperature= 48.73°C, time= 47.28 min and frequency= 130 kHz by RSM optimization. In this optimized condition, the residual amount of Escherichia coli will be (approx. 1.003×10^4 cfu/mL) and in other pronunciation we will receive to 100% of Escherichia coli inactivation goal.

6. CONCLUSIONS

Analysis of variance (ANOVA) showed that in Escherichia coli inactivation the linear effect of frequency and also interaction effect of frequency-time were significant ($p<0.05$). According to the ANOVA, it was seen that regarding antioxidant activity, linear and quadratic effects of time were significant ($p<0.05$). The pH of samples was changed significantly ($p<0.05$) under the linear and quadratic effects of time and frequency and also interaction effect of temperature-frequency. No significant effect of any variables was found on turbidity and total soluble solid of all samples ($p>0.05$). About Browning index of samples the highest level was found in control sample.

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