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Introduction of the Peel of Iranian Pomegranate as a Potential Natural Additive in Food by Phytochemical-based Characterization of Different Genotypes

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Abstract

Over the past decades, the use of natural additives has increased as an alternative to artificial ingredients in the food industry. The purpose of this study was to investigate the potential of pomegranate peel (PP) as a natural food additive. Many factors, including genotype, could affect the quality of PP as a by-product of juice production with many nutritional, functional and anti-infective properties. In this study, the most significant phytochemical characters of thirty Iranian pomegranate peels (IPP) from different genotypes, including total phenolic (TPC) and flavonoid content (TFC), and nine phenolic compounds were determined. The HPLC-DAD-MS results of PPEs revealed nine phenolic compounds in the IPP extracts. Punicalagin β, punicalagin α, and ellagic acid were the main components constituting 20.8–48.7, 13.9–30.1, and 1.6–13.4 µg/mg DW, respectively. The peel of IPP23 (Kabdar-Shirin-e- Behshahr) contained the highest quantity of polyphenolic compounds. Also, TPC and TFC of the peel extracts ranged between 66.38 and 181.41 mg GAE/ g DW and 38.5 to 144.13 mg RE/ g DW, respectively. Eventually, antioxidant potential estimated by the DPPH assay ranged between 4.1 and 14.4 µg/ml. The results showed that the antioxidant property of pomegranate peel extracts is significantly higher than the standard of gallic acid. Also, the peel of the genotypes that had high phenolic compounds were introduced as superior genotypes. The results of HCA showed that, among the studied genotypes, the peel of IPP23 can be introduced as a potential source of natural preservatives in the food industry.

Keywords: Antioxidant, Food preservative, Phytochemical, Polyphenol, Pomegranate

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Introduction

In recent years, natural food additives have been one of the most exciting and widely used areas in the food industry, and consumer demand for healthy food products has increased. To increase the nutritional value, improve the organoleptic properties and increase the shelf life of foods, food additives are used (Kaderides, Kyriakoudi, Mourtzinos, & Goula, 2021). Until now, synthetic antioxidants, like butylated hydroxytoluene (BHT), have been used as additives to delay or inhibit the oxidation of foods. Due to the toxic, and carcinogenicity effect of synthetic antioxidants, the interest in using natural antioxidants has been increased. Natural antioxidants include phenolic compounds of plants such as oregano, rosemary, sage, agricultural wastes, and by-products such as grape pomace, olive leaves, rice husks, etc. (Drevelegka & Goula, 2020; Mourtzinos *et al*., 2016; Nenadis, Kyriakoudi, & Tsimidou, 2013). For example, in the European Union, rosemary extract is used as an additive in several food groups such as seafood, meat and dairy products, edible oils and frying fats (Al-Moghazy, El-Sayed, & Abo-Elwafa, 2022; Kaderides *et al*., 2021).

Pomegranate (*Punica granatum* L.), a medicinal and ancient fruit that belongs to the Punicacea family, is a well-known horticultural crop that is widely grown in semi-arid mild temperate to subtropical regions with hot summers and cold winters (Eikani, Golmohammad, & Homami, 2012). Iran is one of the largest genetic resources and producers of pomegranates in the world. More than 790 genotypes and varieties of pomegranate are distributed across Iran (Zeinalabedini *et al*., 2012). Today, all parts of pomegranate fruit, including peel (exocarp and mesocarp), pulp, and seeds, are used in the food, medicinal, and cosmetic industries (Dhumal, Karale, Jadhav, & Kad, 2014). Annually about 25% of the harvested pomegranate fruit is used for the juice industry and other food products. After juice extraction, a considerable amount of waste materials such as peel and seed are produced (Russo *et al*., 2018; Sood & Gupta, 2015). Pomegranate peel (PP) contain more bioactive compounds such as tannins, flavonoids, and phenolic acids than the edible parts of pomegranate fruit (Fernandes *et al*., 2015; Hernández, Melgarejo, Martínez, Martínez, & Legua, 2011; Parashar, 2010).

PP is a rich resource of bioactive compounds, specifically punicalagin and ellagic acid isomers, which belong to the ellagitannins group. Antioxidant activity and therapeutic effect of PPEs against diabetes, cancer, cardiovascular diseases, inflammation, etc., have proved with scientific evidence (Du *et al*., 2019; Stojanović *et al*., 2017). The presence of natural compounds such as tannins, phenolic acids, and flavonoids in PP acts as antimicrobial and antioxidant agents which prevent the growth of microorganisms, the process of lipid peroxidation, and the elimination of free radicals. It can be used as an additive in food, which increases the stability of food during processing, storage or gastrointestinal digestion conditions. In most studies, no negative effect on sensory properties was observed in foods by adding PPE (Giri, Gaikwad, Raigond, Damale, & Marathe, 2023).

The high area under cultivation of diverse genotypes of pomegranate in Iran leads to access to high volume of PP as by-product. Furthermore, genotype and environmental conditions can influence the chemical composition of PP. The objectives of this study were to determine the highest percentage of polyphenolic compounds and antioxidant activity, and comprehensive phytochemical profiling of thirty well-known Iranian pomegranate genotypes.

Material and Methods

Solvents and reagents

The solvents and reagents used in this study were HPLC or analytical grade. Ethanol, methanol, and trifluoroacetic acid (TFA) used for the extraction and HPLC analyses were purchased from Chemopharma (Vienna, Austria). The reference compounds, such as ellagic acid, punicalagins, rutin, and gallic acid

were purchased from Phytopurify (Chengdu, China). Folin–Ciocalteu, DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium carbonate, sodium nitrite, aluminum chloride, and sodium hydroxide were obtained from Merck (Darmstadt, Germany).

Collection site and plant materials

In the second week of October 2018, thirty different Iranian pomegranate fruit genotypes (Table 1 and Fig. 1) were collected from Yazd Pomegranate Collection, central Iran. In this collection, approximately 790 genotypes from different regions of Iran were collected and planted. From each genotype, five fully mature fruits were manually harvested. Peels were manually separated and then dried in the shade at room temperature for two weeks.

Extraction method

Air-dried, powdered fruit peel (exocarp+mesocarp) of IPPs were extracted by ultrasound-assisted extraction (UAE) method as described by (Pan, Qu, Ma, Atungulu, & McHugh, 2012). For this purpose, 200 mg powdered samples and 10 mL of ethanol-water (70:30, v/v) were placed in test tubes and then were immersed in an ultrasonic bath (Elmasonic P, Germany) under optimal conditions for extraction (4×30 min) with temperature, ultrasonic frequency, and power, at 25°C, 37 kHz and 100% respectively (Rifna & Dwivedi, 2022). The tubes were then centrifuged for 10 min at 4000 rpm and the supernatant was used for further analysis.

Table 1- Thirty different Iranian pomegranate genotypes listed regarding their code

No.	Pomegranate genotype	Code
1	Poust-siyah-dastjerd-shirin-e-Isfahan	IPP1
2	Shirin-Nar-e-Behshahr	IPP2
3	Bavasi-Poust-Sefeed-e-Lorestan	IPP3
4	Poust Sorkh-Daneh-Sefeed-Torsh-e-Khuzestan	IPP4
5	Atabaki-poust-Ghermez-e-Sarvestan	IPP5
6	Siyah-Nar-e-Behshahr Toursh Mazandaran	IPP6
7	Shahvar-poust-Ghermez-e-Shirin	IPP7
8	Torsh-Poust-Coloft-e-Izeh	IPP8
9	Vahshi-Torsh-e-Guilan	IPP9
10	Shirin-Poust-Sefeed-e-Chaharmahal and Bakhtiari	IPP10
11	Goroch-Shahvar-e-Yazdi	IPP11
12	Poust-Ghermez-Chak Chak-e-Ardakan	IPP12
13	Aban-Mahi-Abrandabad-e-Yazd	IPP13
14	Shirin-Pishras-e-Najafabad	IPP14
15	Shoor-Poust-Nazok-Saaghand-e-Yazd	IPP15
16	Vahshi-Kan-Shirin-e-Tehran	IPP16
17	Poust-Ghermez-Torsh-e-Gorgan	IPP17
18	Kodro-Poust-Coloft-e-Kazerun-e-Fars	IPP18
19	Shirin-Poust-Sefeed-e-Shahreza	IPP19
20	Ghermez-Shirin-e-Koohdasht-e-Lorestan	IPP20
21	Sakoli-Sidun-Malas-e-Marvdasht	IPP21
22	Malas-Shahpar-Pishva-Varamin	IPP22
23	Kabdar-Shirin-e-Behshahr	IPP23
24	Togh-Gardani-e-Yazdi	IPP24
25	Agha-Mohseni-e-Gorgan	IPP25
26	Sefeed-Poust-Khosk-e-Bafgh	IPP26
27	Yek-Kilo-Malas-e-Sistan	IPP27
28	Berit-Poust-Ghermez-Malas-e-Fars	IPP28
29	Vashik-Toursh-e-Sistan	IPP29
30	Galou-Koutah-e-Yazdi	IPP30

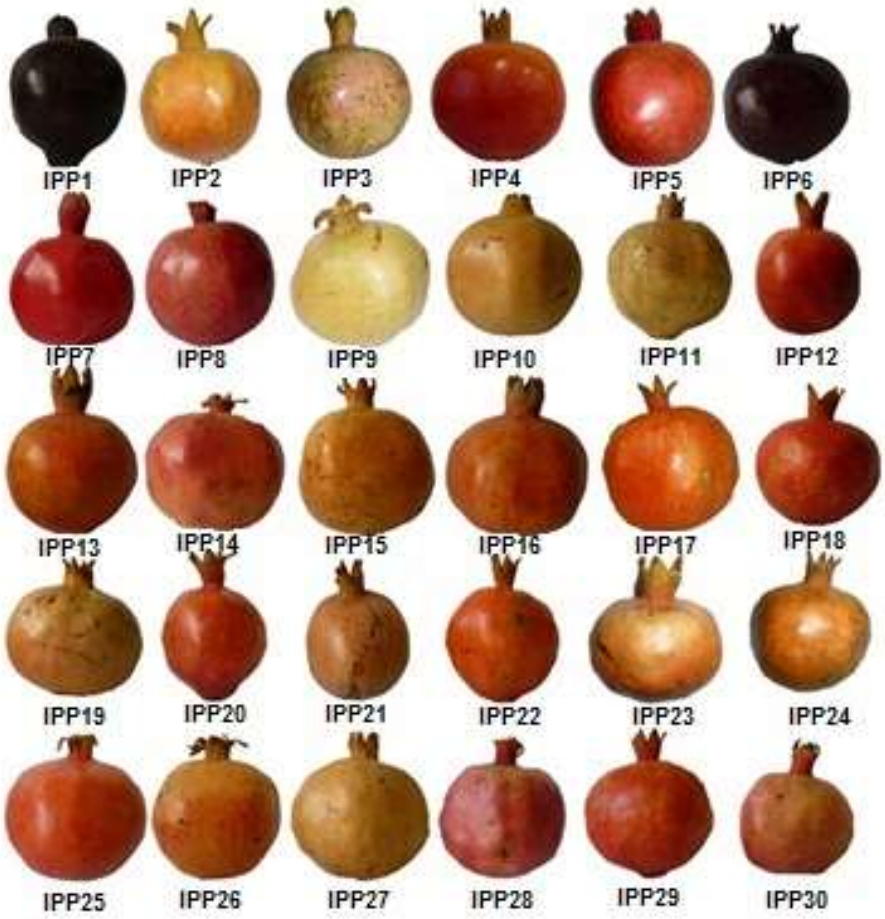

Fig. 1. Thirty Iranian pomegranate genotypes collected from the Agriculture and Natural Resources Research Center of Yazd

Determination of total flavonoid content (TFC) and total phenolic content (TPC)

The method of Shirazi *et al*. (Shirazi, Khattak, Shukri, & Nasyriq, 2014) was employed to determine TFC. 25 µL of extract (1000 ppm), 125 µL of Folin–Ciocalteu solution, and 100 µL of 7.5% sodium carbonate solution were mixed together and placed in the dark at room temperature. After two hours the absorbance of the samples was read at 760 nm. The results were displayed as mg rutin (RE)/ g DW as a mean of five replicates.

A modified method of Folin–Ciocalteu was used for the assessment of TPC in PPE. (Singleton, Orthofer, & Lamuela-Raventós, 1999). 7.5 µL of sodium nitrite (5%) was added to 25 µL of the extract (1000 ppm). Then 7.5 µL of aluminum chloride solution (10%) and 100 µL of sodium hydroxide solution (4%) were added. After 15 minutes, the absorbance of the solutions was read at 510 nm. The reference standard was gallic acid (GAE), and the results were demonstrated as mg GAE/ g DW for five replicates.

DPPH radical scavenging assay

Antioxidant activity of different PPE was measured with DPPH according to the method reported by Ganesan *et al*. (Ganesan, Kumar, & Rao, 2011). In order to measure the antioxidant activity, 1000 µg/ml solution of gallic acid and peel extract of each genotype was prepared. Five different concentrations of extract and standard were prepared in a 96-well plate and DPPH reagent was added to each one. The plates were placed in the dark at room temperature for 30 minutes. The absorbance of each well was read at 517 nm. The mean value

of three replicate was used for the calculation of EC50 (Effective Concentration of 50%, interpreted as a concentration required for 50% scavenging activity) from the dose-response curve. For positive control, a standard of gallic acid was used.

HPLC-DAD and LC-MS analysis of pomegranate peel extracts

20 µL of each sample was injected into a Waters high-performance liquid chromatography (USA) coupled with a photodiode array (PDA) detector. The separation was carried out with a C_{18} Column (Waters SunFire C_{18} Column, 100Å, 3.5 µm, 4.6 mm×150 mm). Data acquisition was made with a DAD detector in the range from 200 to 700 nm, and analytes were recorded at 258, 280, 360 and 520 nm. A gradient elution at 0.5 mL/min to analyzing phenolic compounds in the standards and examined samples was used. The mobile phases, including water with 0.02 % TFA as eluent A and methanol with 0.02 % TFA as eluent B. The samples were eluted by following the gradient program starting with 98% A and 2% B for 5 min, 50% A and 50% B until 30 min, 0% A and 100% B at 38 min, and finally, 98% A and 2% B until 42 min. The standards used for quantitative analysis were: ellagic acid, punicalagins α and β. A mixture of two standard compounds (punicalagin anomers and ellagic acid). The peak areas were plotted versus ppm concentration, with good correlation coefficients (R^2=0.989 and 0.999). PPE samples were also spiked with standards before injection.

LC-MS was used for the characterization of phenolic compounds in PPE using an Agilent HPLC 1200 system (Agilent, Germany) equipped with ChemStation software. The separation was carried out with a C18 Column (Waters SunFire C18 Column, 100Å, 3.5 µm, 4.6 mm×150 mm). The mobile phases, including water with 0.02 % TFA as eluent A and methanol with 0.02 % TFA as eluent B. The samples were eluted by following the gradient program starting with 98% A and 2% B for 5 min, 50% A and 50% B until 30 min, 0% A and 100% B at 38 min, and finally, 98% A and 2% B until 42 min. 20 µL of each sample was injected .A gradient elution at 0.5 mL/min to analyzing phenolic compounds in the standards and examined samples was used. To acquire mass spectra, the HPLC system was coupled to the mass spectrometer (Finnigan™ LCQ™ DECA ion trap). An electrospray ionization device was used for sample analyses (sheath gas: 40 mL min^{-1}, auxiliary gas: 20 mL min^{-1}, spray voltage: 5 kV, capillary temperature: 150°C, capillary voltage: 15 kV, and tube lens: 30 kV). The Xcalibur 2.0 SR2 software (copyright Thermo Electron Corporation 1998–2006) was used for spectra acquisition and processing.

Statistical analysis

To measure the significance of differences among 30 IPPs regarding individual phenolic acids composition, multiple-range tests and one-way analysis of variance (ANOVA) was utilized. Tukey's HSD (Honestly Significant Difference) test was used to discriminate among the means. SPSS 25.0 for Windows were performed for statistical analyses. Hierarchical clustering analysis (HCA) with heatmap based on complete method was performed using "gplots" package in the R program, respectively.

Results and Discussion

Determination of antioxidant activity, TFC, and TPC

TFC and TPC of thirty IPPs are shown in Table 2. The ethanol extract of the PPE showed TPC ranged between 66.4 and 181.4 mg GAE/ g DW. Also, TFC ranged between 38.5 to 144.1 mg RE/ g DW. Among the analyzed genotypes, peel extracts of IPP24 (172.1 ± 0.6 mg GAE/ g DW, 133.5 ± 2.4 mg RE/ g DW), IPP2 (170.1 ± 1.7 mg GAE/ g DW, 144.1 ± 3.1 mg RE/ g DW) and IPP23 (161.5 ± 0.7 mg GAE/ g DW, 131.6 ± 3.1 mg RE/ g DW) showed the highest TPC and TFC content (Table 2). Our results were higher than (89.7- 179.92 mg GAE/g FW) the genotypes reported by Russo *et al*. They reported total phenolic compounds of different

PPE with different methods of extraction (*Russo et al*., 2018). Li *et al*. reported that TPC in PP was between 205.1 and 261.7 mg GAE/g (Li *et al*., 2006). Although a comparison of the values reported in other studies is difficult because they are related to different genotypes, analytical methods, environmental conditions and maturity stages. Our results are similar to values reported by Young *et al*. (52.9-134.2 mg GAE/g) for American genotypes (Young *et al*., 2017), Diamanti *et al*. (150.6 mg GAE/g) for Greek pomegranates (Diamanti, Igoumenidis, Mourtzinos, Yannakopoulou, & Karathanos, 2017), Ali *et al*. (TPC: 103.2 mg GAE/g and TFC: 132.4 mg RE/g) for methanolic peel extract of pomegranate (Ali, El-Baz, El-Emary, Khan, & Mohamed, 2014), while it is lower than the value measured by Hasnaoui *et al*. (208.3-276.3 mg GAE/g) for PPEs of 12 genotypes grown in Tunisia (Hasnaoui, Wathelet, & Jiménez-Araujo, 2014). Antioxidant capacity has been linked to a reduced risk of developing many chronic diseases, such as cancer, diabetes, obesity, and cardiovascular diseases. An effective concentration that requires to increase the initial DPPH concentration by 50 % is defined as EC50 value and better protection has come from a lower EC50 value. (Konsoula, 2016). DPPH scavenging based antioxidant activity was tested for all PPE and GA was used as a reference antioxidant compound. The results expressed as EC_{50} (µg/ml) for studied PPE are summarized in Table 2. There are significant differences in the antioxidant activity between PPEs. DPPH values ranged between 4.1 and 14.4 µg/ml for PPEs. These are in agreement with previously reported studies in comparison with GA (26.9 µg/ml). Among the analyzed genotypes, peel extracts of IPP23 (4.1 µg/ml) and IPP21 (4.4 µg/ml) showed the highest free radical scavenging activity. Our results showed that the peel extracts of the studied Iranian genotypes show an antioxidant capacity comparable to synthetic antioxidants such as gallic acid. Also, PPEs can be used as an alternative antioxidant to protect food against oxidative degradation. These results are close to that of Panichayupakarananta *et al*. work that reported the antioxidant activity (EC_{50}) for two different varieties from Israel and Italy at 3.1 µg/ml and 3.6 µg/ml, respectively (Panichayupakarananta, Issuriya, Sirikatitham, & Wang, 2010). Masci et al. reported that the antioxidant activity of peel extract of the Chinese genotype was 5.8 µg/ml (Masci et al., 2016). Also, Kazemi *et al*. reported higher yield and antioxidant activity of PPEs, and found 5.5 µg/ml as the highest potency of antioxidants by optimization of a pulsed ultrasound-assisted extraction method (Kazemi, Karim, Mirhosseini, & Hamid, 2016). Our finding differs from Indian pomegranate (16.8 µg/ml) reported by Jag Pal. Differences might be due to growing conditions, harvesting, and region (Pal *et al*., 2017). Also, Okonogi *et al*. (Okonogi, Duangrat, Anuchpreeda, Tachakittirungrod, & Chowwanapoonpohn, 2007) reported that PPE had the highest antioxidant activity (IC_{50} of 3µg/mL) among the other eight fruit peel extracts. Thus, it could be concluded that the difference between our obtained data and literature (in terms of TFC, TPC, and antioxidant activity) might be due to differences in fruit ripening, soil composition, latitude, temperature changes, rainfall, and light of different regions. However, this achievement can be used for breeding purposes of this plant (Parcerisa *et al*., 1995).

The highest antioxidant activity of PPE may occur due to its highest polyphenolic compounds, such as ellagitannins, ellagic acids, and gallic acids. Many studies reported that extracts prepared from PP have a phenolic content of 10–45 fold higher than that found in the pulp. The contents of flavonoids and antioxidants were also higher in PPE than in pulp extract. Also, the results of several studies confirm that the antioxidant property of PPE is more than pulp extract (Ali *et al*., 2014; Hasnaoui *et al*., 2014; Russo *et al*., 2018).

Table 2- Total Phenolic content (TPC), Total Flavonoids Contents (TFC) and DPPH Radical Scavenging Assays of PP from different genotypes

Samples	TPC (mg GAE/g)	TFC (mg RE/g)	DPPH EC_{50} (µg/ml)
IPP1	129.8 ± 1.1	96.4 ± 2.6	6.7 ± 0.2
IPP2	170.1 ± 1.7	144.1 ± 3.1	6.1 ± 0.1
IPP3	112.2 ± 2.4	59.3 ± 3.0	10.8 ± 0.1
IPP4	100.1 ± 2.9	56.4 ± 3.6	10.5 ± 0.1
IPP5	89.8 ± 1.3	61.0 ±2.5	9.5 ± 0.1
IPP6	134.4 ± 1.0	84.8 ± 1.9	4.5 ± 0.1
IPP7	86.47 ± 1.0	40.4 ± 3.3	8.0 ± 0.1
IPP8	134.2 ± 0.7	62.7 ± 3.0	7.2 ± 0.1
IPP9	113.3 ± 1.1	71.6 ±1.9	13.6 ± 0.3
IPP10	181.4 ± 2.9	82.3 ± 3.7	14.4 ± 0.3
IPP11	158.8 ± 2.2	95.4 ± 3.3	10.7 ± 0.2
IPP12	89.9 ± 1.0	85.4 ± 0.6	7.5 ± 0.2
IPP13	107.7 ± 0.8	89.8 ± 3.5	7.4 ± 0.2
IPP14	126.9 ± 0.9	77.3 ± 3.1	9.5 ± 0.2
IPP15	114.4 ± 1.0	91 ± 1.8	7.4 ± 0.2
IPP16	112.4 ± 0.9	79.8 ± 2.5	9.5 ± 0.2
IPP17	66.4 ± 0.9	38.5 ± 2.5	5.6 ± 0.3
IPP18	110.1 ± 0.8	83.5 ± 0.1	6.3 ± 0.2
IPP19	113.1 ± 1.4	75.4 ± 0.6	5.5 ± 0.3
IPP20	122.1 ± 0.7	75.2 ± 1.6	6.3 ± 0.2
IPP21	136.5 ± 0.7	66.0 ± 1.0	4.4 ± 0.3
IPP22	112.8 ± 1.7	69.1 ± 1.9	9.3 ± 0.2
IPP23	161.5 ± 0.7	131.6 ± 3.1	4.1 ± 0.3
IPP24	172.1 ± 0.6	133.5 ± 2.4	5.6 ± 0.3
IPP25	87.2 ± 0.4	40.4 ± 3.1	6.5 ± 0.3
IPP26	94.1 ± 1.4	60.4 ± 0.6	6.4 ± 0.2
IPP27	154.6 ± 1.4	97.9 ± 1.9	5.8 ± 0.2
IPP28	103.0 ± 0.5	54.8 ± 1.8	11.2 ± 0.2
IPP29	117.5 ± 1.1	58.9 ± 3.3	5.4 ± 0.3
IPP30	81.2 ± 1.2	53.9 ± 0.6	4.7 ± 0.3
Gallic acid	-	-	26.9 ± 0.3

Chromatographic profiling of pomegranate peel polyphenolic constituents

HPLC-DAD and LC-MS were used for the quantification and metabolite profiling of active ingredients in PPE from various genotypes. Fig. 2 presents data related to the characteristic peaks of PPE. The HPLC-DAD chromatograms and retention time order and their corresponding mass spectrum in the negative mode of ionization were used for peak annotation. Also, punicalagin α/β and ellagic acid are confirmed by authentic reference material.

Based on LC-MS and HPLC-DAD data, nine phenolic compounds, including ellagitannin α/β (1 and 2), two corresponding pedunculagin I isomers (3 and 4), punicalin (5), ellagic acid derivatives (ellagic acid (6), ellagic acid glucoside (7), ellagic acid deoxyhexoside (8), and ellagic acid pentoside (9) were identified (Fig. S1 & S2). The major compounds in the peel extract were quantified in the examined genotypes (Table 3 and S1). Gullon *et al*. determined the antibacterial activity and polyphenolic profile of PP. The HPLC analysis of PP showed eight phenolic compounds that punicagalin and ellagic acid were the main components (Gullon, Pintado, Pérez-Álvarez, & Viuda-Martos, 2016). Identification and quantification of phenolic compounds in different parts of pomegranate were reported by Fischer *et al*. In their study, based on their HPLC-PAD and ESI/MS^n, they

detected 48 compounds. Among them, ellagitannins, gallotannins, hydroxybenzoic acids, gallagyl esters, hydroxycinnamic acids, and dihydroflavonol were identified (Fischer, Carle, & Kammerer, 2011).

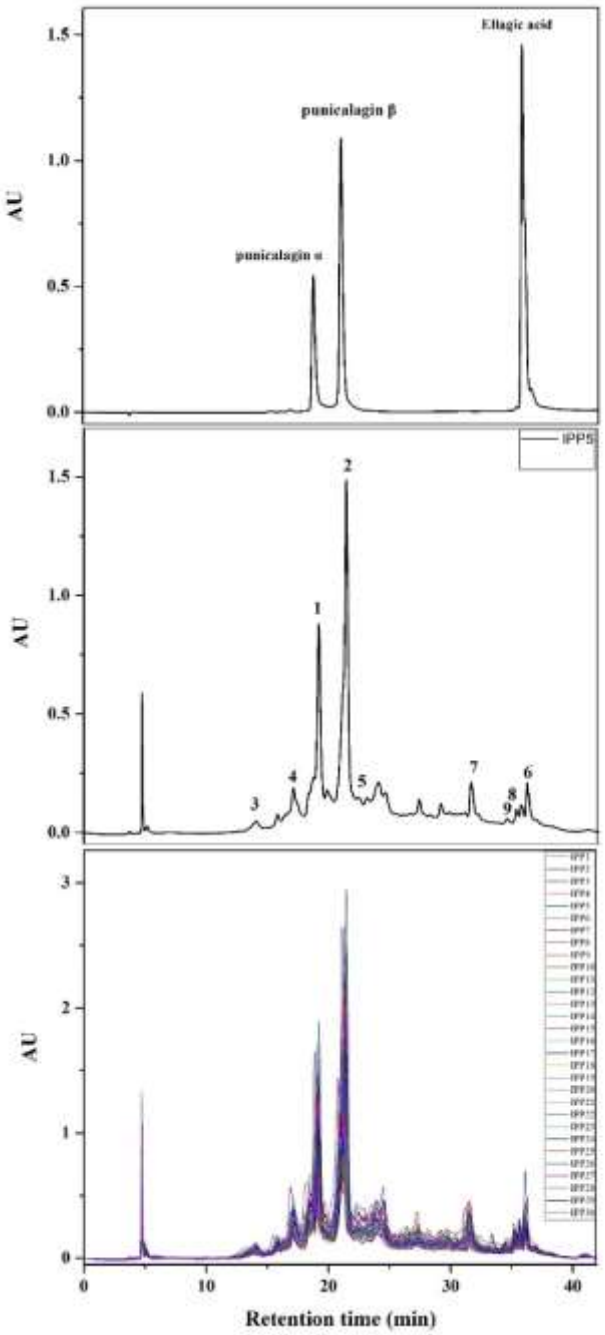

Fig. 2. HPLC-DAD chromatograms of standards and PP extracts in 280 nm

Table 3- Concentration variation of polyphenolic compounds among the peel of thirty studied pomegranate genotypes

Id.	Compound	R_t (min)	λ_{max} (nm)	m/z ([M - H]$^-$)	Concentration (µg/mg dry sample)		
					Range	Average	SD
1	punicalagin α	19.13	258/377	1083	13.9 – 30.1	20.8	4.5
2	punicalagin β	21.30	258/377	1083	20.8 – 48.7	30.6	6.3
3	pedunculagin I isomer	14.04	258/377	783	1.7 – 7.0	3.5	1.3
4	pedunculagin I isomer	18.53	258/377	783	5.3 – 16.9	9.7	2.6
5	Punicalin	22.30	258/377	783	3.4 – 15.7	6.7	3.1
6	Ellagic acid	36.07	254/364	301	1.6 – 13.4	5.9	2.5
7	Ellagic acid glucoside	31.41	254/364	463	2.7 – 20.8	8.8	4.3
8	Ellagic acid deoxyhexoside	35.17	254/364	447	0.7 – 5.1	2.7	1.2
9	Ellagic acid pentoside	34.43	254/364	433	0.1 – 5.9	2.1	1.4

Significant differences were found among genotypes in the studied phenolic compounds ($P<0.01$). Metabolomics as complete metabolite fluctuation found punicalagin β (20.8–48.7 µg/mg DW) followed by punicalagin α (13.9–30.1 µg/mg DW) and ellagic acid (1.6–13.4 µg/mg DW) as main components in PPE samples (Table S1). These results showed that PPE is a rich source of ellagitannins, especially two isomers of punicalagin (α/β). However, the differences with ellagic acid content were extremely high (8-fold). The total content of punicalagins and the content of each isomer was determined by Lu *et al*., and their results showed that the mean value of punicalagin content is 82.4 mg /g for 16 pomegranate genotypes (Lu, Ding, & Yuan, 2008). Our findings also are consistent with the literature (Aqil *et al*., 2012; Kazemi *et al*., 2016; Russo *et al*., 2018).

