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Full Research Paper

Optimization of sugar free dark chocolate product compatible for ketogenic diet and investigating its physicochemical, textural, thermal and sensory properties

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Abstract

There is a challenge in producing a portion both compatible to ketogenic diet and sufficient satiety. This study investigated the possibility of producing sugar-free chocolate product using increasing total fat and protein. The ingredients were chosen such that they do not contain any source of starch and sucrose. The cocoa powder was replaced with cocoa butter substitute (CBS) and sodium caseinate at different levels (0, 5 and 10%) along with constant amount of stevia ketogenic powder and soybean hull as sugar substitute. Results showed that cocoa powder substitution significantly ($p< 0.05$) led to an increase in moisture, water activity, fat and protein and a decrease in ash and carbohydrate amount, respectively. It was also observed that addition of sodium caseinate and CBS made the chocolate softer and to be easily melted ($p< 0.05$). Sensory analysis showed that samples with high protein and fat content got better scores in overall acceptance ($p< 0.05$). Also, principle component analysis showed that the first two components could explain about 81% of total variance. Finally, the best composition was determined by considering both TPA, DSC and sensory properties. This sample contained 5% sodium caseinate and 35% CBS. Moreover, total sugar content and calorie amount of this sample was 2.17% and 547.41 kcal, respectively. The peroxide value of optimized sample was 0.5 meq per kg immediately after production and it reached to 1.13 meq per kg after two months. Consuming 100 g of this chocolate can supply 27% of daily calorie of an adult person (assuming 2000 kcal per day for adults). Consuming this 100 g can also supply 17% and 40% of classic and atkins keto diet. These results showed that, the selected sample with 35% CBS and 5% sodium caseinate could be compatible to ketogenic diet but more clinical research should be done in future.

Keyword: Sugar free, Chocolate, Ketogenic diet, Stevia.

Introduction

Chocolate is a suspension of solid particles including sugar, cocoa and milk ingredients in a continuous fat phase usually cocoa butter (Glicerina et al., 2013). Different kinds of chocolate are assorted according to type of solid particles. Dark chocolate usually contains sugar, high cocoa amount, and less milk ingredients in comparison to milk and white ones. Dark chocolate is a rich source of

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antioxidant due to high cocoa amount in formulation (Afoakwa, 2010). Consuming 100 g of dark chocolate can supply 25% of an adult daily calorie requirement (Zugravu & Otelea, 2019). According to Codex standard, the chocolate should have cocoa butter as main fat ingredient. The chocolate produced with cocoa butter substitute (CBS) is called chocolate product. Hydrogenated palm kernel oil is a kind of cocoa butter substitute. It has sharp melting rate due to its short chain triglycerides like lauric acid (Beckett, 2009).

The ketogenic diet is a kind of eating behavior in which consumption of fat increases, protein amount moderates, and carbohydrate limits to 20 or 50 g per day. The restriction of carbohydrate intake activates the liver to break fat into ketone body and fatty acid. This state is called ketosis (Kalra et al., 2018). In high carbohydrate diet, glucose would generate adenosine triphosphate (ATP) during glycogenesis metabolic pathway but in the ketogenic diet the mechanism is different. Ketones (produced by fat break in liver) generate acetyl coenzyme A (acetyle CoA) during kerbs cycle and energy would be supplied (Clanton et al., 2017). Ketogenic diet is different from low carbohydrate diet. Low carb is kind of diet which was introduced by William Banting in 1863. Carbohydrate amount should be consumed up to 130 g per day in this diet (Watanabe et al., 2020).

The first step to produce a keto-friendly chocolate is sugar substitution. Sugar should be replaced by a low energy sweetener and keto compatible bulking agent. Stevia and alcoholic sugars are the main substitution of sugar in confectionary industry (Sabbaghi, 2021). One such sweetener is the stevia which is a natural sweetener with 300 times sweetness compared to sucrose. Due to its low glycemic index, it can be safe for ketogenic diet (Shah et al., 2010). European Food Safety Authority stated that the stevia consumption is permitted up to 4 mg per day and it is generally recognized as safe (GRAS) (Aidoo et al., 2013). The soybean hull is waste of soybean oil extraction. The hull consists of 8 to 10 percent of whole bean weight. The soybean hull is a rich source of dietary fiber including cellulose, hemi cellulose, lignin, and pectin. It lacks some specific compounds such that the glycogenesis metabolic pathway is not followed (Poore et al., 2012).

Increasing fat content of the chocolate helps it to be more compatible to ketogenic diet. However, different variations of fat amount in chocolate formulation are limited by the dominance of solid's fraction on textural and also other properties of the chocolate (Aidoo et al., 2017). Rezende et al. (2015) declared a direct relationship between fat amount and hardness of the chocolate due to its higher Solid Fat Content (SFC). Azevedo et al. (2017) reported that high fat chocolates were sensed less bitter and also easily melted than low fat chocolate. Aidoo et al. (2015) found out that increasing fat content of sugar free dark chocolate decreased the hardness significantly but the peak temperature was not influenced by this variation. Guinard & Mazzucchelli. (1999) also stated that high fat chocolate samples were faster melting in the mouth according to hedonic sensory test.

Increasing fat amount is not sufficient for the purpose of this study. Ketogenic diet should be enriched with 60- 90% of fat amount (Clanton et al., 2017). In fact, the consumption of food is both for satiety and palatable sense (Holt et al., 1995). There is a reverse relationship between satiety and fat content of food. In other words, the ketogenic diet as a high fat diet is less satiation. Among all macronutrients, protein has more potential action on satiety (Gerstein et al., 2004). So other macronutrient compounds like fiber and protein are needed to be added into ketogenic base formulation to supply satiation sufficiency.

Casein is a type of milk protein existing in 4 types including κ-Casein, β-Casein, αs1-Casein, and αs2-Casein. Sodium caseinate can be immediately dispersed in the fat phase. It is usually used in dairy, meat, and coffee industries (Modler, 1985). Some researchers studied the effect of increasing total protein in the chocolate. Zarić et al. (2015) investigated

the effect of increasing soymilk powder in chocolate formulation. They declared that addition of 20% of soymilk powder changed the optimal producing procedure. These samples would produce in longer milling time with less pre-crystallization temperature. Also, the interaction between soy protein and glycine made more stable gel and T_{index} enhancement. Ashrafie et al. (2014) evaluated the effect of cocoa butter substitution by collagen hydrolysate. Samples with 15 and 20% substitution were the best ones according to textural and sensory experiments.

Therefore, there are limited researches in which the effect of increasing both total fat and total protein were studied. The main purpose of this study was to investigate the effect of increasing total fat and protein and decreasing cocoa powder on physicochemical, textural, thermal and sensory properties of chocolate. Additionally, the acceptable amount of each material will be reported.

Materials and Methods:

Various ingredients include cocoa powder (Altinmarka, Turkey– with 10-12% fat), cocoa butter substitute (Sime-Darby, Malaysia), stevia ketogenic powder (Below, Iran-containing inulin, stevioside and isomalt), soybean hull (Toos-soya, Iran-with 83.7% carbohydrate, 0% sucrose, 1.2% fat, 5.5% ash and 9.2% protein), Polyglycerol polyricinoleate (PGPR) (Kerry, Belgium), the soy lecithin and sodium caseinate (92% protein, 2.9% carbohydrate,0.1% sucrose, 0.9%fat and 4.1% ash) (Toos-Argan, Iran).

Chocolate production

Dark chocolate samples were formulated in factorial design as shown in Table 1. Samples were prepared by semi-industrial ball mill refiner (made by Arman-kherad-toos, Iran) equipped with steel balls 0.92 mm in diameter. First, the melted cocoa butter substitute and emulsifiers were added to ball mill which was rotated at 10 rpm for 5 minutes in 50°C. Then, other mixed ingredients were gradually added to ball mill refiner. The instrument was set at 100 rpm for 4 h. Finally, the mixture was placed into molds with cube shapes and vibrated to remove the bubbles. After cooling the samples to 4°C for 30 minutes, they were wrapped in aluminum foils and stored at 25°C in the incubator for the analysis.

Table 1- The percentage of ingredients in the nine chocolate samples

Sample	Cocoa powder (%)	Cocoa butter substitute (%)	Stevia ketogenic powder (%)	Sodium caseinate (%)	Soy hull (%)	PGPR (%)	Lecithin (%)
1	37	30	25	0	7	0.7	0.3
2	32	30	25	5	7	0.7	0.3
3	27	30	25	10	7	0.7	0.3
4	32	35	25	0	7	0.7	0.3
5	27	35	25	5	7	0.7	0.3
6	22	35	25	10	7	0.7	0.3
7	27	40	25	0	7	0.7	0.3
8	22	40	25	5	7	0.7	0.3
9	17	40	25	10	7	0.7	0.3

Analytical methods

Proximate chemical analysis

The moisture was measured by oven (AOAC, 2000). The water activity analyzer was also used to measure water activity at 25°C (Novasina, Switzerland). The crude protein content was estimated by the Kjeldhal method (AOAC, 2000) and the fat content was measured by Soxhlet extraction (AOAC, 2000). Total minerals were measured as the residue after ashing at 550°C overnight. The carbohydrate content was calculated by

subtracting the sum of moisture, fat, ash and protein content from 100.

Hardness

Textural parameters of the chocolate samples (1.1× 1.1× 1.1 cm^3) were evaluated by TA plus texture analyzer (Lloyd Instruments Ltd, UK) connected to the Nexygen software (version 4.5.1). A trigger force was set on 5 g. The puncture test was done with flat ended probe, 2 mm in diameter, and a 50 N load cell. The probe penetrated each sample to a depth of 5 mm at a constant speed of 1 mm/s at the ambient temperature (Aidoo et al., 2013).

Melting properties

The parameter was evaluated by DSC 214 Polyma (Netzsch, Germany). Five mg of each sample was placed in the instrument's pan. The samples were then heated from 0 to 60°C at a rate of 10°C/min in N_2 stream. Onset temperature (T_{onset}), maximum temperature (T_{max}) and offset temperature (T_{off}) were calculated.by software (Genc Polat et al., 2020).

Sensory evaluation

Chocolate samples (2.5 g) were randomly coded in three-digit cod numbers. Fifteen trained panelists (25- 40 years old including 4 men, 11 women) were selected according to being interested in participating and absence of aversions, allergies or intolerance against chocolate. The evaluations were done at the specific time of 9- 12 A.M. According to Table 2, the sensory attributes of these chocolates including color, hardness, melting rate, bitterness, mouth coating, and casein flavor and overall acceptance were evaluated using a hedonic test 9-point rating scale. The panelists were asked to consume water and cracker between each sample (Rezende et al., 2015).

Table 2- Definition and the main references used in the hedonic test

Attribute	Definition	References
Color	Dark color without any white spots on the surface	Weak: Milk chocolate Strong: 80% Dark chocolate
Hardness	The force required to bite the chocolate using front teeth	Weak: Probiotic cheese Strong: Carrot
Melting rate	The time required for the conversion of solid phase of chocolate into liquid one in the mouth	Weak: Probiotic cheese Strong: Toffee
Bitterness	Characteristic taste of caffeine	Weak: Milk chocolate Strong: 80% Dark chocolate
Casein flavor	Characteristic taste of casein	Weak: Skim milk powder Strong: 99% Dark chocolate
Mouth coating	Sensation of a fat layer in the mouth after the swallowing of chocolate	Weak: Water Strong: Cocoa butter substitution

Ketogenic validation of optimized sample

Reducing sugar content was measured by Lane-Eynon Method (AOAC, 2000). The calorific value was calculated by summing up the samples of multiplication of carbohydrate, protein and fat content by 3.7, 4.36, and 9.02 respectively (Sai et al., 2016).

Oil stability evaluation of optimized sample

The peroxide value was measured for the optimized treatment immediately after production and also after two months (AOAC, 2000).

Data analysis

The effect of cocoa powder, fat and protein amount on the quality of the chocolate samples was analyzed by two-way ANOVA at a

significance level of 95% significance with SPSS version 25 software. All experiments were conducted in triplicates and then mean values were considered. Mean values were compared with Duncan's test at $p<0.05$. Also principal component analysis (with pearson correlation coefficient) was used to determine the relationship between all sensory variables by using SPSS software.

Results and discussion

Proximate chemical analysis

According to Table 3, the moisture content of all samples was in acceptable range of 0.5-1.5 % as Afoakwa mentioned (Afoakwa, 2010). Also, by increasing sodium caseinate, the moisture content is significantly increased ($p<0.05$). This is due to the interaction between protein and vapor molecules which came to the structure during counching. Hydrophilic part of sodium caseinate like carboxyl and phosphoseryl (in B-Cn monomer) creates the hydrogen bond with water and then moisture content would increase (Post et al., 2012; Modler, 1985). As a result of increasing moisture, less water was available in the structure and then water activity was reduced. Ashrafie et al. (2014) found that collagen addition in the chocolate formulation influenced the moisture of chocolate due to the interaction between steam and collagen hydrophilic part but this variation of collagen amount didn't significantly ($p>0.05$) affect the water activity.

Cocoa is a rich source of minerals like Calcium, cupper, etc. (Afoakwa et al, 2007). Analysis of variance showed that both CBS and sodium caseinate had significant impact on ash amount ($p<0.05$). According to Table 3, as the cocoa powder substitution increased, the ash amount is reduced. This reduction was due to the more amount of minerals which existed in the cocoa powder. Onwuka & Abasiekong (2006) substituted the cocoa powder with Bambara Groundnut and Treculia Africana. As cocoa substitution increased, ash amount became more. Because the bambara groundnut and treculia Africana had more ash than cocoa powder.

Total fat content of the chocolate depends on CBS amount and rare quantity of fat in other ingredients. According to Table 3, total fat content of samples with 35 and 40 g of CBS increased 5.54 and 11.72 percent respectively in comparison with 30 g CBS sample. Also, total protein amount of samples ranged between 5.42 and 14.60 gram in 100 g of chocolate. Samples 3, 6, and 9 had the considerable amount of total protein than others. According to Table 3, the carbohydrate amount was differed from 40.81 to 5.66 percent. In fact, samples with less cocoa powder substitution had less carbohydrate amount.

Table 3- The chemical composition of chocolate samples

Sample	Carbohydrate (%)	Ash (%)	Protein (%)	Fat (%)	Water activity (%)	Moisture (%)
1	$55.66\pm 0.55^{Aa\lambda.1}$	$5.61\pm 0.04^{Aa\lambda.1}$	$7.40\pm 0.10^{Aa\lambda.1}$	$30.34\pm 0.44^{Aa\lambda.1}$	$0.21\pm 0.00^{Aa\lambda.1}$	$0.95\pm 0.01^{Aa\lambda.1}$
2	$52.11\pm 0.29^{Aa\lambda.2}$	$5.19\pm 0.62^{Ab\lambda.2}$	$11.32\pm 0.60^{Ab\lambda.12}$	$30.38\pm 0.34^{Aa\lambda.2}$	$0.20\pm 0.00^{Ab\lambda.12}$	$0.97\pm 0.04^{Ab\lambda.12}$
3	$48.66\pm 0.41^{Ac\lambda.3}$	$5.09\pm 0.60^{Ac\lambda.3}$	$14.60\pm 0.04^{Ac\lambda.12}$	$30.66\pm 0.38^{Ac\lambda.23}$	$0.18\pm 0.00^{Ab\lambda.123}$	$0.98\pm 0.04^{Ac\lambda.123}$
4	$51.29\pm 0.27^{Bc\lambda.2}$	$5.16\pm 0.11^{Ba\lambda.2}$	$6.34\pm 0.06^{Ba\lambda.12}$	$36.23\pm 0.28^{Ba\lambda.12}$	$0.21\pm 0.00^{Ab\lambda.12}$	$0.95\pm 0.02^{AB\lambda.1}$
5	$48.13\pm 0.43^{Bb\lambda.3}$	$4.57\pm 0.11^{Bb\lambda.3}$	$10.38\pm 0.10^{Bb\lambda.12}$	$35.93\pm 0.34^{Ba\lambda.23}$	$0.20\pm 0.00^{Ab\lambda.123}$	$0.96\pm 0.01^{ABb\lambda.12}$
6	$45.25\pm 0.31^{Bc\lambda.3}$	$4.27\pm 0.03^{Bc\lambda.3}$	$13.63\pm 0.04^{Bc\lambda.12}$	$35.86\pm 0.31^{Ca\lambda.23}$	$0.19\pm 0.00^{Ab\lambda.23}$	$0.97\pm 0.03^{AB\lambda.12}$
7	$47.00\pm 0.81^{Ca\lambda.3}$	$4.44\pm 0.04^{Ca\lambda.3}$	$5.42\pm 0.63^{Ca\lambda.12}$	$42.16\pm 0.75^{Ca\lambda.23}$	$0.20\pm 0.00^{Ab\lambda.123}$	$0.96\pm 0.05^{Ba\lambda.12}$
8	$43.28\pm 0.44^{Cb\lambda.4}$	$4.06\pm 0.05^{Cb\lambda.4}$	$9.27\pm 0.05^{Ca\lambda.12}$	$42.41\pm 0.08^{Ca\lambda.34}$	$0.20\pm 0.00^{Ab\lambda.23}$	$0.97\pm 0.06^{Bb\lambda.23}$
9	$48.81\pm 0.40^{Cc\lambda.5}$	$3.62\pm 0.34^{Cc\lambda.4}$	$12.61\pm 0.40^{Ca\lambda.2}$	$41.96\pm 0.11^{Ca\lambda.4}$	$0.19\pm 0.00^{Ab\lambda.3}$	$0.98\pm 0.02^{Bc\lambda.123}$

***** Means ± standard deviations from analysis, means within same column with different letters are significantly different ($P<0.05$) from each other for each measured parameter. Uppercase and lowercase letters represent CBS and sodium caseinate, respectively. The letter λ shows the cocoa powder variable.

Hardness

Hardness shows the amount of force needed to penetrate the chocolate structure. It depends on ingredients, polymorphism structure of cocoa butter, and the production method (Rezende et al., 2015). ANOVA showed that both CBS and sodium caseinate significantly ($p<0.05$) influenced the hardness of chocolate samples. According to Fig 1, as cocoa substitution increased, the hardness was reduced. Samples 1 and 9 were the hardest and softest samples, respectively. Addition of CBS to chocolate formulation made more space for solid particles to move. Consequently, the interactions became less and the structure would be softer. Also, as sodium caseinate increased, the cocoa powder and its fiber content were decreased. The less fiber made the texture softer.

There are two kinds of studies about the effect of increasing fat amount on the hardness. The first one relies on increasing solid fat content as a result of increasing fat phase. This SFC increasing would lead to stronger network and harder structure of chocolate. Do et al. (2007) and Rezende et al. (2015) investigated different amount of fat content on the hardness of chocolate. They stated that samples containing more fat content showed more hardness due to the SFC increase and its effect on hardness. The other studies reported an inverse relationship between fat amount and hardness. They rely on the fact that the addition of fat led to increase the fat phase and then solid particles and their concentration would reduce. As a result, the hardness became less as fat amount is increased. Afoakwa et al. (2008) and Aidoo et al. (2017) declared that high fat chocolate samples had less hardness at any particle size or bulking agent concentrations.

The hardness ranged between 30.61- 58.46 N in our study. This amount is near to Do et al. (2007). They investigated the effect of fat increase at 3 levels of 22, 25 and 30% cocoa butter equivalent (CBE) on the hardness of chocolate. The hardness in that study was between 30- 55 N. It can be concluded that the presence of 40% CBS didn't change the minimal hardness in this study. Dewi et al. (2020) reported that hardness was between 15-20 N at 34% CBS in the formulation which was so lower than the current study. The presence of sodium caseinate and soybean hull might be the reason for the suitable hardness even at low fat samples. Also, there was a significant ($p< 0.05$) correlation between hardness and ash ($r= 0.97$), fat ($r= -0.89$) and carbohydrate ($r= 0.95$). It means that as fat content of samples reduced and ash or carbohydrate increased, the hardness would increase.

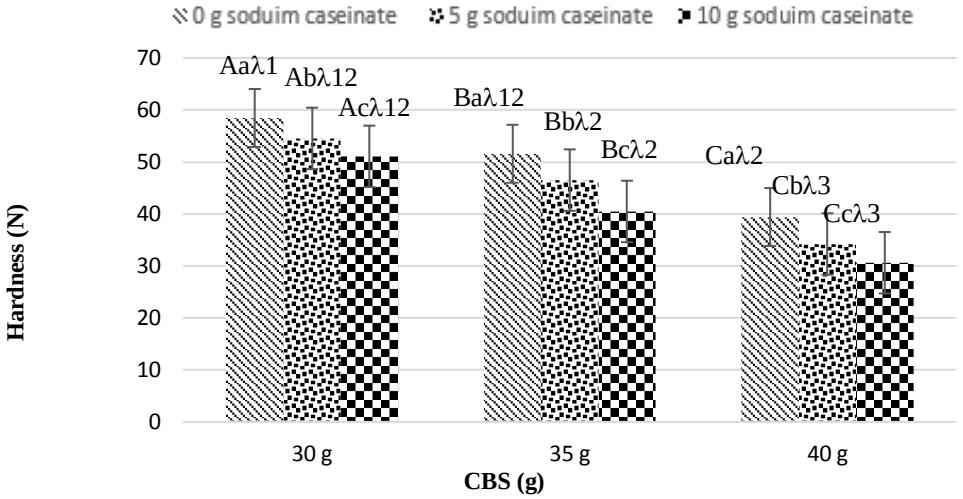

Fig. 1. The effect of CBS and sodium caseinate on hardness of chocolate samples.

*Uppercase and lowercase letters represent CBS and sodium caseinate, respectively. The letter λ shows the cocoa powder variable.

Melting Properties

In the current study, the effect of cocoa powder substitution on thermal properties of chocolate samples was studied. In Table 4, the thermal properties of samples heated from 0 to 60°C were reported. T_{onset}, T_{Peak} and T_{off} values varied between 23.06– 25.43°C, 27.60-29.40°C and 33.40- 29.93°C, respectively. ANOVA showed that increasing CBS from 30% to 40% significantly ($p< 0.05$) reduced all thermal parameters. In fact, the fat phase is responsible for melting behavior of chocolate. When more CBS was added to the formulation, it covered the solid particles. Consequently, the solid's interactions became less and free moving plastic flow is increased. Also, more fat could easily dispersed in salvia and melting procedure could be facilitated. (Afoakwa et al, 2008; Glicerina et al., 2013). Afoakwa et al. (2008) declared that increasing fat content significantly affected only offset temperature. Also, they reported all the parameters above 30° C which were higher than this study.

There is a significant ($p< 0.05$) correlation between peak temperature and ash ($r= 0.84$), fat ($r= -0.90$), carbohydrate ($r= 0.81$), and hardness ($r= 0.89$). It means that as fat content of samples reduced and ash or carbohydrate increased, the peak temperature also increased. As mentioned before, by increasing fat amount, more spaces are created in the structure and as a result particle-particle interactions are reduced. This reduction both facilities first bite (hardness) and melting of the chocolate samples.

Table 4- The effect of CBS and sodium caseinate on melting properties of chocolate samples

Sample	T_{onset} (°C)	T_{max} (°C)	T_{off} (°C)
1	$25.20\pm0.36^{Aa\lambda.1}$	$29.10\pm0.26^{Aa\lambda.1}$	$32.96\pm0.45^{Aa\lambda.1}$
2	$25.43\pm0.41^{Aa\lambda.1}$	$29.46\pm0.10^{Aa\lambda.1}$	$33.40\pm0.26^{Aa\lambda.1}$
3	$25.43\pm0.31^{Aa\lambda.1}$	$29.00\pm0.17^{Aa\lambda.1}$	$33.26\pm0.56^{Aa\lambda.1}$
4	$23.60\pm0.43^{Ba\lambda.2}$	$28.96\pm0.43^{Ba\lambda.2}$	$30.56\pm0.50^{Ba\lambda.2}$
5	$23.80\pm0.66^{Ba\lambda.2}$	$28.40\pm0.26^{Ba\lambda.2}$	$29.93\pm0.11^{Ba\lambda.2}$
6	$23.56\pm0.23^{Ba\lambda.2}$	$28.38\pm0.45^{Ba\lambda.2}$	$30.43\pm0.35^{Ba\lambda.2}$
7	$23.26\pm0.28^{Ca\lambda.3}$	$27.76\pm0.20^{Ca\lambda.3}$	$30.70\pm0.36^{Ca\lambda.3}$
8	$23.06\pm0.30^{Ca\lambda.3}$	$27.60\pm0.34^{Ca\lambda.3}$	$29.96\pm0.61^{Ca\lambda.3}$
9	$23.10\pm0.36^{Ca\lambda.3}$	$27.63\pm0.15^{Ca\lambda.3}$	$30.03\pm0.51^{Ca\lambda.3}$

***** Means ±standard deviations from analysis, means within same column with different letters are significantly different ($P< 0.05$) from each other for each measured parameter. Uppercase and lowercase letters represent CBS and sodium caseinate, respectively. The letter λ shows the cocoa powder variable.