Similar to our results, many studies have also reported that ellagic acid and its derivatives are spread in PP (Gullon *et al*., 2016; Nuncio-Jáuregui *et al*., 2015; Russo *et al*., 2018). Russo *et al*. reported that about 39.7 to 84.2 percent of the main components of PP samples are ellagitannins, which are found at lower percentages also in pomegranate juice and pulp. More than 70% of PP ellagitannins are related to two anomers of punicalagin (α and β) and two isomers of ellagic acid glucoside, which are very close to our results (Russo *et al*., 2018). The content of punicalagin (116.6 mg/g), ellagic acid, and the other ellagic acid derivatives (4.5 mg/g) in PP extracted by pressurized water were reported by Çam and Hışıl (Çam & Hışıl, 2010). Our results for punicalagin correspond to these results for most genotypes, but the sum of ellagic acid was higher than that of the values reported by Çam and Hışıl. It may be attributable to the low solubility of ellagic acid in water compared to ethanol/water, which is used as an extraction solvent in our study. Peel extract of IPP23 showed the highest amount of polyphenolic compounds, such as punicalagin anomers and ellagic acids, than the other studied pomegranate genotypes .

According to several studies, the biological activities of PPE, such as antibacterial or antioxidant properties, anticarcinogenic and antimutagenic properties, are validated by these bioactive compounds (Al-Zoreky, 2009; Wu, Ma, & Tian, 2013). However, the concentration and type of these valuable components depend on different parameters such as genotype analyzed, environmental conditions, maturity stages, etc.

Hierarchical clustering analysis (HCA) with heatmap

A heatmap is a way to visualize hierarchical clustering where data values are transformed to color scale. Also, heatmaps allow us to simultaneously visualize clusters of samples and measured traits. In the present study of HCA with heatmap, the row tree represents the thirty pomegranate genotypes, the column tree represents the measured phenolic compound, and the colors represent the intensities or values

of the data set (Fig. 3)(Shameh, Alirezalu, Hosseini, & Maleki, 2019)

As shown in Fig. 3, the measured phenolic compounds are divided into three groups. The first group includes punicalin, pedunculagin I isomer 1, and ellagic acid glucoside. Punicalagin α and β are in the second group. The third group includes pedunculagin I isomer 2, ellagic acid, ellagic acid pentoside, and ellagic acid deoxyhexoside. The examined genotypes were divided into four groups. The first group had one accession, IPP23 resulting in clustering analysis presented in Figure 3, demonstrating clear discrimination between IPP23 and different genotypes and falls into a separate group. As mentioned in the previous sections, IPP23 has a higher content of polyphenolic compounds like punicalagin α/β, Ellagic acid glucoside, and other punicalagin derivatives. Also, the amount of punicalin in this genotype is low. The second group consisted of 11 genotypes, IPP1, IPP2, IPP6, IPP10, IPP11, IPP15, IPP19, IPP22, IPP24, IPP26, and IPP27. Among the genotypes of this group, IPP1, IPP2, IPP6, IPP15, IPP24, IPP26, and IPP27 are in the separate subgroup. These genotypes have a high content of polyphenolic compounds such as punicalagin and ellagic acid and their derivatives. The genotypes in another subgroup have similar amounts of punicalin and punicalagin β.

The third group included five genotypes, IPP12, IPP13, IPP21, IPP25, and IPP28. The genotypes of this group have the lowest levels of punicalagin α and β, as shown in Table S1. The two genotypes IPP21 and IPP28, which are in a separate subgroup, have high and the same amounts of punicalin and ellagic acid. The last group consisted of IPP3, IPP4, IPP5, IPP7, IPP8, IPP9, IPP14, IPP16, IPP17, IPP18, IPP20, IPP29, and IPP30. The genotypes in this group have the lowest values of punicalin, punicalagin α/β, pedunculagin I isomer1, ellagic acid, ellagic acid and its derivatives and are in a separate cluster. Genotype IPP18, which is in a separate subgroup, has more punicalin than other genotypes.

The results of these classifications showed that punicalagin α/β, ellagic acid had an important role in grouping and differentiation between 30 genotypes.

Conclusion

Following quantitative analysis of the main phenolic compounds in thirty Iranian pomegranates, significant differences in their amount and ratio were observed. Our results showed that IPPs are important sources of phenolic compounds. Among all pomegranate peels, IPP23 (Kabdar Shirin Behshahr) showed the highest quantity of polyphenolic compounds. Considering the parameters measured in the genotypes, IPP23, which has a high amount of measured phenolic compound, can be introduced as the superior genotype. Also, according to the results of this study, PPE had higher antioxidant properties against DPPH than gallic acid standard. It should be noted that the IPP23 extract showed the most significant free radical scavenging capability among other examined genotypes. Here, as all plants were grown at the same location and under the same agrotechnical approach, we can attribute most of the diversity in secondary metabolites to genetic background, even if mediated by the varied susceptibility to the same environmental influences. Eventually, using PP as a valuable natural substance that may act as supplement, prebiotic, food preservative, food additive, stabilizer, and quality-enhancing agent is a new and practical approach to preventing some chronic diseases. Therefore, based on this study, PP can be recommended as a strong source of antioxidants to stabilize food systems.

Conflict of Interest

Authors has no any conflict of interest.

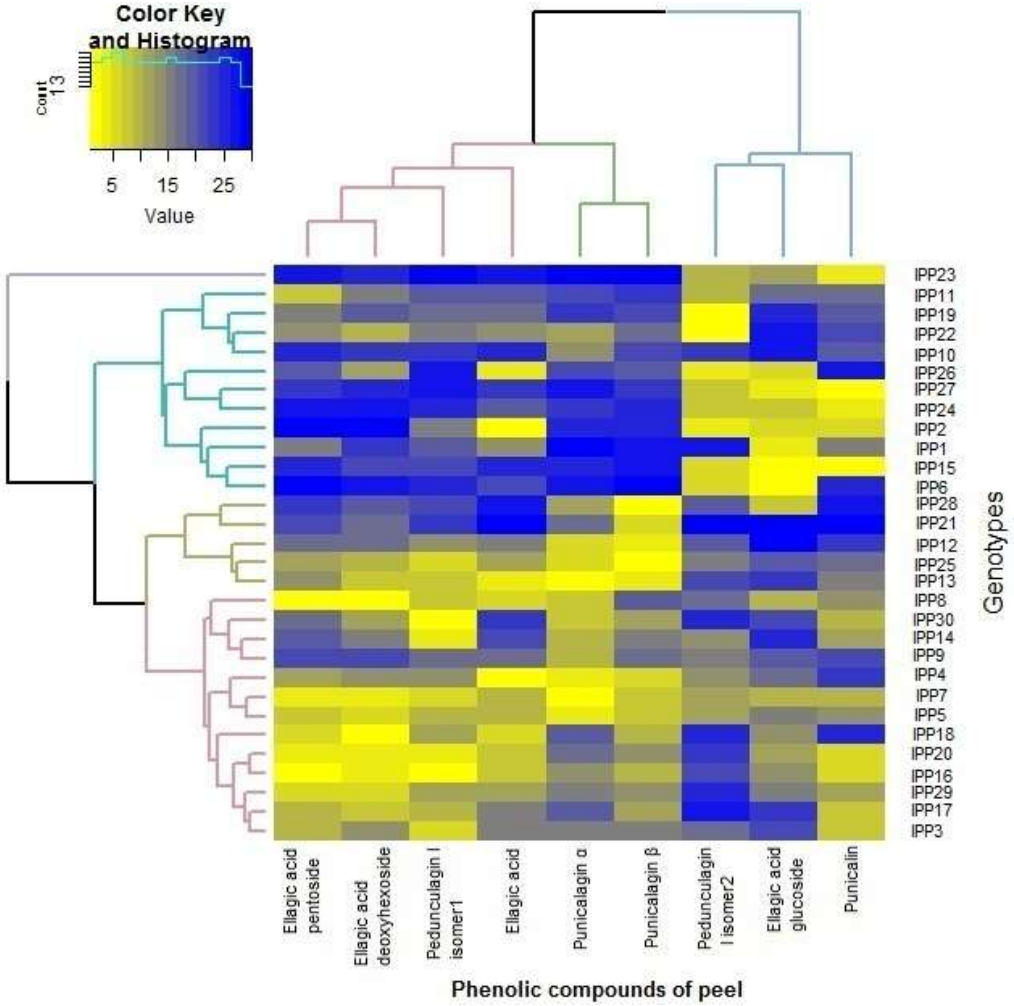

Fig. 3. Hierarchical cluster analysis (HCA) of pomegranate genotypes based on phenolic compounds in peel

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بررسی خصوصیات فیتوشیمیایی پوست ژنوتیپ‌های انار ایرانی و معرفی آن به‌عنوان افزودنی غذایی طبیعی

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چکیده

امروزه، استفاده از افزودنی‌های طبیعی به‌عنوان جایگزینی برای افزودنی‌های سنتزی در صنایع غذایی افزایش یافته است. در این مطالعه به بررسی پتانسیل عصاره پوست انار به‌عنوان یک افزودنی طبیعی غذایی پرداخته شد. پوست انار (*Punica granatum* L.) محصول جانبی میوه انار است که خواص تغذیه‌ای، عملکردی و ضد عفونی کننده‌ای آن در ژنوتیپ‌های مختلف متفاوت است. فعالیت مهار رادیکال آزاد (DPPH)، محتوای فنل و فلاونوئید کل در پوست سی ژنوتیپ انار ایرانی مورد بررسی قرار گرفت. همچنین به‌منظور یافتن تنوع در خصوصیات فیتوشیمیایی پوست ژنوتیپ‌های انار از دستگاه HPLC-DAD-MS استفاده و تعداد نه ترکیب فنلی شناسایی و تعیین مقدار شدند. ترکیبات اصلی پوست انار شامل پونیکالاژین β (۴۸/۷-۲۰/۴۸ میکروگرم بر میلی‌گرم)، پونیکالاژین α (۳۰/۱-۱۳/۹ میکروگرم بر میلی‌گرم) و الاژیک اسید (۱۳/۴-۱/۶ میکروگرم بر میلی‌گرم) می‌باشند. ژنوتیپ IPP23 (کابدار شیرین بهشهر) بیشترین مقدار ترکیبات فنلی در بین سایر ژنوتیپ‌ها را دارد. میزان ترکیبات فنلی کل (۱۸۱/۱- ۶۶/۴ میلی‌گرم گالیک اسید به ازای یک گرم پودر خشک گیاه)، ترکیبات فلاونوئیدی کل (۱۴۴/۱-۳۸/۵ میلی‌گرم روتین به ازای یک گرم پودر خشک گیاه) و خاصیت آنتی‌اکسیدانی عصاره‌های مختلف (۱۳/۹-۳/۸ (میکروگرم در میلی‌لیتر) تعیین شدند. نتایج نشان می‌دهد که خاصیت آنتی‌اکسیدانی عصاره‌های پوست انار به‌طور قابل توجهی بالاتر از استاندارد گالیک اسید است. در نهایت در بین ژنوتیپ‌های مورد بررسی، ژنوتیپ IPP23 به‌عنوان منبع مهم افزودنی‌های طبیعی تعیین شد.

واژه‌های کلیدی: آنتی‌اکسیدان، پلی‌فنل، فیتوشیمیایی، میوه انار، نگهدارنده مواد غذایی

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Effects of *Lactococcus lactis* (*L. lactis*) subsp. *lactis* Supernatant on the Shelf Life of Vacuum-packaged *Oncorhynchus mykiss* Fillets

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Abstract

The present investigation was done to study the effects of *Lactococcus lactis* (*L. lactis*) subsp. *lactis* on the shelf life of the vacuum-packaged *Oncorhynchus mykiss*. Fish fillets were prepared and divided into 5 different treatment groups including control (distilled water), 2% and 4% supernatant, and 10^6 CFU/g *L. lactis* subspecies *lactis*. The pH, Thiobarbituric Acid Reactive Substances (TBARS), Total volatile Nitrogen (TVN), and Peroxide Value (PV) of the fillets were determined on days 0, 5, 10, and 15 while maintained at 4˚C. Protein expression and destruction were analyzed using the SDS-PAGE. The organoleptic assessment was done using five expert sensory panelists. Contents of TBARS, TVN, pH, and PV were increased throughout the storage period (P <0.05). An increase in the concentration of supernatant caused a significant decrease in the content of TBARS, TVN, pH, and PV (P <0.05). The highest and lowest contents of TBARS, TVN, pH and PV on 15th day were belonged to the control (3.367±0.04 mg MDA/kg) and pure bacteria (0.70±0.02 mg MDA/kg), control (87.20±6.40 mg/100g) and 4% supernatant (40.79±0.61 mg/100g), pure bacteria (6.23±0.04) and 4% supernatant (5.44±0.07) and control (12.22±0.01 meq/kg) and 4% supernatant (3.08±0.06 meq/kg) groups, respectively. Protein destruction was lower in the fillet samples treated with pure bacteria and 4% supernatant. The highest scores of the odor, flavor, texture, and color were obtained for fillets treated with 4% supernatant, pure bacteria, pure bacteria, and 4% supernatant and pure bacteria, respectively. The results revealed that treating *O. mykiss* fillets with 4% supernatant and 10^6 CFU/g of pure *L. lactis* subsp. *lactis* can extend the shelf life of *O. mykiss* fillets.

Keywords: Fish, *L. lactis* subsp. *lactis*, *Oncorhynchus mykiss*, Shelf life, Vacuum package

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Introduction

It is well-known that fish is an important source of high-quality animal proteins for human nutrition. Furthermore, fish is a rich source of protein, fatty acids, vitamins, and minerals (Larsen *et al*., 2011). *O. mykiss* or Rainbow trout is one of the most extensively cultured and traded fish in Iran and many other countries of the world. Besides its nutritional value, the large and stable production quantity of rainbow trout makes it an important fish species for the seafood market. It is one of the major sources of protein, minerals, vitamins, and ω-3 long-chain polyunsaturated fatty acids (LC-PUFAs) including eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) which have been demonstrated to have valuable and special health effects to inhibit cardiovascular disease, lower cholesterol levels, and blood viscosity, and reinforce memory and thinking ability for humans (Larsen *et al*., 2011). However, *O. mykiss* fillets are very prone to oxidation and rancidity. The oxidation reactions can cause alterations in the texture, color, and nutritional value of the final product (Chytiri *et al.*, 2004; Fuentes-Amaya *et al.*, 2015). Degradation of polyunsaturated fatty acids (PUFAs) caused by self-acting or enzymatic oxidation throughout different storage circumstances and processing operations can simply result in the development of undesirable oxidation products such as hydroperoxides, peroxides, aldehydes, conjugated dienes/trienes, ketones, and others (Chytiri *et al.*, 2004; Fuentes-Amaya *et al.*, 2015). Additionally, fish are very susceptible to microbial spoilage. The high water activity (a_W) and the presence of corrosive tissues and proteins are the main factors that make it susceptible to chemical and microbial spoilage (Chytiri *et al.*, 2004; Fuentes-Amaya *et al.*, 2015). Therefore, microbiological, enzymatic, and chemical decomposition analyses are used to determine the shelf life of fish and marine products during processing and storage (Chytiri *et al.*, 2004; Fuentes-Amaya *et al.*, 2015).

The competence of numerous techniques of seafood preservation such as low temperature, icing, and suitable packaging such as vacuum packaging, before the addition of synthetic or natural antioxidants, have been applied to monitor the progress of undesirable chemical, oxidative, enzymatic, and microbial changes in the product to increase its shelf-life (Gelman *et al.*, 2001; Giuffrida *et al.*, 2017). Vacuum packing is a technique to interrupt the microbial and chemical spoilage of fish. Vacuum packaging can be defined as the packaging of a product in a high-barrier package from which air is removed to avoid the growth of aerobic spoilage microorganisms, shrinkage, oxidation, and color deterioration (DeWitt & Oliveira, 2016; Özpolat *et al.*, 2014).

Novel research showed that the application of suitable packaging and using appropriate probiotic bacteria especially lactic acid bacteria (LAB) are efficient ways to prevent microbial and chemical spoilage of seafood products. LAB have long been used for changing the aromatic and textural properties of foods and for extending the shelf-life of various products such as milk, meat, poultry, fish, fruits, vegetables, and cereals. In most cases, the production of lactic and acetic acids and the resulting pH decrease are considered responsible for the inhibition of microbial and chemical spoilage (Chowdhury *et al.*, 2016; Nath *et al*., 2014).

LAB are gram-positive microorganisms safely used in the food industry. Certain LAB strains possess probiotic properties for human and animal health (Sałański *et al*. 2022; Fijan, 2014)

Krishnamoorthi *et al.* (2022) isolated bacteriocins producing *Lactococcus lactis* strain CH_3 and reported that bacteriocin has exhibited high antimicrobial, antibiofilm, and DPPH radical scavenging effects.

LAB can produce various antimicrobials including lactic acid, acetic acid, carbon dioxide, hydrogen peroxide, and bacteriocins that can inhibit spoilage and pathogenic organisms leading to extending shelf-life and enhancing the safety of food (Amor *et al.*, 2006) Despite the high importance of prevention from the microbial and chemical spoilage of fish

fillets, there were no previously published data about the application of LAB as an improving factor on the shelf life of fish fillets. Therefore, the present research was done to study the effects of *Lactobacillus lactis* (*L. lactis*) subsp. *lactis* on the shelf life of vacuum-packaged *O. mykiss* fillets.

Material and Methods

Fish Fillet samples

Forty kilograms of *O. mykiss* with an average weight of 600±5 g was randomly obtained from a Rainbow trout farm in Oshnavieh (Northwest of Iran) and immediately transferred to the National Artemia Research Center, Urmia, Iran in the ice box. Sample preparation was done according to the standard protocol of the Ministry of Health and Medical Education, Iran (Standard No. 1803929). All fishes were decapitated and eviscerated. Fish samples were then washed using sterile water. Washed samples were then filleted (100 g fillets) in a sterile hygienic condition.

Bacterial culture and supernatant extraction

L. lactis subsp. *lactis* (PTCC 11454) was prepared from the Persian Type Culture Collection, Research Organization for Science and Technology (IROST), Iran. *Lactobacillus lactis* (*L. lactis*) subsp. *lactis* was cultivated 24 h in Nutrient Broth (NB, Merck, Germany) and stored at -80°C in 50% glycerol until further experiment. The bacterium was then cultured in De Man Rogosa and Sharpe broth (MRS, Merck, Germany) medium according to Abbaspour *et al.* 2019. The bacterium was cultured again on MRS broth medium to obtain a concentration of 10^6 CFU/ml. The supernatant was obtained by refrigerator centrifuge (6000 rpm for 15 min) according to the method described by Scillinger *et al.* (1989) (Schillinger & Lücke, 1989). The supernatant was then filtered by a cellulose acetate filter (0.2 µl mesh size). The pH of the filtered supernatant was then adjusted to 6.5 using sodium hydroxide to hide the pH antibacterial effects (1N, Merck, Germany). The obtained supernatant concentrate was considered 100%. Distilled water was used to prepare 2% and 4% concentrations of bacterial supernatant (Erkan *et al.*, 2007; Sarika *et al.*, 2012; Shamloofar *et al.*, 2015).

Fish fillets inoculation

Fillets of the *O. mykiss* were immersed in 2% and 4% supernatant and live cells (10^6 CFU/ml) of *L. lactis* subsp. *lactis*. Initially, 100 g of fish fillets were immersed in 2% and 4% supernatant and live cells and maintained for 15 min. Therefore, 4 different treatment groups were studied including fish fillet samples treated with 2% and 4% supernatant, pure bacteria, and also those of the control group. Plastic bags were then used for vacuum packaging according to the method described by Tufail et al., 2011. All samples were packed in nylon bags and vacuumed using Multivac-Germany apparatus at room temperature and stored at 4°C. Samplings were carried out on days 1, 5, 10, and 15 after packaging. All samples were analyzed for chemical characteristics (Thiobarbituric Acid Reactive Substances (TBARS), pH, Peroxide Value (PV), and Total Volatile Nitrogen (TVN)) and sensory properties.

TBARS analysis

Lipid oxidation was monitored by the evaluation of thiobarbituric acid reactive substances (TBARS) according to the procedure described by (Salih *et al.*, 1987). Five g of fish fillet was minced and then mixed with 25 mL of trichloroacetic acid (20%) (Sigma Aldrich, USA) and 20 mL of distilled water and then centrifuging for 10 min with the revolving speed of 8000 rpm, and the filtrate was diluted with ultrapure water to 50 mL. The mixture of 10 mL of diluent and 10 mL of TBA solution was heated in a boiling water bath (95–100 °C) for 15 min to develop a pink color and then cooled with running tap water for 5 min. The absorbance of the cooled supernatant was measured at 532 nm by a spectrophotometer (UV-1800, Instruments of Mfg. Co. Ltd.,

Suzhou, China). The amount of TBARS was expressed as mg of MDA/kg sample.

pH analysis

The pH value was determined for the homogeneous mixtures of fish fillets with distilled water (1:10, w/v), using a digital pH meter (713 pH meter, Metrohm Herisau, Switzerland) as described by Benjakul *et al.*, 2006.

PV analysis

The peroxide value of fish fillets was determined by the method described by Shon and Chin, 2008. Briefly, 5 g of fish fillet was heated in a water bath for 3 min at 60 °C followed by thorough mixing through agitation to dissolve the fat and homogenize the sample after the addition of 30 mL of acetic acid-chloroform solution (3:2 v/v). After filtration 0.5 mL saturated potassium iodide solution was added to the filtrate before transferring it into a burette. The sample was titrated against a standard solution of sodium thiosulfate (25 g/L) using starch solution as an indicator. The peroxide value was calculated and expressed in milliequivalent peroxides per kg (meq/kg) of the fish sample using the following formula:

$PV = (S \times N/W) \times 100$

Whereas S= Volume of titration in mL, N= Normality of the sodium thiosulfate solution, W= Sample weight in kg.

TVN analysis

The total volatile basic nitrogen (TVN) of fish fillet was measured according to the method described by (Malle and Poumeyrol, 1989). Briefly, 10 g fish fillet, 1 g magnesium oxide (Merck, Darmstadt, Germany), and 60 mL distilled water were placed in a distilling flask. Samples were boiled and distilled into 40 mL of boric acid (Merck) containing methyl red as an indicator. After the distillation, the contents of the conical flask were titrated with H_2So_4 (Merck) and TVN was expressed as mg of N per 100 g muscle.

Sensory evaluation

Organoleptic analysis was performed according to the method described by Ndaw *et al.* (2008). The cooking procedure was done using liquid vegetable oil (Oila, Iran) and a constant amount of edible salt (Sepidan, Iran). The fresh samples of fish fillets were cooked at 270 °C for 20 min. The odor, texture, flavor, and color of fish fillet samples were evaluated by five expert panelists famed for the organoleptic properties of fish fillet samples. Three to ten-point scales were achieved by panelists according to Codex guidelines for the sensory evaluation of fish and shellfish in laboratories (Codex, 1999).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

SDS-PAGE test was performed according to the method of Lammli (1970) Tissue denaturation of fish fillet samples was tracked using the SDS-PAGE. SDS-PAGE analysis was done using 5% polyacrylamide stacking gel (Thermofisher Scientific, Germany) and 15% resolving gel according to the method described by (Boulares *et al.*, 2013).

Statistical analysis

Results of chemical and sensory analysis were reported as Mean ± Standard deviation (SD). Data were analyzed with SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA) using the analysis of variance test (ANOVA). The Least Significant Differences (LSD) procedure was used to test for differences between means at the 0.05 significance level.

Results

Table 1 represents the results of the TBARS of the fish fillet samples treated with 2% and 4% supernatant, pure bacteria, and control group during storage conditions. The contents of TBARS of all samples were increased after 15 days of cold storage (P <0.05). The highest and lowest contents of the TBARS in fillet samples on the 15^{th} day were related to the control group (3.367±0.04 mg/kg) and pure bacteria group (0.70±0.02 mg/kg). An increase

in the concentration of supernatant caused a significant decrease in the content of TBARS (P <0.05).

Table 2 represents the content of TVN of the fish fillet samples treated with 2% and 4% supernatant, pure bacteria, and control group during the maintenance period. The contents of TVN were increased during cold storage (P <0.05). The highest and lowest contents of the TVN on day 15 were related to fillet samples of the control group (87.20±6.40 mgN/100g) and 4% supernatant group (40.79±0.61 mgN/100g). An increase in the concentration of supernatant caused a significant decrease in the content of TVN (P <0.05).

Table 3 represents the pH of the fish fillet samples treated with 2% and 4% supernatant, pure bacteria, and control group during the maintenance period. The pH values were increased during cold storage (P <0.05). The highest and lowest pH values at day 15 were related to fillet samples of pure bacteria (6.23±0.04) and 4% supernatant (5.44±0.07). An increase in the concentration of supernatant caused a significant decrease in the pH value (P <0.05).

Table 4 represents the PV of the fish fillet samples treated with 2% and 4% supernatant, pure bacteria, and control group during the maintenance period. We found that the content of PV was increased in the maintenance period (P <0.05). The highest and lowest contents of the PV on day 15 were related to fillet samples of the control group (12.22±0.01 meq/kg) and 4% supernatant group (3.08±0.06 meq/kg). An increase in the concentration of supernatant caused a significant decrease in the content of PV (P <0.05).

Table 5 represents the results of the organoleptic tests for different groups of fish fillet samples at the end of the maintenance period. Sensory evaluators gave the highest scores of the odor, flavor, texture, and color to fish fillet samples treated with 4% supernatant (8.00±0.00), pure bacteria (7.00±0.40), pure bacteria, and 4% supernatant (7.75±0.28 and 7.75±0.28, respectively) and pure bacteria (8.50±0.28), respectively.

Fig. 1 represents the SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples on the first day of the maintenance period. There were no significant differences in the diversity of soluble proteins and their expression. A slight difference of about 80 KD was seen between the expression of proteins of fish fillet samples of the control and other groups. Furthermore, a slight difference was seen about 30 KD between the expression of proteins of fish fillet samples of pure bacteria and other groups. Fig. 2 represents the SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples at day 5 of the maintenance period. Severe differences were seen in the diversity of soluble proteins and their expression in 57 KD and higher than 93 KD bands. The severity of expression soluble proteins or their survival amongst other proteins was higher between studied samples. Fig. 3 represents the SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples on day 10 of the maintenance period. Severe differences were also seen in the diversity of soluble proteins and their expression in 57 KD and higher than 93 KD bands. The severity of expression soluble proteins or their survival amongst other proteins was higher between control and supernatant treatments (2% and 4%). Figure 4 represents the SDS-PAGE electrophoretic pattern of soluble proteins from different treatments of fish fillet samples on day 15 of the maintenance period. Severe differences were also seen in the diversity of soluble proteins and their expression especially in the expression of soluble proteins in the ranges between 57 to 93 KD which represented higher proteolysis.