Sensory Analysis

ANOVA showed that all sensory parameters were significantly affected by the cocoa powder substitution ($p<0.05$). As cocoa substitution increased, the panelists' score on the color decreased. It means they prefer darker chocolate samples. Guinard and musschelli, (1999) reported the same result. They stated that higher score was given to dark samples by panelists. Addition of CBS to samples significantly influenced the hardness and melting rate ($p< 0.05$). Generally, samples with 35 and 40% CBS had less hardness and the melting rate scores than samples with 30% CBS. In fact, the panelists prefer softer and easily melted samples. Farzanmehr and Abbasi, (2008) reported the same result. The cocoa substitution had a positive effect on bitterness and casein flavor perception. The panelists liked the dairy flavor of casein. Perception of chocolate bitterness intensity is kind of diversity matter in people. However, this study revealed that panelists liked less bitter chocolate samples. Puchol Michel et al. (2020) reported the same result. They stated that samples containing 70% cocoa were more acceptable in sweetness than 95% cocoa powder. Mouth coating is the layer of fat which

covers mouth surfaces (Tobrica et al., 2016). Mouth coating of sample 7 and then 5 and 6 were the best. According to Table 5, samples with high fat t had more overall acceptance scores. Samples 5 and 7 had the highest overall acceptance scores. In fact, overall acceptance had a significant correlation with the fat content (r= 0.39). In other words, as fat content of samples increased, overall acceptance scores also increased. Rezende et al. (2015) reported the same result in which panelists gave more scores to high fat samples.

Comparing sensory and instrumental data showed an acceptable correlation. The correlation coefficients of hardness and melting variables were 0.88 and 0.70, respectively. Mahdavian and Mazahei (2015) also found high correlation between sensory and instrumental results in chocolate enriched with silver skin of coffee.

Table 5- Sensory characteristics of the chocolate samples

Sample	Overall acceptance	Mouth coating	Casein Flavor	Bitterness	Melting rate	Hardness	Color
1	$5.57\pm0.14^{Aa\lambda.1}$	$5.11\pm0.40^{Aa\lambda.1}$	$4.33\pm0.21^{Aa\lambda.1}$	$5.16\pm0.25^{Aa\lambda.1}$	$6.02\pm0.14^{Aa\lambda.1}$	$5.38\pm0.26^{Aa\lambda.1}$	$6.67\pm0.19^{Aa\lambda.1}$
2	$5.32\pm0.27^{Aa\lambda.1}$	$5.13\pm0.77^{Aa\lambda.1}$	$5.44\pm0.60^{Ab\lambda.1}$	$5.22\pm0.17^{Aab\lambda.12}$	$6.07\pm0.60^{Aa\lambda.1}$	$5.47\pm0.17^{Aab\lambda.12}$	$6.46\pm0.87^{Aa\lambda.1}$
3	$5.38\pm0.21^{Aa\lambda.1}$	$5.15\pm0.30^{Aa\lambda.1}$	$5.58\pm0.19^{Ab\lambda.1}$	$5.40\pm0.16^{Ab\lambda.2}$	$6.18\pm0.16^{Aa\lambda.1}$	$5.45\pm0.19^{Ab\lambda.2}$	$6.32\pm0.05^{Ab\lambda.1}$
4	$6.26\pm0.17^{Ba\lambda.2}$	$5.74\pm0.13^{Ba\lambda.2}$	$5.01\pm0.10^{ABa\lambda.12}$	$5.99\pm0.37^{Ba\lambda.23}$	$7.40\pm0.48^{Ba\lambda.2}$	$5.42\pm0.46^{Ba\lambda.2}$	$6.38\pm0.13^{Ba\lambda.2}$
5	$6.70\pm0.33^{Ba\lambda.2}$	$5.79\pm0.80^{Ba\lambda.2}$	$5.41\pm0.38^{ABa\lambda.2}$	$6.37\pm0.14^{Bab\lambda.3}$	$7.53\pm0.13^{Ba\lambda.2}$	$5.73\pm0.14^{Bab\lambda.23}$	$6.30\pm0.18^{Ba\lambda.2}$
6	$6.46\pm0.14^{Ba\lambda.2}$	$5.93\pm0.18^{Ba\lambda.2}$	$5.73\pm0.183^{ABb\lambda.2}$	$6.39\pm0.12^{Bb\lambda.3}$	$7.36\pm0.31^{Ba\lambda.2}$	$5.70\pm0.10^{Bb\lambda.3}$	$6.27\pm0.34^{Bb\lambda.2}$
7	$6.67\pm0.31^{Ca\lambda.3}$	$6.04\pm0.33^{Ca\lambda.3}$	$4.76\pm0.69^{Ba\lambda.23}$	$6.38\pm0.21^{Ca\lambda.4}$	$7.63\pm0.15^{Ca\lambda.3}$	$6.14\pm0.69^{Ca\lambda.4}$	$6.34\pm0.10^{Ca\lambda.3}$
8	$6.45\pm0.22^{Ca\lambda.3}$	$5.92\pm0.40^{Ca\lambda.3}$	$5.78\pm0.27^{Bb\lambda.3}$	$6.16\pm0.38^{Cab\lambda.4}$	$7.57\pm0.10^{Ca\lambda.3}$	$6.13\pm0.15^{Cab\lambda.4}$	$6.23\pm0.15^{Ca\lambda.3}$
9	$6.36\pm0.75^{Ca\lambda.3}$	$5.96\pm0.14^{Ca\lambda.3}$	$5.89\pm0.14^{Bb\lambda.3}$	$6.47\pm0.12^{Cb\lambda.4}$	$7.08\pm13^{Ca\lambda.3}$	$6.21\pm0.07^{Cb\lambda.4}$	$5.83\pm0.24^{Cb\lambda.3}$

*Means± standard deviations from analysis, means within same column with different letters are significantly different (P< 0.05) from each other for each measured parameter. Uppercase and lowercase letters represent the comparison within the columns in CBS and sodium caseinate content, respectively. The letter λ shows the cocoa powder variable.

Principal Component Analysis (PCA)

In this study, principal component analysis (PCA) was applied to determine the most effective sensory parameters. The component 1 and component 2 had the most variance of 4.13 and 1.54, respectively. These two components could explain about 81% of total variance (eigenvalue >1). Also, Kaiser-Meyer-Olkin index (KMO) was 0.79 which showed sufficiency of analysis.

According to Table 6, as coefficient of each variable became more, the variable was more effective. Melting, bitterness, mouth-coating, and overall acceptance are the most effective parameters in PC1 and color and casein taste were the most effective variables for PC2.

The parameters which were close to each other, had strongly positive relationship. We observed that the hardness, mouth-coating, bitterness, melting an overall acceptance had propensity to be inversely proportional to the color parameter. Due to closer differential of hardness, bitterness, mouth-coating, and melting properties to overall acceptance, they were the most effective parameters for panelist's overall acceptance.

Dewi et al. (2020) investigated the sensory and instrumental properties of sugar free chocolate samples. They declared that particle

size distribution, melting, hardness, luminance (L*) and b* were strongly related to each other and inversely related to moisture and a*. Yeganeh Zad (2012) found that the first three components consisted 94% of total variance. Also, the sweetness, milk taste, hardness, melting, and then bitterness, soy taste, PSD, mouth-coating and at last color were the most effective variables in PC1, PC2 and PC3, respectively

Table 6- Rotated component matrix of first two main components

Sensory Parameter	Component	
	1	2
Color	0.21	0.91
Hardness	0.57	0.60
Melting Rate	0.91	0.24
Bitterness	0.86	0.42
Casein Taste	0.05	0.76
Mouth-Coating	0.84	0.39
Overall Acceptance	0.83	0.37

Selection of the best sample

According to the results, the moisture and a_w of all samples were in acceptable range. The peak temperature and the hardness of samples containing 30 and 35% CBS were closer to defined amount. Also, the overall acceptance showed that samples 5 and 7 had the highest score. By considering these results, sample 5 was the best among other samples.

Calorie amount

Sample 5 contained about 48.13 g carbohydrate, 10.38 g protein and 35.93 g fat. According to Table 7, the total calorie for 100 g of this sample is calculated as follows:

Consuming 100 g of this chocolate can supply 27% of daily calorie of an adult person (assuming 2000 kcal per day for adults). Consuming this 100 g can also supply 17% and 40% of classic and atkins keto diet.

Table 7- Total calorie of sample 5 (35% CBS and 5% sodium caseinate)

Macronutrient	Total amount (g)	Total calorie (kcal)
Fat	35.93	-
Protein	10.38	-
Carbohydrate	48.13	-
Total calorie	-	547.41

Total sugar amount

As mentioned before, the commercial chocolate contains 40- 50% sugar which is not appropriate for ketogenic diet. Reducing sugar content of optimized sample was 2.17%. There is no official standard about legal percent of reducing sugar in keto chocolate products. Famous keto-chocolate brands have at most 3 g reducing sugar in 100 g chocolate.

Peroxide value

The Chocolates may be affected by lipid oxidation. Oxygen which comes from conching process can interact with triglycerides. As shelf life of chocolate is increased, there would be more time for their interaction. Then, aldehyde and ketones are produced and the special odor would be sensed. Measuring peroxide value during shelf life of products help producers to investigate the quality (Williamson, 1998). According to Figure 4, the more shelf time led to more peroxide value. Also, peroxide value was acceptable even after two months. Pandey et al. (2010) and Selamat (1998) reported the same result.

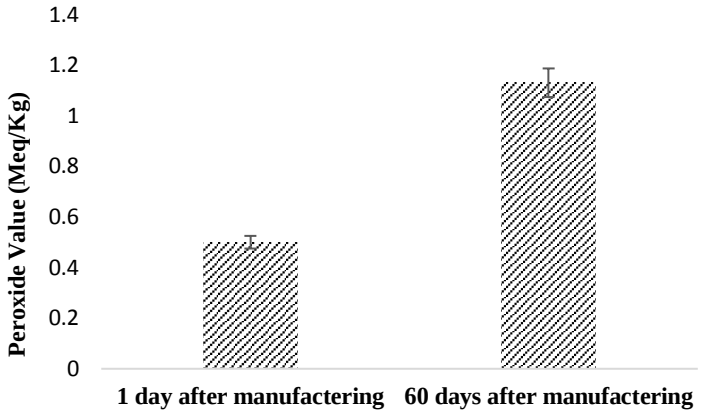

Fig. 4. Peroxide value of optimized chocolate sample

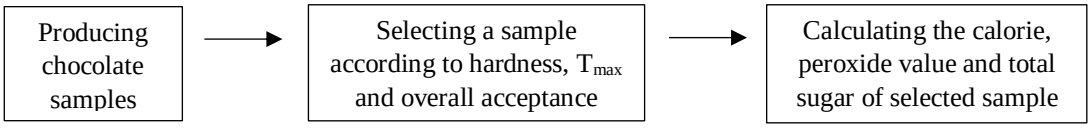

Fig. 5. Configuration map for choosing the best sample

Conclusions

There is a challenge in producing a portion compatible to ketogenic diet with sufficient satiety. This matter led us to produce sugar free chocolate by increasing total fat and protein for ketogenic diet. Increasing both total fat and protein significantly affected all physicochemical and sensory parameters. High fat chocolate samples were softer and easily melted. Panelists gave more scores to sample which contained 35% CBS and 5% sodium caseinate. There was a suitable correlation between sensory and instrumental data. Also, PCA showed a great relationship between overall acceptance of chocolate with hardness, bitterness, mouth-coating and melting properties. The optimized sample was selected by overall acceptance of hedonic test and also by comparing textural and thermal parameters to reliable references. Sugar and calorie content of optimized sample were close to commercial keto-chocolate. The optimized sample could be kept for two months with acceptable peroxide value. These results showed the possibility of producing chocolate which is compatible to ketogenic diet. However, clinical assessment of optimized sample should be done in future researches.

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بهینه‌یابی فرآورده شکلاتی تلخ بدون شکر سازگار با رژیم کتوژنیک و بررسی خصوصیات فیزیکوشیمیایی، بافتی، حرارتی و حسی آن

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چکیده

یکی از چالش‌های طراحی رژیم کتوژنیک، حفظ مقدار کالری عمده هر وعده از چربی و همچنین حفظ مقدار پروتئین مناسب جهت حس سیری می‌باشد. این تحقیق جهت امکان‌سنجی تولید شکلات بدون شکر و با مقدار چربی و پروتئین بالا در مقدار ثابت فیبر سویا و پودر استویا برای افراد ملزم به رژیم کتوژنیک صورت گرفت. پودر کاکائو توسط جایگزین کره کاکائو و سدیم کازئینات هر کدام در سطوح (صفر، ۵ و ۱۰ درصد) جایگزین شد. نتایج نشان داد که جایگزینی پودر کاکائو به‌طور معنی‌داری ($p< ۰/۰۵$) موجب افزایش رطوبت، فعالیت آبی، مقدار چربی و پروتئین کل شد اما مقدار خاکستر و کربوهیدرات کاهش یافت. همچنین افزایش مقدار جایگزین کره کاکائو و سدیم کازئینات به‌طور معنی‌داری ($p< ۰/۰۵$) موجب شد تا همزمان سختی و دمای ذوب نمونه‌های شکلات کاهش یابد. نتایج آزمون هدونیک نشان داد که نمونه‌های با چربی و پروتئین بیشتر، امتیاز بالاتری را کسب کردند. همچنین تجزیه به روش مولفه‌های اصلی نشان داد که دو مولفه اصلی اول ۸۱ درصد کل واریانس داده‌های حسی را تشکیل می‌دهند. در نهایت، با توجه به نتایج آزمون‌های حسی و دستگاهی، بهترین ترکیب شکلات انتخاب شد. این ترکیب حاوی ۳۵ درصد جایگزین کره کاکائو و ۵ درصد سدیم کازئینات بود. مقدار قند کل و کالری نمونه بهینه به‌ترتیب ۲/۱۷ درصد و ۵۴۷/۴۱ کیلوکالری به ازای ۱۰۰ گرم بود. مقدار اندیس پراکسید ۱ روز پس از تولید ۰/۵ میلی‌اکی‌والان در کیلوگرم بود که پس از ۶۰ روز این مقدار به ۱/۱۳ میلی‌اکی‌والان در کیلوگرم افزایش یافت. مصرف ۱۰۰ گرم نمونه بهینه می‌تواند ۲۷ درصد کالری روزانه یک فرد بزرگسال را تامین کند. این نتایج نشان داد که فرمولاسیون بهینه، حداقل شرایط رژیم کتوژنیک را داراست و می‌تواند یک میان وعده مناسب برای افراد ملزم به این رژیم باشد.

واژه‌های کلیدی: بدون شکر، شکلات، رژیم کتوژنیک، استویا.

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Full Research Paper

Nozzle-less electrospinning: Nanoencapsulation of ajwain essential oil using chitosan-gelatin nanofibers

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Abstract

The aim of this research was to investigate the efficiency of nozzle-less electrospinning for encapsulation of ajwain essential oil (as a hydrophobic bioactive) using two hydrocolloids (chitosan/gelatin) in order to enhance its antioxidant properties and stability for food applications. Nanofibers were spun using chitosan/gelatin in ratios of 1:6, 1:8 and 1:10 and ajwain concentrations of 20 and 40%. Solution properties (i.e. viscosity and electrical conductivity) were measured. Encapsulation efficiency and loading capacity data illustrated an enhancement with increasing of essential oil concentration. Fibers diameter and morphology were studied by scanning electron microscopy (SEM). The chitosan/gelatin nanofibers with ratio of 1:6 containing 40% essential oil had the highest encapsulation efficiency (99.9%), loading capacity (39.9%) and the smallest diameter (146 nm). Attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) proved that during electrospinning, no any chemical interaction was occurred between ingredients and differential scanning calorimetry (DSC) data showed that essential oil was well encapsulated in nanofibers. Antioxidant properties were analyzed by 2,2-diphenyl-1-picrylhydrazylradical and approved the efficiency of encapsulation for protection of antioxidants.

Keywords: *Ajwain* essential oil; Antioxidant activity; Encapsulation; Nanofibers; Nozzle-less electrospinning.

Introduction

Oxidation reaction has a series of adverse effects on food quality; thus, it is necessary to develop food packaging material incorporated with natural antioxidants. Hence, in recent years many studies have been conducted on investigating the effects of natural antioxidants such as plant essential oils (Eos) as an alternative to chemical preservatives and synthetic antioxidants for extending the shelf life of food products (Wu et al., 2012)

Trachysper ammi, known as ajwain, is an erect annual herb with striate stem which is traditionally used as a medicinal plant for its antiseptic, appetizer and carminative properties. Thymol, the major phenolic compound presents in ajwain's EO, has been reported as an anti-inflammatory, antifungal, antipyretic, antifilarial, analgesic, antinociceptive and antioxidant agent (Tabatabai et al, 2019). However, EOs are water-insoluble, and biologically sensitive to environmental conditions such as light, oxygen,

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humidity enzymes, alkaline pH and high temperature (Trifković et al., 2014). Recently new methods have been introduced to enhance their sustainability and bioavailability such as encapsulation. Nanoencapsulation process involves entrapment of a bioactive matter within a nanometer size carrier which leads to increase stability, solubility and controlled release of the bioactive compound (Cevallos, Buera, & Elizalde, 2010). Among various nanomaterials such as: nanoparticles, nanoplatelets and nanofibers, the latter with a high aspect-ratio, low density, extremely large surface-to-mass ratio, and superior mechanical performance have been shown to possess excellent mechanical, thermal and electrical properties (Naebe et al., 2007). Among the classical methods to produce nanofibers such as phase separation, drawing, template synthesis, self-assembly, and thermal oxidation, electrospinning is a suitable candidate for establishing fibers due to its attractive features to produce fibers in nanoscale at low cost and its ability to be used for a large variety of materials (Rezaei et al., 2015). Nevertheless, the efficiency of a single-needle electrospinning system is too low for industrial scale. Therefore, nozzle-less electrospinning was investigated in order to enhance productivity in large scale with forming many jets from free surface of polymer solutions (Kostakova et al., 2009).

Chitosan and gelatin are two natural biopolymers used in electrospinning because of their biocompatibility and biodegradability (Ebrahimi et al., 2019). Chitosan, a modified carbohydrate polymer with average molecular weight of 100– 500 kDa, is synthesized through the partial deacetylation of chitin. Chitosan is a cationic polymer with a pKa value of 6.3, that is readily soluble in dilute acid solutions with a pH less than 6 (Voron'ko et al., 2016). It had been widely used in food, cosmetic, biomedical, and pharmaceutical applications due to its excellent properties such as biocompatibility and antibacterial activity. Since the electrospinning of pure chitosan proved to be impossible, due to its structure, viscosity, electrical conductivity, therefore chitosan was mixed with other spinnable biopolymers. Gelatin is a natural biopolymer derived from acid or alkaline hydrolysis of animal collagen (Xu et al., 2020). Gelatin is a typical amphoteric biopolymer with isoelectric point (pI≈ 5.3), which contains both positive and negative charges depending on the functional groups (amino and carboxyl groups) present in the molecule (Zhu et al., 2018). The combination of chitosan and gelatin in the acidic pH, can be successfully applied to synthesize nanofibers with good physical properties by electrospinning (Amjadi et al., 2019; Dhandayuthapani et al., 2010; Xu et al., 2020).

The aims of this study were to encapsulate ajwain essential oil in chitosan/gelatin nanofibers by using nozzle-less electrospinning and investigate their antioxidant properties for its potential application as a natural food additive.

Materials and methods

Gelatin powder (Type A; Bloom number of 220) from bovine and chitosan (molecular mass of 60000- 120000) were obtained from Merck. Ajwain essenential oil was extracted from *Trachysper ammi* which was collected from eastern Esfahan province, Iran. All reagents were at least of analytical grade.

Preparation of solutions for electrospinning

Solvents and solutions were prepared according to our previous work (Vafania et al., 2019).

Solvents were selected based on solubility of different materials. Chitosan and gelatin were soluble in acetic acid and ajwain essential oil was soluble in ethanol. Therefore the solvent consisted of 50% ethanol, 45% acetic acid and 5% deionized water. Chitosan (2% W/V) and gelatin (9% W/V at 40°C) were stirred separately for 24 h and 30 min, respectively. The two obtained solutions were mixed with different ratios (chitosan to gelatin volume ratio of=1:10, 1:8 and 1:6) and then ajwain essenential oil was added to the biopolymer

solution with the ratios of 20 and 40% (V/W of solid biopolymers).

Solution characterization

Important parameters of the solution for electrospinning are viscosity and electrical conductivity which were measured by a viscometer (Brookfield, DV2, USA) using the spindle No. 21 at 50 rpm (shear rate of 46.5 s^{-1}) and an electrical conductometer (Inolab, Germany), respectively. The experiments were performed at 25± 0.5◦C in three replications (Karim et al., 2020).

Nanofiber fabrication

Disk shape nozzle-less electrospinning with 5 disks (Fig. 1) was used to fabricate nanofibers from free surface of chitosan/gelatin solutions containing ajwain essential oil. During the electrospinning process, the voltage was 25 kV, tip to collector distance was 5 cm and rotation speed was 20 rpm. Fibers were collected over an aluminum foil.

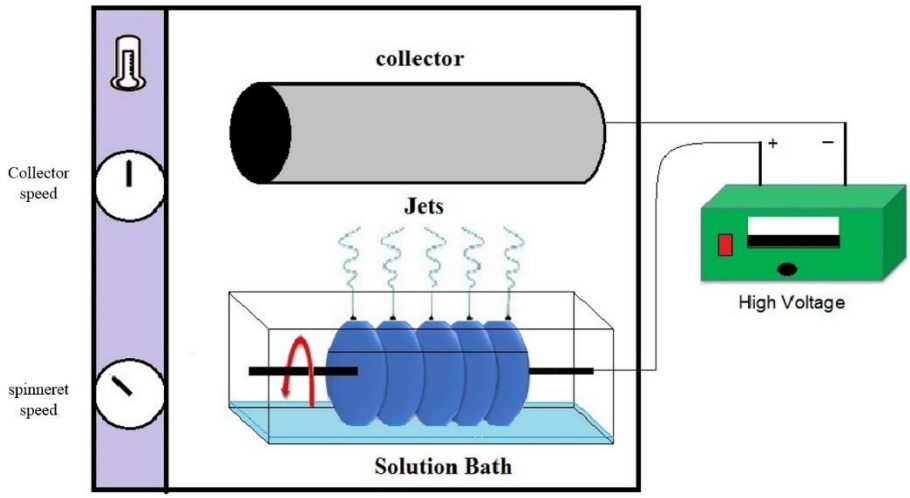


Fig. 1. Schematic of nozzle-less electrospinning.

Scanning electron microscopy (SEM)

The produced fibers were analyzed for their morphologies using a scanning electron microscope (Philips, model X130, Netherlands) after coating with a thin layer of gold. SEM images were used to measure fibers' diameters using ImageJ software (USA).

Determination of encapsulation efficiency and loading capacity

Encapsulation efficiency (EE%) and loading capacity (LC%) were determined by measuring the surface essenential oil. Ethanol was used to extract the surface essential oil from the gelatin/chitosan nanofibers and absorbance was read at 267 nm (T60 UV, England). The EE and LC were determined using following equations (Bashiri et al., 2020; Nahr et al., 2018):

$$\%EE = \frac{\text{Total essence weight} - \text{Free surface essence weight}}{\text{Total essence waight}} \times 100 \quad (1)$$

$$\%LC = \frac{\text{Total essence weight} - \text{Free surface essence weight}}{\text{Total nanofibers weight}} \times 100 \quad (2)$$

Attenuated total reflection Fourier-transform infrared spectroscopy

Attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) is a method to identify functional groups of

organic compounds and changes in chemical structure of gelatin, chitosan, ajwain essential oil and nanofibers (chitosan to gelatin ratio of 1:6 with 40% essential oil). The samples were mixed with KBr (with ratio of 1:100) to prepare tablets. The spectra were acquired at wavenumbers of 4000- 500 cm^{-1} with resolution of 4 cm^{-1} (Broker, model Tensor-27, Germany).

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a common method to investigate thermal behavior of materials. Five mg of samples (gelatin, chitosan and nanofibers containing essential oil) were used for analysis by DSC instrument (Bakher, Germany). Samples were placed into aluminum hermetic crucibles, sealed and analyzed for the temperature range of 25ºC to 350ºC at a heating rate of 10ºC/min.

Antioxidant activity

The antioxidant capacities of ajwain essential oil and nanofiber containing ajwain essential oil were analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. DPPH method is regarded as a fast and extensive approach to assess antioxidant activity and power of combining with free radicals or donating hydrogen in food materials. DPPH radical is a stable free radical with the central atom of nitrogen. DPPH changes from purple to yellow with reduction reaction through capturing hydrogen or electron. To this end, a 0.2 mmol/L DPPH solution was prepared with 1 mg/ml (effective concentrations of EOs in samples) of sample solution prepared in 70 ml/100 ml aqueous ethanol after continuous incubation in a dark space at 4°C. The DPPH radical scavenging activities of the samples were evaluated by measuring absorbance at 517 nm against 70 ml/100 ml aqueous ethanol as a blank. The experiments were performed for different storage periods (1, 6, 12 and 18 days). The antioxidant activity of free essential oils and nanofibers were determined by the following equation (Piran et al., 2012):

$$\text{Antioxidant activity (\%)} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100 \quad (3)$$

Results and discussions

Properties of solutions

Solution viscosity plays an essential role in spinnability of biopolymers. Increasing the concentration of the polymeric solution will lead to an increase in the viscosity, which then increases the chain entanglement among the polymer chains. These chain entanglements overcome the surface tension and ultimately results in uniform bead-less electrospun nanofibers. This viscosity is called critical viscosity at lower which the polymer chains are less involved together and cannot overcome the repulsion forces that leads to formation of particles, instead of fibers. Nevertheless, when the viscosity of solution is too high, electrical field cannot overcome the internal foresees and fibers are not formed again. Hence, it can be concluded that determination of the critical value of the viscosity is essential to obtain bead-less nanofibers (Kurd et al., 2017).

Electrical conductivity of a solution depends upon the type of polymer, solvent and the presenential oil of ions. Increasing the conductivity of the solution to a critical value will not only increase the charge over the surface of the droplet to form Taylor cone but also cause decrease in the fiber diameter (Haider et al., 2018).

Nine series of polymer solutions with different ratios of chitosan to gelatin (1:6, 1:8 and 1:10) with (20% and 40%) and without ajwain essenential oil were prepared and their viscosities and electrical conductivities were measured (Table 1). According to the results a shear thinning behavior was observed in all cases. In fact, an increase in shear rate leads to higher ordering of the polymer chains, which tend to orientate toward the applied stress (Bertolo et al., 2020; Rodrigues et al., 2021). By increasing chitosan volume ratio, electrical conductivity increased as viscosity of the polymer solutions decreased ($P<0.05$). Actually, total concentration of the polymer solution could be dominant with increasing the

ratio of chitosan which played a significant role in decreasing viscosity. On the other hand, the electrical conductivity dramatically increased by increasing chitosan volume ratio because of the fact that electrical conductivity of chitosan solution was higher than gelatin. Liu et al. (2020) indicated that unlike viscosity, the electrical conductivity of the gelatin/chitosan (6:1) solution was higher than that of the gelatin/chitosan (8:1) solution.

Park et al. (2004) reported the same results for silk fibroin/chitosan solutions and indicated that by increasing chitosan ratio, the electrical conductivity of the polymer solution increased and diameter of nanofibers decreased.

On the other hand, the results indicated that viscosity and electrical conductivity of the solutions decreased due to increase of percentage of essential oil ($p<0.05$). According to observation of Moomand and Lim (2015), increasing amount of fish oil in polymer solution caused a reduction in electrical conductivity.

Table 1- Viscosity and electrical conductivity (mean± SD) of solutions for different chitosan to gelatin volume ratios (1:6, 1:8 and 1:10) and amount of ajwain essence (0, 20 and 40%).

Sample number	Chitosan to gelatin ratio	Amount of ajwain essence (%)	Viscosity (cp)	Electrical Conductivity (µS/cm)
1	1:10	0	117 ± 1.41^{a}	480 ± 5^{f}
2	1:8	0	95 ± 2.8^{b}	547 ± 7^{c}
3	1:6	0	66.5 ± 2.1^{d}	883 ± 5^{a}
4	1:10	20	89.5 ± 3.5^{c}	411 ± 5^{h}
5	1:8	20	60.5 ± 2.1^{d}	490 ± 8^{ef}
6	1:6	20	50.0 ± 2.8^{e}	519 ± 10^{d}
7	1:10	40	82.5 ± 4.9^{c}	445 ± 10^{g}
8	1:8	40	63.0 ± 7.1^{d}	504 ± 6^{de}
9	1:6	40	41.5 ± 4.9^{e}	670 ± 11^{b}

* Values with different superscript letters in the same column were statistically significant ($p<0.05$).

Morphology and size of nanofibers

The SEM morphologies of electrospun fibers from the different ratio of chitosan/gelatin containing various concentrations of essenential oil are shown in Fig 2. The results showed that increasing of the chitosan volume ratio had a significant effect on reducing the diameter of the nanofibers due to its low viscosity and higher electrical conductivity. Therefore, the ratio of 1:6 chitosan/gelatin possessed the smallest diameter, with smooth bead-free surface. Similar results were reported for chitosan/gelatin solution by (Ebrahimi et al., 2019). On the other hand, thinner diameters of nanofibers with higher ratio of chitosan were obtained because of its higher electrical conductivity values. Liu et al. (2020) have proved that chitosan has high electric charge density, and the extra charge can lead to the repulsion of the polymer jet, as a result reduced diameters of nanofibers when the ratios of chitosan were increased. Haider et al. (2010) studied electrospinning of biopolymer and reported that by increasing chitosan ratio, the diameter of fibers decreased. On the other hand, increasing the percentage of essential oil had a significant effect ($P<0.05$) on increasing the diameter of nanofiber, which were related to the reduction of electrical conductivity that reduced the elongation of polymer jet through the applied voltage (Charernsriwilaiwat et al., 2013). In our previous report, entrapment of thyme essential oil within the fibers caused an increase of diameter (Vafania et al., 2019). Shao et al. (2018) demonstrated that the incorporation of tea polyphenols can increase the diameter of pullulan-CMC nanofiber.

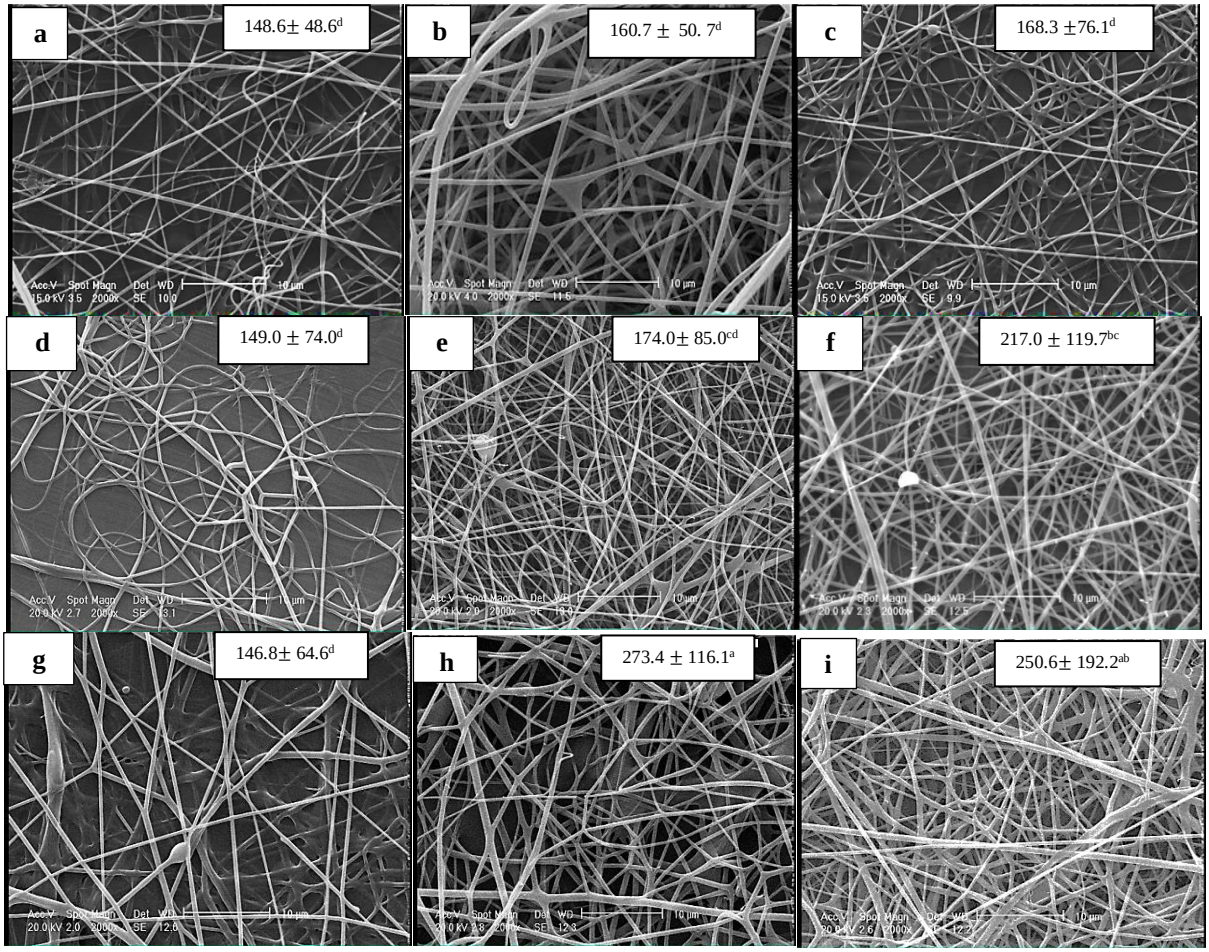


Fig. 2. SEM images of electrospun chitosan/gelatin with different volume ratios (1:6, 1:8 and 1:10) containing ajwain essential oil (%20 and %40): (a) chitosan/gelatin nanofibers in ratio of 1:6 without essential oil (b) chitosan/gelatin nanofibers in ratio of 1:8 without essential oil (c) chitosan/gelatin nanofibers in ratio of 1:10 without essential oil (d) chitosan/gelatin nanofibers in ratio of 1:6 containing 20% essential oil. (e) chitosan/gelatin nanofibers in ratio of 1:8 containing 20% essential oil (f) chitosan/gelatin nanofibers in ratio of 1:10 containing 20% essential oil (g) chitosan/gelatin nanofibers in ratio of 1:6 containing 40% essential oil (h) chitosan/gelatin nanofibers in ratio of 1:8 containing 40% essential oil (i) chitosan/gelatin nanofibers in ratio of 1:10 containing 40% essential oil.

Encapsulation efficiency and loading capacity

The results showed that with increasing percentage of essential oil, encapsulation efficiency and loading capacity increased. These results were in agreement with those found by Rezaei et al. (2016) who claimed with increasing the concentration of vanillin from 1% to 3% (w/w) encapsulation efficiency increased from 68% to 75%. Zhang et al., (2020) studied chitosan-gelatin based edible coating incorporated with nanoencapsulated tarragon essential oils (TEO) and their results about encapsulation efficiency and loading capacity showed that the EE values tended to increase with the increase of the initial content of encapsulated TEO. Also these results were in agreement with the findings of Keawchaoon and Yoksan (2011) reporting on the encapsulation of carvacrol in chitosan nanoparticles and showed that LC% increased by increasing EOs content.

However increasing ratio of chitosan/gelatin did not have significant effect on EE and LC (Fig. 3 A and B). The chitosan/gelatin solution with ratio of 1:6 containing 40% essenential oil had the highest EE (99.9%), LC (39.9%) and the smallest diameter (146 nm), so were

selected to be the optimum formulae for the remaining study.

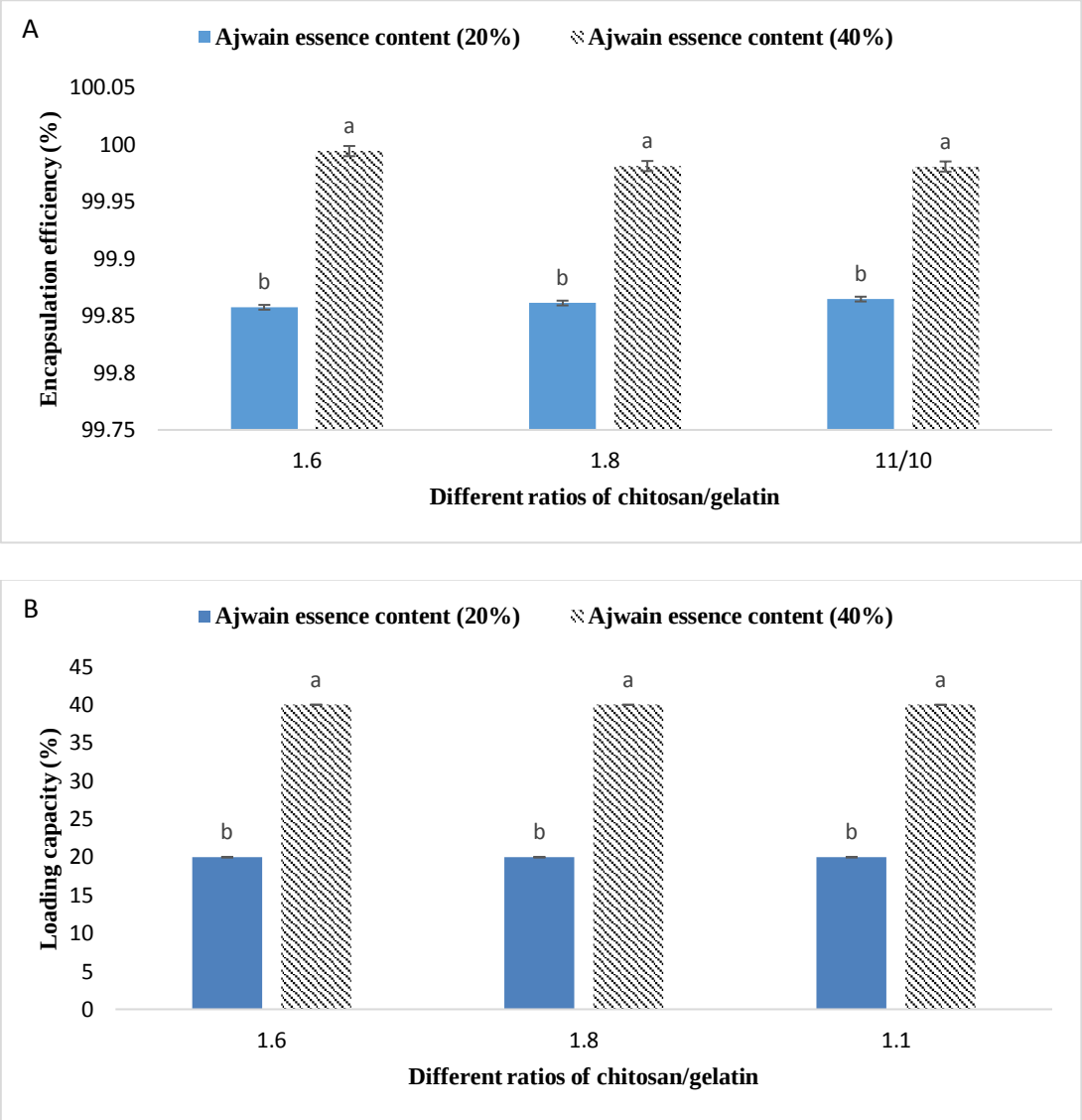


Fig. 3. Encapsulation efficiency (A) and loading capacity (B) of nanofibers (chitosan to gelatin volume ratios of 1:6, 1:8 and 1:10) containing 20 and 40% ajwain essential oil.

ATR- FTIR spectroscopy

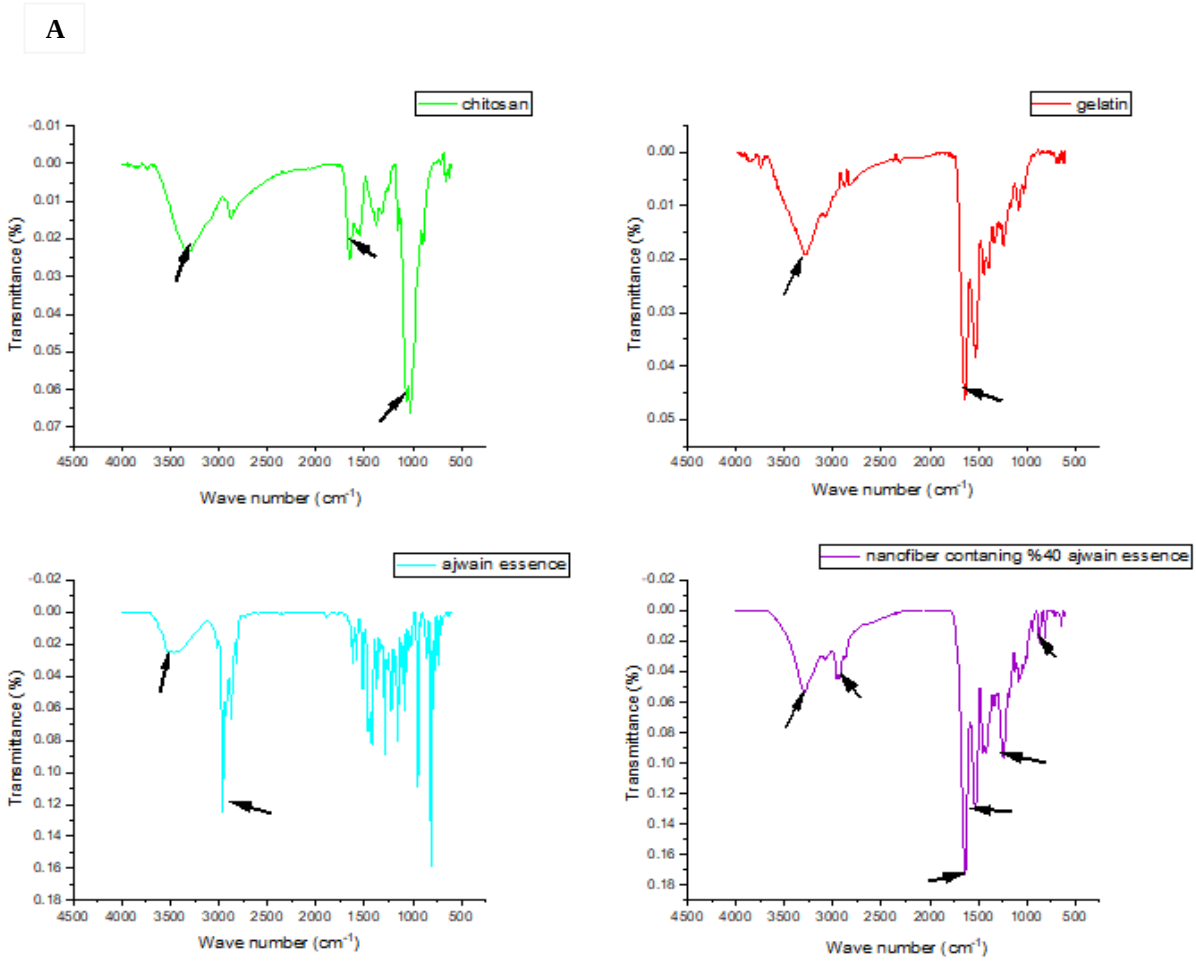
ATR- FTIR analysis was used to identify functional groups and study on any interaction between ingredients (Fig. 4A).

Characteristic peaks of chitosan were around 3500 cm^{-1} and 3200 cm^{-1} that represented the stretching vibration of O–H and N–H bonds, respectively. The C=O stretching (amide I) peak at 1646 cm^{-1} and N–H bending (amide II) peak at 1580 cm^{-1} showed the existence of N-acetylglucosamine. The peak at 1545 cm^{-1} was allocated to strong vibrations of secondary amide. C–O bonds were indicated in 1030–1160 cm^{-1} region. Furthermore, the peak at 2932 cm^{-1} was attributed to the C–H stretch of CH_2 (Altiok et al., 2010). The spectrum of pure ajwain essenential oil showed several characteristic bands, like peaks from 3250 to 3500 cm^{-1} indicated the presence of hydroxy group. The band from 2900 to 2850 cm^{-1} referred to symmetric methyl group. Dimethyl elements can also be indicated at

1370 cm^{-1}. Peaks at 1750, 1640 and 1460 cm^{-1} were attributed to alcoholic functional group. Characteristic phenolic component peaks were detected at 720, 1030 and 1230 cm^{-1} (Chatterjee et al., 2017). The spectrum of nanofibers containing ajwain essential oil indicated that there was no change in the peak intensities and no shift in the wave numbers of ingredient, therefore all interactions were physical.

Thermal analysis

Thermal profiles of the ingredients and produced nanomaterials are depicted in Fig. 4B. For chitosan powder's diagram, two peaks were noticeable, an endothermic at 83°C due to water evaporation and an exothermic at 300°C which could be attributed to the decomposition of chitosan (Guinesi & Cavalheiro, 2006). Gelatin powder indicated three endothermic peaks at 110, 215 and 280°C corresponding to evaporation of water, melting and decomposition, respectively. For the gelatin/chitosan nanofibers containing 40% ajwain essential oil, endothermic and exothermic peaks were observed at 85°C and 279.4°C, that corresponding to water evaporation and decomposition of nanofibers, respectively. The DSC thermogram showed significant reduction in the melting point of electrospun chitosan/gelatin nanofibers compared to pure chitosan and gelatin powders. The decrease in the melting temperature may be attributed to three phenomenon: (i) high surface to volume ratio of the electrospun fibers, (ii) plasticizing effect of a residual solvent in the nanofiber mats on the polymer chains and (iii) modification of the crystalline structure as a result of rapid solidification of polymer solutions in electrospinning.



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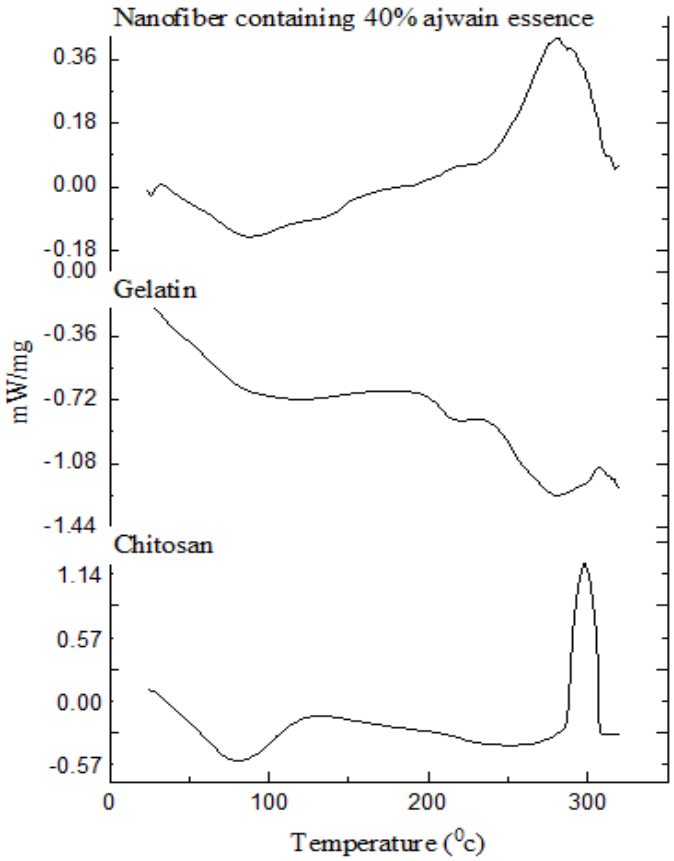

Fig. 4. ATR-FTIR spectrum (A) and DSC thermograms (B) of chitosan/gelatin nanofiber containing ajwain essence and its ingredients.

Antioxidant activity

The radical scavenging activities of the pure essential oil and nanofibers containing essential oil were assessed by the DPPH during 18 days (Fig. 5). For the first day of measurement all the treatments could scavenge free radicals but the pure essential oil showed a higher antioxidant activity than the encapsulated essential oil. This might due to the fact that the phenolic compounds of the pure essential oil were readily available to free radicals. On the other hand, oxidation of some parts of antioxidant compounds during encapsulation could led to lower value of antioxidant activity of encapsulated essenential oil for the first days of storage. During storage both samples showed decrease trends on antioxidant activity. However, after 6th, 12th and 18th days, the encapsulated essential oil exhibited higher antioxidant activity than pure essential oils due to better protecting of phenolic compounds against oxygen and light. The Same results observed in our previous work (Vafania et al., 2019) about encapsulated thyme essential oil. As expected, DPPH radical scavenging activity of the electrospun nanofibers contain thyme essential oil showed the best protecting effect against oxidation than pure one. Gortzi et al., (2008) studied encapsulation of *Myrtus communis* extract in liposomes and reported that its antioxidant as well as its antimicrobial activities were better than pure form.

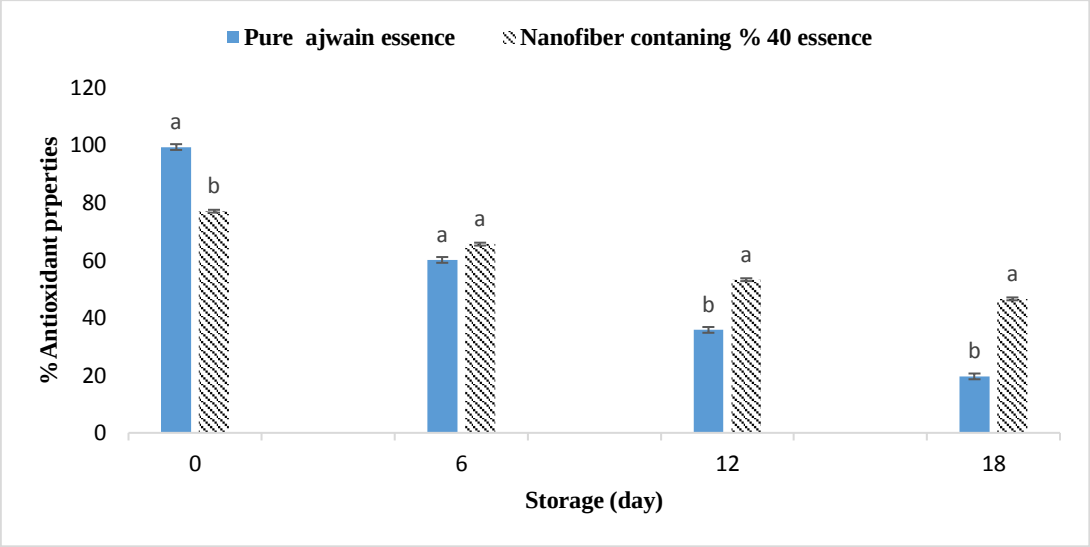

Fig. 5. Antioxidant activity of pure ajwain essence and nanofibers containing 40% ajwain essence during different days of storage.