Table 1- The TBARS of the treated fish fillet samples

Storage period (day)	TBARS (mg/kg)			
	Pure bacteria (10^6 CFU/g)	Supernatant 2%	Supernatant 4%	Control
1	$0.03\pm0/008^{A*d}$	0.015 ± 0.00^{Bc}	0.013 ± 0.00^{Bc}	0.014 ± 0.00^{Bd}
5	$0.052\pm0.00^{Bc**}$	0.330 ± 0.04^{Cc}	0.188 ± 0.05^{Db}	0.914 ± 0.03^{Ac}
10	0.650 ± 0.03^{Cb}	1.520 ± 0.11^{Ab}	1.100 ± 0.09^{Ba}	1.668 ± 0.04^{Ab}
15	0.70 ± 0.02^{Da}	2.770 ± 0.08^{Ba}	0.960 ± 0.08^{Ba}	3.367 ± 0.04^{Aa}

*Dissimilar capital leathers in each row show a significant statistical difference (P <0.05).
**Dissimilar small leathers in each column show significant statistical differences (P <0.05).

Table 2- The TVN of the treated fish fillet samples

Storage period (day)	TVN (mgN/100g)			
	Pure bacteria (10^6 CFU/g)	Supernatant 2%	Supernatant 4%	Control
1	$10.30\pm0.11^{A*d}$	$10.00.\pm0.35^{Ad}$	$8.8\lambda\pm0.31^{Cd}$	9.50 ± 0.50^{Bd}
5	$23.13\pm0.43^{Bc**}$	24.05 ± 0.50^{Bc}	18.86 ± 1.39^{Cc}	42.70 ± 1.05^{Ac}
10	33.50 ± 0.87^{Db}	43.80 ± 0.91^{Cb}	47.25 ± 2.21^{Ba}	55.9 ± 2.10^{Ab}
15	53.25 ± 0.56^{Ca}	$78.6\lambda\pm0.60^{Ba}$	40.79 ± 0.61^{Bb}	87.20 ± 6.40^{Aa}

*Dissimilar capital leathers in each row show significant statistical differences (P <0.05).
**Dissimilar small leathers in each column show significant statistical differences (P <0.05).

Table 3- The pH of the treated fish fillet samples

Storage period (day)	pH			
	Pure bacteria (10^6 CFU/g)	Supernatant 2%	Supernatant 4%	Control
1	$5.36\pm0.13^{A*b}$	4.96 ± 0.07^{Bc}	5.10 ± 0.03^{Bb}	5.10 ± 0.04^{Ba}
5	$6.03\pm0.04^{Aa**}$	5.13 ± 0.03^{Bc}	5.18 ± 0.06^{Bb}	5.26 ± 0.06^{Ba}
10	6.42 ± 0.03^{Ba}	6.72 ± 0.03^{Aa}	5.83 ± 0.03^{Ca}	5.49 ± 0.24^{Ca}
15	6.23 ± 0.04^{Aa}	5.65 ± 0.12^{Bb}	5.44 ± 0.07^{Cb}	5.61 ± 0.16^{Ca}

*Dissimilar capital leathers in each row show significant statistical differences (P <0.05).
**Dissimilar small leathers in each column show significant statistical differences (P <0.05).

Table 4- The PV of the treated fish fillet samples

Storage period (day)	PV (meq/kg)			
	Pure bacteria (10^6 CFU/g)	Supernatant 2%	Supernatant 4%	Control
1	$1.09\pm0.03^{A*c}$	1.17 ± 0.05^{Ac}	0.00	0.00
5	$1.13\pm0.02^{Bc**}$	1.37 ± 0.21^{Bc}	1.08 ± 0.06^{Bc}	3.82 ± 0.13^{Ac}
10	1.53 ± 0.17^{Cb}	3.56 ± 0.13^{Bb}	3.66 ± 0.52^{Ba}	4.20 ± 0.00^{Ab}
15	4.20 ± 0.07^{Da}	7.40 ± 0.11^{Ba}	3.08 ± 0.06^{Cb}	12.22 ± 0.01^{Aa}

*Dissimilar capital leathers in each row show significant statistical differences (P <0.05).
**Dissimilar small leathers in each column show significant statistical differences (P <0.05).

Table 5- The sensory evaluation of the treated fish fillet samples period

Treatments		Organoleptic characters			
		Odor	Flavor	Texture	Color
Pure bacteria (10^6 CFU/g)		$4.25\pm0.25^{B*}$	7.00 ± 0.40^{A}	7.75 ± 0.28^{A}	8.50 ± 0.28^{A}
Supernatant	2%	3.70 ± 0.47^{C}	3.70 ± 0.25^{C}	4.20 ± 0.47^{B}	4.00 ± 0.40^{B}
	4%	8.00 ± 0.00^{A}	6.70 ± 0.25^{B}	7.75 ± 0.25^{A}	8.25 ± 0.25^{A}
Control		3.00 ± 0.00^{C}	3.00 ± 0.00^{C}	3.00 ± 0.00^{C}	3.00 ± 0.00^{C}

*Dissimilar capital leathers in each column show significant statistical differences (P <0.05).

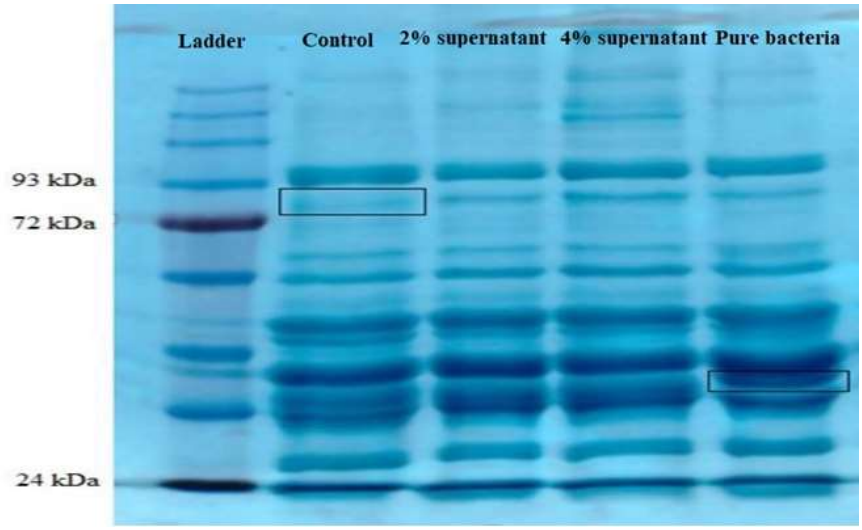

Fig. 1. SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples in the first day of storage period

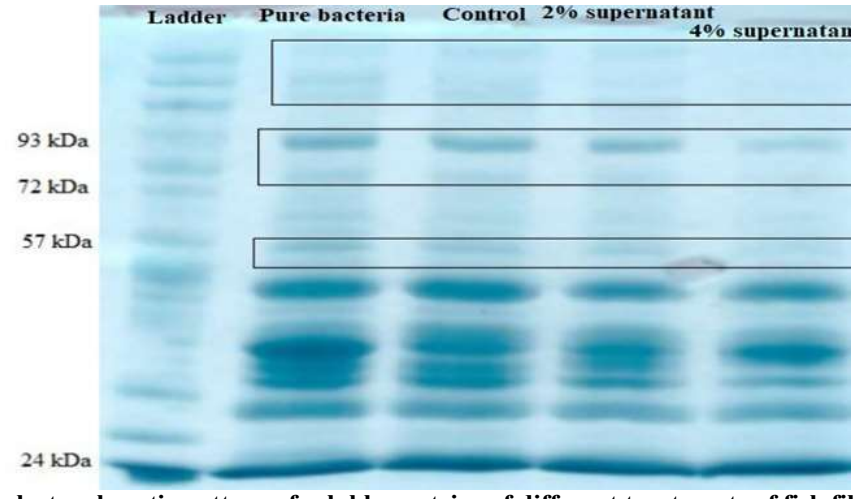

Fig. 2. SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples at day 5 of the storage period

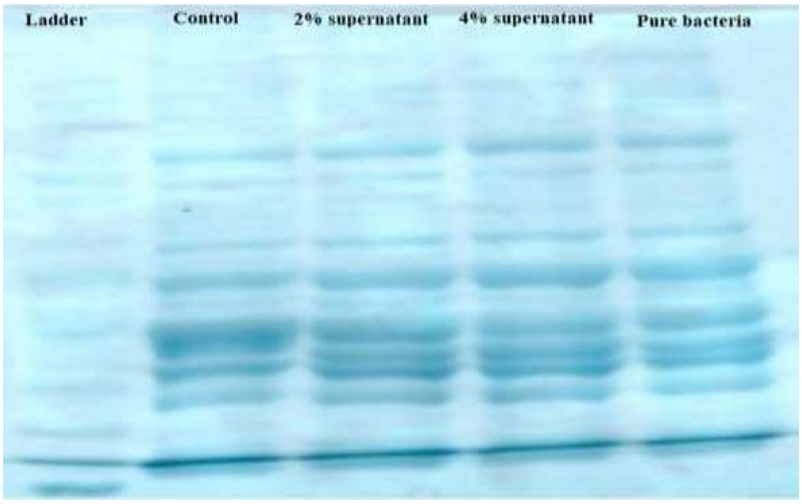

Fig. 3. SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples at day 10 of storage period

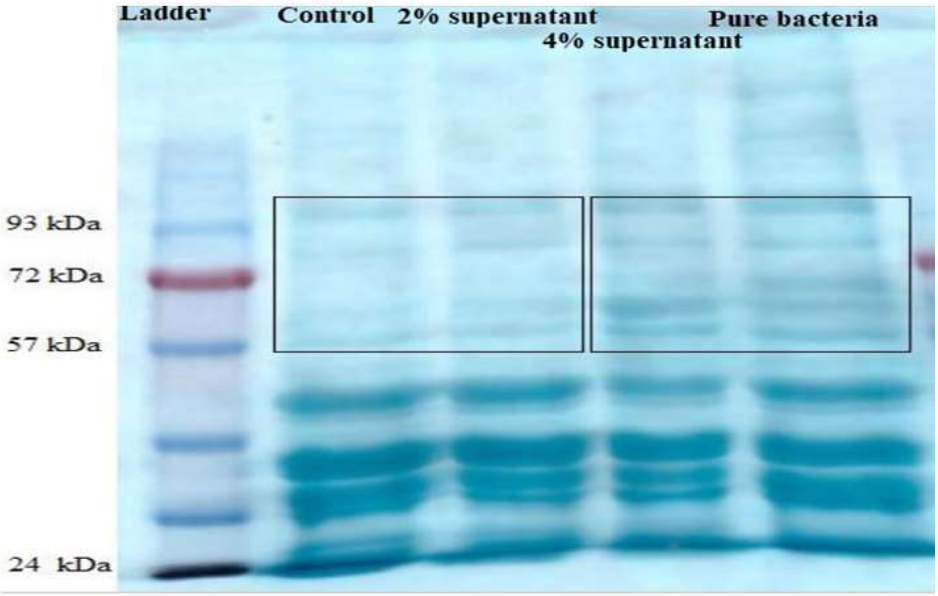

Fig. 4. SDS-PAGE electrophoretic pattern of soluble proteins of different treatments of fish fillet samples at day 15 of the storage period

Discussion

Application of LAB in combination with modified atmosphere packaging and even slight physicochemical treatments and low concentrations of natural traditional preservers may be considered as effective method to increase the shelf life and food safety of fish and marine products through the inhibition of chemical and microbial spoilage without changing the nutritional quality of food products (Pavlićević *et al.*, 2013). The procedure of extension of shelf life by application of natural or controlled microorganisms and their antimicrobial components is recommended by novel scientific reports (Pavlićević *et al.*, 2013).

Abbaspour *et al.* (2018) reported that the number of spoilage bacteria in 4% acid live cells in the storage time of *O. mykiss* fillets was less than human consumption limits (7 logcfu/ml) compared with control and 2% supernatant of *L. lactis* subspecies *lactis* which improved the sensory characteristics of the fillets.

The results revealed that the application of supernatant and pure culture of *L. lactis* subsp. *lactis* caused a significant decrease in the contents of TBARS, TVN, pH, and PV values compared with the control group. Furthermore, the reduction effects were dose-dependent so an increase in the concentration of bacterial supernatant caused a significant decrease in the contents of TBARS, TVN, pH, and PV. Additionally, fillet samples treated with bacterial supernatants and also pure bacteria had lower protein destruction in the SDS-PAGE analysis. Moreover, fillet samples treated with bacterial supernatants and pure bacteria caused an increase in their sensory scores.

The increase in TVN value is attributed to autolytic enzymes and deamination, which lead to the formation of numerous volatile compounds such as dimethylamine, trimethylamine, ammonia, Trimethylamine Oxide (TMAO), hypoxanthine, and non-volatiles histamine, which are shaped by both bacterial and endogenous enzymes. In the treated samples of our study, the values of TVN were lower than in other groups, which may be attributed to the extended lag phase of the spoilage microorganisms as a result of competitive inhibition by LAB and at the same time effect of LAB acidification (Ndaw *et al.*, 2008). Ibrahim & Salha (2009) (Ibrahim & Desouky, 2009) reported that the combined

coating of LAB in tilapia fillets had reduced TVN values. Sudalayandi & Manja (2011) described that out of 7 different species of LAB tested for quality indices reduction, *L. helveticus*, *L. lactis*, and *Pediococcus acidilactici* (*P. acidilactici*) effectively monitored TVN content (Sudalayandi, 2011). Vacuum packaging together with low-temperature preservation is also reported to have the ability to inhibit the growth of spoilage bacteria (Goussault & Leveau, 2006). Similar findings have been reported from Turkey on Bonito fish (Sardasarda) fillets packaged with chitosan film (Alak, 2010).

Oxidative rancidity is one of the most significant factors that effect on the acceptability of the fish throughout storage and processing. As general rule of Standard is that the level of PV in seafood should not exceed 10–20 meq/kg of fat (Lakshmanan, 2000). The control fillet samples of our study harbored the limit of acceptance of PV on day 10 of storage (4.20±0.00 meq/kg). In the presence of *L. lactis* subsp. *lactis* under vacuum, the sample treated with 2% and 4% supernatant and also those treated with pure bacteria harbored the limit of acceptability for PV on day 15 (7.40±0.11, 3.66±0.52 and 4.20±0.07 meq/kg, respectively). Nath *et al.* (2014) reported that the control fillets crossed the limit of acceptance of PV on day 6 of storage (14.11±0.37 meq/kg), while fillet groups treated with *L. sakei* under vacuum packaging crossed the limit of acceptability for PV on day 9 (18.78±0.10 meq/kg). As far as we know, the present research reported the lowest content of PV value in *O. mykiss* fillet samples treated with 4% supernatant of *L. lactis* subsp. *lactis* under vacuum packaging. A probable reason for this finding is the effects of low pH (which occurred by the activity of *L. lactis* subsp. *lactis*) on prevention from the production of PV in fish fillet samples.

We found that the levels of pH in all studied groups have increased during the maintenance period, while the amounts of increase in the fish fillet samples treated with 4% supernatant of the *L. lactis* subsp. *lactis* bacteria were lower than other studied groups. This finding is mainly due to the activity of *L. lactis* subsp. *lactis* and production of acidic products. The pH value should not be above than 4.00-4.50 for soaked fish for products' safety. Acidic conditions make the tissue cathepsins much more active resulting in the degradation of some muscle proteins into peptides and amino acids. These components give the marinade its characteristic flavor and texture. Nath *et al.* (2014) reported that in the presence of *L. sakei* in aerobic or anaerobic (vacuum packaged) conditions all the samples exhibited a lowering of pH values from the initial level of 7.8 which was similar to our findings. Likewise, reductions in pH values were seen in several investigations on marinated anchovies (Sen & Temelli, 2003), sardines (Kilinc & Cakli, 2004), and Pacific saury (Sallam *et al.*, 2007).

TBA test measures malonaldehyde (MDA) produced due to the oxidation of fatty acids with three or more double bonds, and it measures other TBARS such as 2-alkenes and 2, 4-alkadienals. TBA is also usually applied as a quality marker in the fish industry and it has a close association with the sensory properties of fish fillets (Bogdanović *et al.*, 2012). So far, no data have been reported to investigate the effects of *L. lactis* subsp. *lactis* on TBARS content of the vacuum-packaged *O. mykiss* fillets. We found that the fish fillet samples treated with pure *L. lactis* subsp. *lactis* and also those treated with 4% supernatant of *L. lactis* subsp. *lactis* had the lower contents of TBARS. A probable reason for this finding is the effects of low pH (which occurred by the activity of *L. lactis* subsp. *lactis*) on preventing TBARS production in fish fillet samples. Low pH is probably induced by the production of some kinds of acids especially lactic acid, acetic acid, and citric acid due to the activity of *L. lactis* subsp. *lactis* guarantee the higher antioxidant effects which decrease the content of TBARS in fish fillet samples. Furthermore, the lower TBARS values of some treatments might result from the direct microbial utilization of MDA and other TBARS or result from reactions

between TBARS and the amine compounds produced by bacterial metabolism.(Payap & Ommee, 2007). The increase in TBARS values in all treatments is probably due to the induced denaturation of muscle protein, leading to the release of from heme iron, a potential pro-oxidant in the muscle system (Greene & Cumuze, 1981; Payap & Ommee, 2007). Previous research showed that a TBARS value of at least 2.0 mg malonaldehyde/kg is essential for the perception of rancid taste and odors (Greene & Cumuze, 1981). We found that the contents of TBARS in fish fillet samples treated with pure *L. lactis* subsp. *lactis* and also 4% supernatant of *L. lactis* subsp. *lactis* on day 15 of the maintenance period were lower than 1.0 mg malonaldehyde/kg which could indirectly guarantee high scores given to the taste and odor of these treatments.

Sensory evaluation showed that fish fillet samples treated with pure and also 4% supernatant of *L. lactis* subsp. *lactis* harbored the highest scores of odor, flavor, texture, and color. This finding is mainly due to the effects of *L. lactis* subsp. *lactis* on contents of TVN, TBARS, pH, and PV. Similar findings have been reported by (Soltanian *et al.*, 2011; Boulares *et al*., 2013; Chanarat *et al.*, 2014; Adilla *et al.*, 2017 and Rahmatipoor *et al*., 2017).

Conclusion

The present research is the first report of the effects of *L. lactis* subsp. *lactis* on the shelf life of *O. mykiss* fillets. Results showed that using 4% supernatant and also pure *L. lactis* subsp. *lactis* bacteria caused a significant decrease in the contents of TBARS, TVN, pH, and PV values of *O. mykiss* fillets. Contents of the majority of studied chemical parameters were lower than the allowed limit reported by the standard organizations (Tables 1-4). Additionally, levels of protein destruction were studied by the SDS-PAGE in *O. mykiss* fillet samples treated with 4% supernatant and also pure *L. lactis* subsp. *lactis* bacteria were significantly lower than other studied groups. Furthermore, the mean scores given to the odor, flavor, texture, and color parameters were higher in the *O. mykiss* fillet samples treated with 4% supernatant and also pure *L. lactis* subsp. *lactis* bacteria. Therefore, soaking of *O. mykiss* fillet samples on 4% supernatant of *L. lactis* subsp. *lactis* and 10^6 CFU/g of pure bacteria is recommended as efficient method to extend the shelf life of *O. mykiss* fillet samples. However, further studies are required to analyze other chemical, sensory, and microbiological aspects of *O. mykiss* fillet samples treated with other LAB and their metabolites.

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اثرات سوپرناتانت باکتری *Lactococcus lactis* (*L. lactis*) subsp. *lactis* بر ماندگاری فیله قزل‌آلای رنگین‌کمان (*Oncorhynchus mykiss*) بسته‌بندی شده در خلاء

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چکیده

تحقیق حاضر جهت بررسی اثرات *لاکتوباسیلوس لاکتیس* زیرگونه *لاکتیس* بر ماندگاری فیله قزل‌آلای رنگین‌کمان بسته‌بندی شده در خلاء انجام شد. فیله های ماهی تهیه و به ۵ گروه تیمار مختلف شامل کنترل (آب مقطر)، باکتری خالص و ۲ تیمار غوطه‌ورشده در مایع رویی ۲٪ و ۴٪ و $۱۰^{۶}$ *L. lactis* CFU/g زیرگونه *لاکتیس* طبقه‌بندی شدند. مقادیر pH، تیوباربیتوریک اسید (TBARS)، نیتروژن فرار کل (TVN) و مقدار پراکسید (PV) فیله‌ها در روزهای ۰، ۵، ۱۰ و ۱۵ دوره نگهداری در دمای ۴ درجه سانتی‌گراد بررسی شد. بیان و تخریب پروتئین با استفاده از الکتروفورز ژل سدیم دودسیل سولفات-پلی آکریل آمید (SDS-PAGE) آنالیز شد. ارزیابی ارگانولپتیک بااستفاده از پنج ارزیاب حسی خبره انجام شد. مقادیر TBARS، TVN، pH و PV در طول دوره نگهداری افزایش یافته است ($P<0.05$). افزایش غلظت مایع رویی باعث کاهش معنی‌داری در مقادیر TBARS، TVN، pH و PV شد ($P<0.05$). بیشترین و کمترین مقادیر TBARS، TVN، pH و PV در روز ۱۵ به‌ترتیب مربوط به گروه‌های شاهد (۳/۳۶۷±۰/۰۴ میلی‌گرم بر کیلوگرم) و باکتری خالص (۰/۷۰±۰/۰۲ میلی‌گرم بر کیلوگرم)، کنترل (۸۷/۲۰±۶/۴۰ میلی‌گرم بر کیلوگرم) وزن بدن) بود. و ۴٪ مایع رویی (۴۰/۰±۷۹/۶۱ mgN/100g)، باکتری خالص (۶/۲۳±۰/۰۴) و ۴٪ مایع رویی (۵/۴۴±۰/۰۷) و شاهد (۱۲/۰±۲۲/۰۱) meq/kg و ۴٪ مایع رویی (۳/۰۸±۰/۰۶) meq/kg بود. تخریب پروتئین در نمونه‌های فیله تیمار شده با باکتری خالص و ۴ درصد مایع رویی کمتر بود. بالاترین امتیاز بو، طعم، بافت و رنگ به فیله‌های تیمار شده با ۴ درصد مایع رویی (۸/۰۰±۰/۰۰)، باکتری خالص (۷/۰۰±۰/۴۰)، باکتری خالص و ۴ درصد مایع رویی (۷/۷۵±۰/۲۸ و ۷/۷۵±۰/۲۸) و باکتری خالص (۸/۵۰±۰/۲۸) به‌دست آمد. خیساندن فیله‌های *O. mykiss* روی ۴٪ مایع رویی و $۱۰^{۶}$ CFU/g از Subsp. *L. lactis* *لاکتیس* می‌تواند عمر مفید فیله‌های قزل‌آلای رنگین‌کمان را افزایش دهد.

واژه‌های کلیدی: باکتری اسید لاکتیک، بسته‌بندی در خلاء، فیله ماهی، ماندگاری، *Oncorhynchus mykiss*

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Modeling Microbial Population of Coated Sprouted Wheat through Zarrin-Giah Essential Oil in Chitosan Emulsion under Modified Atmosphere Packaging

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Abstract

Due to its health benefits, fresh sprouted cereals are considered popular food source. They are very sensitive and highly susceptible to microbial spoilage during transportation, processing, and storage. This phenomenon makes them potentially high-risk fresh products. This study aimed to assess the effect of emulsion coating consisting of *Dracocephalum kotschyi* essential oil (0, 50, 150, 250, 300 ppm)-chitosan solution (0, 0.3, 0.38, 0.63, 0.75%) during the immersion time (10, 25, 55, 85, 100 s) on the microbial properties of fresh sprouted wheat stored at 4°C. The Response Surface Methodology (RSM) was adopted in modeling the independent variables' effects. The results shown that increase in the essential oil and chitosan solution concentration reduced the microbial spoilage. High concentration of *Dracocephalum kotschyi* oil decreased the fungus population after 12 days. Coating of sprouted wheat at optimized level of independent variables (0.62% chitosan, 57 ppm *Dracocephalum kotschyi* oil and 29.49 s immersion time) reduced the microbial and fungal populations. This treatment can reduce weight loss, and maintain tissue firmness, total phenolic, and ascorbic acid content of the sprouted wheat during cold storage, with no effect on its sensory properties. Our findings indicate that nanoemulsion coating based on chitosan and *Dracocephalum kotschyi* oil at appropriate levels could be beneficial in maintaining sprouted wheat quality and increasing its shelf-life.

Keywords: Badrandjboie-Dennai, Edible coating, Microbiological analysis, Response surface methodology

Abbreviations

RSM	Response Surface Methodology
TPC	Total phenolic content
CCD	Central Composite Design

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Introduction

Food provides components with bioactive properties and various nutrients involved in health improvement and disease prevention (Samtiya *et al*., 2021). Sprouted cereals have been consumed as different types for centuries across the world, especially in Africa and Asia (Marti *et al*., 2018). They are rich sources of proteins, vitamins, minerals and phenols, and bioactive compounds such as glucosinolates, phenolic and selenium all necessary to maintain health (Marton *et al*., 2010). High susceptibility of sprouts to microbial spoilage, due to their high water content (up to 95%) and respiration rate prohibit their long-shelf-life. These features increase the putrefaction and leakage of nutrient rich exudates, tissue damage, and early aging of this product (Turner *et al*., 2020). Different preservation methods like cold storage, chemical immersion, modified atmosphere packaging, and edible coatings are adopted to extend product shelf-life by preventing spoilage, microbial growth and maintaining product freshness. Hurdle technology consists of a combination of preservative methods with synergistic effects on suppressing microbial spoilage, and maintaining nutritional and sensorial properties of perishable products. Combination of edible coating, natural antimicrobial agents like plant essential oil and storage at low temperatures provide the three preventive measures if the shelf-life promotion of the fresh products is sought (Moradi *et al*., 2019; Yavari & Abbasi, 2022).

The edible coating is a thin-layer packaging material consisting of lipids, polysaccharides, proteins, or their combination. Because edible coatings limit gas permeability, they can prevent the oxidative reactions, texture softening, water loss, and microorganisms' proliferation (Benhabiles *et al*., 2013; Eyiz *et al*., 2020). Chitosan is a polysaccharide, obtained by alkaline deacetylation of chitin, with high potential of application as a biodegradable edible coating or film in food packaging (Zhu *et al*., 2008; Saki *et al*., 2019). The degree of deacetylation and the molecular weight of chitosan are highly effective factors on its physicochemical properties, quality, and application. Chitosan-based films and coatings have multi-function positive effects on products (Sridhar *et al*., 2021). Chitosan based edible coating of fresh fruits increase postharvest shelf-life and reduce quality deterioration (Benhabiles *et al*., 2013). Chitosan-based coatings decrease water loss, respiration rate, and microbial contamination of fresh fruits. It has the same effect as modified atmosphere storage in changing the internal gas composition (Petriccione *et al*., 2015).

Incorporating herbal essential oil into edible coatings improves their mechanical, functional, organoleptic, and nutritional features (Rastegar & Atrash, 2021) Essential oils are volatile oily liquid obtained from different plants and applied as food flavorant, antioxidant, antifungal, antiviral, or insecticidal agent (Saki *et al*., 2019; Eftekhari *et al*., 2021). *Dracocephalum* is a genus of flowering plants in the Lamiaceae family with proper source of flavonoids, terpenoids and alkaloids like luteolin, apigenin, oleanolic acid, ursolic acid, geranial, neral, limonene-10-al and rosmarinic acid. *Dracocephalum kotschyi* (locally known as Zarrin-giah or Badrandjboie-Dennaie) contains valuable essential oil that is enriched in different compounds like citral, caryophyllene, terpinyl, acetate, limonene, α-terpineol, δ-3-carene, α-pinene, terpinen-4-ol, geranial, limonene-10-al, 1,1-dimethoxydecane, Gerania, α-pinene), (Heydari *et al*., 2019; Khodaei *et al*., 2018). Phenolic compounds like caffeic acid, chlorogenic acid, phenylpropanoids, and flavonoids in *Dracocephalum* genus contribute to antioxidant and antimicrobial activities. Consumption of *Dracocephalum Kotschyi* essential oil is considered safe (GRAS) for human by the Food and Drug Administration (Heydari *et al*., 2019; Khodaei *et al*., 2018). Food deterioration through spoilage microorganisms during storage has a major

impact on the physicochemical and qualitative properties, and also shelf life of perishables fresh agricultural products (Enyiukwu *et al*., 2020).

The object of this study is to assess a combined preventive approach of chitosan coating incorporated with *Dracocephalum kotschyi* oil as a natural antimicrobial substance to control the microbiological quality and extend the shelf-life of sprouted wheat during cold storage. Response surface methodology is adopted in modeling the changes in microbial population and obtains the best consumption levels of *Dracocephalum kotschyi* essential oil, chitosan and soaking time. The qualitative properties of the treated sample at the best conditions was compared to the control sample.