Conclusion

The extracted ajwain essenential oil was encapsulated in nanofibers of gelatin/chitosan in ratios of 1:6, 1:8 and 1:10 and ajwain concentrations of 20 and 40% using nozzle-less electrospinning. High encapsulation efficiency and loading capacity confirmed the suitability of encapsulation process. The results of SEM, ATR-FTIR and DSC showed that nanofibers of chitosan/gelatin with ratio of 1:6 containing 40% essence had an appropriate diameter and high stability. Antioxidant activities of the pure and encapsulated essential oils were compared. Results from this study supported the use of nanofiber for protection of antioxidants and application of nozzle-less electrospinning technique to facilitate the use of EOs in food preservation.

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الکتروریسی بدون نازل: نانوانکپسولاسیون اسانس زنیان با استفاده از نانوالیاف کیتوزان-ژلاتین

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چکیده

هدف از این تحقیق بررسی کارایی الکتروریسی بدون نازل برای انکپسوله کردن اسانس زنیان (به‌عنوان یک زیست فعال آبگریز) با استفاده از دو هیدروکلوئید (کیتوزان/ژلاتین) به‌منظور افزایش خواص آنتی‌اکسیدانی و پایداری آن برای کاربردهای غذایی بود. نانوالیاف با استفاده از کیتوزان/ژلاتین در نسبت‌های ۱:۶، ۱:۸ و ۱:۱۰ و غلظت‌های ۲۰ و ۴۰ درصد زنیان ریسیده شدند. خواص محلول (ویسکوزیته و هدایت الکتریکی) اندازه‌گیری شد. داده‌های کارایی انکپسولاسیون و ظرفیت بارگذاری مبین بهبود با افزایش غلظت اسانس بود. قطر و مورفولوژی الیاف با میکروسکوپ الکترونی روبشی مورد بررسی قرار گرفت. نانوالیاف کیتوزان/ژلاتین با نسبت ۱:۶ حاوی ۴۰ درصد اسانس دارای بیشترین کارایی انکپسولاسیون (۹۹/۹٪)، ظرفیت بارگذاری (۳۹/۹٪) و کمترین قطر (۱۴۶ nm) بودند. طیف‌سنجی فروسرخ با انعکاس کلی ضعیف شده (ATR-FTIR) ثابت کرد که حین الکتروریسی، هیچ برهمکنش شیمیایی بین مواد تشکیل‌دهنده رخ نداده است و داده‌های کالریمتری روبشی افتراقی (DSC) نشان داد که اسانس به‌خوبی در نانوالیاف محصور شده است. خواص آنتی‌اکسیدانی توسط آزمون DPPH تجزیه و تحلیل شد و کارایی کپسولاسیون برای محافظت از آنتی‌اکسیدان‌ها را تأیید کرد.

واژه‌های کلیدی: اسانس زنیان، فعالیت آنتی‌اکسیدانی، انکپسولاسیون، نانوالیاف، الکتروریسی بدون نازل.

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Full Research Paper

Synbiotics as potentially growth promoter substitution for improving microbial and oxidative stability of Japanese quail meat

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Abstract

The use of antibiotics in livestock breeding, especially poultry, leads to an increase in antibiotic resistance and human disorders. Therefore, researchers are seeking a good substitute to improve gut microbial balance, growth performance, and meat quality of livestock. The present study was aimed to investigate the effect of diets containing different levels (0, 90, and 100%) of probiotic Fermacto (F), prebiotic Primalac (P), and their mixture on chemical, microbial, and sensory properties of Japanese quail meat. The F_{100} sample showed the highest color and odor scores; whilst, juicier feature was more dependent on prebiotic level. $F_{100}P_{100}$ and $F_{90}P_{90}$ ranked the highest meat flavor and overall acceptance scores, respectively. The lowest number of microorganisms and total coliforms were observed in $F_{90}P_{100}$ during storage. In general, the addition of synbiotics to the diet of Japanese quail led to improve in meat quality and decrease in microbial contamination besides controlled oxidation during refrigeration.

Keywords: Japanese quail; Antibiotic; Synbiotic; Poultry; Meat quality.

Introduction

Nowadays, as a result of increasing population growth and food-borne illnesses, access to healthy and safe foods has become one of the main human concerns (Severino et al., 2015; Timmer, 2017). Protein sources such as meat play a key role in human nutrition (De Smet & Vossen, 2016). However, the prevalence of cardiovascular diseases and diabetes in today's societies caused by eating unhealthy foods such as red meat has increased the demand for white meat (Bronzato & Durante, 2017; Khademipoor et al., 2017).

Poultry meat in comparison to other domestic animals has only 3.5 to 5% fat, which mostly includes unsaturated fatty acids (Marcinčák et al., 2008). Quail is a valuable and economical bird with significantly high breeding level in many countries, which can be a potential substitute to chicken due to some

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characteristics such as rapid growth, delicacy, low feed intake, early onset of lay, high egg production, short generation and incubation periods, high resistance to many common diseases, cardiac friendly, and high quality meat and eggs (Panda et al., 2017). Poultry breeding, at an industrial scale, results in an increase in many microbial diseases and subsequently antibiotic usage. It was reported that excessive consumption of antibiotics and the presence of drug residues in poultry carcasses can threaten human health (Ashraf et al., 2018; Mehdi et al., 2018; Muaz et al., 2018). Therefore, many researches have focused on introducing antibiotic substitutes (Barbieri et al., 2015; Danka et al., 2007; Mehdi et al., 2018; Nasehi et al., 2015). In an attempt to replace antibiotics (avilamycin+ sodium monensin), the effect of probiotics (*Bacillus amyloliquefaciens*) and organic acids (lactic, acetic, and butyric acid), indivitually or combined, on intestinal anaerobic bacteria, allometric growth of digestive organs, intestinal morphometrics, and broiler chicken performance was studied by Barbieri et al. (2015). In another study, probiotic protexin (in place of antibiotics) was successfully used in Japanese quail feed and its effects on meat properties were examined by Nasehi et al. (2015). The probiotic controlled microorganism activity, reduced oxidation reactions, increased water-holding capacity, and improved meat color index. Probiotics and prebiotics are interesting alternatives that can improve gut microbial balance and natural defense system of animals by suppressing the growth of pathogenic bacteria (Roberfroid, 2000).

Probiotics are health-promoting micro-organisms that exist in the large intestine as a natural microbiota, and can lead to a significant health effect on the host through maintaining and improving the microbial balances of the intestine, if they reach the minimum amount of 10^7 CFU/mL. Lactobacillus and Bifidobacterium strains are the most important probiotics, having some functionalities include anticarcinogenic, antimutagenic, antiseptic activities, immune stimulation, lowering serum cholesterol, increasing nutritional value, and treating diarrhea and gastrointestinal tract infections via preventing the attachment of gastrointestinal pathogens and producing antibacterial compounds (Pandey et al., 2015; Tripathi & Giri, 2014). Prebiotics, as indigestible and bioactive compounds, do not hydrolyzed in the stomach and small intestine and selectively stimulate the growth or activity of health-promoting bacteria in colon, thereby improving the host health (Bigliardi & Galati, 2013; Gibson & Roberfroid, 1995). Synbiotics are a mixture of probiotic microorganisms and prebiotic compounds that together render more health effects (Pandey et al., 2015). To the best of our knowledge, there are very few studies on the effect of synbiotic diets based on Primalac and Fermacto on the physicochemical, sensory and microbial characteristics of Japanese quail meat. The aim of this study was therefore to investigate the effect of diets containing different levels of probiotic, prebiotic, and synbiotic on oxidative stability, microbial, and sensory properties and shelf-life extension of Japanese quail meat.

Materials and methods

Probiotic Fermacto (containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Enterococcus faecium*) and prebiotic Primalac (fermentation product of *Aspergillus orizae*) were provided from Star-Labs (USA) and PET-AG, Ltd (UK), respectively. Butylated hydroxyl toluene (BHT), trichloroacetic acid (TCA), and malondialdehyde were purchased form Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were analytical grade and purchased from Merck (Darmstadt, Germany).

Quail management

A total of 405 quail chicks (1-day-old) were allocated to 9 treatments with 3 replicates of 15 chicks each based on a full-factorial completely randomized design. The experimental treatments were included tree levels of 0, 90, and 100% of the recommended levels of

Fermacto and Primalac for the initial and growth periods (Table 1). These supplements were added to the quail diet based on NRC (National-Research-Council, 1994). Two quails of each replicate were randomly chosen and slaughtered after 42 days of breeding. The slaughtered quails were de-skinned, trimmed into thigh and breast muscles, and then transferred to the laboratory under cold storage. Next, the thigh muscles were manually deboned, coded, and stored in refrigerator until further analysis at 1, 4, and 7 days of storage.

Table 1- Diet formulations used in this study.

Treatment	Fermacto+ Primalac (%)	Code
1 (Control)	0	F_0P_0
2	0+ 90	F_0P_{90}
3	0+ 100	F_0P_{100}
4	90+ 0	$F_{90}P_0$
5	90+ 90	$F_{90}P_{90}$
6	90+ 100	$F_{90}P_{100}$
7	100+ 0	$F_{100}P_0$
8	100+ 90	$F_{100}P_{90}$
9	100+ 100	$F_{100}P_{100}$

pH measurement

The pH of quail meats was measured based on the method of Brannan (Brannan, 2009). Briefly, 5 g of sample was mixed with 50 mL of distilled water and homogenized in a blender. Afterwards, the pH of homogenized dispersion was read at room temperature using a pH-meter (Metrohm, 827 pH lab, Switzerland).

Water holding capacity (WHC)

The quail thighs were grinded in a laboratory blender for 5 sec to create a homogeneous paste under sterile conditions. One g of the obtained paste was completely covered with a Whatman No 1 filter paper and then centrifuged at 1400 rpm for 4 min. The meat sample was separated from filter paper and weighed. Afterwards, the sample was dried in an oven at 90°C for 5 min and then re-weighed. Finally, WHC of samples was calculated according to the following equation (Nasehi et al., 2015):

$$WHC\ (\%) = [(A_1 - A_2)/A_0] \times 100 \quad (1)$$

Where, A_0, A_1, and A_2 are the weight of sample before centrifugation, after centrifugation, and after drying, respectively.

Oxidative stability

One g of the paste of quail thighs was mixed with 2.5 mL of 0.8% butylated hydroxyl toluene (BHT) and 4 mL of 5% trichloroacetic acid (TCA) solution. The suspension was centrifuged at 3000 rpm for 3 min. The upper phase was discarded and the lower phase was reached to 5 mL with 5% TCA. 2.5 mL of the obtained solution was charged with 1.5 mL of 0.8% BHT in a screw-capped tube. Afterwards, the tube was placed in water bath at 70°C until the color change was observed (~30 min). The tube was then immediately cooled in an ice bath followed by storing it at ambient temperature. Thiobarbituric acid (TBA) values of the meat samples were spectrophotometrically measured at 521 nm and expressed as mg malondialdehyde/kg of meat (Botsoglou et al., 1994).

Sensory evaluation

The sensory properties of the cooked meat such as color, odor, juiciness, tenderness, taste, and overall acceptability were evaluated by 10 panelists based on a 7-point hedonic scale test (score 1 for dislike extremely and score 7 for like extremely).

Color evaluation

The lightness (L), redness (a), and yellowness (b) of the meat samples were measured by a Chroma meter (CR-400, Konica Minolta, Japan).

Microbiological analyses

The meat samples were pasted by a laboratory meat grinder under aseptic conditions. The serial dilutions were made by 0.1% peptone water. For coliforms enumeration in meat samples, one mL of 10^{-2} dilution was poured into petri-dish using pour plate method and then charged with violet red bile agar medium. In order to determine total microbial count, 0.01 mL of each ten-fold dilution was spread on plate count agar plates. The plates were then incubated at 35- 37°C for 24 h and the results were reported as $\log_{10}$ colony forming units (CFU) per gram of meat sample (log CFU/g).

Statistical analysis

Data were analyzed using a factorial experiment in a randomized complete block design with SAS software (version 9.1). Duncan's multiple range test was used to compare the means at 5% significance level ($p\leq0.05$).

Results and discussion

Changes in pH

The metabolic process stops due to bird slaughtering and ceasing of blood flow. However, some processes continue for moments that lead to glycogen breakdown in an anaerobic pathway and production of lactic acid, which the latter reduces pH of tissues (Asghar et al., 1991). As can be seen in Fig. 1, the highest pH value belonged to $F_{100}P_{100}$ treatment and the lowest pH belonged to F_0P_{90} on the first day of storage. Increasing probiotic content resulted in an increase in pH value of meat samples. As storage time increased to 4 days, $F_{100}P_{100}$ and $F_{100}P_0$ treatments showed the highest and lowest pH values, respectively. Also, the control sample was not significantly differed from other treatments ($p\leq0.05$). Different levels of probiotic, prebiotic, and synbiotic did not have a significant effect on meat pH at 7 days of refrigeration. The results indicated that the presence of probiotic and prebiotic in the quail diet reduced the amount of stored glycogen due to the fact that polysaccharide levels were not sufficient to undergo anaerobic degradation, lactic acid production, and finally pH decrement. On the other hand, pH increment during storage could be attributed to amino acids-deamination and ammonia rescue. Effect of a diet containing different levels of organic selenium and vitamin E (Senobar et al., 2012) and *Mentha pulegium* and *Zataria multiflora* extracts (Khademipoor et al., 2017) on the quality of Japanese quail meat did not show any significant change in meat acidity.

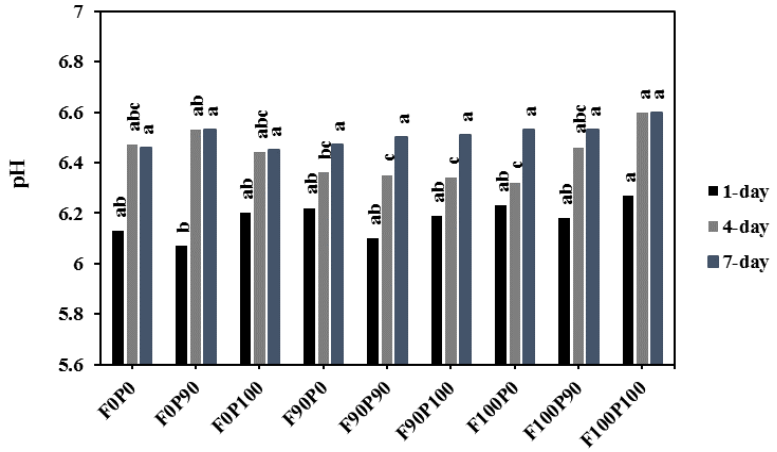

Fig. 1. Changes in pH of quail meat samples during cold storage. Common superscript letters on each day show significant difference between samples ($p\leq0.05$).

WHC

The effect of synbiotic diets on WHC of meat samples is given in Fig. 2. The results showed that there was no significant difference between all samples at 1 and 4 days of storage, however; most of the treatments had a higher WHC than the control. Nasehi et al. (2015) reported that the addition of probiotic protexin to the Japanese quail diet did not significantly change WHC of the samples (Nasehi et al., 2015). WHC was significantly influenced by different levels of probiotics and prebiotics at 7 days of refrigeration. $F_{90}P_0$ had the highest WHC, while $F_{100}P_{100}$ presented the lowest content. WHC of meat samples decreased as a function of prebiotic level. It is worth noting that WHC plays an important role in meat processing industry and its lower content increases economic losses, negative effects on the technological characteristics of meat products, and decreases sensory features, especially the appearance of fresh meat (Schäfer et al., 2002). The reduction in WHC is mainly due to the myofibril shortening, pH decrement, myosin denaturation, and actomyosin formation (Senobar et al., 2012). Therefore, as the results showed, the lack of pH reduction may lead to a dry-firm-dark (DFD) meat with high WHC.

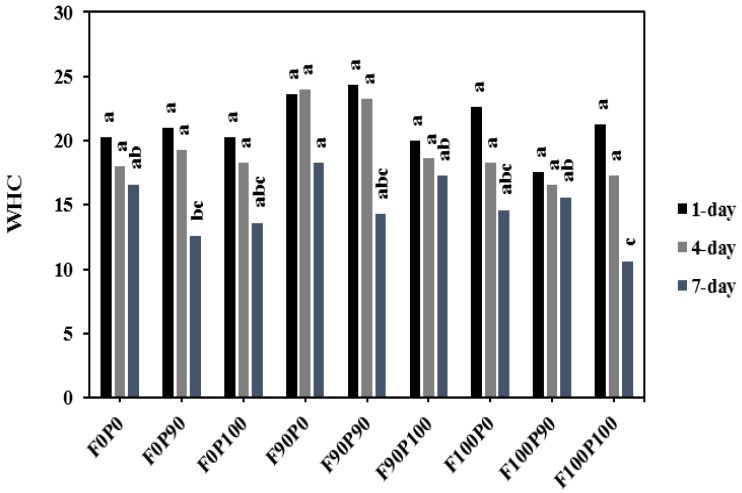

Fig. 2. Changes in water holding capacity (WHC) of quail meat samples during cold storage. Common superscript letters on each day show significant difference between samples ($p \leq 0.05$).

Oxidative stability

Lipid oxidation is one of the main problems in the meat industry that leads to flavor loss and reduced nutritional value of many meat products (Senobar et al., 2012). Although, free radicals are known as pro-oxidants of lipid oxidation in meat, fat content, and fatty acid profile can also influence the lipid oxidation during meat storage (Kim et al., 2002). As shown in Fig. 3, TBA value of all samples increased as a function of storage period and this effect was more pronounced in the control sample (F_0P_0). F_0P_{90} significantly had the highest TBA on first day of storage. The lowest TBA was observed in $F_{90}P_{100}$ treatment. After 4 days of storage, $F_{100}P_{100}$ and $F_{90}P_0$ presented the highest and lowest TBA value, respectively. Control sample showed significantly higher oxidative compounds in meat stored for 7 days and the lowest TBA was for $F_{90}P_{90}$. Lower oxidation in quail meat fed with probiotic and prebiotic could be ascribed to antioxidant compounds produced by probiotics and their storage in meat tissues. In addition, the possible reaction of malondialdehyde with muscle components such as proteins, amino acids, myosin, and formation of carbonyl compounds could induce irregular changes in oxidation

extent (Soglia et al., 2020). Nasehi et al. (2015) reported a decrease in TBA and an increase in sensory features of fresh-fried quail meat fed with probiotics (Nasehi et al., 2015). Additionally, in another research, fat oxidation was significantly decreased in broiler chicken meat fed with rosemary powder and vitamin E (Eftekhari et al., 2010).

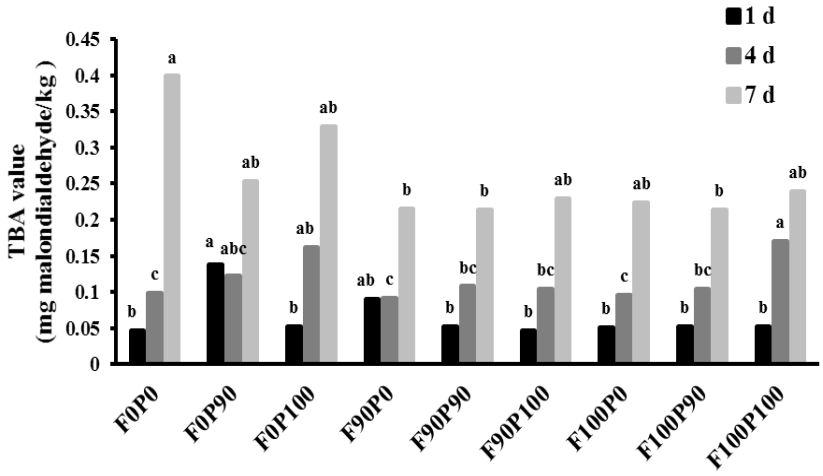

Fig. 3. Thiobarbituric acid (TBA) value of quail meats during cold storage. Common superscript letters on each day show significant difference between samples ($p \leq 0.05$).

Sensory properties

Fig. 4 shows the effects of different levels of feeding with probiotics, prebiotics, and synbiotic on sensory properties of quail meat. The color of samples was not significantly different, except for $F_{90}P_{100}$ and $F_{100}P_{90}$ which ranked lower scores among treatments. $F_{100}P_0$ received the highest score. $F_{100}P_0$ showed higher acceptance than other treatments in terms of odor. However, it did not show any significant difference with the control sample. The juiciness was mainly related to prebiotic content and the highest score was for F_0P_{90}, but there was no significant difference between the treated and control samples. Although, there were no significant differences between treatments in terms of tenderness, the highest score was for $F_{90}P_{90}$ and $F_{100}P_{100}$.

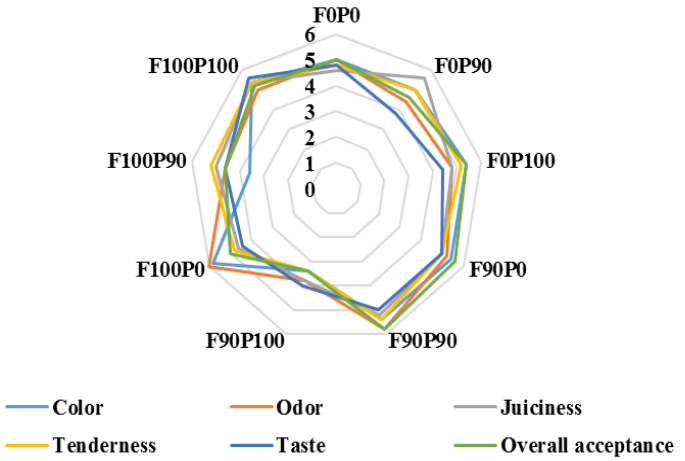

Fig. 4. Sensory properties of quail meats after feeding with different levels of probiotic and prebiotic.

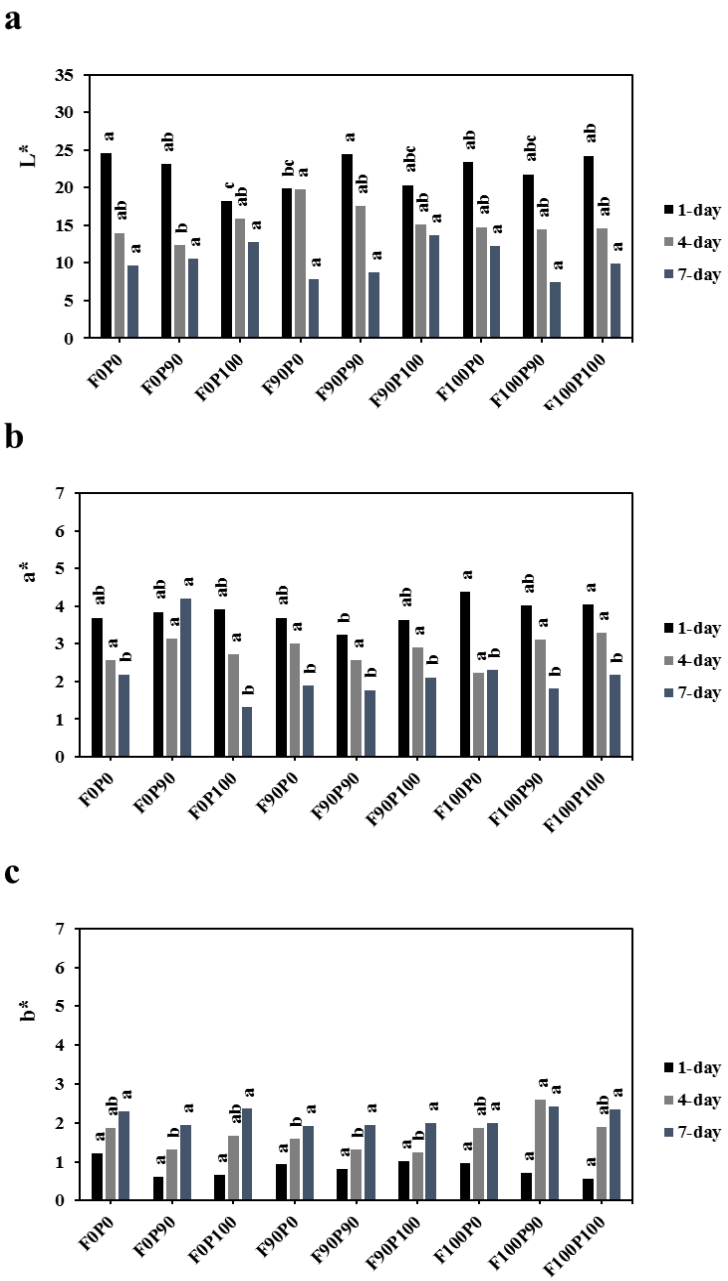

Fig. 5. L* (a), a* (b), and b* (c) indices of quail meat samples during cold storage. Common superscript letters on each day show significant difference between samples ($p \leq 0.05$).

The taste acceptance of $F_{100}P_{100}$ was considerably high and other treatments had no significant difference with the control sample. The results revealed that almost all sensory characteristics improved as the level of probiotic increased. WHC increment leads to improvement of redness, juiciness, and tenderness of meat samples. As Emadzadeh et al. (2011) stated that meat juiciness plays a key role in improving meat texture and overall acceptability (Emadzadeh et al, 2011). Fig. 4 shows the overall acceptance of meat samples. Although, no significant differences were observed between treatments, $F_{90}P_{90}$ ranked the highest score. It is noteworthy that probiotics can utilize prebiotics and produce some flavors such as acetaldehyde, diethyl acetate, and

acetoin which can be stored in meat tissues and then released by cooking.