Materials and methods

Materials

Dracocephalum kotschyi was purchased from Research Center for Medicinal Plant Resources, Isfahan, Iran. The *Dracocephalum kotschyi* essential oil was extracted by steam distillation in Clevenger apparatus (AVZH-CLNGR, Pioneers of Iranian Nanomaterials, IRAN) and dried over anhydrous sodium sulfate and refrigerated at 4 ℃ in dark glass containers. The quantitative and qualitative analyses of *Dracocephalum kotschyi* oil was run by applying gas chromatography coupled to mass spectrometry (GC/MS), (Agilent 6890N, USA), (Martucci *et al*., 2015). The primary components of this oil consist of Perillaldehyde (18.53%), Carvacrol (12.99%), α-Pinene (10.42%), Eugenol (10.11%), E-β Damascenone (8.64%), Geraniol (7.82%), Limonene (7.63%) and Terpinen (5.78%).

The Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-dichloroindophenol, sodium carbonate, trichloroacetic acid and methanol were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Other chemicals were purchased from Merck Co. (Darmstadt, Germany).

Sprouting of wheat seeds and assessment of their qualitative properties

The sprouting process follows the method described by Khaskheli *et al*. (2019), with slight modifications. First, the seeds were soaked for 10 min in a 0.07% sodium hypochlorite sanitizers solution, and were then immersed in distilled water for 6 h. Water was then removed and the seeds were spread on moist filter paper, and finally they were incubated in a growth chamber (Teifazma, TAT-J55, Iran) at 29 ± 2 °C and 85% humidity for 72 h.

The moisture, protein, lipid, carbohydrate, crude fiber, and ash content of sprouted wheat were measured in accordance to the AOAC 945.38 standard method.

The Mg, Mn, K, Ca, Mg, Fe, Zn content of sprouts were determined by applying the atomic spectrometry (Shimadzu AA-6200, Japan).

Preparation of *Dracocephalum kotschyi* oil-chitosan nanoemulsions

To prepare chitosan dispersion, it was weighted (Zurich 4000C, Switzerland) according to Table 1 and added to a beaker containing acetic acid (0.5 %v/v). The solution was heated to 45 °C, mixed on a magnetic stirrer (Heidolph-MR 3001, Germany) at 10,000 rpm for 10 min to complete dissolution and the pH was adjusted to 5.2 with NaOH (Petriccione *et al*., 2015; Maleki *et al*., 2018). The *Dracocephalum kotschyi* oil (0-300ppm), as the oil phase, the Tween 80 (100 ppm) and chitosan dispersions (0-0.75% w/w) in deionized water as the aqueous phase were subjected to sonicatation at 20 kHz (Fisher Scientific, 705 Sonic Dismembrator, USA) to form nanoemulsions (Rashid *et al*., 2020; Kotta *et al*., 2015).

Treating sprouted wheat by applying *Dracocephalum kotschyi* oil-chitosan nanoemulsions

Sprouted wheat was immersed in the *Dracocephalum kotschyi* oil-chitosan nanoemulsions and then dried at 25 ± 1 °C. The samples were packed under modified atmosphere (30% O_2 and 70% N_2) in polyethylene bags, and stored at 4 ±1 °C, 80-

85% relative humidity for 12 days (Maleki *et al*., 2018; Moradi *et al*., 2019).

Microbiological analysis

Total microbial count was determined by applying the tablet colony counting method (standard plate count) according to Yavari & Abbasi (2020). Briefly, 10 g of the sample was immersed in 90 ml saline solution and vortexed (Seward Medical, London, U.K.) for 2 min. The samples were analyzed for total microbial counts by incubation (B Series Incubator, BINDER Inc., USA) at 37 °C for 48 h on plate count agar (PCA). Data was expressed as log colony forming units (CFU)/g sample.

The total yeast and mold count were determined according to Maleki *et al*. procedure. A 20 g sample and 180 ml of 0.1% peptone water were homogenized into the stomacher bags (Seward Medical, London, U.K.) for 1 min. At this stage, the serial decimal dilutions were prepared, and 100 µl of the diluted sample was spread on potato dextrose agar (PDA), and plates were incubated at 25 °C for 5 days.

Experimental design

The central composite design (CCD) in Response Surface Methodology (RSM) was applied to assess the independent variables' effects [chitosan solution concentration 0-0.75% (X_1) and *Dracocephalum kotschyi* oil concentration 0-300 ppm (X_2) in *Dracocephalum kotschyi* oil-chitosan nanoemulsions and immersion time 10-120 s (X_3)] and their interactions on responses. 20 treatments with six central points as shown in Table 1 were developed. The independent variables' function in a second-order polynomial equation is expressed as follows:

Table 1- Effect of independent variables on microbiological qualities **of wheat sprouts**

Run	Variables			Response			
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
1	0.13	50	25	10.22	7.86	11.91	5.70
2	0.13	250	25	10.08	7.77	11.78	0.00
3	0.63	50	25	9.67	7.30	11.51	6.48
4	0.63	250	25	10.02	7.65	11.83	6.40
5	0.13	50	85	10.20	7.77	11.96	6.30
6	0.13	250	85	10.10	7.80	11.90	6.30
7	0.63	50	85	9.70	7.39	11.35	0.00
8	0.63	250	85	10.23	7.93	11.93	6.78
9	0.38	0	55	10.08	7.71	11.81	0.97
10	0.38	300	55	8.30	7.08	11.26	6.30
11	0.00	150	55	10.04	7.80	11.90	6.30
12	0.75	150	55	9.47	7.32	11.29	6.00
13	0.38	150	10	9.87	7.45	11.66	6.60
14	0.38	150	100	10.04	7.78	11.88	6.48
15	0.38	150	55	9.93	7.58	11.70	5.70
16	0.38	150	55	9.90	7.69	11.71	6.00
17	0.38	150	55	9.92	7.66	11.59	5.70
18	0.38	150	55	9.81	7.66	11.65	6.18
19	0.38	150	55	10.01	7.71	11.54	6.18
20	0.38	150	55	10.04	7.55	11.64	5.70

X_1: chitosan solution concentration (%), X_2: *Dracocephalum kotschyi* oil concentration in *Dracocephalum kotschyi* oil-chitosan nanoemulsions (ppm), X_3: immersion time (s), Y_1: microbial count, Y_2: total yeast and mold count, Y_3: microbial count after 12 days, Y_4: total yeast and mold count after 12 days.

$$Y=a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \quad (1)$$

where a_0 is the constant, a_1, a_2 and a_3 are the linear, a_{11}, a_{22} and a_{33} are the quadratic and a_{12}, a_{13} and a_{23} are interactive coefficients. The coefficients of the response surface equation were determined in Design-Expert 7.1.1 software environment (Rezvain *et al.*, 2020; Mazrouei Sebdani & Abbasi, 2023).

Comparison of qualitative features of optimal and control samples

After modeling and optimizing the independent variables' effect on dependent variables change, the optimum and control samples were assessed according to the instructions for quality characteristics such as pH, weight loss (%), ascorbic acid (mg/100g), total phenolic compounds (mg GAE/100g), firmness (N), and total microbial count (log CFU/g) at intervals 0, 4, 8, 12, 16 and 20 days. The sensory assessment was evaluated immediately after production.

pH

The samples' pH was assessed according to Maleki *et al.* (2018) procedure through a pH meter (Metrohm Ltd. Herisau, Switzerland).

Weight loss

The samples' weight loss was determined by weighing the samples on the initial day and after storage time by a digital balance (Zurich 4000C, Switzerland). Weight loss was determined through Eq. (2) (Maleki *et al.*, 2018; Rastegar & Atrash, 2021):

$$\text{Weight loss } (\%)=(W_a-W_b/W_a)100 \quad (2)$$

where W_a is the sample weight on the first day, and W_b is the weight after storage time.

Firmness

The sprouts' firmness was determined by the texture analyzer (Brookfield AMETEK CT3-115 LFRA, USA) equipped with a 39 mm cylindrical probe. The samples were penetrated for 40 mm at a 60 mm s^{-1} speed, and the force volumes were expressed as newton (N), (Maleki *et al.*, 2018; Moradi *et al.*, 2019; Eyiz *et al.*, 2020).

Ascorbic acid

The level of ascorbic acid in the samples was determined based on the titrimetric method by 2,6-dichlorophenolindophenol. First 10g of the sample was blended with 50 ml of 5% w/v trichloroacetic acid, the mixture was then poured into a 100 ml volumetric flask, shaken, filtered, made up to volume and then, 10 mL of solution was titrated against 2,6-dichloroindophenol until the solution color changes into pink for 15 s. The Ascorbic acid content was expressed as mg/100g weight basis of sample (Huang *et al.*, 2017; Eyiz *et al.*, 2020).

Total phenolic content (TPC)

The extraction of phenolic compounds was done according to Rastegar & Atrash (2021) procedure. To prepare the methanol extract, 5.0 g of sprouted sample was homogenized by 15 ml of methanol (80%) and centrifuged (10,000×g) for 15 min. The supernatant was applied in analyzing total phenolic content and antioxidant activity. The TPC was determined according to the colorimetric Folin–Ciocalteau method. First, 0.125 ml of the methanol extract was mixed with 1.5 ml Folin–Ciocalteu reagent (1:10 diluted), and after 5-6 min, 1.25 ml of Na_2CO_3 (7.5% w/v) was added and the mixture was kept at room temperature for 60 min. The absorbance was recorded at 760 nm by UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan). The results were represented as mg gallic acid equivalent (mg 100 g–1of the dry weight), (Nouri & Abbasi, 2018).

Statistical analysis

The obtained data were analyzed in a completely randomized design through SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The means were compared by applying LSD at $p < 0.05$ level. Statistical analysis of sensorial properties was run through Kruskal–Wallis test. All tests were performed at least in three repetitions and Values are presented as means ± SD

Results and Discussion

Proximate compositions and functional compounds of wheat sprouts

Seed germination is a proper process that affects composition and bioavailability of the seeds' content. The concentration of compounds in sprouted wheat are tabulated in Table 2. The high moisture content in the product reduces shelf life and increases spoilage probability. This product is an important resource of protein, dietary fiber, and minerals like potassium, calcium, magnesium, manganese and iron.

The results indicated that the sprouted wheat is a proper source in providing nutritional requirements of human body. According to available records, germination is known as a proper process affecting the composition and bioavailability of seeds components. The sprouted wheat is introduced as a rich source of protein (13.24%), fat (3.89%), total phenolic (3.2 mg GAE/g), minerals and vitamins (Ghavam *et al*., 2021). Sprouted grains and beans like black mung bean are a great source of phenolic acids, antioxidants like vitamins C and E, beta-carotene and flavonoids, including hydroxybenzoic acids, hydroxycinnamic acids and C-glycosidic (Feng *et al*., 2018).

Table 2- Proximate compositions of wheat sprouts

Components	Value
Moisture (%)	47.31±1.2
Protein (%)	11.69±0.93
Lipid (%)	2.50±0.70
Crude fiber (%)	3.49±1.80
Ash (%)	0.50±0.50
Ascorbic acid (mg/100g)	1.72±0.09
Na (mg/kg)	19.99±0.04
K (mg/kg)	6993±0.04
Ca (mg/kg)	79.13±0.90
Mg (mg/kg)	129.40±0.03
Mn (mg/kg)	10.98±1.10
Fe (mg/kg)	10.30±0.22
Zn (mg/kg)	8.92±0.06

All values represent the mean of three replicates expressed as mean±SD.

Microbial analysis

Fresh fruit and vegetables' deterioration is due to spoilage caused by microorganisms. The effect of *Dracocephalum kotschyi* essential oil, chitosan concentrations, and soaking time on total microorganism and yeast and mold count of sprouted wheat after production and at the 12th storage day was assessed. Quadratic models were developed to describe the independent variables' influence on microbial analysis. Soaking Time had no significant effect on total microorganism count. The linear terms of chitosan and *Dracocephalum kotschyi* oil concentration had significant effect on bacterial count after production and at the 12^{th} storage day ($P< 0.0001$). A remarkable decrease was observed in the bacterial population after production at the highest concentration of chitosan, Table 3. The Interaction of independent variables had synergistic effect on the bacterial count reduction, Fig. (1A-C). The contribution of *Dracocephalum kotschyi* essential oil on inhibiting the microorganism growth is due to the existence of compounds like perillaldehyde, carvacrol, limonene, E-β Damascenone, and geraniol with antibacterial properties (Ghavam *et al*., 2021). Ghavam *et al*. found considerable antimicrobial effect of *Dracocephalum kotschyi* oil on different microorganisms like *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Aspergillus niger*, *Shigella dysenteriae*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The antimicrobial action of chitosan is associated with the formation of a

polyelectrolyte composition, because its protonated amine groups selectively bind to the microorganisms' negatively charged cell surface, cause the loss of components and inhibit microbial growth (Pizato *et al*., 2022). Chitosan films with 20% carvacrol (the main terpene of oregano essential oil) reduced the *Pseudomonas fragi*, *Shewanella putrefasciens*, and *Aeromonas hydrophila* growth (Gutierrez-Pacheco *et al*., 2020). The antibacterial activity of chitosan films against *Staphylococcus aureus* and *E. coli* increased after adding 10 $mg \cdot mL^{-1}$ carvacrol (Gutierrez-Pacheco et al., 2020). Chitosan film incorporated with *Thymus piperella* essential oil reduced the *Serratia marcescens* and *Listeria innocua* growth (Gutierrez-Pacheco *et al*., 2020). The coating nanoparticle of chitosan on fresh-cut apples acts as a barrier to moisture and a well-dispersed coating with a greater antimicrobial impact on microorganisms (Pilon *et al*., 2015).

Table 3- Regression coefficients of predicted polynomial models for assessing responses

Coefficient	Responses			
	Y_1	Y_2	Y_3	Y_4
X_0	10.50^{***}	8.00^{***}	12.33^{***}	6.00^{***}
X_1	-0.57^{**}	-1.24^{**}	-1.41^{*}	2.90^{ns}
X_2	$-5.48\times10^{-3***}$	-3.35×10^{-3ns}	$-4.87\times10^{-3**}$	-4.28×10^{-3ns}
X_3	–	$+2.13\times10^{-3*}$	–	-0.042^{ns}
X_{12}	$+5.64\times10^{-3**}$	$+4.75\times10^{-3**}$	$+5.51\times10^{-3**}$	$+0.061^{***}$
X_{13}	–	–	–	$-5.23\times10^{-4***}$
X_{23}	–	–	–	-0.21^{***}
X_{11}	-1.13^{**}	–	–	–
X_{22}	$+1.42\times10^{-5**}$	$+8.39\times10^{-6*}$	$+1.23\times10^{-5**}$	$-1.80\times10^{-4***}$
X_{33}	–	–	–	$+4.23\times10^{-4*}$
Lack of fit	0.487^{ns}	0.204^{ns}	0.126^{ns}	0.127^{ns}
R^2	0.86	0.85	0.81	0.88
CV (%)	0.84	1.06	0.81	7.88

CV: Coefficient of variation
Ns: Not significant ($p > 0.05$).
*: Significant at $p \leq 0.05$.
**: Significant at $p \leq 0.01$.
***: Significant at $p \leq 0.001$.

X_1: Chitosan concentration (%), X_2: *Dracocephalum kotschyi* oil concentration in chitosan (ppm), X_3: Immersion time (s)

Y_1: Microbial count, Y_2: Total yeast and mold count, Y_3: Microbial count after 12 days, Y_4: Total yeast and mold count after 12 days

The sprouted wheats' fungus count was reduced after production by increasing chitosan concentration. A considerable reduction in fungus population was observed at the 12th storage day at highest *Dracocephalum kotschyi* essential oil concentration, Table 3. The variables' interaction at the middle levels had reducing effect on product fungus immediately after production, Fig. (1B). At the highest levels of chitosan concentration and immersion time, fungus population decreased at the 12th storage day, Fig. (1E). The interaction effect of *Dracocephalum kotschyi* oil concentrations with chitosan and immersion time was proper in reducing the fungus population at the 12th storage day, Fig. (1D, F). Edible coatings or films with essential oils can increase the product shelf-life and control the microbial quality of fruits and vegetables. Chitosan with lemon essential oil had a positive effect in reducing the fungal growth on strawberries stored at 5ºC (Perdones *et al*., 2012). The application of pectin with lemon essential oil was efficient in reducing the growth of molds and yeasts in strawberries stored under refrigeration (Pizato *et al*., 2022). Chitosan with and without oregano essential oil had fungicidal activity against *Botrytis cinerea*, *Penicillium sp*., *Rhizopus stolonifer*, and *Alternaria alternata*.

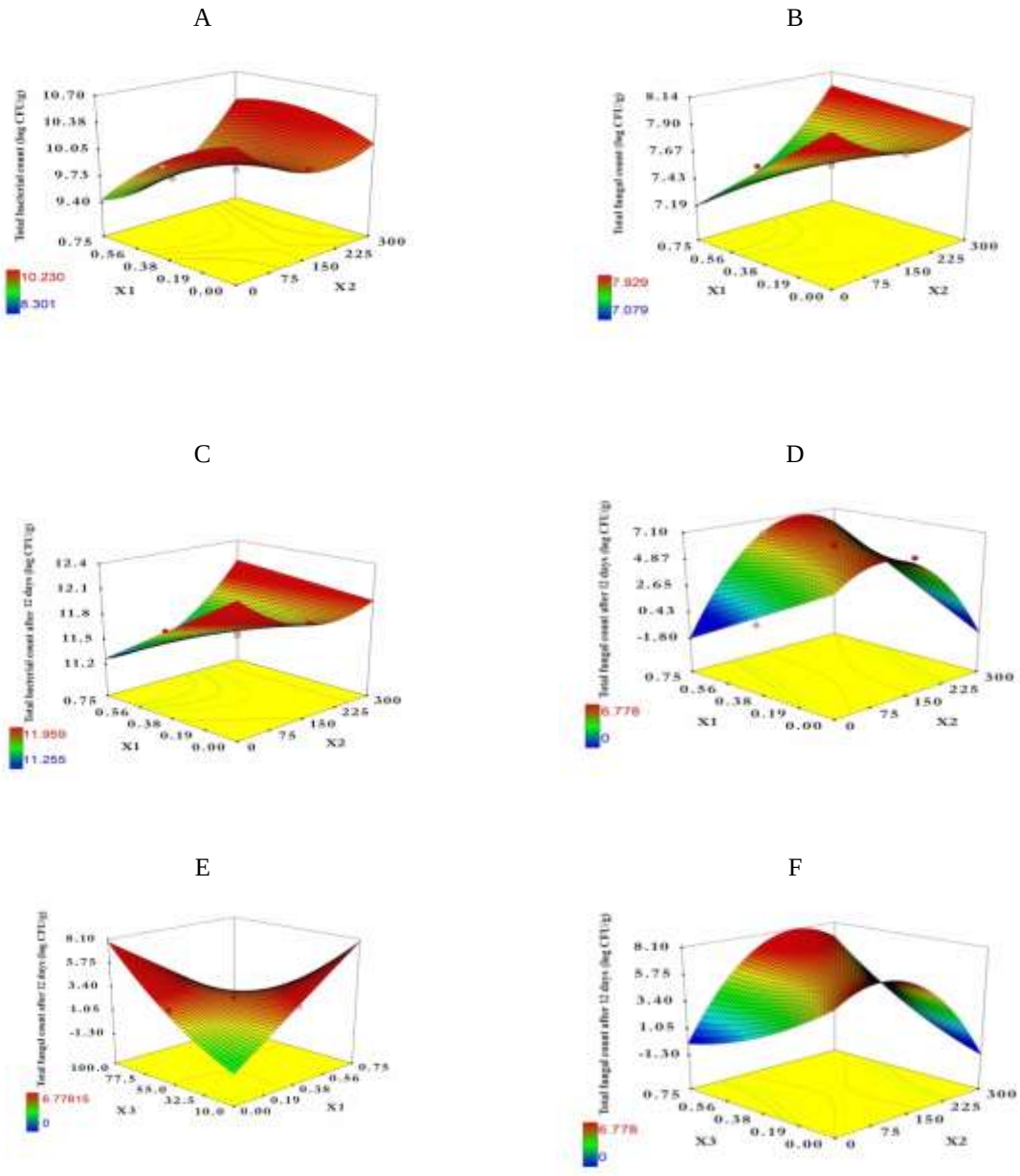

Fig. 1. Response surface plots for the interaction effects of independent variables on the microbial changes of wheat **sprouts**

X_1: Chitosan concentration (%), X_2: *Dracocephalum kotschyi* oil concentration in chitosan (ppm), X_3: Immersion time (s)

Inhibitory effect of chitosan coating on fungal growth follows two mechanisms: 1) chitosan may induce the synthesis of chitinase in plant tissues, which degrades microbial cell walls, and 2) polycationic nature of chitosan may scavenge the major anionic species on the surface of fruits that alter the membrane cell permeability and disorganize molecule with morphological and structural changes in fungal cells (Maleki *et al.*, 2018; Gutierrez-Pacheco et al., 2020).

Molds have an absolute requirement for O_2, and packaging in low O_2 and high CO_2 condition extremely control the fungal growth.

Therefore, the atmospheric change from aerobic to anaerobic situation in coating is not proper for molds and yeasts activity (Maleki *et al*., 2018; Thirupathi Vasuki *et al*., 2023).

The antimicrobial activity of *Dracocephalum kotschyi* oil is due to the effect of different compounds like limonene in disrupting the cell membrane complex of microorganisms (Ghavam *et al*., 2021), and reduce of O_2 diffusion through coating and allows a higher CO_2 concentration surround the products mainly because of the resistance to gas diffusion as a result of the lipophilic nature of *Dracocephalum kotschyi* oil (Moradi *et al*., 2019; Mohammadi *et al*., 2021).

Optimization of sprouted wheat coating through *Dracocephalum kotschyi* oil-chitosan emulsions

The objective of this study was to determine the optimal values of the independent variables in sprouted wheat treatment to improve microbial quality. After modeling, the numerical optimization method was applied to achieve the optimum conditions (minimum microbial and fungal counts) of the product. Coating wheat sprouts with nanoemulsion containing 57 ppm *Dracocephalum kotschyi* oil in 0.62% chitosan solution with 29.49 s immersion time provided the best condition for treating this product and controlling the microbial activity. The predicted results correspond to the experimental results obtained at laboratory validated the RSM models are shown in the Table 4.

Table 4- Validation of RSM models

Responses	Predicted value	Experimental value	Error percentage
Total bacterial count after production (log CFU/g)	9.63	9.54	0.94
Population of fungus after production (log CFU/g)	7.30	7.15	2.09
Total bacterial count at the 12th storage day (log CFU/g)	11.40	11.41	0.08
Population of fungus at the 12th storage day (log CFU/g)	5.69	5.18	9.84

Comparison of qualitative and microbial characteristics of coated wheat sprouts and control during storage

The best level of independent variables was selected to control the sprouted wheat microbial quality. The effect of treating wheat sprouts at optimal conditions during the storage time was assessed on the quality properties like the pH, weight loss, ascorbic acid, total phenol contents, firmness, and total microbial and fungal count of the product.

The coated wheat sprouts had lower pH than the uncoated sample at the beginning of production. During storage, the control pH decreased until the 8th day and then increased. This phenomenon is due to the sprouts spoilage and formation of alkaline autolysis compounds and fungal metabolites (Huang *et al*., 2017; Maleki *et al*., 2018). Changes in the coated sample pH in the storage period were lower than that of the control sample, therefore, the pH of the coated sample was lower than that of the control at the 12th, 16th and 20th of the storage days, Fig. (2A). Changes in pH are associated with 1) the effect of treatments on the respiration rate and 2) metabolic activity that change the biochemical components of the products (Maleki *et al*., 2018). The difference in the loss of water during storage time is other reason of changes in pH (Benhabiles *et al*., 2013). The pH changes of Chinese kiwi fruit have been attributed to the respiration consumption in living cells and oxidation that are responsible of organic acids reduction (Huang *et al*., 2017).

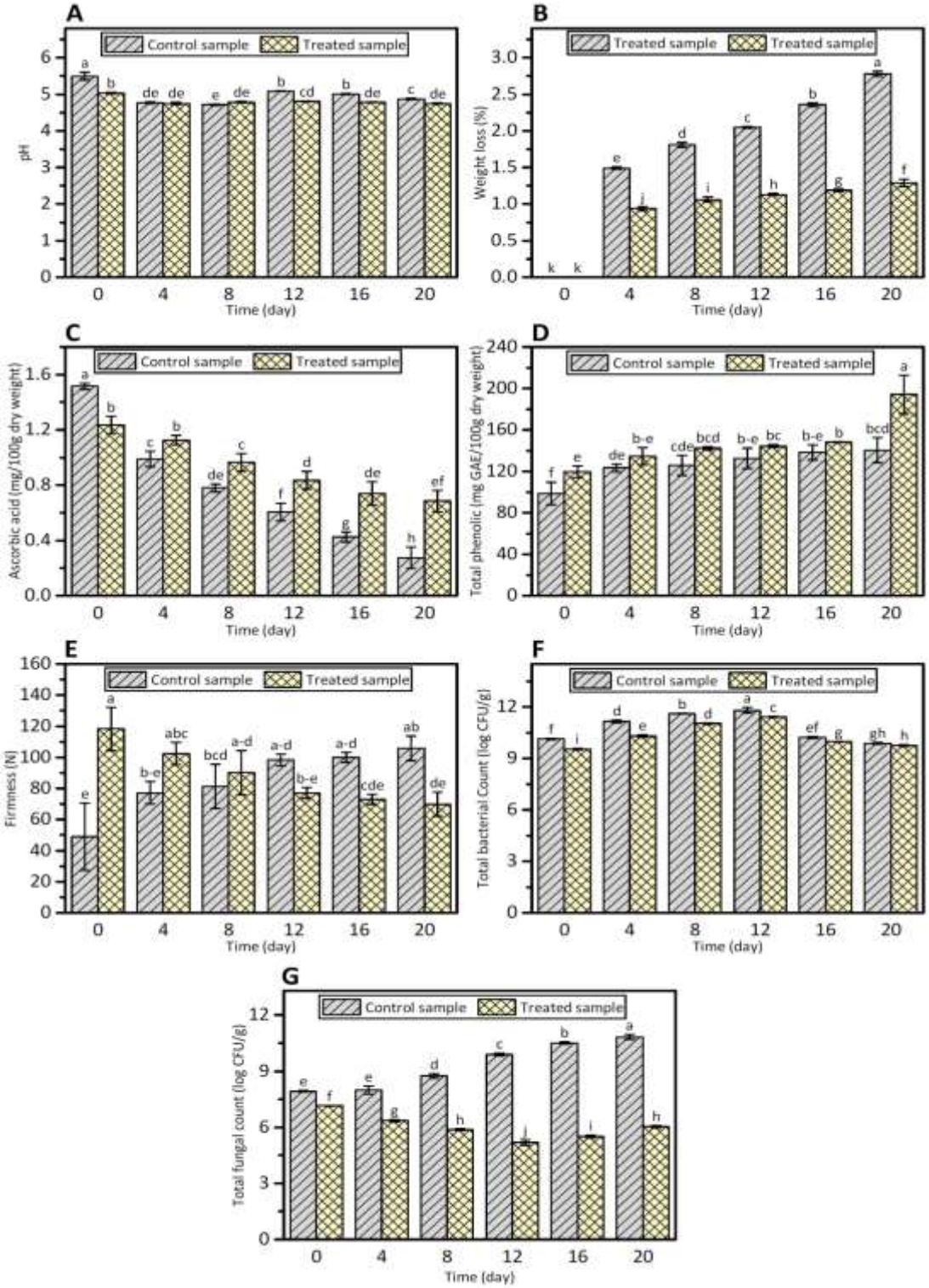

Fig. 2. The qualitative and microbial properties of the coated and uncoated sprouted wheat during storage at 4 ± 1 °C

Product weight loss is one of the most important factors limiting the fresh vegetables and fruits' shelf-life in storage period (Huang *et al*., 2017; Maleki *et al*., 2018). The weight loss of coated sample after 4th, 8th, 16th and 20th days, was lower than the uncoated product, Fig. (2B).

At the last days of product storage (days 12th, 16th, 20th), the weight loss in both the treated and control samples increased considerably. This phenomenon is attributed to transpiration and respiration of product, due to a sharp increase in microbial and fungal growth. Chitosan-based coating reduced respiration and oxidation reactions during storage period by hindering water and gas exchange (Zhu *et al*., 2008). The *Dracocephalum kotschyi* oil incorporated into the chitosan coating as a polysaccharide-based edible coating formed a barrier layer on the surface of the sprouts that reduced the sprouts surface evaporation and respiration rate (Moradi *et al*., 2019). Incorporating of Mentha spicata essential oil as an antioxidant agent into coating of strawberries reduced O_2 diffusion, diminished the oxidative stress, decreased the rate of water loss and consequently reduced fruit senescence (Ghavam *et al*., 2021).