Changes in meat color

Meat lightness varied from 24.59 to 48.7 over different storage days (Fig. 5).

*L** index of samples on 1st day, except for F_0P_{100} and $F_{90}P_0$, did not differ significantly with the control (Fig. 5a). *L** value on 4 and 7 days was not significant among treatments. The reduction in *L** value of quail meat over time could be ascribed to water reduction which resulted in surface dryness and darkness. By studying the effect of diets containing probiotics on characteristics of Japanese quail meat during storage period, Nasehi et al. (2015) observed a decrease in *L** value of samples.

$F_{100}P_0$ and $F_{90}P_{90}$ showed significantly higher and lower *a** value on 1st day, respectively (Fig. 5b). However, there was no significant difference between other treatments. In addition, quail meats fed with 100% probiotic had relatively higher *a** index (higher redness). Redness of treatments did not differ significantly at 4 days of storage. However, at 7 days, $F_{90}P_0$ presented significantly higher *a** value than other treatments. *a** value decreased as a function of storage time. It was reported that the increase in contents of antibiotic and probiotic resulted in a non-significant increase in redness compared to the control (Nasehi et al., 2015). The increase in redness of some samples than the control could be due to antioxidant activity of probiotics and prebiotics (Das & Goyal, 2015; He et al., 2015). Meat redness depends on the presence of ferric iron and its oxidation state. Lipid autoxidation and subsequently oxymyoglobin oxidation to metmyoglobin have been related to color deterioration in meat (Nieto et al., 2010). Therefore, antioxidant compounds can decrease the red color degradation via controlling the rate of oxidative reactions. The reduced rates of lipid oxidation and metmyoglobin formation in meat supplemented with antioxidants have been reported in the literature (Lauzurica et al., 2005; Nieto et al., 2010).

Evaluation of *b** value of meat samples showed that there was no significant difference between treatments at both 1 and 7 days of storage (Fig. 5c). However, *b** value at 4 days was notably higher in treatments containing 100% probiotic and especially in 90% prebiotic ($F_{100}P_{90}$) compared to other samples. *b** value of all treatments increased as refrigeration period increased. Carotenoids are responsible for yellow color of foods and an inverse relationship is observed between red color and yellow color; an increase in redness will result a decrease in yellowness (Marconi et al., 2000). Khademipoor et al. (2017) showed that the addition of *Mentha pulegium* or *Zataria multiflora* powder to Japanese quail diets does not affect their color meat (Khademipoor et al., 2017).

Microbial load

The influence of diets containing probiotic, prebiotic, and synbiotic on microbial load of quail meat is presented in Fig. 6. Results showed that total microbial count was not affected by the experimental variables on 1st day (Fig. 6a). On the contrary, significant changes were observed at 4 and 7 days of storage. It can be seen that control sample on 4th day of refrigeration had no significant difference compared to the other treatments. The highest number of microorganisms was found in treatments rich in prebiotic and prebiotic, i.e., $F_{100}P_{100}$ and the lowest microbial count was observed in $F_{90}P_{100}$ treatment. On 7th day, control treatment did not differ significantly with other samples, except for $F_{90}P_{100}$, which had the lowest microbial count. The highest number of microorganisms was also found in probiotic-free treatments. The results of total coliforms of quail meats during storage are provided in Fig. 6b. The highest total coliform belonged to F_0P_{100} on 1st day, $F_{90}P_{90}$ on 4th day, and F_0P_{90} on 7th day. In addition, $F_{90}P_{90}$, $F_{90}P_{100}$, and control had the lowest coliforms at 1, 4, and 7 days, respectively. Nasehi et al. (2015) showed that the addition of probiotic lowered microorganism activity in fresh meat and total

bacterial count in quails fed with antibiotic or probiotic was relatively lower than that of the control sample during storage (Nasehi et al., 2015). They also claimed that probiotic protexin like virginamycin was able to control the activity of microorganisms during cold storage, but the supplements did not play an effective role in controlling coliform activity. Similarly, a significant reduction in total microbial count in Japanese quail meats fed with probiotics was reported by Javadi et al. (2012). The reduction in microbial count by probiotics could be due to several mechanisms such as maintaining natural microbiota of the intestine via eliminating competitive factors, replacing the metabolic process through increasing the activity of digestive enzymes and reducing the activity of bacterial enzymes, improving food absorption and digestion, and stimulating the immune system (Javadi et al., 2012).

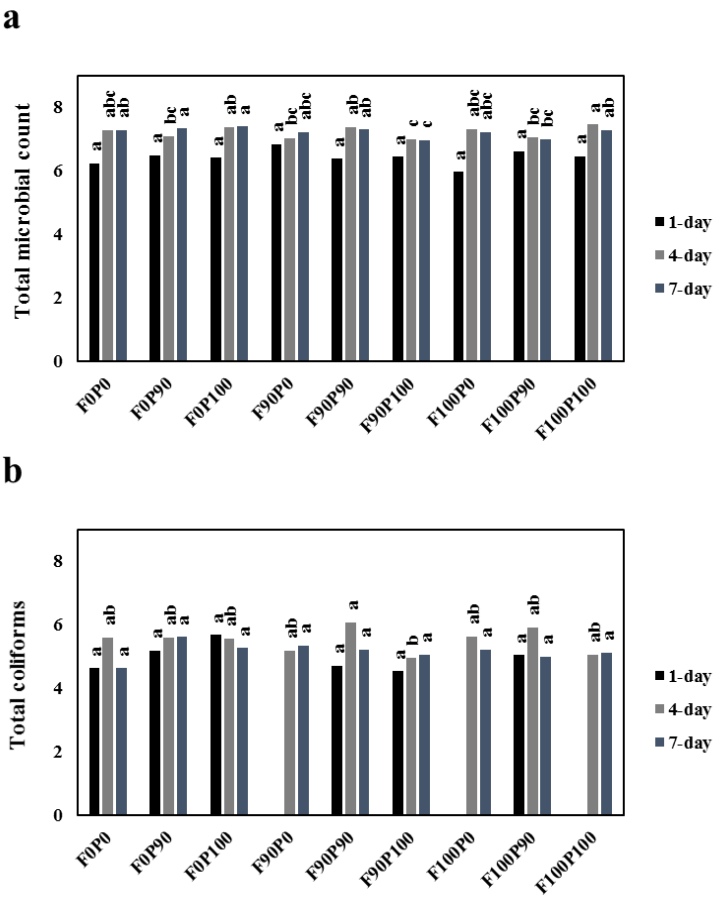

Fig. 6. Total microbial count (a) and coliforms (b) of quail meat samples during cold storage. Common superscript letters on each day show significant difference between samples ($p \leq 0.05$).

Probiotic microorganisms can probably produce some bactericidal and bacteriostatic compounds in intestine such as lactoferrin, lysozyme, hydrogen peroxide, and organic acids, which potentially have antimicrobial effect toward pathogenic bacteria (Kabir et al., 2005). The addition of prebiotics galactomannans, oligosaccharides, and arabinoxylans to broiler chicken diet, reduced *Salmonella typhimurium* in ileum and cecum and increased Lactobacillus and Bifidobacterium species in cecum (Faber et al., 2012). Some oligosaccharides, especially galactomannans may directly prevent pathogenic bacteria binding to epithelial cells in small intestine and inhibit their proliferation in the gastrointestinal tract. Moreover, Kalsum et al. (2012) stated that probiotic *Lactobacillus*

fermentum can inhibit the growth of pathogenic bacteria such as *S. typhimurium* and *Escherichia coli* in quail via producing antibacterial compounds (Kalsum et al., 2012).

Conclusions

The present research shows that probiotic and prebiotic addition did not influence pH of quail meats. Moreover, most treatments presented non-significantly higher water holding capacity than the control after 4 days of storage. Quails fed with $F_{90}P_{100}$ showed the lowest meat oxidation in fresh state. However, oxidation increased as a function of refrigeration and its rate was lower in probiotic-rich samples. Probiotic had more positive effects on color and odor scores; whilst, meat juiciness influenced more by prebiotic addition. It was shown that $F_{100}P_{100}$ and $F_{90}P_{90}$ treatments received higher taste and overall acceptance than others, respectively. Synbiotic effect on microorganisms of meat showed that $F_{90}P_{100}$ had lower total microbial count compared to other treatments after 4 and 7 days of cold storage. Total coliforms of meat samples affected remarkably at initial storage periods and the lowest number was accounted for $F_{90}P_{100}$ treatment. Taking everything into consideration, synbiotic addition to Japanese quail diet can improve meat quality and control lipid oxidation along with microbial contamination during cold storage. In this study, the $F_{90}P_{100}$ feeding treatment was introduced as the best treatment with high-quality meat.

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سین‌بیوتیک‌ها به‌عنوان جایگزین بالقوه محرک رشد برای بهبود پایداری میکروبی و اکسیدشوندگی گوشت بلدرچین ژاپنی

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چکیده

استفاده از آنتی‌بیوتیک‌ها در پرورش دام به‌ویژه طیور منجر به افزایش مقاومت آنتی‌بیوتیکی و اختلالات انسانی می‌شود. بنابراین، پژوهشگران به دنبال یک جایگزین خوب برای بهبود تعادل میکروبی روده، عملکرد رشد و کیفیت گوشت دام هستند. مطالعه حاضر با هدف بررسی تأثیر جیره‌های حاوی سطوح مختلف (صفر، ۹۰ و ۱۰۰ درصد) پروبیوتیک فرماکتو (F)، پری‌بیوتیک پریمالاک (P) و مخلوط آنها بر ویژگی‌های شیمیایی، میکروبی و حسی بلدرچین ژاپنی انجام شد. گوشت نمونه F100 بالاترین امتیاز رنگ و بو را نشان داد. در حالی که ویژگی آبداری به سطح پری‌بیوتیک وابسته‌تر بود F100P100 و F90P90 به‌ترتیب بالاترین امتیاز را در طعم گوشت و پذیرش کلی کسب کردند. کمترین تعداد میکروارگانیسم و کلی‌فرم کل درF90P100، طی مدت نگهداری مشاهده شد. به‌طور کلی افزودن سین‌بیوتیک‌ها به جیره بلدرچین ژاپنی علاوه بر کنترل اکسیداسیون در یخچال منجر به بهبود کیفیت گوشت و کاهش آلودگی میکروبی شد.

واژه‌های کلیدی: بلدرچین ژاپنی، آنتی‌بیوتیک، سین‌بیوتیک، طیور، کیفیت گوشت.

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Full Research Paper

Microwave and traditional brewing methods of Iranian black tea: bioactive compounds, antioxidant activity and heavy metals

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Abstract

Black tea, which is obtained from the leaves of small tree *Camellia sinensis*, is a popular drink that has been consumed for centuries all around the world. In this study, a sample of Iranian black tea was brewed by two methods of microwave and traditional brewing and their extracts were then assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) assays to determine their phenolic and flavonoid contents as well as heavy metal (HM) (copper, nickel, chromium, cadmium and lead) content. It was observed that there is a direct relationship between the antioxidant activity with their phenolic and flavonoid contents. The highest and lowest antioxidant activities were observed for samples brewed by microwave at 360 W for 2.5 min and 900 W for 7.5 min, respectively. As the brewing power and time increased, the antioxidant activity decreased. Brewing tea by microwave and traditional methods caused a significant reduction in the amount of heavy metals, which was lower than the allowable limit according to the Iranian national standards. These results demonstrate the importance of exposure time and radiation power when tea is prepared by microwave.

Keywords: Black tea; Tea extract; Brewing; Antioxidant; Heavy metals.

Introduction

Tea is the most broadly used aromatized beverage worldwide and its consumption has a long record. As a drink, it originates using green leaves of *Camellia sinensis*, which contain compounds such as polyphenols, caffeine, and catechins, that possess medicinal properties. For example, a group of catechins that are extracted from *Camellia sinensis* green leaves are shown to exhibit anti-cancer effects and extend the life of healthy cells. Due to its nutritional, specific taste, agricultural and financial value, both tea leaves and its extract are used in several industries, including food, cosmetics and beverages (Chen et al., 2020; Ghasemzadeh-Mohammadi et al., 2017; Li et al., 2020). Because of the importance of these elements, many studies have been carried out on tea plants (Wang et al., 2020; Zhang et al., 2020). Besides the presence of the antioxidant compounds, other ingredients such as essential elements that exist in the tea plant have both

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disadvantages and advantages for the human body. For example, it has been found that aluminum is a critical agent increasing the effect of the dementia of Alzheimer's illness (Fung et al., 2003). On the other hand, presence of heavy metals (HMs) was already proved in many studies (Idrees et al., 2020; Seenivasan et al., 2008). Soil, nutrients, fertilizers, and pesticides used during plant growth are some of the sources of HMs, which can persist in tea and affect human health. Therefore, it is a matter of concern to study concentration of these elements in teas. Most of the organics in tea are heat-sensitive and for their extraction, low-temperature preparations are required in order to prevent the possibility of their hydrolysis or thermal degradation (Pereira & Meireles, 2007). One of the recently used extraction techniques is Microwave-Assisted Extraction (MAE) technology which is a speedy and well-organized technique designed for rapid extraction of compounds from solid matrices. Because of the shorter extraction time, it is supposed that antioxidant contents of tea can be preserved more effectively. Moreover, it can be regarded a greener method based on environmental standards (Ahmad et al., 2021; Farahmandfar, Asnaashari, & Bakhshandeh, 2019). To the best of our knowledge, MAE as a novel brewing approach, and parameters affecting it (such as microwave time and power) on the extraction of antioxidant ingredients and HM content has not yet been studied for the Iranian black tea. This research aimed to reveal how MAE and the conventional tea brewing method can affect the Iranian black tea extract. MEA parameters which can affect the extraction were also studied.

Materials and methods

The tea sample in this study was obtained from a local market in Sari (north of Iran) close to a tea farm and processing factory (Rasht-Gilan). Folin-Ciocalteu reagent was obtained from Merck-Millipore (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrochloric acid (HCl) and tert-butylhydroquinone were purchased from Sigma-Aldrich (St. Louis, MO). For the ferric reducing/antioxidant power (FRAP) method, sodium phosphate buffer (pH 6.6), potassium ferricyanide, ferric chloride (Sigma), and trichloroacetic acid (Merck-Millipore) were used. All other chemicals were of analytical grade (Merck) and used without further purifications.

Tea brewing

For traditional brewing, 2.00 g of the finely powdered tea sample was poured into 100.0 mL distilled water to prepare a 2% w/v mixture and kept at 100°C for 20 min. For MAE, the same amount of tea was poured into 100.0 mL of distilled water and was subjected to three 900, 600, and 360 W power settings in a microwave oven (LG company, Korea, Model MH8265 DIS), for three different times of 2.5, 5.0 and 7.5 min (Spigno & De Faveri, 2009).

Phenolic content

In order to determine phenolic content of the tea extract, a previously developed method (Farahmandfar, Asnaashari, Asadi, & Beyranvand, 2019; Farahmandfar et al., 2017) was employed with small modifications. Briefly, 100.0 µL of the tea sample was added to 500.0 µL of 10% w/v folic acid. The solution was then mixed with 1.000 mL distilled water, held at ambient temperature for 1 min, mixed with 1.5 mL of 20% w/v sodium carbonate, and transferred to a test tube. After keeping the mixture for 2 h in a dark place at 25°C, to determine its phenolic content, the absorbance of the solution was recorded at 760 nm by a spectrophotometer (T80+ UV/Vis spectrophotometer, Purkinje General, Malaysia), against standard solutions.

Flavonoid content

The colorimetric aluminum chloride technique was applied for flavonoid determination of tea sample (Asadi & Farahmandfar, 2020; Farahmandfar et al., 2017).

Briefly, 0.5 mL solution of the sample was blended with 1.5 mL of methanol, 0.1 mL of

10% w/v aluminum chloride, 0.1 mL of 1.0 mol/L potassium acetate, and 2.8 mL of distilled water. After holding the mixture in a dark place at room temperature for 30 min, the absorption of the samples was recorded at 415 nm to evaluate its flavonoid content. Quercetin was used to construct a standard calibration curve, and the results were presented in mg of quercetin per 100 g of extract.

DPPH

Anti-radical activity of the extract using DPPH was studied according to Farahmandfar and Aziminezhad (2021) with slight changes. For this purpose, different concentrations of the prepared extract were added to 2.7 mL of DPPH radical (6×10^{-5} mol/L) solution and using a spectrophotometer, radical reduction of DPPH samples was measured by adsorption monitoring in 517 nm. The inhibitory effect was expressed as a percentage and calculated according to Eq. (1).

$$\text{Radical Scavenging activity\%} = A_{DPPH}-A_s/A_{DPPH}\times100 \quad (1)$$

Where, A_s and A_{DPPH} are the absorbance of the sample including the extract and absorbance of DPPH solution, respectively.

FRAP

The reducing power of the samples was carried out according to Farahmandfar, Tirgarian, Dehghan, and Nemati (2020). To carry out this test, 2.5 mL of the extract with different concentrations (500, 1000, 1500, 2000 and 2500 mg/kg) were mixed with 2.5 mL of mixture of sodium phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL potassium ferricyanide solution (1% w/v) and incubated at 50 °C for 20 min. After that, 2.5 mL of 10% w/v trichloroacetic acid solution was added to the samples to stop the reaction. Sample was then placed in a centrifuge (FAR TEST, Model VS 4000 C, Iran) at 3000 rpm for 8 min. 5.0 mL of the supernatant was taken and diluted with 5.0 mL of distilled water containing 1.0 mL of 0.1% w/v ferric chloride solution. Finally, the absorbance of the solution was recorded at 700 nm.

Heavy metal determination

For dry digestion of dry Iranian black tea, a method suggested by Pourramezani et al (2019) was followed. 5.0 g of the sample was burnt in an electric furnace at 450± 5°C for 8 h. The ash was dissolved in 5.0 mL of concentrated hydrochloric acid to be digested and then the mixture was diluted by distilled water to 50.0 mL and its HM content was determined by flame atomic absorption spectroscopy (Perkin-Elmer, model 100, USA) Lead (Pb), copper (Cu), cadmium (Cd), nickel (Ni) and chromium (Cr) were determined separately. The HM determination for the extract was performed according to Ting et al. (2013). 10.0 g of powdered tea was poured into 200 mL of water in a 500 mL round flask and stirred at 250 rpm for 1 h using a rotary (REMI, Model RS-24 Plus, India) at 25°C. This blend was then filtered using a vacuum pump (EYELA, A1000, Japan) and the collected herbaceous extract was analyzed by atomic absorption spectrometer. Concentration of each HM was obtained using Eq. (2).

$$C = \frac{Gs\times v}{w} \quad (2)$$

C= metal concentration in solid sample (mg/kg)

Gs=metal concentration in digested solution (mg/L)

V=dilution volume (50 mL)

W= weight of dry sample (10.0 g)

Statistical analysis

In our investigation, we managed the statistical analysis of the data with SPSS software v. 16, applying a completely randomized design in addition to a one-way analysis of variance. Later, Duncan's test was carried out to correlate the mean values of the samples (three replicates) at a confidence interval of 95%.

Results and discussion

Total phenolic and flavonoids content

Phenolic compounds are secondary metabolites of plants that have at least one hydroxyl group in their aromatic ring. Through

donating electrons, these compounds act as anti-radical agents and a barrier to oxidative disease progression (Dai & Mumper, 2010). Flavonoids are classified as phenolic compounds which typically have a 15-carbon structure, two phenolics, and one heterocyclic ring. Studies have indicated that plant-based flavonoids could prevent *Helicobacter pylori* infections (Ahmed & Eun, 2018; Ardalani et al., 2020). As depicted in Table 1, the tea sample obtained by MAE method could make a significant difference in terms of the phenolic and flavonoid contents compared to tea sample obtained from the traditional method ($p<0.05$). The total phenolic and flavonoid content of tea sample brewed in the microwave oven (except 900 W; 7.5 min) was significantly higher than that of traditional method. So, it can be concluded that microwave radiation can have a positive effect on the extraction of bioactive compounds. The infusion treatments in the microwave oven heated for 2.5 min at 360 W had the highest total phenolic (3224.9 mg GAE/100 g of extract) and flavonoid content (2237.4 mg QE/100 g of extract) while those heated at 900 W for 7.5 min had the lowest value. Spigno and De Faveri (2009) showed significantly greater amounts of the phenolic compounds extracted in a short time through tea brewing using MAE compared to the traditional extraction method. The findings of Krishnan and Rajan (2016) are in consistent with our observations which showed that MAE is more efficient in flavonoids extraction. Studies on brown microalgae, banana peel, tea, and pomegranate peel also supported the higher effectiveness of the MAE compared to the traditional technique (Kaderides et al., 2019; Yuan et al., 2018). MAE might be more effective because the heat is transferred to the sample through the dual mechanism of ion conduction and bipolar rotation (Pasrija & Anandharamakrishnan, 2015), and because radiation destroying the plant matrix and releasing the plant compounds into the solvent (Cassol et al., 2019; Routray & Orsat, 2012). We observed that as the power and the time of the microwave process increases, the total phenolic and flavonoid content of tea extract decreases. Higher radiation power and longer extraction time also significantly decreased the bioactive components content. As a result, phenolic and flavonoid compounds might be degraded if they are radiated for a long-term in a microwave oven (Oussaid et al., 2018; Routray & Orsat, 2012). Destruction of antioxidant compounds during the microwave process occurs at high brewing time and with intense radiation power.

Table 1- Phenolic and flavonoid content of tea determined after extracting by two brewing methods.

Treatment	Power (W)	Temperature (˚C)	Time (min)	Total phenolic content (mg GAE/100 g extract)	Flavonoid content (mg QE/100 g extract)
Microwave	900		7.5	2540.22± 3.00 ^{i}	1764.99± 2.08 ^{i}
	900		5.0	2870.22± 0.61 ^{f}	1992.69± 0.42 ^{f}
	900		2.5	3011.79± 3.83 ^{d}	2090.38± 2.66 ^{d}
	600		7.5	2820.39± 0.71 ^{g}	1958.31± 0.49 ^{g}
	600		5.0	3011.89± 2.72 ^{d}	2090.44± 1.89 ^{b}
	600		2.5	3150.88± 0.53 ^{b}	2186.35± 0.37 ^{d}
	360		7.5	2968.18± 1.52 ^{e}	2060.28± 1.05 ^{e}
	360		5.0	3022.71± 1.11 ^{c}	2097.91± 0.77 ^{c}
	360		2.5	3224.90± 0.60 ^{a}	2237.42± 0.42 ^{a}
Traditional		100	20.0	2690.86± 2.04 ^{h}	1868.93± 1.42 ^{h}

* Mean± SD
* Means with different letters within column indicate significant differences at P< 0.05

DPPH radical scavenging activity

The antioxidant activity of tea samples for two brewing methods including MAE and traditional brewing methods can be seen in Table 2. Analysis of variance showed a significant difference between the antioxidant activity of tea sample obtained by MAE method and tea sample obtained using the traditional method ($p<0.05$). MAE showed higher antioxidant activity in all concentrations compared to the traditional brewing methods. The microwave oven is known as an appropriate equipment for phenolic compounds extraction compared to the conventional method (Yuan et al., 2018). The rotation of water molecules in the electric field of the microwave oven produces heat which helps to release the phenolic compounds more efficiently (Cassol et al., 2019). Janda et al. (2020) determined antioxidant activities of five coffees with different brewing methods. They tested AeroPress, drip, espresso machine, French press, and simple infusion methods. The results revealed that the sort of brewing techniques had a significant influence on the antioxidant activities of the samples. The lowest radical scavenging activity was reported for the coffee from the espresso machine, and the highest value was related to the AeroPress brewing method. The antioxidant activities for all treatments, DPPH free radical inhibition were enhanced as the concentration of tea extract was increased from 500 to 2500 mg/kg. The highest level of the free radical inhibition was observed at 2500 mg/kg sample brewed at 360 W for 2.5 min. Researchers declared higher concentrations of the extracts showed higher antioxidant activity levels and confirmed the results of this research (Calderón-Oliver & Ponce-Alquicira, 2021; Farahmandfar, Naeli, Naderi, & Asnaashari, 2019; Rehder et al., 2021; Yuan et al., 2018)

Table 2- DPPH radical scavenging activity (%) in tea extract derived from the conventional and microwave brewing methods.

Treatment	Power (W)	T (˚C)	Time (min)	The concentration of sample extract				
				500 (mg/kg)	1000 (mg/kg)	1500 (mg/kg)	2000 (mg/kg)	2500 (mg/kg)
Microwave	900		7.5	9.88± 0.01^{i}	25.56± 0.03^{i}	32.20± 0.04^{i}	46.37± 0.05^{i}	50.54± 0.06^{i}
	900		5	11.15± 0.00^{f}	28.85± 0.01^{f}	36.34± 0.01^{f}	52.34± 0.01^{f}	57.05± 0.01^{f}
	900		2.5	11.71± 0.01^{d}	30.29± 0.04^{d}	38.17± 0.05^{d}	54.96± 0.07^{d}	59.91± 0.08^{d}
	600		7.5	10.96± 0.00^{g}	28.35± 0.01^{g}	35.72± 0.01^{g}	51.44± 0.01^{g}	56.07± 0.01^{g}
	600		5	11.71± 0.01^{d}	30.30± 0.03^{d}	38.17± 0.03^{d}	54.97± 0.05^{d}	59.92± 0.05^{d}
	600		2.5	12.24± 0.00^{b}	31.67± 0.01^{b}	39.90± 0.01^{b}	57.46± 0.01^{b}	62.63± 0.01^{b}
	360		7.5	11.53± 0.01^{e}	29.84± 0.02^{e}	37.60± 0.02^{e}	54.15± 0.03^{e}	59.02± 0.03^{e}
	360		5	11.74± 0.00^{c}	30.39± 0.01^{c}	38.29± 0.01^{c}	55.13± 0.02^{c}	60.09± 0.02^{c}
	360		2.5	12.52± 0.00^{a}	32.41± 0.01^{a}	40.83± 0.01^{a}	58.80± 0.01^{a}	64.09± 0.01^{a}
Traditional		100	20	10.45± 0.01 ^{h}	27.06± 0.02^{h}	34.09± 0.03^{h}	49.09± 0.04^{h}	53.51± 0.04^{h}

* Means with different letters within column indicate significant differences at $P<0.05$

* Mean± SD

Ferric reducing/antioxidant power

Reducing power is often used as an indicator of electron donation which is an important mechanism to determine the antioxidative activity of the phenolic compound. The existence of reductants such as antioxidants in the tested materials reduces ferric ions (Fe^{3+}), so decreasing the capacity of antioxidant compounds is an index of its antioxidative activity. The effect of reducing power is shown in Table 3. In this study, the extract obtained from MAE indicated significant differences in antioxidant activities compared with the tea sample obtained from the traditional brewing

method ($p<0.05$). The results revealed that the reducing power of Fe^{3+} was in correlation with the concentration of the sample. The tea sample under the 360 W, 2.5 min, (2500.0 mg/kg) condition, using microwave brewing method gave the maximum reduction power of 4.712%. For all treatments, the reducing powers were enhanced as the concentration of tea samples was increased from 500.0 to 2500.0 mg/kg. Yuan et al. (2018) stated the highest iron (III) reduction power and the highest DPPH radical inhibition, in the case of various brown macroalgae species extracts, reported for the treatments obtained by MAE.

Table 3- Ferric reducing antioxidant power in tea extract derived from the conventional and microwave brewing methods

Treatment	Power (W)	T (˚C)	Time (min)	Ferric reducing antioxidant power (%)				
				500 (mg/kg)	1000 (mg/kg)	1500 (mg/kg)	2000 (mg/kg)	2500 (mg/kg)
Microwave	900		7.5	1.74± 0.00^{i}	2.17± 0.00^{i}	3.11± 0.00^{i}	3.38± 0.00^{i}	3.72± 0.00^{i}
	900		5	1.97± 0.00^{f}	2.44± 0.00^{f}	3.51± 0.00^{f}	3.82± 0.00^{f}	4.19± 0.00^{f}
	900		2.5	2.06± 0.00^{d}	2.57± 0.00^{d}	3.69± 0.00^{d}	4.01± 0.00^{d}	4.40± 0.01^{d}
	600		7.5	1.93± 0.00^{g}	2.40± 0.00^{g}	3.45± 0.00^{g}	3.75± 0.00^{g}	4.12± 0.00^{g}
	600		5	2.06± 0.00^{d}	2.57± 0.00^{d}	3.68± 0.00^{d}	4.01± 0.00^{d}	4.41± 0.00^{d}
	600		2.5	2.16± 0.00^{b}	2.68± 0.00^{b}	3.85± 0.00^{b}	4.19± 0.00^{b}	4.60± 0.00^{b}
	360		7.5	2.03± 0.00^{e}	2.53± 0.00^{e}	3.63± 0.00^{e}	3.95± 0.00^{e}	4.34± 0.00^{e}
	360		5	2.07±0.00^{c}	2.57± 0.00^{c}	3.70± 0.00^{c}	4.02± 0.00^{c}	4.42± 0.00^{c}
	360		2.5	2.21±0.00^{a}	2.75± 0.00^{a}	3.94± 0.00^{a}	4.29± 0.00^{a}	4.71± 0.00^{a}
Traditional		100	20	1.84±0.00^{h}	2.29± 0.00^{h}	3.29± 0.00^{h}	3.58± 0.00^{h}	3.93± 0.00^{h}

* Means with different letters within column indicate significant differences at $P<0.05$
* Mean± SD

Heavy metals content

Some of the required daily elements may be supplied through the regular consumption of tea. The proper function of the human body requires the proper function of important enzymes, which require the participation of some HMs that can be found in tea (e.g., copper, iron, manganese, and zinc) (Atasoy et al., 2019; SeyyediBidgoli et al., 2020).

However, other elements, such as Cr, Cd, Ni, and Pb, can have adverse or toxic effects on human health (Atasoy et al., 2019). The concentrations of HMs copper, nickel, chromium, cadmium, and lead in dry tea and its extracts obtained from various tea brewing methods are shown in Table 4. Data were obtained following the standard method suggested by AOAC (AOAC, 2020). Calibration curve of each heavy metal was drawn with working standard solution before testing. As can be seen, the concentration of Pb in all samples ranged from 0.04 to 3 mg/kg. The maximum amount of Pb belonging to the dry sample was higher than the allowable limit recommended by Iranian national standard (Std. No.623) which is 1.0 mg/kg. In a study by SeyyediBidgoli et al. (2020) the concentration of Pb in Iranian black tea was determined as 0.125± 0.103 mg/kg which was lower than our report which is due to the difference in samples examined.

Ni concentration ranged from 0.22 to 3.60 mg/kg. The maximum amount of Ni belonging to the dry sample. Researchers also reported Ni value around 0.097± 0.078 mg/kg in their study (SeyyediBidgoli et al., 2020). Nickel is a toxic metal and there is no safe limit for it according to the national standard of Iran. The concentration of Cu value in this study was determined between 0.15 to 77.50 mg/kg. The highest concentration of Cu was detected for the dry sample, which was higher than the allowable limit according to the Iranian national standards (50 mg/kg).

Table 4- The heavy metals concentrations in mg per kilogram

Treatments	Power (W)	Temperature (˚C)	Time (min)	Cu (mg/kg)	Ni (mg/kg)	Cr (mg/kg)	Cd (mg/kg)	Pb (mg/kg)
Microwave	900		7.5	0.22	0.22	0.001>	0.01	0.10
	900		5	0.19	0.53	0.001>	0.02	0.20
	900		2.5	0.39	0.32	0.001>	0.04	0.07
	600		7.5	0.15	0.51	0.001>	0.01	0.14
	600		5	0.28	0.88	0.001	0.04	0.14
	600		2.5	0.16	0.44	0.001>	0.02	0.13
	360		7.5	0.28	0.48	0.001>	0.03	0.21
	360		5	0.33	0.38	0.001>	0.05	0.12
	360		2.5	0.33	0.23	0.001>	0.04	0.04
Traditional		100	20	0.30	0.38	0.001>	0.03	0.11
Dry sample				7.75	3.60	1.72	1.42	3.00

In a study by SeyyediBidgoli et al. (2020) the concentration of Cu in Iranian black tea was reported as 0.173± 0.107 mg/kg. The concentration of Cd in all of our samples was between 0.01 to 1.42 mg/kg. The highest concentration of Cd was also calculated for the dry sample which was higher than the allowable limit recommended by Iranian national standard (0.1 mg/kg). In contrast with this study, SeyyediBidgoli et al. (2020) recorded a lower concentration for Cd for dry black Iranian tea (0.045± 0.064 mg/kg). Concentration of Cr in all samples ranged from 0.001 to 1.72 mg/kg. The highest concentration of Cr was also recorded for the dry sample. which was higher than the allowable limit recommended by the Iranian national standards (4 µg/kg). Ghale Askari et al. (2020) reported the concentration of Cr in their research on dry black Iranian tea as 0.4 mg/kg which was lower than our study.

Conclusions

According to the findings of this study, the microwave method was more efficient than the conventional method of brewing in terms of extracting the greatest amount of phenolic compounds and antioxidants from Iranian black tea. On this process, it was discovered that time and microwave power played a significant role. Additionally, because the procedure takes less time with the microwave approach, it can be considered a good way to extract antioxidants from tea. It was also found that concentration of Cu, Cd, Ni and Pb HMs the examined tea was higher than maximum contamination level suggested by the Iranian standards; therefore, preventive measures should be taken such as cultivating plants away from industrial zone and roads and avoiding the use of excessive contaminated chemical fertilizers.

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روش‌های دم کردن مایکروویو و سنتی چای سیاه ایرانی: ترکیبات زیست فعال، فعالیت آنتی اکسیدانی و فلزات سنگین

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چکیده

چای سیاه که از برگ درختچه *Camellia sinensis* به‌دست می‌آید، نوشیدنی محبوبی است که قرن‌هاست در سراسر جهان مصرف می‌شود. در این تحقیق، نمونه‌ای از چای سیاه ایرانی به دو روش مایکروویو و دم‌آوری سنتی تهیه شد و عصاره های حاصل از آن با استفاده از آزمون‌های دی فنیل پیکریل هیدرازیل (DPPH) و احیاء کنندگی آهن/ قدرت آنتی‌اکسیدانی (FRAP) و محتویات فنولیک و فلاونوئید و همچنین میزان فلزات سنگین (HMs) (مس، نیکل، کروم، کادمیوم و سرب)، مورد ارزیابی قرار گرفت. مشاهده شد که بین فعالیت آنتی‌اکسیدانی با محتوای فنلی و فلاونوئیدی آنها رابطه مستقیم وجود دارد. بیشترین و کمترین فعالیت آنتی‌اکسیدانی برای نمونه‌های دم‌آوری شده توسط مایکروویو به‌ترتیب در نسبت توان ۳۶۰ وات- زمان ۲/۵ دقیقه و توان ۹۰۰ وات- زمان ۷/۵ دقیقه گزارش شد لذا با افزایش توان و زمان دم کردن، فعالیت آنتی‌اکسیدانی کاهش یافت. دم کردن چای به روش مایکروویو و سنتی باعث کاهش قابل توجهی در میزان فلزات سنگین که کمتر از حد مجاز طبق استانداردهای ملی ایران بود، گردید. این نتایج اهمیت زمان قرار گرفتن در معرض اشعه و قدرت تشعشع را هنگام تهیه چای با مایکروویو نشان می‌دهد.

واژه‌های کلیدی: چای سیاه، عصاره چای، دم‌کردن، آنتی‌اکسیدان، فلزات سنگین.

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Full Research Paper

Numerical calculation of the lethality of becteria in bottled milk under cold plasma treatment
Running title: Effect of cold plasma on milk bacteria

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Abstract

In recent years, cold plasma is one of the expected alternatives for post-harvest treatments and post-harvest management of products. A surface discharge plasma system was used for investigating the destruction time of *Bacillus cereus*, *Bacillus coagulans*, *Bacillus stearothermophilus*, and *Clostridium botulinum* in bottled milk. The simulation was performed by COMSOL a3.5 software for a two-dimensional geometry. The collected experimental data were simulated in COMSOL software. The *k* factor of microorganism deactivation data was used to validate the simulated data. Results showed that the production of reactive oxygen species during plasma treatment increases with time and extends to the entire container. The concentration of reactive oxygen species (at the output of the plasma probe) at the beginning of the production was high, and at the end when they leave the free surface of the milk, the concentration decreased. Increasing the initial temperature of milk sample, from 50 to 80℃, can cause significant changes in the amount of ozone from 125 mol/m^3 to 266 mol/m^3, respectively (p <0.05). However, voltage changes in these two temperatures did not show a significant effect on ozone concentration. Also, immediately upon the initiation of plasma treatment, plasma destruction begins where the concentration of active species is higher. It is shown that among the four studied bacteria, *Bacillus stearothermophilus* has the highest resistance against cold plasma, and after that other bacteria have shown similar resistance. Finally, it can be concluded that the deep plasma treatment in bottle can make it possible to overcome the surface limitation of cold plasma treatment.

Keywords: Cold plasma; Milk; Sterilization; Pasteurization.

Introduction

All necessary nutritional compounds and energy for human can be found in milk (Balthazar et al., 2017). On the other hand, Milk is high in water and this makes it suseptible to bacterial deterioration and some bacteria such as *Escherichia coli*, Salmonella, *Listeria monocytogenes*, can easily grown in milk. This leads to decrease in nutrional and quality characteristics of milk (Dash & Jaganmohan, 2022). One of the ways to reduce these unacceptable changes, is using thermal processes such as pasteurization and sterilization. Thermal processing can inactivate the microorganisms but also can exert negative effect on milk. For example, some interactions, changes in protein structure, browning

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reactions, nutritional loss which ultimately reduces the qaulity of processed milk (Eazhumalai et al., 2021; Sharma & Singh, 2022; Wu et al., 2021).

In some cases, the interaction between serum proteins and proteins of fat glubule membrane was reported due to thermal processing (Kim & Jiménez-Flores, 1995). Also, the increasing of heating temperature can staibize proteins with long chains. It is reported that thermal processing of milk can change the second structure of proteins (Farrell Jr et al., 2001).

In recent years, cold plasma is considered one of the the expected alternatives for post-harvest treatments and post-harvest management of products (Misnal et al., 2022). Cold plasma technology (CP) is a non-thermal physical process that has a high potential for application in the food industry (Jiang et al., 2016). Because this technology can easily be used on a large scale and does not leave any dangerous chemical residues, while it destroys or inactivates pathogens without thermal damage to food (Misnal et al., 2022).

Cold plasma showed a high effieciency of inactivation of microorganisms (Niveditha et al., 2021; Soni et al., 2021).

Table 1- Physical characteristics of milk and ozone

Property	The amount of characteristics	Unit	Refrence
	Milk		
Density	((0.3*T[1/degC])+(0.03*T^2[1/degC^2])+(0.7*4.1)+(0.01*4.1^2)+1034.5)[kg/m^3]	kg/m³	(Bakshi & Smith, 1984)
Viscosity	(2721.5/T[1/degC])+()^2.8 (0.1*4.1)-8.9	Pa·s	(Bakshi & Smith, 1984)
Relative penetration	60		(Ghanem, 2010)
	Ozone		
Ozone density	2.14	kg/m³	(Wang et al., 2020)
The speed of ozone gas monement in fluid	0.003	m/s	(Wang et al., 2020).
Effective diffusion coeficient of ozone gas in fluid	1.74×10^{-9}	m^2/s	(Wang et al., 2020).
Ozone gas movement vector	8.33×10^{-6}	-	(Wang et al., 2020).
Diameter of ozone bubbles	3.21	mm	(Wang et al., 2020).

By cold plasma technology, it is possible to processing foods under low temperature which is a high advantages of cold plasma treatment in food technologies. Also, this technology seems to be environmentaly fraindely. But it has some limitations such as the deactivation of microorganisms with CP, is a surface treatment and is not suitable to process the entire volume of material. Some studies numerically investigated the effect of cold plasma treatment in model systems (Ranjbar Nedamani, 2022; Ranjbar Nedamani & Hashemi, 2022). Numerical calculation can reduce the number of experiments and can make it possible to investigate a wide range of factors or conditions regardless the costs of experimental activities. Tabibian (2019) nnumericaly studied the CFD (computational fluid dynamics) modeling of fludiezed bed reactors combined with cold plasma jet for treatment of particles (Tabibian, 2019). Also Wang et al. investigated the deactivation of yeast in a model media by

numerical calculations (Wang et al., 2020). The aim of this work is to investigate the effect of cold plasma technology on bacterial deactivation in milk through a needle plasma in bottled milk.

Materials and method

Problem definition

The physical model of the plasma system

A surface discharge plasma system was used for this purpose. The reactor of this system was a quartz cylinder with a diameter of 1 cm and a height of 25 cm. A steel cover with a thickness of 1 mm and a height of 25 cm was used on the inner surface of the reactor and as a high-voltage discharge electrode. The liquid inside the bottle (milk) was also considered the neutral electrode (Wang et al., 2020). Electric discharge was performed to the electrode with the studied frequency and voltage. Plasma produces active species such as hydrogen peroxide, ozone, hydroxyl radicals, and oxygen radicals. Since ozone monitoring during operations is easy and a suitable indicator to check plasma conditions, ozone concentration was used as a simulation index in this study.

Definition of variables

Physical characteristics of milk

To perform the simulation, some physical characteristics of milk and variables such as ozone movement speed in the fluid, ozone effective diffusion coefficient, ozone gas density, ozone gas bubble diameter, and ozone movement vector in the fluid were used according to Table 1.

Simulation and Governing equations

The simulation was performed by Comsol a3.5 software for a two-dimensional geometry as shown in Fig. 1. Four modules of laminar bubble flow, dilut species transport (for air injected between electrodes in the valve), dilute species transport (to remove bacteria or test compound in the valve), and electric field (plasma generator) were solved.

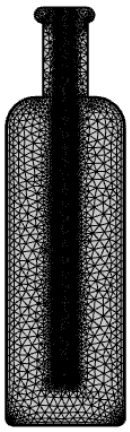


Fig. 1. The two-dimensional geometry of the milk bottle inside which the plasma generator system is placed.

Calculation of the the process destroying factor of K for pathogen deactivation in milk

During thermal processes, milk acts as a complex system of different compounds. A large amount of chemical, physical, and biochemical reactions occur in it. Some of these changes are of great importance because they can change the characteristics of milk. Others may change the nutritional value of milk and even increase its biological safety (De Jong, 2008).

Since in plasma treatment the non-thermal condition was used to destroy the microorganisms, it is believed that the incidence of temperature-affected reactions in milk will decreased. The reactions which may occure in milk during processing, can be divided into five categories: (1) destruction of microorganisms, (2) inactivation of enzymes,

(3) denaturation of proteins, (4) loss of nutritional compounds, and (5) formation of new compounds. Most of these reactions can be represented by a simple one-step irreversible reaction as A→B. by a standard reaction rate equation in the form of equation (1):

$$r_A = -kC_A^n, \quad r_B = -r_A \qquad (1)$$

Where r is the reaction rate (mol/m^3.s), k is reaction rate constant (m^3/mol.s), and n is reaction degree. The way in which the reaction rate constant is affected by temperature, is important in determining the extent of final transformations caused by heat treatment. For this reason, equations 2 and 5 were used.

$$k_{Bacillus\ Stearothermophilus} = 101.15 \exp\left(\frac{-345.4}{8314T}\right) \qquad (2)$$

$$k_{Clostridium\ Botulinum} = 107.5 \exp\left(\frac{-351}{8314T}\right) \qquad (3)$$

$$k_{Bacillus\ Coagulans} = 151.29 \exp\left(\frac{-509}{8314T}\right) \qquad (4)$$

$$k_{Bacillus\ Cereus} = 91.92 \exp\left(\frac{-294.5}{8314T}\right) \qquad (5)$$

Laminar bubble flow

This module was used to simulate the movement of ozone bubbles from the plasma reactor inside the valve and equations 6 to 9 were solved by the software.

$$\emptyset_l \rho_l \frac{\partial u_l}{\partial t} + \emptyset_l \rho_l (u_l.\nabla) u_l = \nabla.[-pI + \emptyset_l(\mu_l + \mu_T)(\nabla u_l + (\nabla u_l)^T)] + \emptyset_l \rho_l g + F \qquad (6)$$

$$\rho_1 \nabla.(u_l) = 0, \qquad u_l = u \qquad (7)$$

$$\frac{\partial \emptyset_g \rho_g}{\partial t} + \nabla. N_{\rho_g \emptyset_g} = -m_{gl}, \qquad \emptyset_g \rho_g = rhogeff \qquad (8)$$

$$N_{\rho_g \emptyset_g} = \emptyset_g \rho_g u_g, \qquad u_g = u_l + u_{slip} - \mu_T \frac{\nabla \emptyset_g}{\rho_l \emptyset_g} \qquad (9)$$

where l and g are related to liquid (milk) and gas (ozone), respectively.

The density of gas is negligible compared to the density of milk. Laminar flow equations were used to solve the rising of ozone bubbles inside the bottle. The density, diameter, and diffusion coefficient of ozone bubbles were considered according to Table 1.

Transport of diluted species

The deactivation of microorganisms depends on the amount of ozone and ions - formed by the plasma. The rate of this reaction can be calculated according to Fick's law in the form of equations 10 and 3-25:

$$\frac{\partial c_i}{\partial t} + \nabla.(-D_i \nabla c_i) + u.\nabla c_i = R_i \qquad (10)$$

$$N_i = -D_i \nabla c_i + u c_i \qquad (11)$$

During the simulation, the value of R_i (reaction rate) was defined according to equation 12:

$$R_i = -k_{reac} c_{o_3} \qquad (12)$$

Initial and boundary conditions

In solving this problem, the outer boundary at the top of the plasma-generating reactor was considered a free surface. For the simplicity of the calculations, the surface motions of the fluid were neglected. The border of entering ozone into the valve was considered at the end of the

reactor. The ozone flow rate was calculated according to equation 13:

$$n.N_1 = n.\left(uc_{0.j}\right) \quad (13)$$

Solving the problem

The COMSOL a3.5 software was used to solve four modules based on laminar flow. A system of Intel® Core TM i5-4300U, 2.50 GHz, RAM 4 GB, and Windows 10 64-bit were used for this purpose. The relative tolerance of solving the problem was 0.01 and data recording was done for ten minutes at one minute intervals. The normal mesh was used for geometry and the fine mesh was used for the reactor in 2D space as shown in Fig. 1.

Verification of the simulated model

The collected experimental data were simulated in Comsol software. The *k* factor of microorganism deactivation data were used to validate the simulated data. After investigating the best fitted simulated data through comparing the R^2, the accuracy of simulation was also validated.

Statistical analysis

To check the validity of the model and to detect regression coefficients and statistical significance, an analysis of variance was performed by ANOVA in Design Expert. The equation of the line, regression coefficients, and lack of fit were analyzed by statistical parameters R^2, *p-value* (at the level of 0.05), and *Adj-*R^2 .

Results and discussion

Verification of the simulated model

In microbial inactivation, if a semi-logarithmic plot of microbial population is plotted, a linear plot with slope *k* is obtained (Ibarz & Barbosa-Cánovas, 2002). Process temperature can change the slope of this graph. But in this study, the effect of the number of ions formed during plasma treatment and especially the amount of ozone concentration should be considered as an indicator to check the amount of air ionization. In particular, temperature changes can affect the amount of ozone gas produced by plasma treatment (Ranjbar Nedamani & Hashemi, 2022). Figure 2 shows the pre-treatment results for fitting the experimental and simulated data. The value of R^2= 0.9802 indicates the proper fit of these data with each other. In this way, the simulation conditions are well adapted to the real conditions and it will be possible to change the parameters in the simulation with the least error of the output data.

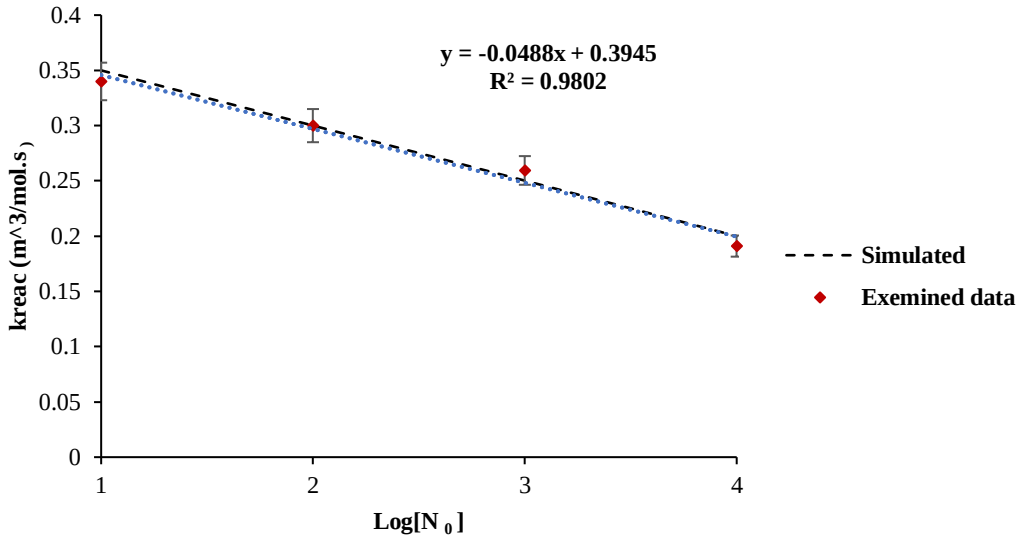
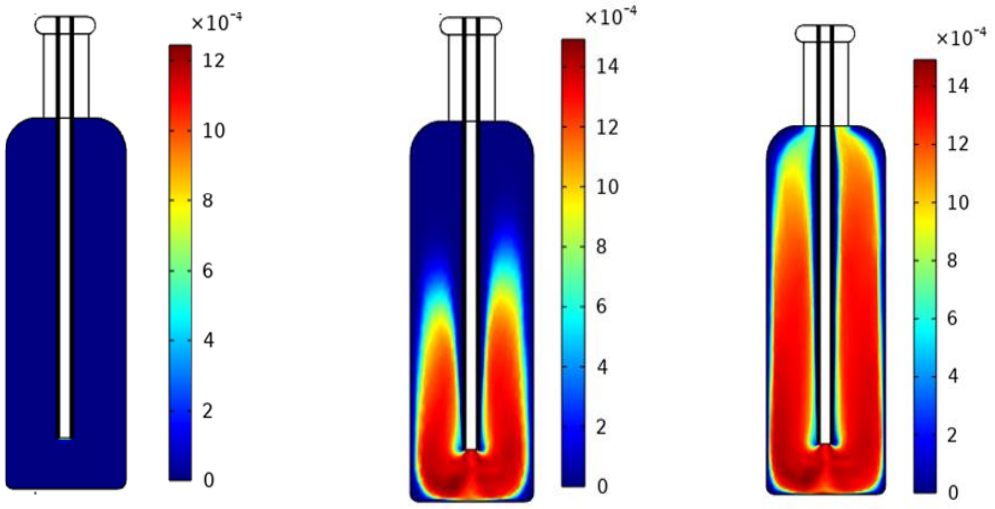


Fig. 2. Fitting the simulated and experimental data to verify the simulation.