The ascorbic acid content of uncoated sample decreased during storage, while, in the coated sample, did not change until the 4th day.However, ascorbic acid content decreased on 8th, 12th, 16th and 20th storage days. Spontaneous oxidation in the presence of oxygen is the main reason of ascorbic acid deterioration during storage, which was reduced in the treated sample (Moradi *et al*., 2019). Change in the ascorbic acid content in the coated sample was lower than that of the control sample Fig. (2C). Coating materials form a protective membrane between sprouted wheat and its surrounding atmosphere which decrease moisture transfer, O2, and CO2 exchange, and finally prevents the ascorbic acid oxidation. *Dracocephalum kotschyi* oil in chitosan coatings increased their antioxidant activity thus, maintaining the ascorbic acid in the coated product during storage (Moradi *et al*., 2019). Toğrul & Arslan (2004) found that coating tangerines with Carboxymethyl cellulose prevented ascorbic acid loss during storage. Oms-Oliu *et al*. coated pear slices with alginate-containing films and reported that the ascorbic acid content and antioxidant activity remained unchanged at the end of storage period. Bilbao-Sainz *et al*. reported that a layer-by-layer alginate and fungal chitosan based edible coating preserves the ascorbic acid content in fruit bars.

During cold storage, TPC of coated samples was higher than that in the uncoated one. TPC of the coated and uncoated samples in the 20th day of storage followed an ascending trend, Fig. (2D). Edible coatings are effective in reducing oxygen supply for enzymatic oxidation of phenolic compounds by providing a barrier layer to gas exchange on product surface (Moradi *et al*., 2019). *Dracocephalum kotschyi* oil-chitosan emulsion provides a semi-permeable barrier for gas exchange, which lowers the reduction rate in metabolism, oxidative and browning reactions. Preservation of phenolic compounds in sprouts treated with chitosan coating at the end of storage was promoted by reducing polyphenol oxidase activity and actualizing the phenylalanine ammonia lyase (PAL) (Moradi *et al*., 2019). Chitosan, arabic gum and alginate coatings had a positive effect on preserving total phenolics of carambola fruit during storage (Gol *et al*., 2015).

There was a significant difference in firmness of coated and uncoated samples. Firmness in coated sample was higher than the uncoated sample after production and at 4th day ($P< 0.05$). The coating material formed a compact structure and increased the product hardness (Bibao-Sainz *et al*., 2018). At the end of the storage period, firmness of the coated sample was almost similar to the uncoated one upon production. Firmness of the coated sample is due to the activity of texture hydrolyzing enzymes and respiration decreased on 12th, 16th and 20th day, Fig. (2E), while, the firmness of the uncoated sample increased on 8th, 12th, 16th and 20th of storage days. This phenomenon is attributed to more water evaporation and weight loss impact from sprouted wheat surface on increasing firmness. Textural properties of products are one of the primary criterions that limit the shelf-life of fruits and vegetables which are affected by different factors. Water evaporation and weight loss are the major

reason of increasing product firmness. Appropriate coatings with control weight loss can reduce the firmness of product more than control in storage period (Rastegar & Atrash, 2021). Softening is mainly due to cell structure deterioration by pectin hydrolysis in the cortical parenchyma cell wall and middle lamella of fruits and vegetables, This process involves depolymerization and decomposition of the chain length of pectin substances and leads to an increase in pectinesterase and polygalacturonase activities (Moradi *et al*., 2019). Microorganisms' activity is the other factor affecting on the texture changes of agriculture products. Edible coatings based on protein and polysaccharide can limit gas exchanges on fruit and vegetable surface causing a rise in carbon dioxide and a fall in oxygen concentrations that reduce pectinesterase and polygalacturonase activities in the texture of the coated products (Moradi *et al*., 2019). Antimicrobial compounds can inhibit microorganisms' growth and prevent the texture decomposition in fresh product. Carvacrol, limonene, eugenol, geraniol, E-β damascenone, and α-pinene in *Dracocephalum kotschyi* oil have unique antimicrobial properties. The impact of these factors causes texture change in the fresh product during storage.

Edible sprouted wheat has an ample surface area with high moisture content and nutrients, forming a proper environment for microorganisms' growth (Moradi *et al*., 2019). Microbial population, especially fungus increased during storage period, while, the coated sample had lower bacterial and fungus than uncoated Fig. (2 F-G). *Dracocephalum kotschyi* oil and chitosan suppressed microbial spoilage in sprouted wheat not only with their antimicrobial properties but their promotion of decay resistance through preserving the phenolic compounds and ascorbic acid (Saki *et al*., 2019). Applying different types of essential oil can be recommended as a safe method for extending shelf life of fruits and vegetables by controlling fungal decay without any harmful effects on products (Jhalegar *et al*., 2015). In this context, Chitosan-based edible coating containing 0.6% cinnamon inhibit the microorganism growth on fresh-cut potatoes (Wang *et al*., 2011).

In the sensory assessment, no considerable difference was observed between coated and uncoated samples as to odor, flavor, color, texture and overall acceptance ($P > 0.05$), Table 5. The results revealed beneficial effects of this treatment in terms of delay in microbial spoilage without significant changes in the organoleptic properties of the product. In the other study, Guerra *et al*. found that the coated cherry tomatoes with chitosan and peppermint oil had not significant difference in sensorial scores than the control after 24 days of storage. Combination of chitosan and thymol had a positive influence on sensorial properties of fresh fig (*Ficus carica* L.) fruit during 20 days storage at 6 °C (Saki *et al*., 2019). The color of strawberry did not change significantly by coatings with *Aloe vera* gel enriched with basil (*Ocimum basilicum* L.) essential oil, and the glossy surface was the main reason for the high acceptance of the coated fruit (Mohammadi *et al*., 2021).

Conclusion

The *Dracocephalum kotschyi* oil-chitosan nanoemulsions have a positive effect on wheat sprouts' quality by reducing bacterial and fungal growth. The effects of variables' interactions on product quality protection are higher than their independent effects.

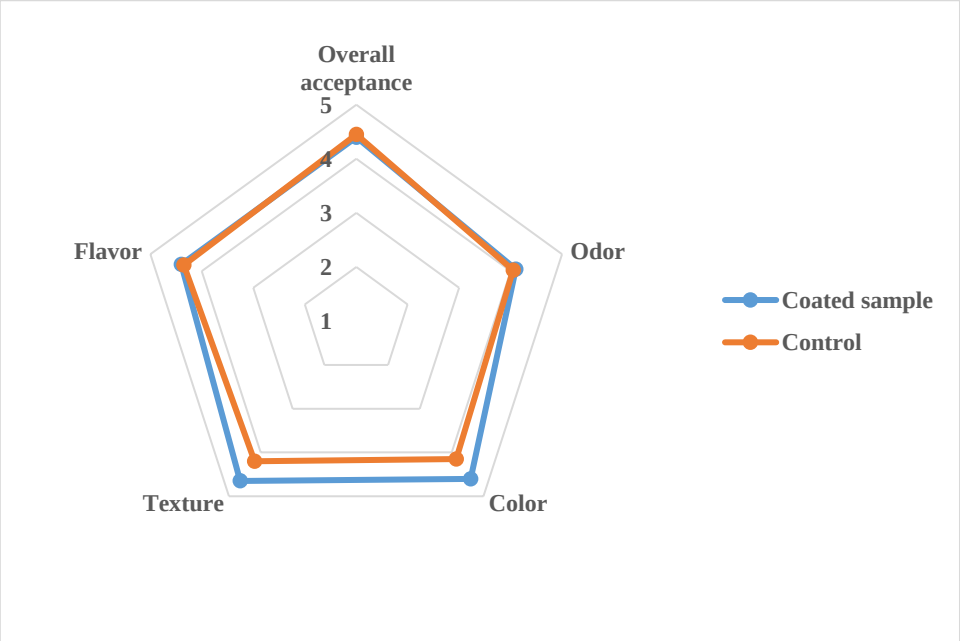

Fig. 3. Effect of edible coating based on chitosan and *Dracocephalum kotschyi* oil on flavor, odor, color, texture and overall acceptability of sprouted wheat stored at 4 °C after 20 days

The best edible coating is formulated at 0.62% chitosan, 57 ppm *Dracocephalum kotschyi* oil and 29.49 s immersion time which extend the product shelf-life. The TPC and ascorbic acid contents of coated sample are higher than that of the control. The treated sprouted wheat has significant low weight loss, and microbial populations than the uncoated sample during storage time. The sensory evaluation of the coated sample received the highest score for overall quality. Application of this emulsion is promising in preserving the safety and quality of sprouted wheat as a valuable and perishable product during cold storage. Despite considerable findings of this article, further studies on combination of new treatments are required to preserve quality of sprouted cereal.

Declarations

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Data availability: The datasets generated and/or analyzed in this study are available from the corresponding author upon request.

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Informed consent: Not applicable

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مدلسازی جمعیت میکروبی جوانه گندم پوشش داده شده با امولسیون حاوی اسانس زرین‌گیاه در کیتوزان تحت بسته‌بندی اتمسفر اصلاح شده

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چکیده

غلات تازه جوانه‌زده به‌دلیل فواید سلامتی بخشی که دارند، منبع غذایی محبوب و پرطرفدار به شمار می‌روند. آنها بسیار حساس بوده و در طول حمل و نقل، فرآوری و نگهداری بسیار مستعد به فساد میکروبی هستند. هدف از این پژوهش بررسی تأثیر پوشش نانوامولسیون حاوی کیتوزان (۰/۷۵-۰ درصد) و اسانس زرین گیاه (۳۰۰-۰ ppm) در زمان غوطه‌وری (۱۰-۱۲۰ ثانیه) بر خواص میکروبی گندم تازه جوانه‌زده طی نگهداری در دمای ۴ درجه سانتی‌گراد می‌باشد. مدلسازی اثر متغیرهای مستقل بر کیفیت محصول توسط روش سطح پاسخ انجام شد. نتایج نشان داد که افزایش غلظت اسانس و محلول کیتوزان باعث کاهش فساد میکروبی محصول می‌شود. غلظت بالای اسانس زرین‌گیاه جمعیت قارچی را پس از ۱۲ روز کاهش داد. پوشش‌دهی گندم جوانه‌زده در سطح بهینه متغیرهای مستقل (۰/۶۲ درصد کیتوزان، ۵۷ ppm، و ۲۹/۴۹ ثانیه زمان غوطه‌وری) موجب کاهش معنی‌دار جمعیت میکروبی و قارچی محصول گردید. این تیمار توانست افت وزن را کم کند و سفتی بافت، محتوی فنول کل و اسید آسکوربیک گندم جوانه‌زده را در طول دوره نگهداری سرد حفظ کند، بدون آنکه تأثیری بر خواص حسی آن داشته باشد. یافته‌های این تحقیق نشان داد که پوشش نانوامولسیونی مبتنی بر کیتوزان و اسانس زرین‌گیاه در سطوح مناسب می‌تواند در حفظ کیفیت و افزایش عمر ماندگاری گندم جوانه زده مفید و مؤثر باشد.

واژه‌های کلیدی: آنالیز میکروبی، بادرنجبویه دنایی، پوشش خوراکی، روش سطح پاسخ

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Evaluation of Physicochemical and Textural Properties of Low-Fat and Low Sodium Imitation Pizza Cheese

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Abstract

The increasing growth of cardiovascular diseases, high blood pressure, and hardening of the vessel walls as well as obesity in many countries has made low-fat and low sodium pizza cheese one of the subjects of study all over the world. The effects of four important independent variables including inulin (0-0.025 %), pre-gelatinized starch (0-0.5 %), NaCl (0.35-1%), and KCl (0.35-1%) were studied. The fat content of imitation pizza cheese was significantly decreased to 11.91% with the increased levels of inulin and starch substitution ($p<0.05$). Also, its moisture and pH values were significantly different ($p < 0.05$). The increased levels of pre-gelatinized starch and inulin reduced hardness (from 5.04 to 3.55) and adhesiveness (from 4368.89% to 1640.54%), however, increased cohesiveness (from 0.365 to 0.43) and springiness (from 0.456 to 0.545). NaCl and KCl increased the hardness of the product. Inulin and starch led to decrease the a* value. The b* value decreases with the increase of inulin and increases with the increase of modified starch. The formulation containing 0.19% inulin, 0.4% pre-gelatinized starch, 0.35% NaCl, and 0.50% KCl was found as the optimal formulation for low-fat imitation cheese. Results of scanning electron microscope (SEM) images revealed that inulin crystals were accumulated in the continuous phase, which this can lead to important changes in the sensory and textural properties. The study concludes that inulin or starch can be used to replace up to 3.6% of fat in the imitation pizza cheese and 0.35% NaCl-0.50% KCl to lower the sodium content of the product.

Keywords: Imitation pizza cheese, Inulin, Optimization, Pre-gelatinized starch

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Introduction

Fat consumption is directly related to various diseases such as obesity, diabetes, hardening of blood vessels and blood pressure. In recent years, the demand for low-fat products has increased dramatically. Hence, the food industry is interested in reducing the fat content and using fat substitutes. In addition, it is important to improve the organoleptic characteristics and shelf life of the products (Shehata *et al.*, 2022). The development of flavor, texture and appearance of cheese is largely influenced by fat. Imitation pizza cheese is a relatively high-fat product, containing 20-27% fat (Bi *et al.*, 2016). Therefore, there is potential for formulating low-fat versions of this product. However, the decrease of fat from cheese leads to defects in texture, yield and taste, such as rubbery texture, lack of taste, bitterness, bad taste, poor meltability and unfavorable color (Żbikowska *et al.*, 2020). It is not easy to produce low-fat cheese with desirable characteristics. The challenge of using fat substitutes in cheese while maintaining the same functional and organoleptic properties of cheeses has attracted a lot of attention (de Souza Paglarini *et al.*, 2021). Fat substitutes are compounds with a protein or carbohydrate structure that can replace all or part of the fat and mimic some of its functional properties. They include starch, inulin, polydextrose and carboxymethyl cellulose. Inulin is a plant storage carbohydrate that has a linear chain of fructose molecules linked by 1-20 β-fructosyl bonds (Ruiz-Moyano *et al.*, 2019). Inulin also acts as a prebiotic, enhancing the probiotic bacteria in the human gut's microbiota and improving immune functions and calcium absorption (Tsatsaragkou *et al.*, 2021). Some of the benefits of prebiotic food include normalizing intestinal movements, helping to maintain intestinal health, reducing cholesterol levels, helping to control blood sugar levels and helping to achieve a healthy weight (Rosa *et al.*, 2021). To date, inulin has been successfully used as a partial fat replacer in fermented sausages (Glisic *et al.*, 2019), yogurt (Żbikowska *et al.*, 2020), soft white cheese (Shehata *et al.*, 2022), rice muffin (Amorim *et al.*, 2021), sponge cake (Krupa-Kozak *et al.*, 2020), biscuits (Tsatsaragkou *et al.*, 2021), and cookie (da Silva & Conti-Silva, 2018).

High amounts of sodium increase the risk of heart attack and high blood pressure. Different types of emulsifying salts such as: phosphate, citrate, tartrate, ammonium or potassium are used in the production of imitation cheese. The problem of high blood pressure caused by high sodium consumption should be reduced by using potassium chloride in food as a substitute for sodium chloride (Ayyash *et al.*, 2011). Replacing sodium chloride with potassium chloride has a significant effect on sensory properties such as bitterness, saltiness, hardness in the process of cheese. The aim of this study was to investigate the physicochemical, textural and sensory characteristics of imitation pizza cheese as a result of replacing potassium chloride with sodium chloride and using different amounts of inulin and modified starch as a fat substitute.

Materials and Methods

Materials

Hydrogenated soybean oil was prepared from Behpak Co. (Behshahr, Iran). Pre-gelatinized starch was obtained from Food Science and Technology Research Institute, ACECR (Mashhad, Iran). Inulin, sodium caseinate (90% protein), and all the solvents used in this research were procured from Sigma-Aldrich (USA) and Merck (Germany) Chemical Companies.

Preparation of low- fat and low sodium imitation pizza cheese

Imitation cheese was prepared using the method described by Hennelly *et al.* (2006) with some modification. Briefly, 52 % w/w water, 14 % w/w hydrogenated soybean oil and 7 % w/w canola oil were mixed at 50 °C. Sodium chloride, potassium chloride, potassium sorbate and sodium phosphate were added to the water and oil mixture and mixing was done at 50 rpm and 80 °C for 1 minute (the

temperature and stirring speed remained constant until the end of the production process). Then 24.5 % w/w sodium caseinate was added to the mixture, and the agitation was continued for about 15 min. After producing a homogeneous mass, pre-gelatinized starch (0-0.5 % w/w) and inulin (0-0.25 % w/w) were added and the mixing process was continued for 2 min. Citric acid (0.5 % w/w) was added and the final mixing was performed for 2 min. The produced imitation pizza cheeses were packaged in polypropylene containers and stored at 4 °C. The Formulation of produced imitation cheese was based on 52 % water, 24.5 % sodium caseinate, 21 % oil, 0.2 % potassium sorbate, 1.3 % sodium chloride, 0.5 % citric acid, and 0.5 % disodium phosphate (without considering independent variables).

Chemical analysis

pH

pH value was measured using a pH-meter (Cole-Parmer, EW-35419-10, USA) on a homogenate cheese/water (1:1) slurry (AOAC, 2000).

Dry matter (DM)

Imitation cheese samples were dried in oven and DM content was measured using the method described by Bermúdez-Aguirre & Barbosa-Cánovas (2012).

Fat

Fat content of prepared imitation cheese was measured using Gerber method (Kleyn *et al.*, 2001).

Salt

Salt measurement was done by potentiometric titration method (AOAC, 2000).

Color analysis

The color properties of prepared imitation cheese samples (L*, a* and b* values) were studied using the method developed by Cunha *et al.* (2010).

Textural analysis

Textural parameters were measured using the method described by Kiziloz *et al.* (2009) with some modification. Briefly, the texture was evaluated by using TA Plus texture analyzer (QTS25, CNS FARANEL, UK) equipped with a computer programmed with NEXYGEN 3 software. A cylindrical probe (35 mm in diameter) was attached to a 5 Kg compression load, while the target value was set at 20 mm with the speed of 100 mm/min. The Samples (15×15×15 mm^3) were placed into cylindrical vessel. The probe was set to penetrate in the samples up to 50% of their initial height. The texture profile analysis (TPA) that used in this study was based on the evaluation of instrumental hardness (the peak force estimated during the first compression cycle), instrumental cohesiveness (the ratio of the positive force area during the second compression to that during the first compression), instrumental adhesiveness (the negative force area of the first compression cycle), and springiness (the height or deformation food that goes back to the previous state during the end of first compression cycle and starting the second cycle).

Determining microstructure

Samples were prepared for SEM by treating with OsO_4 (2%) for 24 h and followed by trimming to a 1-mm in thickness. In order to dissolve lipids, the samples were washed using toluene and followed by drying for overnight. SEM Images were taken from surface of the samples coated with gold at x500 and x300 magnifications by scanning electronic microscopy (SEM) (Cambriidge S360, US) (Marcellino & Benson, 1992).

Sensory evaluation

The sensory properties of the product were evaluated by 10 trained individuals in terms of color, flavor, sweetness, hardness, and overall acceptance on a 5-point hedonic scale. In this test, the excellent sample scored 5, good 4, average 3, bad 2, and very bad 1(Kiziloz *et al.*, 2009).

Statistical analysis

A three level, four variables box behnken design was used to optimize with respect to four independent variables, namely inulin, starch, NaCl, and KCl. Table 1 shows the factors and their levels. The multiple regression equation was employed to fit the second-order polynomial equation based on the observed results as follows:

$$Y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} x_i + \sum_{i=1}^{4} \beta_{kii} x_i^2 + \sum_{i<j=2}^{4} \beta_{kij} x_i x_j$$

Where Y represents the predicted response; $\beta_{k0}, \beta_{ki}, \beta_{kii}$ and β_{kij} represent regression coefficients; and x_i, x_j are the coded independent factors. The models were compared based on R^2, R^2-adj, and R^2-pred. R^2 values closer to 1, indicate that the model is more accurate (Yolmeh & Najafzadeh, 2014). After selecting the most accurate model, the statistical significance of regression coefficients was investigated using the analysis of variance (ANOVA) by Dunkan's test at 95% confidence level. Surface plots were used to study the interactive effects of the independent variables (Yolmeh & Jafari, 2017).

The aim of the optimizing formulation of imitation cheese was to maximize the L*, b*, cohesiveness, springiness, and to minimize fat and adhesiveness with the same weight (w= 1). The validity of the optimum formulation was examined by the desirability values of the responses that ranged from 0 to 1. Values of desirability close to 1 indicate the most desirable and valid optimal formula (Ghorbannezhad *et al.*, 2016).

Results and Discussion

Fitting the response surface models

According to the design used in this study, 30 experiments were carried out and the observed results are shown in Table 1.

The values of R^2, R^2-adj and R^2-pred revealed that 2FI model was more adequate than other models for DM value of prepared imitation cheese samples; however, for fat and L* value, quadratic model was suitable. Cubic model had more accuracy on the other responses of prepared imitation cheese samples (Table 2). Lack-of-fit values of the selected models were insignificant (P>0.05) that shows suitability of the models to predict the responses (Table 3).

The significance of the selected models was evaluated through analysis of variance (ANOVA). A small P-value and a large F-value for each term in the models would show a much effect on the response (Esmaeili *et al.*, 2015). Therefore, quadratic term of NaCl (C^2), linear term of starch (B), (C^2), linear term of NaCl (C), interaction between starch and NaCl (BC), quadratic term of starch (B^2), interaction between quadratic term of inulin and KCl (A^2D), A^2D, A^2D, and C^2 had the most effect on pH, DM, fat, L*, a*, b* values, hardness, cohesiveness, springiness, and adhesiveness of low-fat imitation cheese, respectively (Table 3).

Effects of independent variables on the responses

pH

pH is an important characteristic that affects almost all quality parameters of cheese, including taste, texture, and appearance. So that the structure of cheese largely depends on the physicochemical state of the protein. It also depends on pH and ionic composition (Chatli *et al.*, 2019). Adding of dietary fiber like starch and inulin has a significant effect ($P < 0.05$) on various physicochemical parameters such as dry matter, fat, pH, texture and color (Fig. 1). pH value of low-fat imitation cheese was initially increased by adding starch up to 0.3%, but subsequently decreased to 5.66 (Fig. 1 (f)). On the other hand, the pH was increased to 5.7 by increasing NaCl content (Fig. 1 (a, c)).

Table 1- The formulation and the experimental data for the responses

Formulation	Independent variables						Dependent variables						
	Inulin (A)	Starch (B)	NaCl (C)	KCl (D)	pH	DM (%)	Fat (%)	L*	a*	b*	Hardness (N)	Cohesiveness	Springiness (mm)	Adhesiveness (Nm)
1	0	0	0.675	0.675	5.6	50.3	22	76.7	-2.4	17.2	1.611	0.410	0.5136	906.94
2	0.25	0	0.675	0.675	5.5	56.2	11	73.4	-1.9	19.1	3.204	0.431	0.5311	1486.45
3	0.125	0.25	0.35	0.35	5.7	56.9	12	74.7	-2.2	17.8	3.246	0.430	0.5351	1781.57
4	0.125	0.25	1	1	5.8	54.9	11	73.2	-2.1	19.2	3.40	0.446	0.5544	1287.14
5	0.125	0.25	0.35	1	5.9	58.7	11	72.8	-2.1	18.4	5.033	0.401	0.4905	4207.32
6	0.125	0.25	0.675	0.675	5.8	55.7	13	74.0	-2.1	18.2	5.737	0.434	0.5356	4986.85
7	0.25	0.5	0.675	0.675	5.7	54.9	11	75.2	-2.1	18.7	4.868	0.432	0.5456	2949.05
8	0	0.5	0.675	0.675	5.6	56	11	74.9	-2.2	18.1	4.354	0.419	0.5302	3376.75
9	0.125	0.25	1	0.35	5.7	56.6	5.6	73.4	-1.9	19.0	3.825	0.423	0.5317	2569.21
10	0.125	0.25	0.675	0.675	5.7	54.6	11	75.1	-2.1	18.3	3.777	0.405	0.5065	1551.24
11	0	0.25	0.675	1	5.5	54.6	12	74.7	-2.3	18.4	5.837	0.453	0.5545	3014.23
12	0.25	0.25	0.675	1	5.6	53.9	16	75.2	-2.1	18.5	4.901	0.457	0.5683	2599.84
13	0.125	0	0.35	0.675	5.9	59.1	10	72.2	-2.3	17.5	3.256	0.410	0.5106	1946.66
14	0.25	0.25	0.675	0.35	5.7	57.5	7.2	72.6	-2.5	17.4	4.111	0.400	0.5023	3011.95
15	0	0.25	0.675	0.35	5.9	57.2	12	73.9	-2.1	18.3	6.668	0.365	0.4556	5655.23
16	0.125	0	1	0.675	5.6	49.8	13	74.6	-2.5	18.9	9.495	0.507	0.6337	4051.85
17	0.125	0.5	0.35	0.675	5.8	57.3	7.2	73.2	-2.3	18.1	3.5033	0.411	0.5101	1811.50
18	0.125	0.25	0.675	0.675	5.6	52.1	10	72.7	-2.4	18.3	6.085	0.446	0.5541	2630.29
19	0.125	0.25	0.675	0.675	5.4	53.9	7.2	73.5	-2.4	18.2	7.162	0.443	0.5544	2429.96
20	0.125	0.5	1	0.675	5.6	54.5	12	73.5	-2.4	18.5	4.742	0.478	0.6019	1803.64
21	0	0.25	1	0.675	5.6	52.3	13	72.5	-2.5	18.2	5.689	0.441	0.5472	2616.72
22	0	0.25	0.35	0.675	5.8	52.6	4.8	71.1	-2.3	19.3	5.379	0.464	0.5752	3059.71
23	0.125	0	0.675	1	5.6	53.2	12	74.3	-2.4	18.4	9.960	0.476	0.5956	5958.05
24	0.125	0.25	0.675	0.675	5.9	57.4	6	74.3	-2.2	17.8	2.328	0.381	0.4766	1239.42
25	0.25	0.25	1	0.675	5.7	52.8	9	73.5	-2.6	18.1	4.445	0.448	0.5409	1614.61
26	0.125	0.25	0.675	0.675	5.7	57.8	14	73.5	-2.3	17.4	1.355	0.359	0.4365	693.42
27	0.125	0	0.675	0.35	5.7	58.2	12	73.5	-2.3	18.1	7.439	0.443	0.5571	6153.85
28	0.125	0.5	0.675	0.35	5.7	53.1	6.8	72.6	-2.2	18.5	4.477	0.413	0.5111	1647.70
29	0.125	0.5	0.675	1	5.3	50.5	15	72.1	-2.8	17.2	4.711	0.436	0.5468	1501.29
30	0.25	0.25	0.35	0.675	5.7	52.7	7.2	72.2	-2.5	17.8	5.394	0.401	0.5012	3894.85

Table 2- The statistics of the four fitted models

Models	Statistics	Responses									
		pH	DM	Fat	L*	a*	b*	Hardness	Cohesiveness	Springiness	Adhesiveness
Linear											
	R^2	71.76	46.09	26.74	30.74	40.14	26.69	19.31	19.71	35.24	30.35
	R^2-adj	62.11	35.31	17.26	19.08	28.79	18.46	12.63	12.62	26.73	16.15
	R^2-pred	46.75	31.62	9.02	10.12	10.33	7.68	5.58	4.22	19.27	7.45
2FI											
	R^2	62.21	84.69	42.94	38.51	61.84	42.19	57.57	44.71	47.71	35.61
	R^2-adj	46.12	76.25	37.08	27.98	55.25	36.65	42.76	31.57	39.57	21.08
	R^2-pred	13.80	57.50	23.32	13.55	35.11	24.41	29.61	20.67	11.80	9.24
Quadratic											
	R^2	85.99	71.91	90.15	67.82	67.34	57.32	55.94	27.32	68.03	74.62
	R^2-adj	73.26	59.94	78.64	55.26	58.33	58.32	24.46	19.54	57.38	60.35
	R^2-pred	58.54	34.68	54.67	44.08	25.16	48.25	18.91	9.13	39.26	49.61
Cubic											
	R^2	89.62	69.03	71.83	87.65	88.64	80.32	75.45	68.45	78.48	88.90
	R^2-adj	80.15	46.87	59.46	82.74	73.69	64.31	68.78	59.14	76.94	91.72
	R^2-pred	69.67	22.18	34.08	24.73	61.43	38.87	50.34	40.02	46.31	88.65

pH value of low-fat imitation cheese was initially increased by adding KCl. The pH value was gently increased and then decreased by increasing inulin content. Increasing the initial pH and decreasing the final pH could be related to the nature of inulin and hydrolysis of inulin, respectively (Fig. 1 (d)) (Mensink *et al.*, 2016). The results showed that lowest pH (5.2) and highest pH (5.39) were belonged to full fat cheese and higher concentration of inulin, respectively. Abbasi & Nateghi (2022) reported that the pH of low-fat pizza cheese decreased with increasing the amount of pregelatinized corn starch in the formulation, which could be related to the increase in the acidity of the cheese in these conditions. Shabani *et al.* (2013) showed that the addition of white cheese as a substitute in processed pizza cheese caused a significant decrease in pH. The reason was that white cheese had a lower pH than processed pizza cheese. The pH range of 5.1-6.6 is the best choice for processed cheese, as it promotes protein formation and hydration, emulsifier solubility, and calcium ion confinement. Similar research results show that cheese samples having higher concentration of fiber had less acidity due to increased pH. The pH values of low-fat cheeses were slightly higher than that of full-fat cheese throughout cheese ripening (Karahanal, 2011; Lashkari *et al.*, 2014).