Reactive species concentration

Figures 3 and 4 show the changes in ozone concentration as an indicator of reactive species production during plasma treatment. According to Fig. 3, the production of reactive oxygen species during plasma treatment increases with time and extends to the entire container. These active species come from the surface of the milk in the bottle to the outside of the bottle in the form of bubbles, and after production, they quickly leave the radical state to react on microorganisms or possibly other consumption compounds due to their short life span. Thus, the concentration at the beginning of the production of reactive oxygen species (at the output of the plasma probe) was high, and at the end when they leave the free surface of the milk, the concentration decreased. This bubbly movement towards the top of the active species causes the flow and smooth corrosion of the milk inside the bottle, but the speed of displacement is small due to the low speed of air injection into the probe, and it will not cause turbulence flow and had no affect on the fat cells of the milk.



(a **(b)** **(c)**

Fig. 3. The gas component (ozone) inside the milk bottle at time zero (a), 5 minutes (b), and 10 minutes (c).

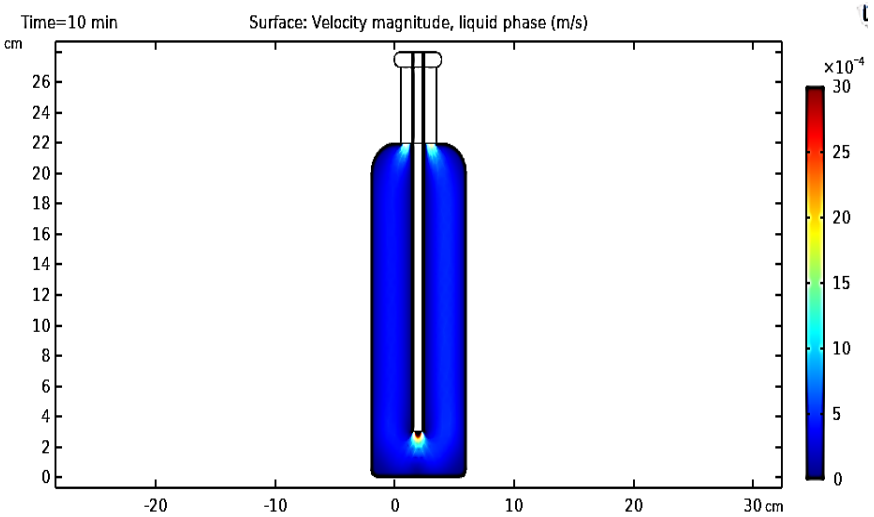


Fig. 4. The speed of milk movement during plasma treatment (m/s), Y and X axis are height and width of geometry, respectively.

Fig. 5. shows the changes in ozone concentration during plasma treatment. As displayed in this figure, it is necessary to achieve complete lethality of the microorganism, it is better to carry out plasma treatment continuously; because the effect of plasma treatment will be practically lost when the active species of oxygen is used up or expires.

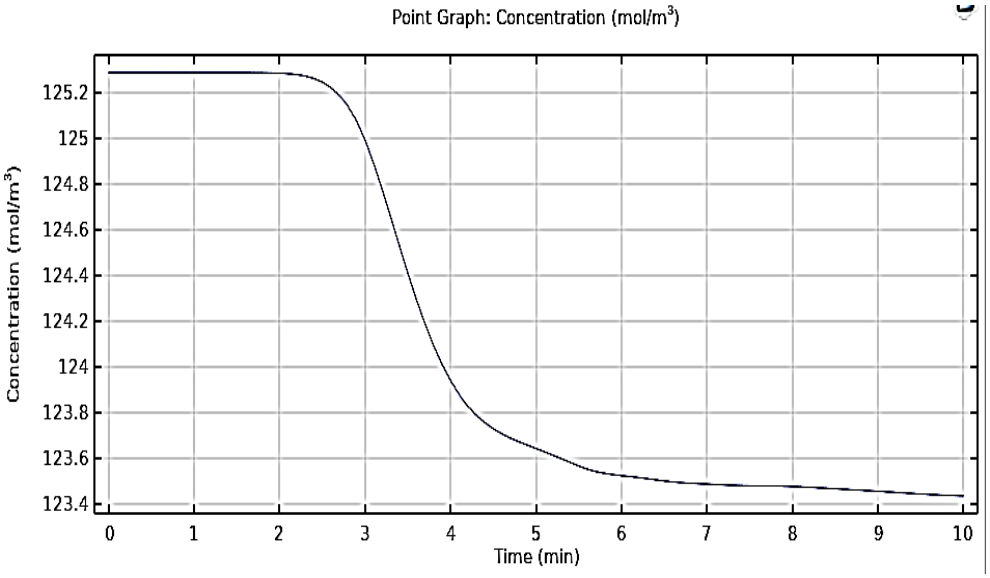


Fig. 5. Changes in ozone concentration inside the milk bottle during plasma treatment

Fig. 6. shows the relationship between ozone concentration and changes in temperature and voltage studied in this study. According to the figure, changes in initial temperature had a greater effect on the amount of active species production than changes in voltage. Increasing the initial temperature of the milk sample, from 50 to 80℃, can cause significant changes (p<0.05) in the amount of ozone from 125 mol/m^3 to 266 mol/m^3, respectively. However, voltage changes in these two temperatures did not show a significant effect on ozone concentration.

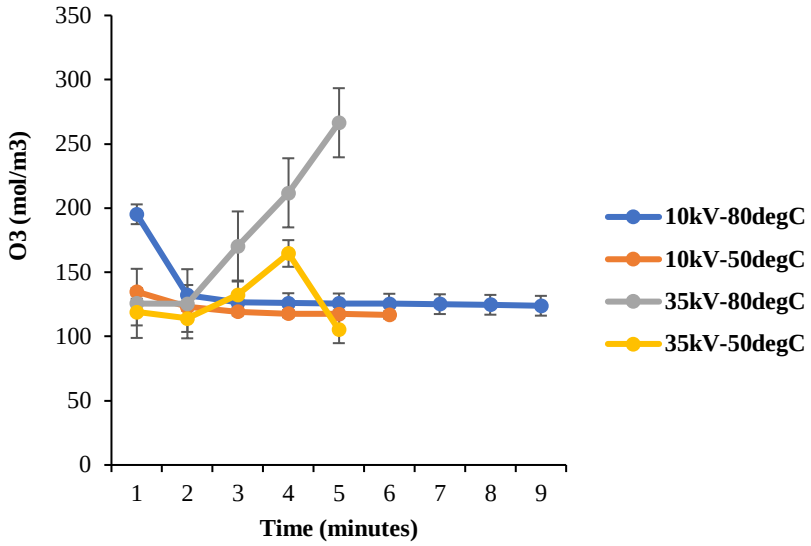
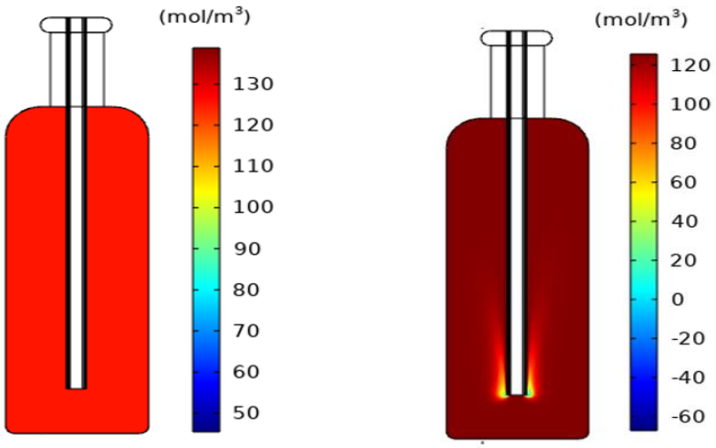


Fig. 6. The amount of ozone concentration produced at different voltages and temperature.

Temperatures of cold plasma treatment

The study of Bahreini et al. (2021) on the inactivation of microorganisms in milk also did not show a relationship between voltage and destruction rate (Bahreini et al., 2021). In a previous study on the inactivation of yeast, it was also found that ozone concentration has a direct relationship with temperature (Ranjbar Nedamani, 2022). The reason for this was perhaps due to the selection of high voltages for operation. Study by Aslan (2016) showed that the effect of voltage on sterilization can be investigated by changing the applied voltages to 1.5 kV, 3 kV, and 5 kV with a fixed frequency of 500 Hz for 3 minutes (Aslan, 2016). He reported that under the same application conditions, the level of bacterial growth obtained at voltages of 3 kV was significantly (p<0.05) lower than that at 1.5 kV and 5 kV. Therefore, it can be concluded that applying a low voltage to the plasma to inactivate all bacteria in the milk, had low effect. Hence, the plasma microbial inactivation efficiency improved and increased with applying higher voltage. In this regard, Wu et al. (2021) reported that a voltage of more than 70 V with 120 seconds of treatment was similar to the ultra-high temperature (UHT) sterilization process and better than pasteurization (Wu et al., 2021). They also reported that all bacteria in the plasma treated group showed varying degrees of destroying and even broken mycelium morphology. Since bacteria had a broken morphology, the bacterial cell can leak out and this will lead to the destruction of the microorganism. These changes in the microorganism concentration inside the milk bottle during plasma treatment are shown in Fig. 7.



(a **(b)**

Fig. 7. Microorganism concentration inside the milk bottle at zero time (a) and immediately after the start of plasma treatment (b).

As shown in the Fig. 7, immediately upon the initiation of plasma treatment, plasma destruction begins where the concentration of active species is higher. With the development of active species, the destruction of microorganisms develops throughout the bottle. The feature of using a long cylindrical probe that is placed in the center of the bottle is that in this way, the speed of distribution of plasma species from depth to the surface will be wide. While other plasma treatment methods, because plasma is considered a surface disinfection method, have limitations and are unable to treat samples with a depth greater than a certain limit, especially if the sample is liquid and with a large volume. In addition, Fig. 8. shows the inactivation time of *Bacillus cereus*, *Bacillus coagulans*, *Bacillus stearothermophilus*, and *Clostridium botulinum* during treatment of bottled milk with cold plasma.

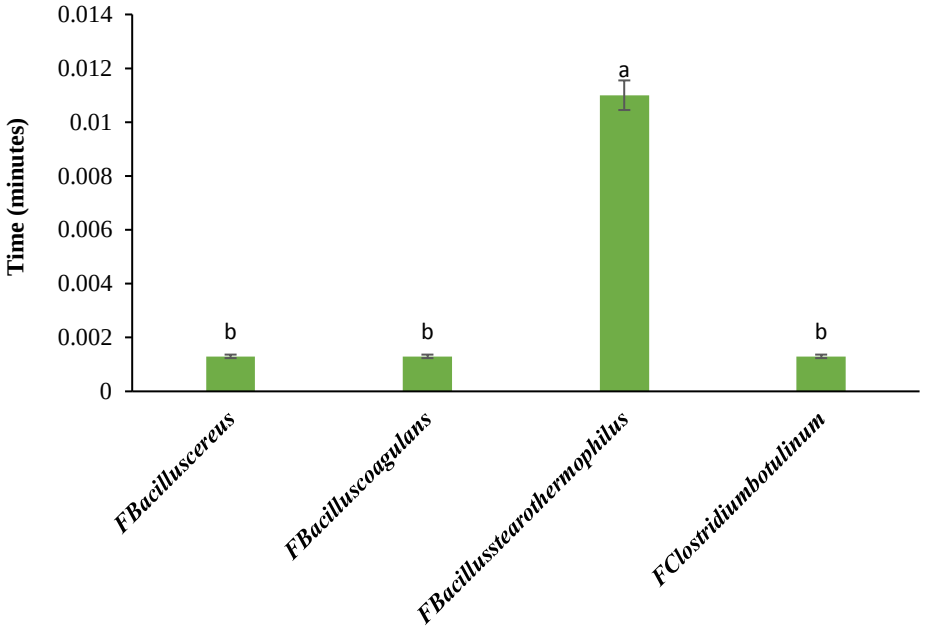


Fig. 8. Inactivation time of studied microorganisms in milk.

It is shown in the figure that among the four studied bacteria, *Bacillus stearothermophilus* has the highest resistance against cold plasma, and after that other bacteria have shown similar resistance. Although many studies have focused on the decontamination ability of cold plasma technolog, limited research has been reported on the effect of this technology on cow's milk. Wu et al. (2021) investigated the destruction of *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* with the help of DBD plasma (Wu et al., 2021). They reported that after 120 seconds of treatment at 80 V, the inactivation rate of all three bacterial species was 100%. Gurol et al. (2012) also reported that treatment with corona-type plasma at a voltage of 9 kV was able to destroy 54% of the microbial population of *Escherichia coli* within 3 minutes (Gurol et al., 2012). Kim et al. (2015) investigated the rate of destruction of *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella typhimurium* using encapsulated-DBD plasma during periods of 5 and 10 minutes. They reported that the treatment was able to reduce the microbial population by 20 log CFU/ml after 10 minutes (Kim et al., 2015). Bahreini et al. (2021) also reported that by using the plasma jet system in the inactivation of *Staphylococcus aureus* and *Escherichia coli* in milk, voltage changes during plasma treatment did not affect the microbial population of *Escherichia coli* (Bahreini et al., 2021). Cold plasma has different effect on different types of microorganisms. Since the microorganisms had determined and specific characteristics, the CP treatment showed different effects on microorganism even in the same strains. The stationary phase of microorganism growth or spores are more resistance to CP (Liao et al., 2017). Also, the gram-negative bacteria are more sensetive than gram-positive bacteria to CP treatment (Liao et al., 2017; Schlüter & Fröhling, 2014). Because in the outer part of gram-positive membrane, there is a thick peptidoglycon structure. On the other hand, in gram-negative bacteria, the elecrostatic force of CP overcomes the strenght of its membrane tensile and thus the cell wall of bacteria will destroyed (Nishime et al., 2017). The nature of material which used in CP treatment affect the process effeciency. The CP is a surface treatment in most solid food materials and the active produced species penetrate to solid food based on the water content, prosity and also physici-chemical properties (Surowsky et al., 2013). But in liquid foods, more volume should be treated with CP and if it is possible, the penetration depth of reactive species had low importance on process efficiency. Kim et al. (2015) reported that they found a strong relationship between the ability of these species to inactivate the microorganisms because with the increase in atomic oxygen concentration, the level of inactivation of microorganisms also increased. Lioa et al. (2018) have reported that in addition to the direct effect of these active species in the inactivation of microorganisms, the formation of antimicrobial compounds such as hydrogen peroxide, hydroperoxide radicals, and ozone also destroy microorganisms (Liao et al., 2018). What should be noted here is that the amount of milk fat has no effect on the inactivation of microorganisms in milk. There is limited studies regarding the effect of milk fat content on microbial deactivation of milk. Gurol et al. (2012) reported that fat content of milk, had no effect on the susceptibility of *Escherichia coli* by plasma treatment, thus revealing the potential advantage of this disinfection system for possible use in fat-content food materials. They suggested in pulsed electric field processig of milk, fat can make a protection against pulsed electric and thus can had a reverse effect on microorganisms. But in cold plasma treatment, there is no pulsed electric effect of field and only the gas species can attak the micribial cell and destroy it. Thus it can be concluded that the atmospheric conditions of CP operation had a great effect on the final efficiency of it (Surowsky et al., 2014).

Conclusion

It is important to know that the CP is an surface treatment and before selecting the type

of CP apparatus, the type of food material should be considered. Also, the type of microorganism, the microbial population, and the growth phase of microorganism are effective on the final inactivation effeciency of CP. To stablish a widespread adoption of cold plasma in dairy industry, there is some limitations. We know that the thermal processes are the validated classic methods in food industry and all of the food machinary are designed based on thermal processes. Replacing the CP treatment with conditional thermal processes in food industry needs to overcome or determining the CP limitations. Different studies are trying to find the CP effect, its limitation, and its benefits in different food products or raw material. It can be concluded that this new technology can be applied to reduce the cost of processing and preserving the nutritional and physicochemical characteristics of food.

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Data Availability Statement

Data will be made available on request.

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محاسبه عددی کشندگی باکتری در شیر بطری شده تحت تیمار پلاسمای سرد

آزاده رنجبر ندامانی[1*]

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چکیده

در سال‌های اخیر، پلاسمای سرد یکی از جایگزین‌های مورد انتظار برای تیمارهای پس از برداشت محصولات هستند. در این مطالعه، یک سیستم تخلیه سطحی برای جستجوی زمان نابودی *باسیلوس سرئوس*، *باسیوس کوآگولانس*، *باسیلوس استئاروترموفیلوس* و *کلستریدیوم بوتولینیوم* در شیر بطری شده استفاده شد. شبیه سازی توسط نرم‌افزار COMSOL ورژن ۳/۵a برای یک هندسه دو بعدی اجرا شد. داده‌های آزمایشی جمع‌آوری شده در نرم‌افزار شبیه‌سازی شدند. فاکتور k حاصل از داده‌های غیرفعال‌سازی میکروارگانیسم برای تأیید داده‌های شبیه‌سازی استفاده شد. نتایج نشان دادند تولید گونه‌های فعال اکسیژن طی تیمار پلاسمای سرد، با افزایش زمان افزایش می‌یابد و در کل ظرف پخش می‌شود. غلظت این گونه‌ها در ابتدای تولید یعنی در لحظه خروج از پروب پلاسما، بالا بوده و در انتها که سطح آزاد شیر را ترک می‌کنند، کاهش می‌یابد. با افزایش دمای اولیه نمونه شیر از ۵۰ به ۸۰ درجه سانتی‌گراد، می‌توان تغییرات بارزی در مقدار ازون مشاهده کرد. اما تغییرات ولتاژ در این دو دما اثر بارزی بر غلظت ازون نداشت. همچنین بلافاصله با آغاز تیمار پلاسما، تولید پلاسما نیز آغاز شده و میزان غلظت گونه‌های فعال در آن لحظه بیشترین مقدار است. نشان داده شده است که در بین چهار باکتری مورد مطالعه، *باسیلوس استئاروترموفیلوس* بیشترین مقاومت را در برابر پلاسمای سرد داشته و باکتری‌های دیگر بعد از آن قرار می‌گیرند. در نهایت می‌توان نتیجه گرفت که تیمار پلاسما در عمق بطری، این امکان را ایجاد می‌کند که محدودیت کاربرد سطحی تیمار پلاسمای سرد رفع شود.

واژه‌های کلیدی: پلاسمای سرد، شیر، استریلیزاسیون، پاستوریزاسیون.

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Full Research Paper

Development of Mozzarella cheese freshness indicating film by embedding purple carrot extract in gelatin and Persian gum matrix

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Abstract

This study aimed to evaluate the effects of intelligent pH-sensitive composite film based on gelatin and Persian gum incorporated with purple carrot extract (PCE) on the freshness of wrapped mozzarella cheese. In this regard, the color, pH, yeast and mold count of control and treatments wrapped with intelligent pH-sensitive composite film during 60 days were evaluated. The results showed that the pH significantly reduced in wrapped cheese with and without PCE (control) samples during storage. However, this reduction was more pronounced in the control sample ($P<0.05$). Additionally, the application of composite film on cheese affected the color during storage. It was observed that L* and *a** values of the composite film-wrapped cheese were significantly higher than the control sample, but the *b** values were significantly lower than the control sample. Moreover, poor microbial growth (yeasts and mold) was observed in cheese samples wrapped by composite film with purple carrot compared to the control. Also, the pH of the composite film with extract significantly decreased from 6.33 to 4.85 during storage ($P < 0.05$), which showed the changes of color from purple to pink. After 40 days, the color changed to pink, indicating the end of the cheese storage. Therefore, it was concluded that the pH-sensitive film, while being an effective method to improve the shelf life of mozzarella cheese, can also use as an indicator for freshness.

Keywords: Gelatin; Mozzarella cheese; Persian gum, pH-sensitive film; Purple carrot extract.

Introduction

Edible coatings and films are thin layers of various natural materials, such as polysaccharides, proteins, and lipids which coat the surface of fruits and vegetables (Al-Hassan & Norziah, 2012). They act as an excellent physical barrier to water and simple gases such as CO_2 and O_2, leading to reduction of deterioration and oxidation, and moisture loss (De Pilli, 2020; Suhag et al., 2020). Polysaccharide and protein coatings and films can effectively extend the shelf life of different foods due to their hydrophilic nature. Also, it has been reported that coating\film made of lipid components such as paraffin and wax can significantly reduce water loss from fruits and vegetables because they provide a more effective barrier toward water evaporation

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(Jafarizadeh Malmiri et al., 2012). Therefore, development of coatings and films from both hydrophobic and hydrophilic biomolecules with suitable nutritional and sensorial characteristics could be a useful packaging technique to preserve perishable food products and enhance their shelf life (Jafarizadeh et al., 2011). The different features of coatings materials like type, amount, density, viscosity, and surface tension as well as the techniques of coatings applications, directly influence edible coatings performances (Zhong et al., 2014).

Persian gum is one of the main carbohydrates recommended for food applications in Iran. Recently some researchers reported that this gum had good properties in the production of edible films (Pak et al., 2020; Tabatabaei et al., 2022).

Gelatin is one of the main proteins used in the production of film and coating all around the world. Application of these ingredients in the composite films can improve the physicochemical, mechanical, and structural properties (Akrami-Hasan-Kohal et al., 2020). The intelligent coating is a type of system applied for packaging of food. In this system, some changes during the shelf life was monitored. Sensors, data carriers, and indicators are important parts of this method for evaluation the quality of food products. Usually, this method is based on data collection and evaluation of some food parameters including pathogens, carbon dioxide, oxygen, temperature, freshness, leakages, pH-level (Azman et al., 2022; Schaefer & Cheung, 2018).

PCE (purple carrot extract) is an ingredient with pH-sensitive properties. The color of this extract is depended on the pH of the solution and can be changed from purple to pink at different pHs. Purple carrots (*Daucus carota subsp. Sativus var. Atrorubens Alef*) grown in the Middle East and Europe. The acylated anthocyanins in purple carrots are a natural pigment suitable for coloring foods (Arab et al., 2023). One of the main ingredients in intelligent films is pH-sensitive materials. Therefore, this extract can act as a part of this film.

Cheeses are a valuable source of nutrients and probiotics which have been an important part of dairy consumption around the world. Soft cheese products are prone to microbial spoilage by a wide range of microorganisms, resulting in short shelf life. Fresh Mozzarella cheese is a white cheese of the pasta filata family of Southern Italy origin, which is cut and produced in various shapes. Owing to high moisture content, fresh Mozzarella considered highly perishable product with a short shelf life of approximately 5 to 7 d. Moreover, the growth of spoilage microorganisms such as Pseudomonas *spp* and coliforms is another main factor limiting Mozzarella cheese shelf life (Altieri et al., 2005). Therefore, several attempts have been conducted to introduce effective techniques that can extend the shelf life of this popular cheese. In this regard, the application of edible coatings and films on cheeses could be a promising strategy for cheese preservation and extension of their shelf life (Costa et al., 2018). However, limited studies and few commercial examples have been found regarding various coating materials and also technologies applied to cheese products (O'Callaghan & Kerry, 2016). Thus, the objective of the present study is to evaluate the effects of an intelligent pH-sensitive composite film based on gelatin and Persian gum incorporating PCE on the freshness of mozzarella cheese.

Materials and methods

Bovine gelatin (bloom 200) was purchased from Gelatin Halal Co. (GHC, Tehran, Iran) and Persian gum was obtained from a local market in Shiraz (Fars Province, Iran). Glycerol as the plasticizer was prepared from Emboy Kimya (Turkey). Purple carrots were purchased from a local market in Bandar Abbas (Iran). Ethanol, HCL, and NaOH were from Merck CO. (Germany) and YGC was prepared from Hi-Media, (India).

Extract preparation

The ripe purple carrot was used for anthocyanin extraction according to the method

of Wang et al. (2021). First, 100 g of carrot cuts was mixed with 500 mL of 80% ethanol solution containing 1% HCL (12 M) for 24 h at 4°C. Then the mixture was centrifuged at 8000 g for 15 min at 4°C to obtain anthocyanin-rich extract.

Film preparation

Gelatin, Persian gum and glycerol solutions were obtained by dissolving each of them in 100 cc of a solution containing 45 cc of PCE and 55 cc of distilled water with ratios of 5, 5 and 3% w/v respectively. The prepared solutions were mixed by a magnetic stirrer for 6 h. Films were prepared by pouring 20 ml of the mixture into the Petri dishes. In order to make a thin layer, the solutions were spread on the surface by a glass rod with a 10-centimeter diameter and then put aside to dry at ambient temperature for 48 h and 24 h in the desiccator (Arab et al., 2023).

Determination of pH-sensitivity of film

In order to investigate color changes, the film was flooded with an aqueous media with different pHs from 2 to 12 for about 15 min. The color change was recorded by Minolta colorimeter (CR-20, Konica Minolta, Inc., Tokyo, Japan). The color parameters of cheese samples in terms of lightness (L^*), redness (a^*), and yellowness (b^*) were determined, then the color difference (ΔE) for the film at different pHs compared to a constant pH (pH =2) was calculated through the following equation:

$$\Delta E = [(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2]^{1/2} \quad (1)$$

L*, a* and b*(Color indexes of the film at different pH)

L_0, a_0 and b_0 (Color indexes of the film at pH equal to 2)

Cheese preparation

Finger mozzarella cheese was prepared by adding mesophilic-thermophilic starter culture (Chr-Hansen Company) to the high-fat milk which was heated at 85°C for 15 s. After cheese milk coagulation, whey was separated from the freshly formed curd in a special cheese vat to obtain curd with 48% dry matter. The dry curd was maintained until the desirable pH of 5.2 was reached.

Then the curd was transferred to the cooker stretcher at 75°C equipped with a direct heating system and two spirals with reverse rotational directions. The other ingredients were added to the curd in this stage. The subsequent stage was transferring curd to Mulder for 10 min. Then, the curd was immersed in cold water at 1- 3°C to stabilize the structure. Finally, mozzarella cheese was packed and kept in cold storage (Bermúdez-Aguirre & Barbosa-Cánovas, 2012).

Film application

Two stripes of the prepared edible film (9.5 x 7.5 cm) were heat sealed on three sides to form the pouch, cheese sample was then placed into the pouch followed by air removal under vacuum. The automatic machine equipped with a vacuum pump (60 cm/Hg- 0.80 bar/11.6 PSI) was used to completely seal the pouch. PE with the mentioned dimensions was also used as secondary packaging to cover the mozzarella cheese samples. All cheese wrapping samples were stored at 4°C.

Color of cheese

The color parameters of cheese samples in terms of lightness (L^*), redness (a^*), and yellowness (b^*) were determined using a Hunter colorimeter (Model CR- 300, Minolta Camera Co, Tokyo, Japan) as described by Dai et al. (2018).

pH of cheese samples

The pH of cheese samples was measured using a digital pH meter and titration method according to Iran's national standard No. 2852 (Öztürk & Güncü, 2021).

pH of film samples

The film samples (1 g) were mixed with 9 mL of deionized water for 1 min. pH values were measured at room temperature using a

digital pH meter by direct immersing the electrode into the mixture.

Microbiological analysis

Determinations of yeast and mold counts were performed by inoculating the required dilutions in YGC (Hi-Media, India) and incubated at 28°C for 72-120 h, to form visible colonies. Results were interpreted as the presence or absence of yeast and molds in 1g of the sample (Tirloni et al., 2019).

Statistical analysis

All experiments were conducted in triplicates. The design of the completely randomized analysis of variance (ANOVA) procedure was conducted using SAS Statistical Software (Version 9.1 SAS Institute Inc., 2000; Cary, NC, US). Duncan multiple range tests were performed to compare mean values at (p<0.05).

Results and discussion

pH sensitivity of film

The pH response and ΔE of PCE-loaded film are shown in Fig. 1 and Table 1 respectively. Film samples were shown pH- sensitivity and notable color changes as affected by different buffer solutions..

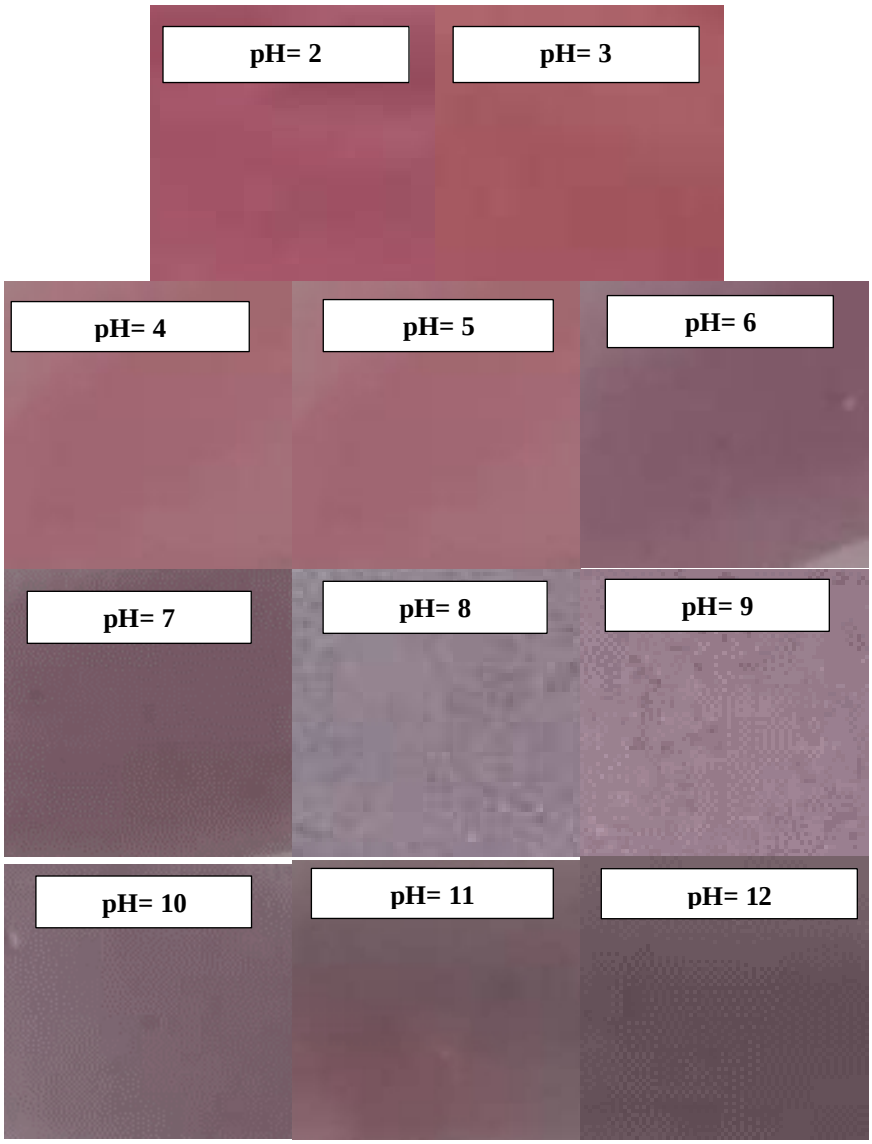

Fig. 1. Color of composite film based on gelatin and Persian gum incorporated with PCE in different pHs.

Table 1- Color difference (ΔE) of composite film based on gelatin and Persian gum incorporated with PCE in different pHs.

pH	L*	a*	b*	ΔE
2	46 ± 1^{a}	35 ± 1^{a}	11 ± 3^{a}	-
3	47 ± 1^{a}	33 ± 1^{b}	5 ± 1^{b}	6.403^{a}
4	50 ± 2^{b}	25 ± 2^{c}	4 ± 1^{b}	8.602^{b}
5	60 ± 2^{c}	21 ± 1^{d}	3 ± 1^{b}	10.817^{c}
6	43 ± 2^{d}	19 ± 1^{e}	2 ± 1^{b}	17.146^{d}
7	40 ± 2^{e}	18 ± 1^{e}	0 ± 1^{c}	3.742^{e}
8	55 ± 2^{f}	15 ± 1^{f}	-1 ± 1^{d}	15.330^{f}
9	57 ± 1^{f}	13 ± 1^{g}	-1.3 ± 0.3^{g}	2.844^{g}
10	45 ± 2^{d}	12 ± 1^{h}	-1.5 ± 0.2^{g}	12.043^{h}
11	35 ± 2^{g}	11 ± 1^{h}	-1.8 ± 0.2^{g}	10.054^{i}
12	38 ± 2^{h}	10 ± 1^{h}	-2.0 ± 1.0^{h}	3.169^{j}

Different letters in each column indicate significant differences ($P < 0.05$).

By increasing pH values, the color of the film turned from pink to blue/purple. The structural changes of anthocyanin at different pHs causing the color changes. Anthocyanins exist basically in cationic form (red or pink flavylium cation) at pH 2– 3. The purple color observed at high pH (pH 4– 6) due to the generation of carbinol pseudo-base and the blue-colored observed at pH> 7 due to the formation of quinoidal bases (Koosha & Hamedi, 2019). Other researches also showed the pH sensitivity of biopolymer-based films incorporated with extracts of purple potato, red cabbage, blackberry pomace, and purple and black eggplant (Yong, et al., 2019). Changes in the color of the sample were validated by measuring the ΔE of samples. The difference ($p< 0.05$) in the amount of ΔE indicates the color change of the film at different pH. The results proved that PCE-loaded film could act as a pH indicator.

Color of cheese

The observed values for *L**, *a**, and *b** are represented in Fig. 2, 3, and 4 respectively. Color is considered one of the critical characteristics of cheese, which affects consumer acceptability and taste perception. Also, the color could be an excellent indicator for consumers to evaluate the freshness and quality of various products (Dong, et al., 2020). At the beginning of the storage, *L** values for all treatments were between 59- 70 and 76- 82 for the control. This value declined in all samples after 60 days of storage, which could be attributed to microorganism growth and lipid oxidation in products (Cerqueira, et al., 2009; Huang et al., 2018).

Similar results were reported regarding the significant reduction of *L** value during 10 days of storage in cheese coated with sodium alginate, the sample coated with sodium alginate containing 1% *Pimpinella Saxifraga* essential oil, and the control sample (Ksouda, et al., 2019). Mei et al. (2015) also reported that the growth of mold and yeast during 20 days of refrigeration storage on the cheese surface, caused *L** value reduction in uncoated and coated bod liong cheese samples (a type of semi-hard cheese) with chestnut starch-chitosan mixture and chestnut starch-chitosan enriched with pine fruit essential oil or *Cornus officinalis* fruit.

As illustrated in Fig. 3 and 4, the *a** values for the wrapped cheese with the composite film were significantly higher than the control, although the *b** values were lower in the control sample. Despite the protective effects of films in preventing the growth of microorganisms and the antioxidant properties of film, these samples had lower *L** and *b** values than the control sample, while the values of a* were higher compared to the control sample. Several studies proved that wrapping materials had major effects on the discoloration of food items during shelf life. Pena-Serna et al. (2016)

indicated that Mongolian cheeses coated with zein and zein-xanthan solutions were more subjected to color changes compared to uncoated samples. They stated that increasing *b** and *a** values and decreasing *L** values immediately after wrapping were related to the yellowish color of the wrapping solutions. Ramos et al. (2012) also reported higher color changes in cheese samples coated with whey-gum protein isolate. Similarly, previous research on coated Queso Blanco cheese (a type of fresh cheese with a soft texture) containing flax oil (QB-FO) with whey protein isolate (WPI) and also WPI containing oregano essential oil reported a significant reduction of *L** values during the storage period compared to the control sample. Moreover, they reported the *b** values of wrapped samples increased drastically during the storage and were significantly higher than QB-FO at the end of the storage period (Gurdian, et al., 2017). Thus, selecting the proper materials for coating and film preparation effectively improves the color of this well-known dairy product and hence directly affects consumer acceptance.

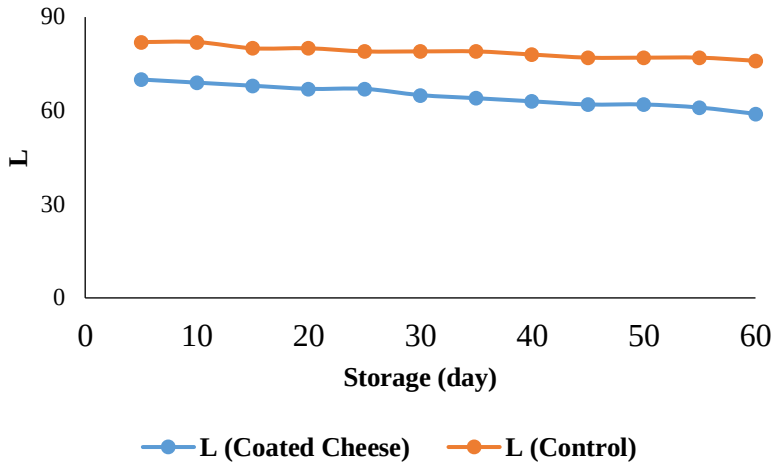

Fig. 2. *L values of mozzarella wrapped with the composite film based on gelatin and Persian gum incorporated with PCE, and control during 60 days of storage.**

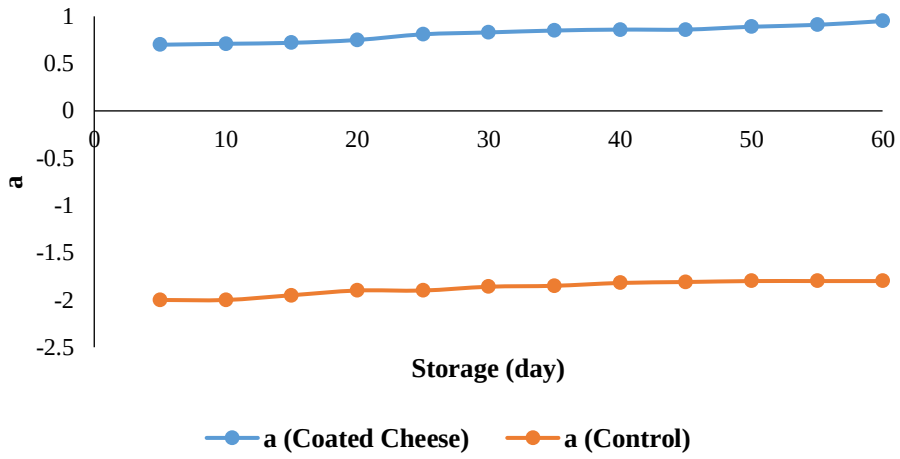

Fig. 3. *a values of mozzarella wrapped with the composite film based on gelatin and Persian gum incorporated with PCE, and control during 60 days of storage.**

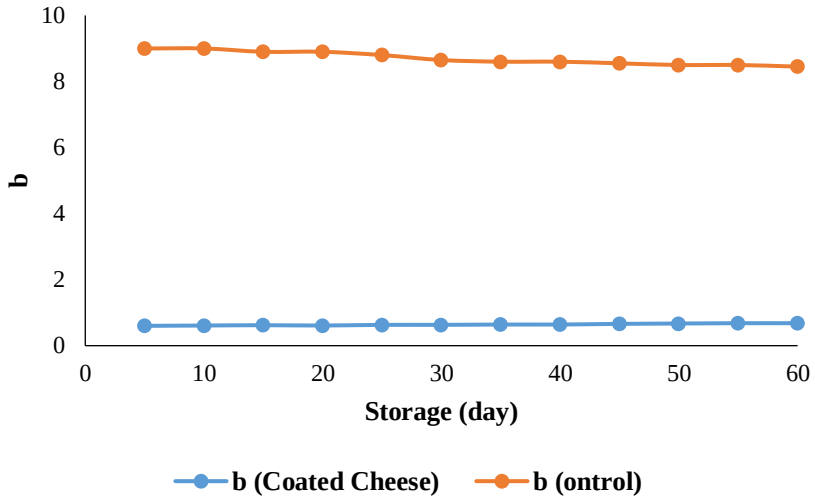

Fig. 4. *b values of mozzarella wrapped with the composite film based on gelatin and Persian gum incorporated with PCE, and control during 60 days of storage.**

pH of cheese

The pH values as an important quality parameter of cheese products were monitored during refrigerated storage. The results of many studies revealed that pH changes can directly affect the structural properties and chemical composition of cheese. The pH reduction throughout the storage significantly promotes mineral solubility and also changes casein micelles structure, leading to subsequence changes in the nature and intensity of protein interactions in cheeses (Pastorino et al., 2003). Moreover, Hayaloglu (2016) reported that the flavor and total microbial counts of cheeses are also drastically affected by the pH values. Thus pH can be a good indicator of various bacterial growth, including coliforms or pathogenic bacteria.

The pH values of wrapped cheese with the composite film and control cheese during 60 days in the refrigerator can be seen in Fig 5. The pH standard of mozzarella cheese is between 5.1 and 5.3. Regardless of the film types, the pH levels decreased in both cheese samples which was significant for the control sample ($P<0.05$). This reduction can be attributed to the conversion of lactose into lactic acid by increasing lactic acid bacteria activities such as *Lactococcus* and *Lactobacillus* in cheese during storage (Evert-Arriagada et al., 2018). The present results were in good agreement with previous reports by Ramos et al. (2012) of pH reduction in whey protein isolate coating of semi-hard cheese due to lactic acid production. Mahcene et al. (2021) also reported that microbial fermentation and peptides production caused a pH decrease in freshly coated homemade cheeses with sodium alginate containing essential oils. Similar changes in pH were found for ricotta cheese prepared by thermal coagulation method and treated with a chitosan-whey protein mixture during the first 6 days of storage. The production of amino acids and Free Fatty Acids during proteolysis and lipolysis were the main reason for this reduction. However, no significant differences were observed between the coated and control samples (Di Pierro et al., 2011). Moreover, Amjadi, et al. (2019) stated that the release of CO_2 as a result of the subsequent metabolism of lactate as well as the decarboxylation of amino acids led to pH reduction on the surface of the white cheese.

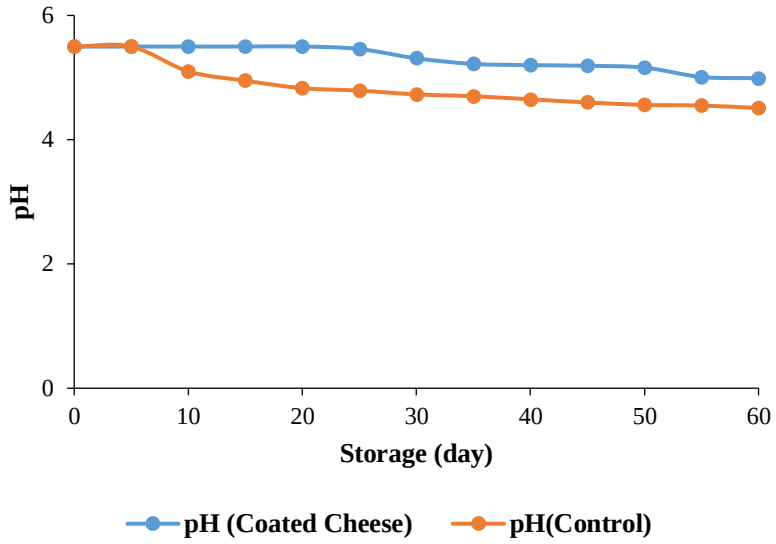

Fig. 5. pH of mozzarella wrapped with the composite film based on gelatin and Persian gum incorporated with PCE, and control during 60 days of storage.

pH of the film

Figure 6 shows the pH values for the composite edible film during 60 days of storage. The pH of the film significantly decreased from 6.33 to 4.85 during the storage period ($P<0.05$). As described previously in section 3.1, the pH reduction of the film could be related to producing free fatty acids and amino acids (Mahcene, et al., 2021; Pirsa, Karimi Sani, Pirouzifard, & Erfani, 2020). The color of films turned to pink at pH 5.

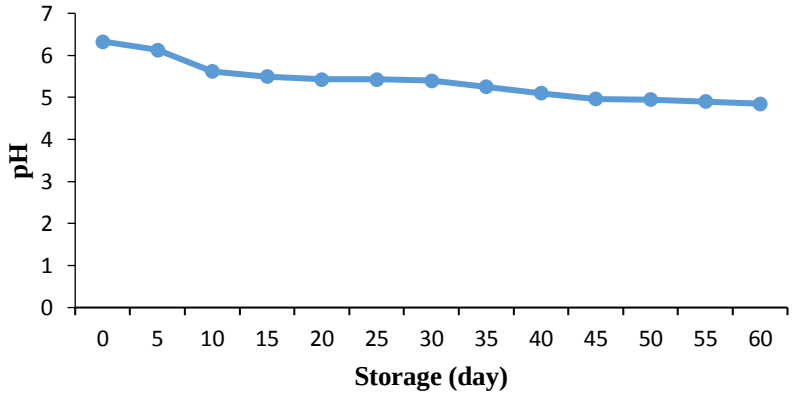

Fig. 6. pH of the composite edible film based on gelatin, Persian gum incorporated with PCE during 60 days of storage.

Yeast and mold

Yeasts and mold contaminations are common threats to dairy products which promote some defects such as off-odor and off-flavor (Trmčić, et al., 2016). In fact, suitable acidity conditions and high water activity of cheese surface made it a great medium for various microorganisms (Proulx, et al., 2017). Since yeasts are capable of growth even under anaerobic conditions, monitoring yeast existence in dairy products is very critical (Mileriene, et al., 2021). Microbial deterioration may occur during the manufacturing, storage, and even transportation of both raw milk and final cheese products (Ferrão, et al., 2016).Besides, the serious health

issues of consuming spoiled cheese, the identification of microbial contamination in cheese products could be a major financial loss for dairy factories. Thus, any successful efforts to inhibit mold and yeast growth effectively enhance the handling, marketing, and shelf life of cheese and could be a great opportunity for exporting this dairy product (Trmčić, et al., 2016). The application of edible film or wrapping to cover chees surface is a desirable approach to extend the shelf life and protect the cheese from further microbial contaminations (Kumar, 2019). Table 2 shows mold and yeast count during 60 days of storage. Based on the results, the control was more exposed to microbial growth compared to the wrapped cheese with composite film. Moreover, the storage time had a significant effect on mold and yeast proliferation in the control sample ($P<0.05$). Although, mozzarella cheese wrapped with composite films was completely resistant to microbial contamination, and the total counts of yeast and mold were not changed during storage. Similarly, Youssef et al. (2016) reported that soft Egyptian cheese coated with chitosan and carboxymethyl cellulose had no mold contamination, while a significant proliferation of 2.06 log CFU/g was observed for the control sample. Several studies also confirmed the effectiveness of edible coating and film application in preventing microbial growth on cheeses (Ramos, et al., 2012; Resa et al., 2016).

Table 2- Mold and yeast count (Log CFU/g) in mozzarella wrapped with the composite film based on gelatin and Persian gum incorporated with PCE and, control during 60 days of storage

Day	Wrapped Cheese	Control
0	<10	<10
5	<10	<10
10	<10	1
15	<10	1.69
20	<10	1.90
25	<10	2.30
30	<10	2.81
35	<10	2.84
40	<10	2.91
45	<10	2.91
50	<10	2.92
55	<10	2.96
60	<10	2.97

Conclusion

The effects of intelligent pH-sensitive composite film prepared by gelatin, Persian gum, and PCE on the properties of wrapped mozzarella cheese were studied. Results showed that the pH significantly reduced in wrapped cheese with and without PCE (control) samples during storage. However, this reduction was more pronounced in the control sample. Additionally, the application of composite film on cheese affected the cheese's color during its storage. It was observed that a^* values of the composite film-wrapped cheese were significantly higher than the control sample. Moreover, poor microbial growth (yeasts and mold) was observed in wrapped cheese by film composite with purple carrot compared to the control. After 40 days, the color changed to red, which means the end of shelf life of cheese. Finally, the results showed that this system can be used as a good intelligent pH-sensitive film for food products.

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ساخت فیلم نشان‌دهنده تازگی پنیر موزارلا با جاسازی عصاره هویج بنفش در شبکه ژلاتین و صمغ ایرانی

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چکیده

مطالعه حاضر با هدف بررسی تأثیر استفاده از فیلم کامپوزیتی حساس به pH مبتنی بر ژلاتین و صمغ ایرانی همراه با عصاره هویج بنفش (PCE) بر تازگی پنیر موزارلا انجام شد. در این راستا، رنگ، pH و تعداد مخمر و کپک نمونه شاهد و نمونه بسته‌بندی شده با فیلم کامپوزیتی حساس به pH در مدت ۶۰ روز مورد ارزیابی قرار گرفت. نتایج حاضر نشان داد که pH در نمونه‌های پنیر بسته‌بندی شده با و بدون PCE (شاهد) در طول نگهداری کاهش معنی‌داری داشت، اما این کاهش در نمونه شاهد بیشتر بود ($p <$ ۰/۰۵) علاوه بر این، استفاده از فیلم کامپوزیت بر روی رنگ پنیر در طول نگهداری تأثیر می‌گذارد. مشاهده شد که مقادیر L^* و a^* پنیر بسته‌بندی شده با فیلم کامپوزیت به‌طور قابل توجه بالاتر از نمونه شاهد بود، اما مقادیر b^* به‌طور قابل توجه کمتر از نمونه شاهد بود. علاوه بر این، در پنیر بسته‌بندی شده توسط کامپوزیت فیلم حاوی هویج بنفش نسبت به شاهد، رشد میکروبی ناچیزی (مخمرها و کپک) مشاهده شد. همچنین در طول نگهداری pH فیلم کامپوزیت حاوی عصاره به‌طور قابل توجهی از ۶/۳۳ به ۴/۸۵ کاهش یافت ($p <$ ۰/۰۵) که منجر به تغییر رنگ از بنفش به صورتی شد. پس از ۴۰ روز، رنگ به صورتی تغییر کرد که نشان‌دهنده پایان نگهداری پنیر است. بنابراین نتیجه‌گیری شد که فیلم حساس به pH در عین حال که یک روش موثر برای بهبود ماندگاری پنیر موزارلا است می‌تواند نشان دهنده تازگی آن نیز باشد.

واژه‌های کلیدی: ژلاتین، پنیر موزارلا، صمغ ایرانی، فیلم حساس به‌pH، عصاره هویج بنفش.

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