Table 3- Variance analysis of the responses

	pH				DM				Fat				L*			
Source	**DF**	**Mean of squares (MS)**	**F**	**P**	**DF**	**MS**	**F**	**P**	**DF**	**MS**	**F**	**P**	**DF**	**MS**	**F**	**P**
Model	22	0.012	6.23	0.0256	14	5.24	2.72	0.033	14	16.611	2.71	0.040	14	1.63	2.98	0.0283
Inulin (A)	1	0.003	1.57	0.2651	1	0.19	0.48	0.500	1	14.963	2.44	0.142	1	0.34	0.62	0.4466
Starch (B)	1	0.036	18.83	0.0074	1	17.3	9.00	0.008	1	24.083	3.93	0.069	1	0	0	1
NaCl (C)	1	0.036	18.83	0.0074	1	13.67	7.12	0.016	1	10.830	1.77	0.206	1	11.41	20.88	0.0005
KCl (D)	1	0.036	18.83	0.0074	1	9.74	5.07	0.038	1	38.163	6.23	0.026	1	0.6	1.1	0.3126
A^2	1	0.039	20.44	0.0063	1	–	–	–	1	0.017	0.01	0.958	1	0.067	0.12	0.7321
B^2	1	0.06	31.14	0.0025	1	–	–	–	1	4.388	0.71	0.413	1	2.45	4.48	0.0542
C^2	1	0.062	32.21	0.0024	1	–	–	–	1	46.354	7.56	0.016	1	1.22	2.24	0.1584
D^2	1	0.036	18.88	0.0074	1	–	–	–	1	1.7142	0.28	0.606	1	0.01	1.68	0.217
AB	1	0.054	28.31	0.0031	1	2.96	1.54	0.2316	1	30.25	4.93	0.045	1	0.053	0.097	0.7606
AC	1	0.046	24.03	0.0045	1	0.72	0.38	0.5479	1	10.240	1.67	0.219	1	1.03	1.89	0.193
AD	1	0.036	18.95	0.0073	1	2.48	1.29	0.2717	1	19.360	3.16	0.099	1	0	0	1
BC	1	0.037	19.04	0.0073	1	0.008	0.44	0.5181	1	0.810	0.13	0.722	1	0.02	0.39	0.5445
BD	1	0.037	19.15	0.0072	1	3.69	1.92	0.184	1	16.810	2.74	0.121	1	0.98	1.79	0.2034
CD	1	0.062	32.15	0.0024	1	0.044	0.023	0.8814	1	10.240	1.67	0.218	1	2.84	5.2	0.0402
A^2B	1	0.039	20.18	0.0064	–	–	–	–	–	–	–	–	–	–	–	–
A^2C	1	0.04	20.78	0.0061	–	–	–	–	–	–	–	–	–	–	–	–
A^2D	1	0.045	23.22	0.0048	–	–	–	–	–	–	–	–	–	–	–	–
AB^2	1	0.037	19	0.0073	–	–	–	–	–	–	–	–	–	–	–	–
AC^2	1	0.037	19	0.0073	–	–	–	–	–	–	–	–	–	–	–	–
AD^2	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B^2C	1	0.037	19	0.0073	–	–	–	–	–	–	–	–	–	–	–	–
B^2D	1	0.037	19	0.0073	–	–	–	–	–	–	–	–	–	–	–	–
BC^2	1	0.039	19	0.0073	–	–	–	–	–	–	–	–	–	–	–	–
BD^2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C^2D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CD^2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A^3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B^3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Residual error	5	0.002	–	–	17	1.92	–	–	13	6.128	–	–	13	0.55	–	–
Lack of fit	2	0.002	1.14	0.427	14	0.001	0.37	0.914	10	7.017	2.21	0.278	10	0.65	3.47	0.167
Pure error	3	0.002	–	–	3	3.99	–	–	3	3.166	–	–	3	0.19	–	–
Total	29	–	–	–	29	–	–	–	29	–	–	–	29	–	–	–

Table 3- Variance analysis of the responses (Continued)

	a*				b*				Hardness				Cohesiveness			
Source	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P
Model	22	0.024	5.46	0.034	22	0.23	18.24	0.0022	22	1.3	6.73	0.0217	22	0.028	5.1	0.0393
Inulin (A)	1	0.005	1.28	0.31	1	0.260	20.21	0.0064	1	0.04	2.27	0.1921	1	0.057	25.29	0.004
Starch (B)	1	0.065	14.72	0.0122	1	0.76	59.99	0.0006	1	4.71	24.33	0.0044	1	0.280	39.07	0.0015
NaCl (C)	1	0.065	14.72	0.0122	1	0.76	59.99	0.0006	1	0.004	24.33	0.0044	1	0.440	39.07	0.0015
KCl (D)	1	0.065	14.72	0.0122	1	0.76	59.99	0.0006	1	0.004	24.33	0.0044	1	0.440	39.07	0.0015
A^2	1	0.160	35.88	0.0019	1	0.79	62.56	0.0005	1	4.73	24.43	0.0043	1	0.440	47.43	0.001
B^2	1	0.067	15.24	0.0114	1	1.46	116.07	0.0001	1	0.006	32.62	0.0023	1	0.530	39.21	0.0015
C^2	1	0.065	14.72	0.0122	1	0.79	62.75	0.0005	1	0.004	24.33	0.0043	1	0.440	44.45	0.0011
D^2	1	0.200	43.41	0.0012	1	0.850	67.65	0.0004	1	0.005	24.94	0.0041	1	0.500	39.09	0.0015
AB	1	0.067	15.18	0.0115	1	1.12	88.52	0.0002	1	4.96	25.63	0.0039	1	0.440	42.1	0.0013
AC	1	0.073	16.55	0.0096	1	1.27	100.5	0.0002	1	6.18	31.91	0.0024	1	0.470	39.36	0.0015
AD	1	0.076	17.22	0.0089	1	0.93	73.64	0.0004	1	7.8	40.29	0.0014	1	0.440	46.91	0.001
BC	1	0.016	37.22	0.0017	1	0.78	61.54	0.0005	1	0.005	25.68	0.0039	1	0.530	41.22	0.0014
BD	1	0.065	14.72	0.0122	1	0.79	62.28	0.0005	1	4.72	24.36	0.0043	1	0.460	39.55	0.0015
CD	1	0.066	14.86	0.012	1	0.89	70.55	0.0004	1	6.97	35.98	0.0018	1	0.440	39.51	0.0015
A^2B	1	0.066	15.06	0.0116	1	1.11	88.14	0.0002	1	8.09	41.77	0.0013	1	0.440	41.61	0.0013
A^2C	1	0.076	17.27	0.0089	1	1.24	97.93	0.0002	1	6.32	32.64	0.0023	1	0.470	40.06	0.0015
A^2D	1	0.085	19.21	0.0071	1	0.84	66.74	0.0004	1	10.02	51.72	0.0008	1	0.450	62.15	0.0005
AB^2	1	0.066	14.93	0.0118	1	1.04	82.31	0.0003	1	4.72	24.35	0.0043	1	0.700	39.96	0.0015
AC^2	1	0.066	14.93	0.0118	1	1.04	82.31	0.0003	1	4.72	24.35	0.0043	1	0.450	39.96	0.0015
AD^2	0	–	–	–	0	–	–	–	0	–	–	–	0	–	–	–
B^2C	1	0.066	14.93	0.0118	1	1.04	82.31	0.0003	1	4.72	24.35	0.0043	1	0.450	39.96	0.0015
B^2D	1	0.066	14.93	0.0118	1	1.04	82.31	0.0003	1	4.72	24.35	0.0043	1	0.450	39.96	0.0015
BC^2	1	0.066	14.93	0.0118	1	1.04	82.31	0.0003	1	4.72	24.35	0.0043	1	0.450	39.96	0.0015
BD^2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C^2D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CD^2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A^3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B^3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Residual error	5	0.0044	–	–	5	0.013	–	–	5	0.190	–	–	5	0.011	–	–
Lack of fit	2	0.0021	0.36	0.725	2	0.020	4.98	0.111	2	0.056	1.79	0.308	2	0.011	0.99	0.466
Pure error	3	–	–	–	3	0.005	–	–	3	0.150	–	–	3	0.011	–	–
Total	29	–	–	–	29	–	–	–	29	–	–	–	29	–	–	–

Table 3- Variance analysis of the responses (Continued)

	Springiness				Adhesiveness			
Source	**DF**	**MS**	**F**	**P**	**DF**	**MS**	**F**	**P**
Model	22	0.0020	14.15	0.004	22	0.00003	24.37	0.0011
Inulin (A)	1	0.0034	24.56	0.0043	1	0.000002	0.29	0.611
Starch (B)	1	0.0088	63.19	0.0005	1	0.0003	228.48	< 0.0001
NaCl (C)	1	0.0088	63.19	0.0005	1	0.0000002	228.48	< 0.0001
KCl (D)	1	0.0088	63.19	0.0005	1	0.0000002	228.48	< 0.0001
A^2	1	0.0096	68.22	0.0004	1	0.0000002	229.54	< 0.0001
B^2	1	0.0120	85.3	0.0002	1	0.0000003	292.91	< 0.0001
C^2	1	0.0110	81.81	0.0003	1	0.0000003	321.4	< 0.0001
D^2	1	0.0100	72.2	0.0004	1	0.0000002	234.43	< 0.0001
AB	1	0.0100	70.61	0.0004	1	0.0000002	236.45	< 0.0001
AC	1	0.0100	71.12	0.0004	1	0.0000002	229.47	< 0.0001
AD	1	0.0090	64.09	0.0005	1	0.0000002	238.58	< 0.0001
BC	1	0.0120	88.78	0.0002	1	0.0000002	241.99	< 0.0001
BD	1	0.0089	63.33	0.0005	1	0.0000002	255.95	< 0.0001
CD	1	0.0089	63.65	0.0005	1	0.0000002	229.76	< 0.0001
A^2B	1	0.0094	66.96	0.0004	1	0.0000002	–	–
A^2C	1	0.0160	114.48	0.0001	1	0.0000002	–	–
A^2D	1	0.0160	116.44	0.0001	1	0.0000003	–	–
AB^2	1	0.0095	68.05	0.0004	1	0.0000002	–	–
AC^2	1	0.0095	68.05	0.0004	1	0.0000002	–	–
B^2C	1	0.0095	68.05	0.0004	1	0.0000002	–	–
B^2D	1	0.0095	68.05	0.0004	1	0.0000002	–	–
BC^2	1	0.0095	68.05	0.0004	1	0.0000002	–	–
BD^2	–	–	–	–	–	–	–	–
C^2D	–	–	–	–	–	–	–	–
CD^2	–	–	–	–	–	–	–	–
A^3	–	–	–	–	–	–	–	–
B^3	–	–	–	–	–	–	–	–
Residual error	5	0.0007	–	–	5	0.00001	–	–
Lack of fit	2	–	4.16	0.087	2	0.00094	0.84	0.514
Pure error	3	–	–	–	3	0.00001	–	–
Total	29	–	–	–	29	–	–	–

Dry matter (DM)

With the increase of starch, the DM of low-fat imitation cheese decreased from 56.7673 to 54.7% (Fig. 2 (a) and (b)). Moghise *et al.* (2022) reported that DM of Feta cheese was decreased by adding inulin/kefiran to it, which was due to increasing moisture content. However, Rafiei *et al.* (2022) observed that moisture content of Mozzarella cheese was decreased by adding rice starch hydrocolloid to it. de Souza Paglarini *et al.* (2021) shows that cheese samples with dietary fiber and low fat have a higher percentage of dry matter. Abbasi & Nateghi (2022) and Świąder *et al.* (2021) reported similar results in the production of functional low-fat yogurt.

Fat

Fat is responsible for sensory attributes including aroma, texture, and flavor of cheese. Fat affects the cheese texture by filling the interstitial spaces in the protein and mineral matrix (Lashkari *et al.*, 2014). The lowest and highest amount of fat (5.6 % and 22%) was detected in samples containing 0.13 % inulin and without inulin and starch dietary fibers, respectively. As the concentration of starch and sodium chloride increased, the fat content of imitation pizza cheese decreased.

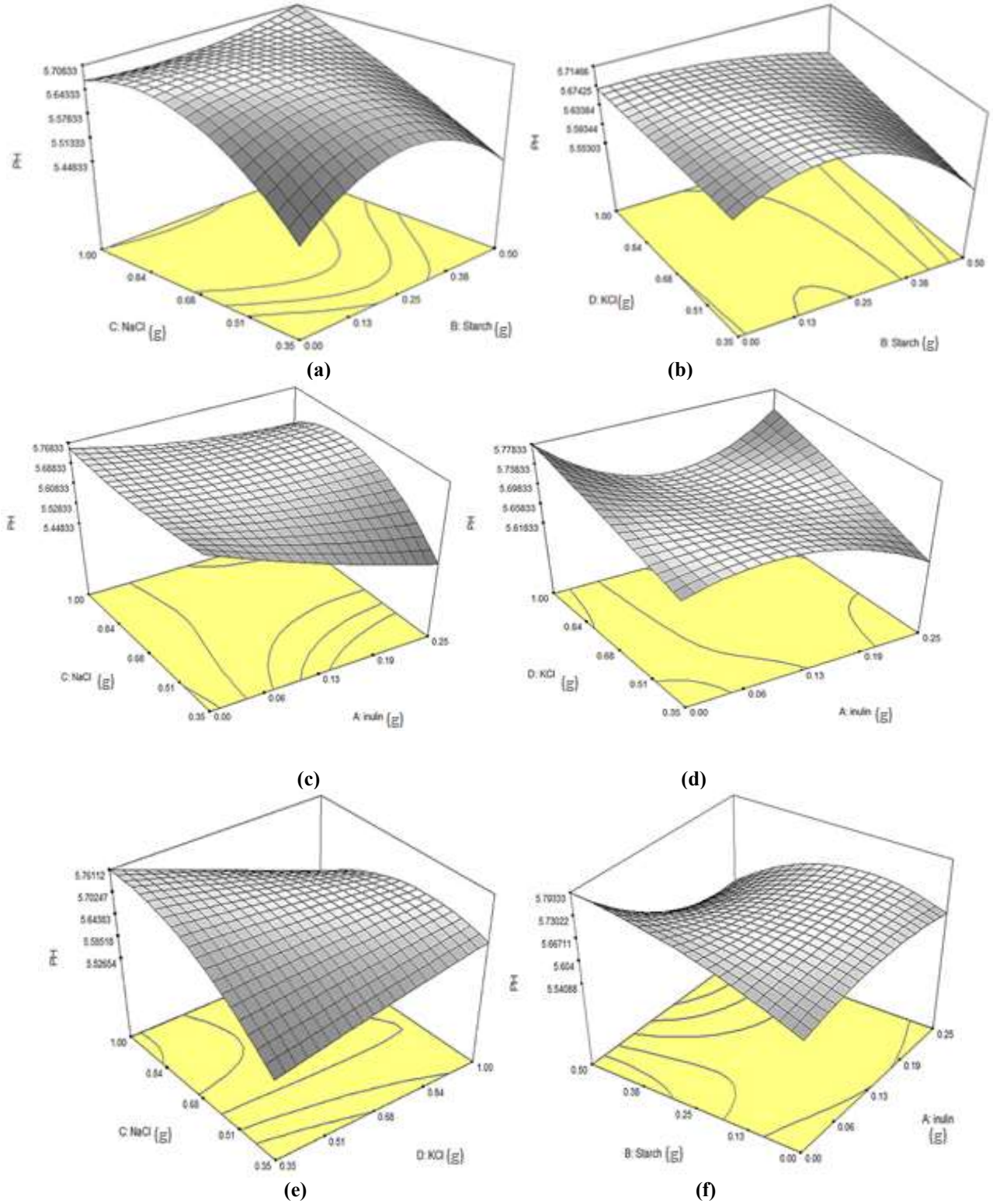
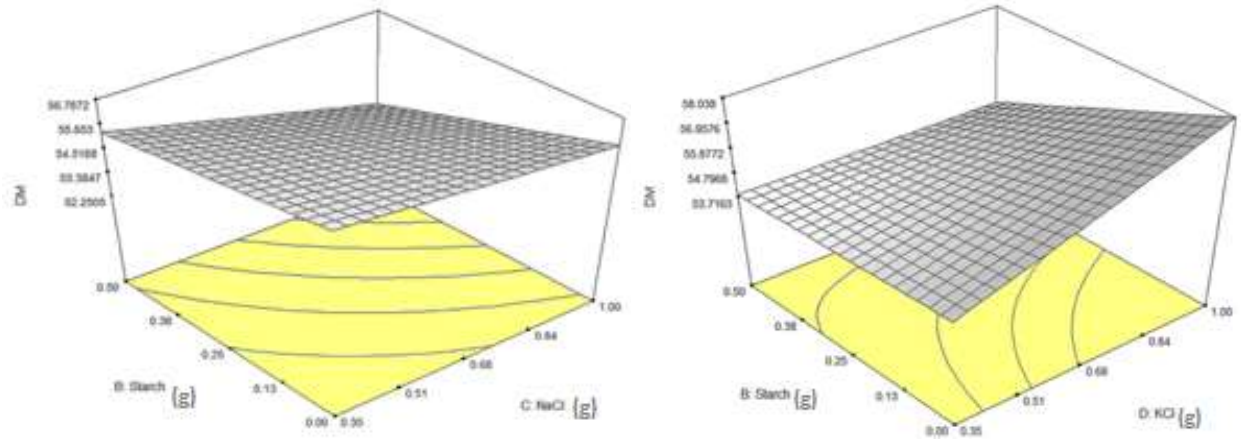


Fig. 1. Effect of starch and NaCl (a), starch and KCl (b), inulin and NaCl (c), inulin and KCl (d), NaCl and KCl (e), starch and inulin (f) on the pH of imitation cheese



(a) **(b)**

Fig. 2. Effect of starch and NaCl (a), starch and KCl (b) on the DM of imitation cheese

An increase in starch and potassium chloride variables resulted in a decrease in cheese fat. The increase in inulin and starch caused an increase (13.7627%) and then a decrease in fat content (11.91117%). Fig. 3 (c) shows that the increase in inulin (0.13%) resulted in an 11.9117% increase in fat and then decreased. The increase in sodium chloride (1%) resulted in an increase in the fat content. In general, decreasing NaCl levels resulted in a decrease in fat, protein, ash, sodium and pH, and an increase in moisture and l-lactic acid. As moisture increases, fat and protein decrease due to a dilution effect (Rulikowska *et al*., 2013). Results obtained from the effect of inulin and potassium chloride showed that the increase of each of these two parameters led to the decrease of fat content. With the simultaneous increase of inulin and potassium chloride, the fat content decreased. The reduction of fat with the increase of inulin is attributed to the replacement of inulin with sodium caseinate in the product. The reason for the reduction of fat with the increase of starch is the presence of pectin in the structure of starch, which replaces fat in imitation cheese (Hennelly *et al*., 2006). Mounsey & O'Riordan (2008) studied the influence of pre-gelatinized maize starch on the rheology; microstructure and processing of imitation cheese, and reported that this replacement caused a decrease in protein and increase the stability of fat globules. Fadaei *et al.* (2012) studied the chemical characteristics of low-fat wheyless cream cheese containing inulin as fat replacer. The results showed that it is possible to make a wheyless cream cheese with lower fat content and desirable attributes using inulin (10%) as fat replacer, and that inulin and stabilizers can improve chemical properties of low-fat whey less cream cheese. The results of Borges *et al.* (2019) showed that reduced-fat Frescal sheep milk cheese containing 5 %w/w inulin (as fat replacer) has sensory and textural attributes. Lashkari *et al.* (2014) investigated the effect of fat replacement with tapioca starch on the structure and sensory characteristics of Feta cheese. The results showed that the percentage of hardness increased with the reduction of cheese fat. Abbasi & Nateghi (2022) used pre-gelatinized corn starch to improve the sensory and physicochemical properties of low-fat pizza cheese. The results showed 67.56% of low-fat milk powder, 27.93% of fat and 4.5% of starch were the optimal quantities in the formulation. Melt ability, acidity, elasticity, firmness, total soil matters, flavor, texture and total acceptance were 4.08, 0.46, 13.91, 14.11, 49.68, 3.85, 4.26 and 3.78 respectively. The optimal formulation showed the highest acceptance rate among the treatments.

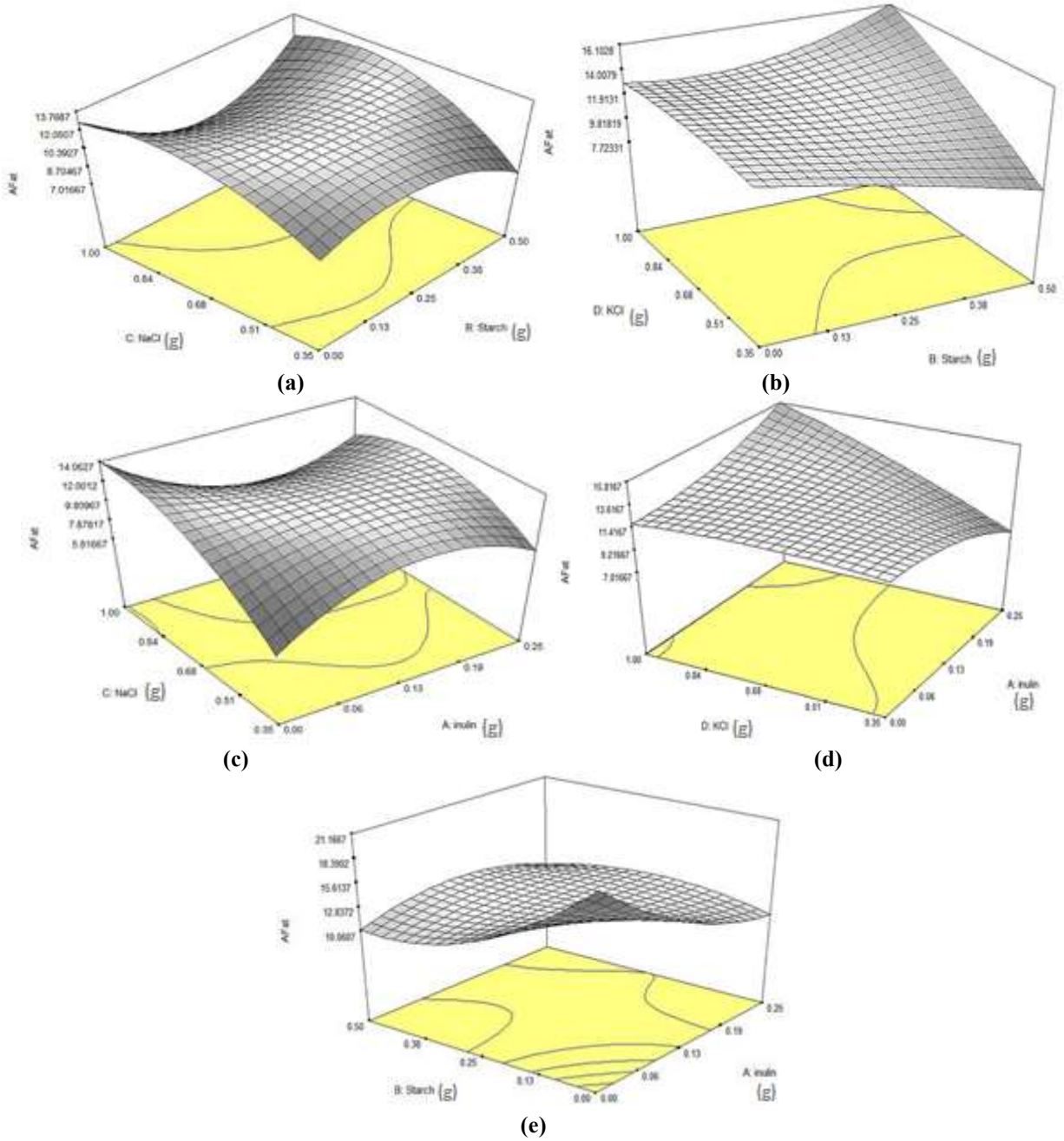


Fig. 3. Effect of starch and NaCl (a), starch and KCl (b), inulin and NaCl (c), inulin and KCl (d), starch and inulin (e) on the fat percentage of imitation pizza cheese

Salt

In cheese, salt reduction is still a challenging task, because sodium chloride has multiple and essential functions such as increasing the flavor and aroma of cheese, adjusting the final pH, water activity and texture of the product, as well as affecting microbial growth (Lavasani, 2022). The increase of potassium chloride resulted in a significant decrease in the amount of salt in the product ($p<0.05$), while the salt content of the product increased with the increase in sodium chloride. Fig.3 shows the simultaneous effect of two variables (sodium chloride and potassium chloride) on the amount of salt in the product. The percentage of salt in imitation pizza cheese was reduced ($p<0.05$) by the combination of sodium chloride and potassium chloride. Dorosti *et al.* (2010) evaluated the effect of

partial replacement of NaCl with KCl on the characteristics of Iranian white cheese. They showed that reducing sodium chloride by 50% has no significant effect on cheese quality. The partial replacement of NaCl with KCl is not able to significantly change the acid number and textural characteristics of the cheese samples. Rulikowska *et al.* (2013) evaluated the effect of reducing sodium chloride on Cheddar cheese quality. The results showed that salt reduction has an adverse effect on the taste and texture of cheddar. Salt reduction led to a simultaneous decrease in pH, a slight decrease in buffering capacity, and an increase in water activity and the growth of starter and non-starter lactic acid bacteria, which led to an increase in proteolysis. Mohammadzadeh (2020) analyzed the impact of replacing sodium chloride with potassium chloride on certain quality indices of fish sauce from Caspian Sea fish. Between two replacement concentrations of KCl, 50% has better quality than 25% in terms of total nitrogen, formaldehyde nitrogen, and amino nitrogen. Lavasani (2022) studied the quality and composition of Iranian low-salt UF-white cheese. The results showed that KCl did not significantly affect the moisture, dry matter, fat, total nitrogen/dry matter, and water soluble nitrogen of cheeses. Sensory evaluation showed that as the concentration of KCl increased, the cheese gradually became less acceptable. Treatments contained more potassium chloride were crumblier and less firm. The aroma evaluation of cheese samples revealed that acetaldehyde, ethanol, acetoin, and diacetyl had a significant decrease in their amounts during storage. According to the results, reducing sodium by up to 50% did not have a significant impact on the quality and composition of Iranian low-salt UF-white cheese.

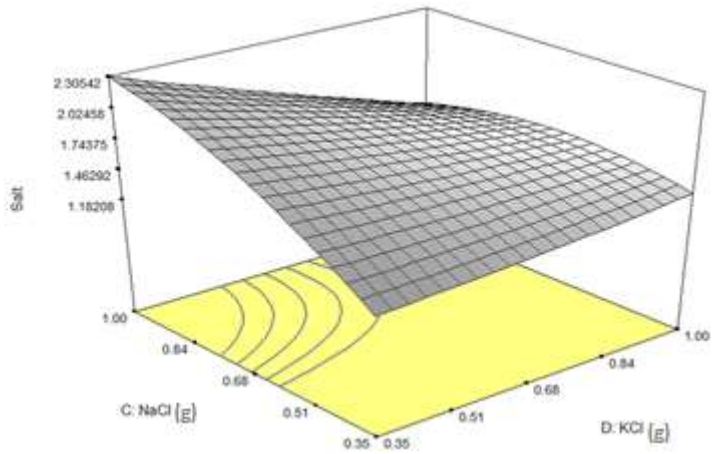
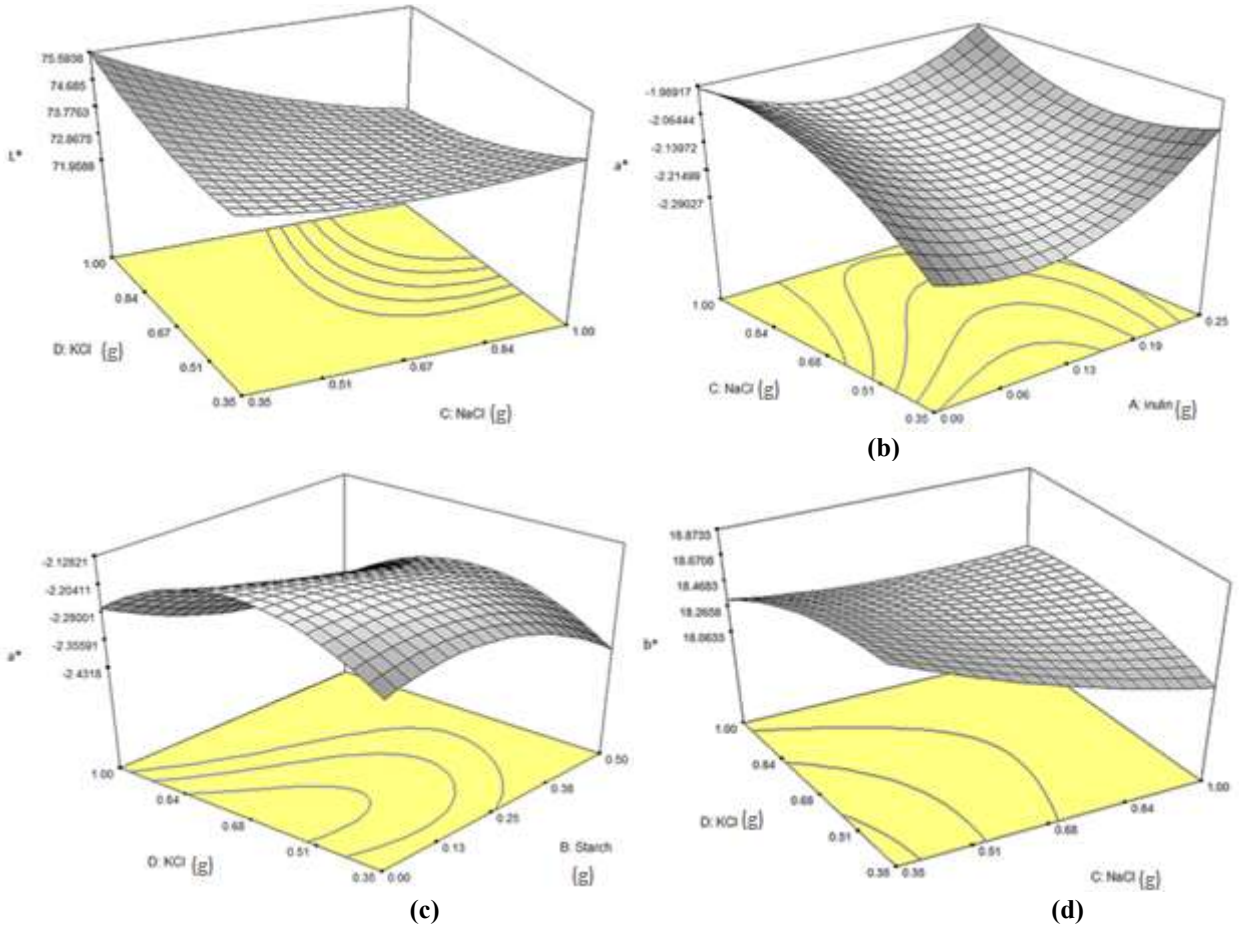


Fig. 4. Effect of two variables (NaCl and KCl) on the salt of imitation pizza cheese

Color parameters

Increasing NaCl content led to reduce L* value of low-fat imitation cheese samples (Fig. 1 (g)). On the other hand, the L* value was gently increased by increasing KCl content at low levels of NaCl contents. However, at the higher content of NaCl, the L* value significantly decreased. This is attributed to natural color of sodium caseinate (yellow to opaque) and KCl (white) (Hogan *et al.*, 2001). Fig. 5 (h) shows the interactive effect of inulin and NaCl on the redness of low-fat imitation cheese. The a* value was initially decreased by increasing inulin content and followed by significant increase. On the other hand, the redness was increased by adding NaCl. According to Fig. 1 (i), the a* value was initially increased by increasing starch and KCl content, but subsequently reduced. Juan *et al.* (2013) reported that low-fat cheeses showed less lightness than full-fat cheeses, with inulin cheese having the lowest amount. In 6 days of

storage, inulin cheeses showed the highest yellowness values. However, these instrumental color differences were not recognized by the panelists. Jayarathna *et al.* (2022) showed that sausages containing 2% inulin had lower lightness (L*) values than the control ($p<0.05$). During storage, the value of L*, pH, and water holding capacity decreased and the values of redness (a*) and yellowness (b*) increased in all samples. Fig. 1 (k) shows the interactive effect of NaCl and KCl on b* value of low-fat imitation cheese. The b* value was increased by decreasing NaCl and KCL content (Fig. 1 (k)). Due to its particulate nature, inulin can act as light scattering centers and increase the turbidity of cheeses. High-fat cheeses had less redness and yellowness than low-fat cheeses.



(b) **(c)** **(d)**

Fig. 5. Effects of the independent variables on color parameters of imitation pizza cheese (a: L* value; b and c: a* value; d: b* value)

Textural properties

Hardness

Hardness of low-fat imitation pizza cheese was initially increased by decreasing starch content to 0.25%, and then remained constant. On the other hand, the hardness was decreased by adding inulin at low concentration of starch (Fig. 6 (a)). As is shown in Fig. 6 (b), the hardness was increased by increasing KCl and NaCl content. The hardness is attributed to the stability of emulsion, so that each emulsion-weakening agent caused in the reducing hardness (Hennelly *et al.*, 2006). Similarly, the increasing hardness was observed for mozzarella cheeses containing fat replacers reported by Rafiei *et al.* (2022) and Moghise *et al.* (2022), respectively. Borges *et al.* (2019) reported that the hardness of Frescal sheep milk cheese increased significantly with the addition of inulin ($p<0.05$). A higher ratio of moisture to

protein reduces the bond between fat and casein. Diamantino *et al.* (2019) showed that by increasing the level of starch, cheese became harder and the maximum hardness was observed in cheese with higher concentration of starch. Cheese containing starch has more hardness as compared to other treatments due to crystal formation. The increase in the hardness of cheese containing starch is due to the change in protein matrix compactness, because the addition of starch increased the water-binding capacity of protein matrix. In a similar study, Sołowiej *et al.* (2015) used inulin and whey protein polymers in low -fat cheese and found that hardness increases with increasing level of inulin. Inulin can probably act as a stabilizer because of its ability to water binding. Therefore, the water molecules become immobilized and cannot move freely among the other molecules in the mixture. This improves the consistency of the mixture and thus increases the hardness.

Cohesiveness

Fig. 6 (c) shows the interactive effect between inulin and NaCl content on cohesiveness of imitation cheese. The cohesiveness was initially increased by adding NaCl up to about 0.7%, but subsequently reduced. However, this trend was reversed at higher inulin content. On the other hand, the cohesiveness of imitation cheese was increased by adding inulin. Juan *et al.* (2013) reported that the mean amount of cohesiveness and chewiness of low-fat fresh cheeses was higher than that of high-fat cheeses. As is shown in Fig. 6 (d), the cohesiveness was remarkably increased by increasing starch and NaCl content. Butt *et al.* (2020) found that imitation cheese replaced with pregelatinized starches was more cohesive with improved melting properties compared to the control. It corresponded to the results of Moghiseh *et al.* (2021), and Diamantino *et al.* (2019) in Mozzarella, and cheddar reduced-fat cheeses, respectively.

Springiness

Fig. 6 (e) shows the interactive effect between inulin and starch content on springiness of imitation cheese. The springiness was increased by increasing inulin content at low content of starch. However, the opposite is true when increasing inulin content at high levels of starch. Moghiseh *et al.* (2021) reported that Less springiness and greater cohesiveness of mozzarella cheese at high level of inulin can be due to the increase in moisture and protein content, which lead to the hydration phenomenon of caseins and the formation of a firm and less plastic structure. The springiness was increased by increasing starch content (Fig. 6 (e)). Juan *et al.* (2013) found that reduced-fat cheeses have a higher value of springiness than full-fat cheese. According to Fig. 6 (f), springiness of imitation cheese was increased by increasing NaCl and decreasing KCl content. This result was in agreement with Koca & Metin (2004) for low-fat fresh kashar cheese. Kiziloz *et al.*, (2009) studied development of the structure of an imitation cheese with low protein content, and reported that hardness; cohesiveness and springiness of the cheese were affected positively by κ-carrageenan and negatively by α-amylase.

Adhesiveness

As shown in Fig. 6 (g), adhesiveness of low-fat imitation cheese was increased by increasing NaCl content. At low levels of NaCl, the adhesiveness was initially decreased by increasing inulin content, but subsequently increased. However, it was unlike at high levels of NaCl by increasing inulin content (Fig. 6 (g)). The adhesiveness was increased by adding starch and KCl (Fig. 6 (h)). Fat reduction with incorporated fat mimetics can increase protein-water interactions and increase cheese adhesiveness. As a result, by reducing the cheese fat, its hardness, springiness, consistency and chewiness increased.

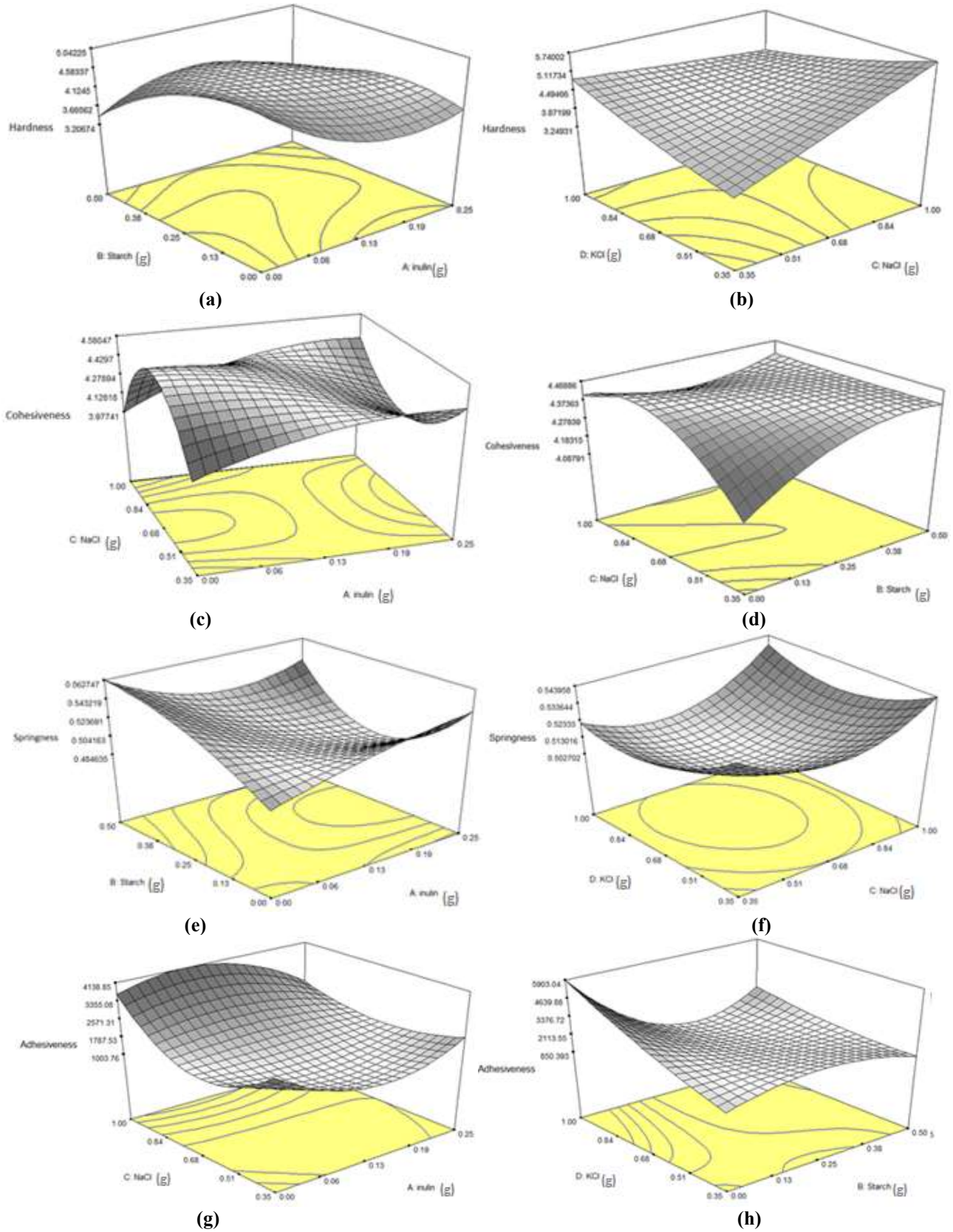


Fig. 6. Effects of the independent variables on textural properties of imitation pizza cheese

Diamantino *et al.* (2019) stated that the role of fat is so important that even if moisture is higher in low-fat cheddar cheese, the texture will be hard due to the denser protein matrix with less open spaces. Oliveira *et al.* (2011) found that when the starch level in Edam green cheese increases, the intermolecular interaction also increases, resulting in a denser three-dimensional matrix that affects the textural properties of low-fat cheese.

Sensory evaluation

The aroma of imitation pizza cheese increased significantly from 6.14 to 7.39, with an increase in inulin content. The amount of aroma increased and then decreased as starch content increased (Fig. 7(a)). The results showed that the simultaneous effect of starch and sodium chloride reduced the aroma of imitation pizza cheese. Also, the aroma of imitation cheese decreased with the increase of starch and potassium chloride. When potassium chloride alone increased, there is an increase in aroma, but when sodium chloride alone increased, there is a decrease in aroma the imitation cheese texture decreased from 7.81 to 7.09-7.27% as inulin or starch increased. The texture score was significantly affected by reducing fat and increasing inulin and modified gelatin starch. The texture of the imitation pizza cheese containing inulin and starch was harder than that of the control cheese. The treatments with the lowest and highest amount of fat were scored lower. Some of the examined samples were found to be too soft or have an unfavorable hardness, according to the panelists. The sensory evaluation revealed that the increase in inulin resulted in a decrease in color index of imitation cheese. The color did not change significantly due to the increase of sodium chloride. Based on the results, the increase in starch caused a decrease in the color index. Cheeses with less potassium chloride are more palatable because they have more sodium (NaCl). The taste of salts is influenced by the nature of their cations and anions. The salts become bitter as their molecular weight increases for cation and anion. The aroma and taste scores differ between treatments due to the concentration of potassium chloride in combination with sodium chloride. Potassium chloride has an inherent bitterness due to the presence of potassium ions, the higher concentration, the more noticeable this bitterness will be. Mazaheri Nasab *et al.* (2012) reported that partial substitution of fat by carrageen and whey concentrate in low fat mozzarella cheese could produce a low fat product with desirable sensory properties. According to Sadrolodabaee & Shahabad (2014), cheese sample with 1% of mono and diglycerides had a higher overall acceptance rate than other samples. Pishelmi *et al.* (2017) utilized pre-gelatinized starch in the formulation of low-fat stirred yogurt and found that an increase in pre-gelatinized starch content led to an increase in overall acceptance scores. Heydari & Razavi (2021) observed that creaminess was improved by applying high pressure on corn and waxy corn starches, which are novel fat replacements. Abbasi & Nateghi (2022) showed that the apparent desirability of this cheese decreased as an increase in starch content occurred. Low-fat pizza cheese with only 3% starch, 75% milk powder, and 34% fat received the highest score.

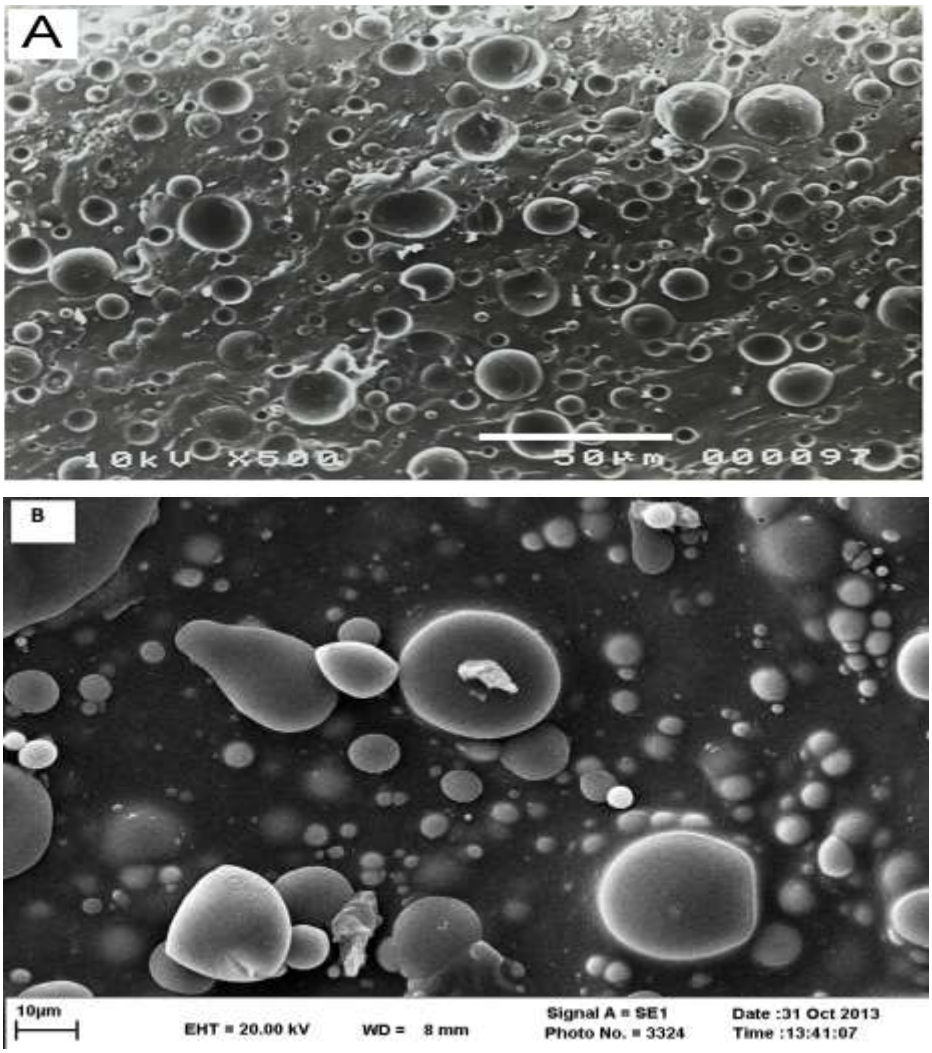
Optimization

The numerical optimization technique performed to optimize the formulation, when weight and importance values for all of responses were equal (Yolmeh *et al.*, 2014). The fat content, L*, b*, cohesiveness, springiness and adhesiveness attributes were considered for the optimization formulation of imitation cheese. The formulation containing 0.19% inulin, 0.4% starch, 0.35% NaCl, and 0.50% KCl was found as the optimal formulation. The fat content, L*, b*, cohesiveness, springiness and adhesiveness were acquired 4.67, 74.07, 18.72, 4.32, 0.52, and 3879.52 respectively, as the predicted results whose composite desirability values were equal to 0.83. The experimental results of fat content, L*, b*, cohesiveness, springiness and adhesiveness at the optimum formulation

were 4.94, 72.67, 17.04, 3.96, 0.42, and 3914.65, respectively.

Scanning electron microscope (SEM) images

As shown in Fig.7, there were many large and small particles in the imitation cheese, which indicated that inulin crystals are accumulated in the continuous phase (Fig.7 (b)). Therefore, the effective volume fraction was increased, which can lead to important changes in the sensory and textural properties. There are particles of gelatinized and immersed starch granules in the continuous phase in samples containing starch and without inulin. As well as fat globules remains small and uniform by increasing the starch content (Fig.7 (c)). Disruption of fat globules was remarkably increased by increasing inulin and starch contents (Fig.7 (d)). Karami *et al.*, (2009) studied microstructural properties of fat during the accelerated ripening of ultrafiltered-Feta cheese. They showed through scanning electron microscopy images that with an increase in lipase levels from 2 to 6 g 100 kg^{-1} of retentate, disruption of fat globules increased significantly.



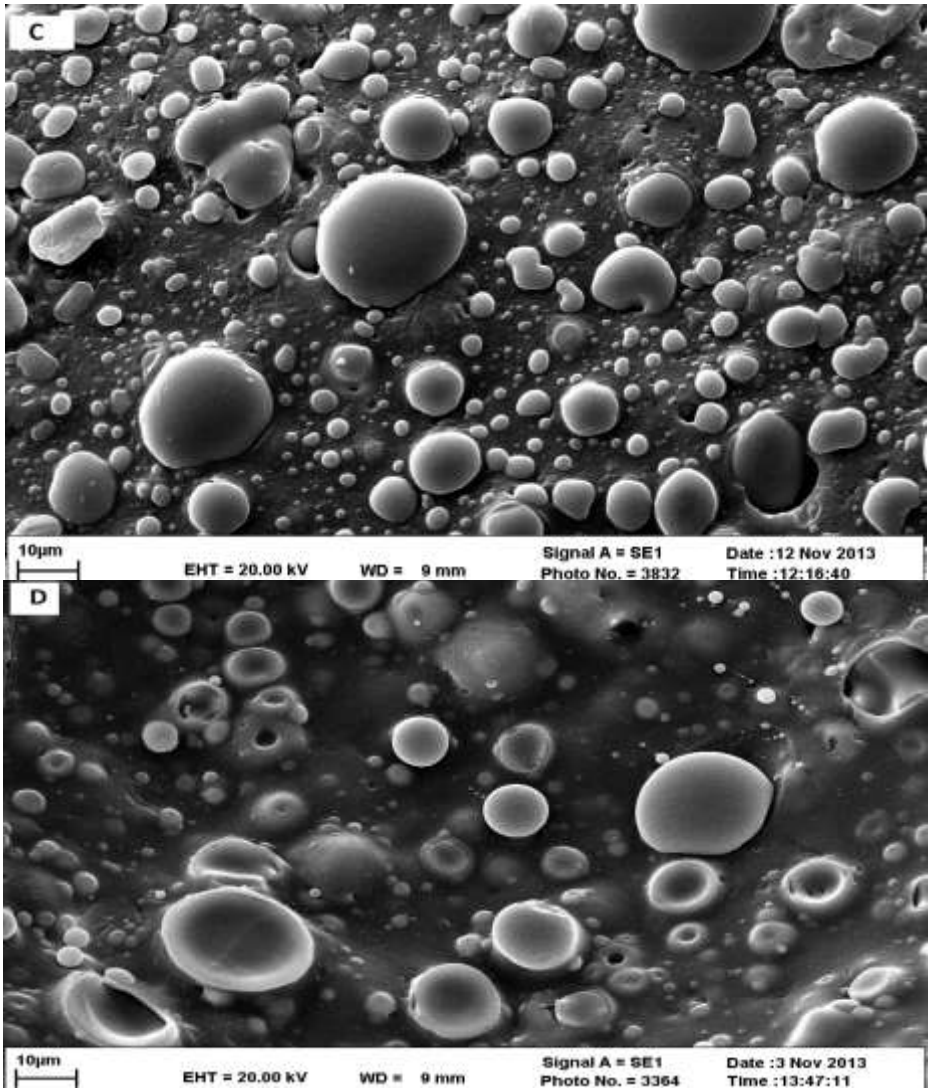


Fig. 7. SEM images of control sample (A), imitation cheese containing inulin (B), imitation cheese containing starch (C), imitation cheese containing inulin-starch (D)

Conclusion

Fat consumption is directly related to various diseases such as obesity, diabetes, hardening of blood vessels and blood pressure. The development of flavor, texture and appearance of cheese is largely influenced by fat. Imitation pizza cheese is a relatively high-fat product, containing 20-27% fat. Also, high amounts of sodium increase the risk of heart attack and high blood pressure. In this study, low-fat and low sodium imitation pizza cheese was properly developed and RSM was successfully applied for optimizing its formulation. The formulation containing 0.19% inulin, 0.4% starch, 0.35% NaCl, and 0.50% KCl was found as the optimal formulation of imitation cheese. At the optimal formulation, the fat content, L*, b*, cohesiveness, springiness and adhesiveness were measured were 4.94, 72.67, 17.04, 3.96, 0.42, and 3914.65, respectively. The replacement of fat by increasing the concentration of inulin or pre-gelatinized starch had a significant effect on the properties of imitation pizza cheese. With increased levels of inulin or starch, the resultant imitation cheeses had less hardness and adhesiveness; however, their cohesiveness and springiness were higher.

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ارزیابی ویژگی‌های فیزیکوشیمیایی و بافتی پنیر پیتزای تقلیدی کم چرب و کم‌سدیم

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چکیده

رشد روزافزون بیماری‌های قلبی عروقی، فشار خون بالا، سفت شدن دیواره رگ‌ها و همچنین چاقی در بسیاری از کشورها، پنیر پیتزای کم‌چرب و کم‌سدیم را به یکی از موضوعات مورد مطالعه در سراسر جهان تبدیل کرده است. اثرات چهار متغیر مستقل شامل اینولین (۰/۰۲۵-۰ درصد)، نشاسته پیش‌ژلاتینه (۰/۵- ۰ درصد)، NaCl (۱-۰/۳۵ درصد) و KCl (۱-۰/۳۵ درصد) مورد بررسی قرار گرفت. محتوای چربی پنیر پیتزای تقلیدی با افزایش سطوح جایگزینی اینولین و نشاسته به‌طور قابل توجهی به ۱۱/۹۱ درصد کاهش یافت ($P < ۰/۰۵$). همچنین مقادیر رطوبت و pH آن به‌طور معنی‌داری متفاوت بود ($p<۰/۰۵$). افزایش سطوح نشاسته پیش‌ژلاتینه شده و اینولین باعث کاهش سختی (از ۵/۰۴ به ۳/۵۵) و چسبندگی (از ۴۳۶۸/۸۹ به ۱۶۴۰/۵۴ درصد) شد، اما چسبندگی (از ۰/۳۶۵ به ۰/۴۳) و فنریت (از ۰/۵ به ۰/۴) را افزایش داد. افزودن NaCl و KCl، سختی محصول را افزایش داد. افزودن اینولین و نشاسته، a* را کاهش داده‌اند. b* با افزایش اینولین کاهش می‌یابد و با افزایش نشاسته اصلاح شده افزایش می‌یابد. فرمول حاوی ۰/۱۹ درصد اینولین، ۰/۴ درصد نشاسته پیش‌ژلاتینه، ۰/۳۵ درصد NaCl و ۰/۵ درصد KCl به‌عنوان فرمولاسیون بهینه برای پنیر تقلیدی کم‌چرب یافت شد. نتایج تصاویر میکروسکوپ الکترونی روبشی (SEM) نشان داد که کریستال‌های اینولین در فاز پیوسته انباشته شده‌اند که می‌تواند منجر به تغییرات مهمی در ویژگی‌های حسی و بافتی شود. این مطالعه به این نتیجه رسید که اینولین یا نشاسته را می‌توان برای جایگزینی تا ۳/۶ درصد از چربی موجود در پنیر پیتزا تقلیدی و ۰/۳۵ درصد NaCl و ۰/۵ درصد KCl برای کاهش محتوای سدیم محصول استفاده کرد.

واژه‌های کلیدی: اینولین، بهینه‌سازی، پنیر پیتزای تقلیدی، نشاسته پری‌ژلاتینه

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Effects of Aqueous Extracts of Propolis on Total Polyphenol Content and Antioxidant Activity of Raw Milk

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Abstract

In recent years, the use of propolis in food products has received attention owing to its functional role. This study was conducted to investigate the effect of different concentrations of propolis extract on the total polyphenol content and antioxidant activity of raw milk. For this purpose, an aqueous extract of dry propolis was prepared and stored in dark-colored bottles at 4 °C until the day of experiments. The propolis extract was added to raw milk in concentrations of 0, 4.7, 9.1, 16.6, and 28.5%. Total phenolic content and antioxidant activity were measured using the colorimetric Folin-Ciocalteu method and DPPH assay, respectively. Measurements were performed on the first day at zero hour and after 6 and 24 h of treatment, and the storage temperature was maintained at 5°C until analyzed. The amount of total polyphenol increased with the increase in the concentration of propolis extract in the treated milk in 0 hour; accordingly, the lowest and the highest amounts of total polyphenol were related to control milk and milk containing 28.5% propolis extract, respectively. A decreasing trend was observed in total polyphenol in the control sample and raw milk containing 4.7% propolis extract during 24 hours. There was an increase in total polyphenol content in raw milk containing 9.1% and 16.6% propolis extracts, the trend of changes in raw milk containing 28.5% extract was insignificant. The addition of propolis extract caused an increase in the antioxidant activity and total phenolic content in raw milk. According to the results, it is recommended to carry out more studies to clarify the functions of propolis's total polyphenol content and its interaction with milk proteins.

Keywords: Antioxidant activity, Aqueous extract, Propolis, Raw milk, Total polyphenol

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Introduction

Milk and dairy products are important suppliers of a wide range of essential nutrients, some especially important at certain stages of life (Givens, 2020). Recently, various studies have been implemented to enhance the nutritional value of this food or its products (Givens & Kliem, 2009). The complex biochemical composition, high water activity, and nutrient content of milk serve as an ideal medium for microorganisms that cause milk deterioration (Fusco *et al*., 2020), and the application of heat treatment in milk affects the flavor, and the milk proteins (Bezie, 2019). Orcho *et al*. examined the shelf life extension ability and antioxidant activity of the ethanolic extracts of *Moringa stenopetale*, *Artemesia anua*, and *Mentha spicata* to preserve milk. All the plant extracts showed suitable antioxidant activities, and *Moringa stenopetale* had the most effect on extending shelf life of raw milk samples (Orcho *et al*., 2023). The vitamins, such as E and C, beta-carotene, and the enzyme system in the milk provide a possible complex set of pro-oxidative and anti-oxidative reactions (Zulueta *et al*., 2007). Currently, we are faced with increasing controversial research on the antioxidant capacity of milk (Gülcin, 2012).

The antioxidant activity of propolis has been reported in various studies (Kasiotis *et al*., 2017; Özkök *et al*., 2021). More than 300 compounds, including different flavonoids, polyphenolic esters, terpenoids, steroids, amino acids, and caffeic acids and their esters, as well as mineral compounds, have been reported in propolis (Mouhoubi-Tafinine *et al*., 2016). Today, considerable attention is conferred on the activity and content of the bioactive compounds of propolis (Andrade *et al*., 2017). It has attained wide acceptance across numerous countries as a diet supplement that enhances health (Azemin *et al*., 2017). The antimicrobial activity of propolis against food spoilage microorganisms, such as *Bacillus cereus* (Kim & Chung, 2011) and *Escherichia coli* (Tosi *et al*., 2007), is confirmed, and this feature can contribute to the maintenance of greater nutritional value.

The different concentrations of propolis have improved the milk production, fatty acid composition, and antioxidant capacity of milk in dairy cows (Santos *et al*., 2019). Using Brazilian red propolis, as a substitute for potassium sorbate preservative, its antioxidant activity was significantly increased during yogurt storage (Aguiar *et al*., 2014). The addition of aqueous extract of propolis in concentrations of 1%, 2%, and 3% was accompanied by an increase in phenolic and flavonoid compounds, and antioxidant activity in raw milk (El-Deeb, 2017). Also, the adding 0.5 and 1% of propolis extracts to milk increased the shelf life of raw and pasteurized milk (Shaban *et al*., 2021). The propolis can be used as an antioxidant, where lipid autoxidation may reduce the sensory quality and the nutritional value of food (Pobiega *et al*., 2019).

In recent years, consumer demand has grown for raw milk and dairy products manufactured from unpasteurized milk (McLauchlin *et al*., 2020). During pasteurization, approximately 5-15% of whey protein is denatured (Deeth & Lewis, 2017). Superoxide dismutase activity and glutathione level as antioxidant parameters are affected by the heat treatments (Martysiak-Żurowska *et al*., 2019). The current research aimed to investigate the effect of extract of propolis on the total polyphenol content and antioxidant activity of raw milk.

Materials and Methods

The milk was obtained by hand milking on a livestock farm after washing the cow's udders. Samples were transported to the laboratory in sterile bottles away from light and heat. Folin-Ciocalteau reagent, 2, 2-diphenyl-1-picryhydrazyl free radical (DPPH), gallic acid, and methanol were from Merck Company (Darmstadt, Germany). Other chemicals were of the highest commercial grade and used without further purification.

Although the alcoholic extraction of propolis contains high phenolic compounds, this method has disadvantages, such as strong residual flavor and intolerance of some consumers to alcohol (Pobiega *et al*., 2019). In this study, the

aqueous extract was chosen. For this aim, dry propolis (brown with green veins) purchased from a beekeeper in the Otaghvar district of Langarud County, northern Iran, in the summer. It was transferred to the laboratory in a completely sterile condition. Then, 5 g, 10 g, 20 g, and 40 g propolis were weighed in separate Erlenmeyer flasks, and 100 ml of deionized water (65°C) was added to the flasks. After shaking for two hours, centrifugation was performed at 1500 rpm for 5 min. The supernatant was separated and stored in dark-colored bottles (Said *et al.*, 2006). Extract was added to raw milk with concentrations of 4.7%, 9.1%, 16.6%, and 28.5%. The sampling of raw milk and propolis extract was performed by previously sterilized instruments in an autoclave at 121°C for 15 min.

Measurement of total polyphenols content

Polyphenol was measured according to the colorimetric Folin-Ciocalteu method. For this purpose, 1 ml of the control sample and milk samples containing propolis extracts were pipetted into a 100 ml volumetric flask and diluted with distilled water, and mixed well. In control samples, 1 ml of water was added separately to two test tubes. Moreover, 1 ml of the diluted extract was poured into two separate tubes, and 5 ml of Folin-Ciocâlteu reagent solution was added to all the tubes and mixed well. After 3-8 min, 4 ml of a sodium carbonate solution was added to all the tubes and then capped. The tubes were placed at ambient temperature for 60 min; then, the optical absorption was measured by a 2100 UV/VIS spectrophotometer (UNICO, USA) at 765 nm (Singleton & Rossi, 1965).

Measurement of antioxidant activity

Antioxidant activity was measured using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay. This method is one of the most popular and frequently employed methods to evaluate the antioxidant capacity of foods (Pyrzynska & Pękal, 2013). After preparing different concentrations of propolis extract, 1 ml of the sample was mixed with 3 ml of methanolic DPPH radical solution, and stirred vigorously. The reaction mixture was kept at room temperature (23°C) for one hour in the darkness. Absorbance was measured at 517 nm by a 2100 UV/VIS spectrophotometer (UNICO, USA). Methanolic DPPH solution and methanol were used as standard and to zero the spectrophotometer, respectively. Finally, the antioxidant activity was obtained using the following formula:

$$\% \text{ DPPH}^{\cdot} \text{ scavenging activity} = (1 - [A_s / A_c]) \times 100,$$

Where A_s and A_c are the absorption of the sample solution and methanolic DPPH solution, respectively (Li *et al.*, 2009). It should be noted that the measurements were taken on the first day at the zero hour and after 6 and 24 h of treatment, and the storage temperature was maintained at 5°C until analyzed.

Data Analyses

This study was conducted based on a random design, and the collected data were analyzed in SPSS software (version 16). Measurements with three repetitions were performed. Data were reported as mean ± standard deviation. A p-value<0.05 was considered a significant level.

Results and Discussion

In this study, the total phenolic content of the extracts was determined by comparison with a calibration curve of gallic acid as a standard (the standard curve equation: $y= 0.0118x+ 0.0434$, $r^2 = 0.9992$). The comparison of the mean and standard deviation of total polyphenol and antioxidant activity of different concentrations of propolis extract is presented in Table 1. There was a significant difference in total polyphenol and antioxidant activity of the studied treatments ($p<0.001$).

The comparison of the total polyphenol content and antioxidant activity (µg/ml) of the studied samples during 24 hours is tabulated in Tables 2 and 3, respectively. The total polyphenol content and antioxidant activity of the propolis extract-treated milk sample was higher than the control in all three times of examinations (p=0.001).

Table 1- Mean and standard deviation of total polyphenol content and antioxidant activity of different concentrations of propolis extract

Group	Total polyphenol content(mg/g)	Antioxidant activity (µg/ml)
4.7% extract	2.512±0.047[a]	8.501±0.100[a]
9.1% extract	4.203±0.551[b]	11. 926±0.490[b]
16.6% extract	7.106±0.010[c]	17.671±0.068[c]
28.5% extract	11.670±0.005[d]	27.302±0.152[d]

Table 2- Mean and standard deviation of total polyphenol content (mg/g) in the propolis extract-treated milk during the storage period

Treatment	Storage Period			p-value 1
	0 hour	6 hour	24 hour	
Control sample	0.693±0.001[aA]	0.504±0.010[aB]	0.422±0.005[aC]	0.010
4.7% extract	1.491±0.003 [bA]	1.453±0.010 [bB]	1.421±0.001 [bC]	0.010
9.1% extract	1.582±0.021 [cA]	1.621±0.010 [cB]	2.094±0.001 [cC]	0.001
16.6% extract	2.216±0.003 [dA]	2.092±0.021 [dB]	2.251±0.001 [dC]	0.030
28.8% extract	2.257±0.002 [eA]	2.206±0.020 [eA]	2.173±0.001 [eA]	0.150
p-value 2	0.001	0.001	0.001	

p-value$_1$: Significant difference in one treatment over time
p-value$_2$: Significant difference in different treatments at a time

Table 3- Mean and standard deviation of antioxidant activity (µg/ml) in the propolis extract-treated milk during the storage period

Treatment	Storage Period			p-value 1
	0 hour	6 hour	24 hour	
Control sample	16.063±0.003[aA]	15.608±0.010[aB]	14.923±0.008[aC]	0.020
4.7% extract	34.680±0.002[bA]	33.863±0.011 [bB]	32.495±0.001 [bC]	0.010
9.1% extract	36.662±0.010 [cA]	37.391±0.015 [cB]	37.094±0.001 [cC]	0.001
16.6% extract	41.216±0.003 [dA]	41.078±0.001 [dB]	40.865±0.001 [dC]	0.040
28.8% extract	42.278±0.001 [eA]	42.216±0.020 [eA]	42.193±0.002 [eA]	0.189
p-value 2	0.001	0.001	0.001	

p-value$_1$: Significant difference in one treatment over time
p-value$_2$: Significant difference in different treatments at a time

In the present study, the total polyphenol content and the antioxidant activity of selected concentrations of propolis aqueous extract were evaluated. High TPC is generally regarded as an indication of high total antioxidant capacity (Li *et al.*, 2009). The results of the present study were in line with those of previous studies reporting the antioxidant activity of propolis extract (Devequi-Nunes *et al.*, 2018; Mohammadzadeh *et al.*, 2007).

Propolis is rich in phytochemicals (Abdullah *et al.*, 2020), and flavonoids, polyphenols, carboxylic acids, quercetins, fatty acids, cinnamic acid, esters, and terpenoids are its most important bioactive compounds (Sawicka *et al.*, 2012). It contains various compounds with biological activities, such as antioxidant, antibacterial, and anti-inflammatory. These properties would make it an ideal candidate which could be used as a beneficial ingredient (Irigoiti *et al.*, 2021).

The effect of collection time (Isla *et al.*, 2009), the geographical region, and plant species (Alvear *et al.*, 2021) on propolis polyphenol has been confirmed. The extraction method affects the antioxidant activity of propolis extract; accordingly, the highest activity was observed in methanolic extract (Esfandiarifard, 2021). Therefore, all the mentioned factors were influential in the results.

The total polyphenol content was obtained at 0.69 mg/g in the control sample at zero hour. These phenolic compounds can be derived from food and/or catabolism products of amino acids (Lopez & Lindsay, 1993). Different

concentrations of aqueous propolis extract led to an increase in the total polyphenol content in treated milk. The interaction between propolis extract polyphenols and milk casein might be the main reason. The results of previous studies have shown that phenolic compounds have a high affinity with milk proteins, especially casein (Arfaoui, 2020; Yildirim-Elikoglu & Erdem, 2018). Since caseins are proteins rich in proline, they have a high affinity for the hydroxyl group of polyphenolic compounds (Yuksel *et al.*, 2010). It has been shown that the formation of the casein-flavonoid complex can increase the absorption of flavonoids by biological membranes (do Nascimento *et al.*, 2022). It seems that adding propolis to milk can enhance its health effects due to its polyphenol content and antioxidant activity.

Based on the results, a significant decrease was observed in the total polyphenol content of the control sample and raw milk containing 4.7% propolis extract, during 24-hour storage. This result agreed with previous studies indicating the decrease of total polyphenols in yogurts enriched with fruits containing high polyphenols during storage (Arfaoui, 2020; Sánchez-Bravo *et al.*, 2018). A slight decrease in the total phenolic content in dragon fruit treated with 0.5% ethanol extracts has been reported (Zahid *et al.*, 2013). A significant increase was found in total polyphenol content in the treatments of 9.1% and 16.6% propolis extracts within 24 hours. It seems that 9.1% and 16.6% propolis extracts are suitable for further use. The results highlight the necessity of further studies to clarify the functions of total polyphenol of propolis and its interaction with milk proteins.

This work has limitations that must be mentioned. In this study, only DPPH assay was used to evaluate antioxidant activity. Furthermore, we measured total polyphenol content and antioxidant activity during a short-term storage period. Unlike several works that have used the effects of ethanol extraction, the current study investigated the effect of aqueous propolis extract. For further studies, it is suggested to investigate the effect of different extraction methods on sensory properties, microbial and physicochemical characteristics, and their potential as a natural preservative.

Conclusion

The present study showed the effect of different concentrations of aqueous propolis extract on the increase of total polyphenol content and antioxidant activity of raw milk. It seems that one of the advantages of using propolis extract relates to its higher antioxidant capacity. Therefore, it is recommended to be used for fortifying milk by conducting more studies.

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اثرات عصاره آبی برەموم بر محتوای پلی‌فنل کل و فعالیت آنتی‌اکسیدانی شیر خام

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چکیده

در سال‌های اخیر استفاده از برەموم در محصولات غذایی به‌دلیل نقش عملکردی آن مورد توجه قرار گرفته است. این مطالعه به‌منظور بررسی اثر غلظت‌های مختلف عصاره برەموم بر میزان پلی‌فنل کل و فعالیت آنتی‌اکسیدانی شیر خام انجام شد. برای این منظور عصاره آبی برەموم خشک تهیه و تا زمان آزمایش در قوطی های تیره رنگ در دمای ۴ درجه سانتی‌گراد نگهداری شد. عصاره برەموم در غلظت‌های ۰، ۴/۷، ۹/۱، ۱۶/۶ و ۲۸/۵ درصد به شیر خام اضافه شد. محتوای فنلی کل با استفاده از روش رنگ‌سنجی فولین سیوکالتیو اندازه‌گیری شد. براساس یافته‌ها، میزان پلی‌فنل کل با افزایش غلظت عصاره برەموم در شیر تیمار شده در ساعت صفر افزایش یافت. بر این اساس، کمترین و بیشترین مقدار پلی‌فنل کل به‌ترتیب مربوط به شیر شاهد و شیر حاوی ۲۸/۵ درصد عصاره برەموم بود. در طول ۲۴ ساعت، روند کاهشی در پلی‌فنل کل در نمونه شاهد و شیر خام حاوی ۴/۷ درصد عصاره برەموم مشاهده شد. در حالی‌که میزان پلی‌فنل کل در شیر خام حاوی ۹/۱ و ۱۶/۶ درصد عصاره برەموم افزایش یافت، روند تغییرات شیر خام حاوی ۲۸/۵ درصد عصاره معنی‌دار نبود. افزودن عصاره برەموم باعث افزایش فعالیت آنتی اکسیدانی و محتوای فنلی کل شیر خام شد. با توجه به نتایج، توصیه می‌شود مطالعات بیشتری برای روشن شدن عملکرد محتوای پلی‌فنل کل برەموم و برهمکنش آن با پروتئین‌های شیر انجام شود.

واژه‌های کلیدی: برەموم، پلی‌فنل کل، شیر خام، عصاره آبی، فعالیت آنتی‌اکسیدانی

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Optimization of Oleofoam and Protein-Polysaccharide Ratios for Enhanced Physicochemical Characteristics of A/O/W Double Emulsion: Potential Applications in the Food Industry

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Abstract

Preparing air-in-oil-in-water (A/O/W) double emulsion involves two key steps: oleofoam formation and dispersion of the oleofoam in an aqueous solution containing protein as an emulsifier and hydrocolloid as a thickening agent. This study aimed to investigate the effect of oleofoam level and varying concentrations of protein-polysaccharide ratios on the thermal stability, encapsulation yield and rheological properties of A/O/W double emulsion. An oleofoam was obtained using a lipophilic emulsifier (distilled monoglyceride MG) and sunflower oil at 5°C with maximum stability. Two levels of oleofoam (20% and 25 wt %) were added to an aqueous solution containing different concentrations of sodium caseinate (SC) (5, 8, and 10 wt %) and kappa carrageenan (KC) (0.4 and 0.8 wt %). Results indicate that oleofoam level did not significantly affect air encapsulation efficiency and particle size, while protein-polysaccharide ratios could significantly impact all properties of A/O/W double emulsion. Increasing the concentration of sodium caseinate and kappa carrageenan improved thermal stability and encapsulation yield while simultaneously reducing particle size. All A/O/W emulsions exhibited shear thinning behavior among the range of shear rates studied, indicating significant potential for food applications.

Keywords: A/O/W double emulsion, Oleofoam, Protein-polysaccharide ratios, Rheological properties

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Introduction

Oleofoam is a type of foam that has attracted increasing attention from researchers and industry professionals in recent years. This unique foam is formed by the stabilization of air in the oil phase using oil-soluble surfactant molecules like fats (triglycerides) and fatty acids/alcohols/esters, which can form solid crystals with varying shapes and structures. These crystals can interact with the surrounding air medium to create a strong network of interlocking crystal particles, resulting in a foam-like structure (Binks & Vishal, 2021). Oleofoams have a wide range of properties that make them a promising candidate for various applications, from food products to cosmetics and health care; it is a cheap novel system with low-fat content and calories, pleasant texture, long-term stability and microbial spoilage resistance (Fameau & Binks, 2021; Murray, 2020). This structured oil helps to follow the growing trend of creating "clean label" products (Fameau & Binks, 2021) and to decrease the utilization of saturated or trans-fatty acids in food products (Qiu *et al.*, 2021). Recently, oleofoam has successfully been used as an alternative fat to produce low density sponge cakes (Wetlaufer & Floter. 2022) and aerated mayonnaise sauces (Saremnejad *et al.*, 2019).

Foamed emulsions (emuleofoam) come in different types, depending on the continuous phase surrounding the bubbles. These include aqueous foams with no oil, water-in-oil emulsions, bicontinuous oil and water phases, and oleo foams (Salonen, 2020). Recently a new type of air-in-oil-in-water (A/O/W) double emulsion has been investigated. A two-step preparation process is employed to produce A/O/W double emulsions; the initial step involves the aeration of the oil phase that contains endogenous crystals which come from the olegelator. Ensuring an optimal solid fat content is essential for the stabilization of air bubbles while maintaining the desired fluidity of the oil phase in the following step. Subsequently, the oleofoam is progressively incorporated in an external aqueous phase containing an emulsion stabilizer and thickening agent at such large concentrations (Goibier *et al*., 2019). Food scientists will find these novel emulsions highly intriguing as they can decrease fat intake.

Previous studies have investigated the optimization of A/O/W emulsions by employing various emulsifiers and thickeners. Burn *et al.* (2015) successfully developed a stable A/O/W emulsion using oleofoam with rapeseed oil, mono- and diglycerides, and hydrophilic emulsifiers such as sodium caseinate or gum Arabic. Goibier *et al*. (2019) focused on enhancing A/O/W emulsions by utilizing an oleofoam composed of anhydrous milk fat and sodium caseinate as the sole emulsifier. Qiu *et al.* (2021) achieved the stabilization of oleofoam containing medium-long chain diacylglycerol and β-sitosterol through the incorporation of gum Arabic and xanthan gum. These studies collectively contributed to advancing the quality and stability of A/O/W emulsions for diverse applications through the strategic selection and combination of emulsifiers and thickeners, but they did not clarify the effect of each component (oleofoam, emulsifier and thickener) on the emulsion properties.

Emulsions' stability depends on synthetic surfactants or natural molecules (biopolymers) like proteins and polysaccharides (Seddari & Moulai-Mostefa, 2015). The intermolecular interaction between protein and polysaccharide plays a vital role in enhancing the stability of the A/O/W emulsion. The mechanism behind A/O/W double emulsions phenomenon stability involves biopolymer adsorption, usually protein, followed by adding polysaccharides. The polysaccharide does not interact with the adsorbed biopolymer, but it increases the viscosity of the continuous aqueous phase. In the process of emulsification, the protein tends to create small droplets. In contrast, the polysaccharide produces a more robust and compact network (Paraskevopoulou *et al.*, 2005), stabilizing the A/O/W emulsion. The main objective of this study was to explore the potential of A/O/W double emulsions as potential fat substitutes in food applications. To

achieve this, a series of double emulsions were prepared by systematically varying the concentrations of sodium caseinate (5, 8, and 10 wt. %) and kappa carrageenan (0.4 and 0.8 wt. %) within two different levels of oleofoam (20 and 25 wt. %). The study was focused on investigating the impact of these three factors on various properties of the double emulsions, including their microstructure, bubble size distributions, rheological behavior, and thermal characteristics. By analyzing these properties, we aimed to gain scientific insights into the suitability of these double emulsions as potential fat substitutes in food products.

Materials and Methods

Materials

Distilled monoglyceride (MG) was obtained from Palsgaard, with a melting point of 65 °C. The sunflower oil used in the study was purchased from the local market in Iran and has a relative density of 0.918 and a viscosity of 58 cP at 20°C. Dodecyl sulfate (SDS) (C12H25O4SNa), Tween 80, and sodium azide (NaN3) were procured from Sigma-Aldrich, France. Sodium caseinate (SC) from the Dutch DMW Company and kappa-carrageenan (KC) from Negin Khorak Pars in Iran were also obtained for the research.

Oleofoam formation

Monoglycerides are amphiphilic molecules (they have both hydrophilic and hydrophobic regions). The glycerol backbone is hydrophilic, while the fatty acid chain is hydrophobic. This unique structure allows monoglycerides to interact with different components in food systems. They also can form a protective layer around air bubbles, preventing coalescence and improving the stability of foams and aerated products (Binks & Vishal. 2021). The oleofoams were prepared by dissolving 10 wt. %of monoglyceride (MG) in sunflower oil at 80°C for 5 minutes and then cooling it to room temperature. The resulting mixture was whipped at two different temperatures (25°C and 5°C) using a 5-speed mixer (Gosonic, model No. GHM-818, 250W, China) at the highest speed for 30 minutes (Saremnejad *et al*., 2019).

Oleofoam characterization

The oleofoam was observed using an Olympus BX41 microscope (Olympus BX41, Japan) equipped with a digital camera (Canon EOS 1000D); then, about 500 bubbles from three pictures were used to measure the bubble size and distribution using image analysis software (ImageJ 1.50f for Windows). Foamability parameter was measured in the production time using Eq (1) (Saremnejad *et al*., 2019). The over-run of the oleofoam is calculated using Eq. (2) (Liu *et al*., 2021).

Foamability = (The volume of foam / the initial volume of liquid) × 100 (1)

$$\% \text{ Overrun} = \frac{(\text{Voil+air})-(\text{Voil})}{(\text{Voil})} * 100 \quad (2)$$

Where (V_{oil}+V_{air}) equals the volume of oil plus entrapped air bubbles, (V_{oil}) is the volume of the oil which was warmed to (80 ± 2°C) in order to fully release air bubbles.

Oil drainage

Oil drainage from oleofoam samples was measured after storage at 25°C for 1 h, 24 h, 72 h, 7 days, and 28 days. Samples of approximately 10 mL were placed in a centrifugal tube. After each storage period, the amount of drained oil was measured in triplicate and calculated using Eq. 3 (Alhasan *et al*., 2023).

Oil drainage (%) = V (drained oil)/ V (oleofoam) x 100 **(3)**

A/O/W double emulsion formation

To prepare sodium caseinate (SC) emulsifiers, controlled concentrations of emulsifiers 5%, 8%, and 10% were dissolved in water containing sodium azide via magnetic stirring at room temperature. Kappa-carrageenan (KC) with percentages of 0.4% and 0.8% were dissolved in distilled water at 8۵°C using magnetic stirring for 30 minutes. The resulting solutions were stored for 12 hours at 5°C to complete hydration. The A/O/W double emulsion was prepared by combining oleofoam solutions with different concentrations of 10

%MG (20% and 25%) prepared at a controlled temperature of 5°C. The oleofoam solutions were mixed with the aqueous phase (SC-KC) in a 1:1 weight ratio, following the method described by (Brun *et al*., 2015) and (Qiu *et al*., 2021) with modifications. The mixed solutions were then sheared using a digital high-shear mixer dispersion; It must be noted that a cylindrical probe with a diameter of 59.7 mm (bob) inside another cylinder (cab) with a diameter of 65.7 mm was used to achieve proper emulsion formation and maintain the integrity of the multiple structures at a speed of 1500 rpm for 60 se. at 25°C to reduce the droplet size in the final multiple A/O/W emulsions. After shearing, the resulting emulsion was promptly refrigerated for further analysis.

A/O/W double emulsion characterization

Encapsulation yield of air bubbles

The total content of oil droplets containing air bubbles was determined after mixing using Eq. (4) (Goibier *et al*., 2019).

$$EY = \frac{V1-V2}{V\,foam \times overrun} \times 100 \qquad (4)$$

Where V1 is the volume of the emulsions after centrifuging, V2 is the volume of the emulsion in the absence of air bubbles, and V foam is the foam volume initially introduced in the double emulsion.

Optical microscope observation

The globule size distribution of A/O/W emulsions was measured using a Laser Particle Sizer ANALYSETTE (Fritsch, Germany), and the optical microscope utilized in the study was an (Olympus BX41, Japan), which was equipped with a Canon EOS 1000D digital camera. Photomicrographs were captured to visualize the oil droplets containing air bubbles in the A/O/W emulsions. Certain emulsions had high viscosity, necessitating their preparation by previous research (Goibier *et al*., 2019).

Thermal Stability

The thermal stability of the A/O/W emulsions was analyzed according to the method of Liu *et al*. (2021) with slight modifications. Ten mL of each sample (F0) of The A/O/W emulsions were then transferred into tightly sealed tubes with plastic caps and maintained at 75 °C for 5 min. The oil that separated from the emulsion (F1) was subsequently measured. The stability of the emulsion was calculated using the following equation (5):

$$\text{Stability} = (F1 / F0) \times 100 \qquad (5)$$

Rheological properties

A Bohlin rotational viscometer (Bohlin Model Visco 88, Bohlin instruments, UK) was employed to evaluate the rheological properties of the samples. The measurements were conducted at a constant shear rate of 50 (s-1) and over a range of 14-400 (s^{-1}) at 25 °C.

Statistical analysis

Experiments were performed in triplicate. The collected data were analyzed by one-way analysis of variance (one-way ANOVA). Significant differences in means were compared using Duncan's test at a 5 % significance level using the SPSS 16.0 statistical software (SPSS Inc., Chicago, USA)

Results and Discussion

Oleofoam

Foamability

The foamability of oleogels, prepared with a 10% of MG at 25°C and 5°C, was evaluated in this study. Fig. 1, Demonstrates that the sample prepared at 5°C exhibited higher formability and overrun percentage compared to the sample prepared at 25°C throughout the preparation process. This indicates that the formability and overrun of the oleogels were significantly influenced by the temperature of the preparation. Lower temperature has been observed to have a stabilizing effect on air bubbles in oleofoams, as it leads to higher solid fat content. Mishra *et al*. (2020) reported that at lower temperatures, specifically 5°C, there was an increase in overrun. This increase in overrun can be attributed to the formation of α crystals, which subsequently undergo a transformation into β crystals during the aeration process.

These findings highlight the role of temperature in influencing the crystal structure and stability of oleofoams. In this study, both samples demonstrated high overrun, with the sample prepared at 5°C exhibiting an overrun value of 140%, higher than the other sample which had an overrun value of 120%. This result indicates that lower temperatures contribute to an increased overrun, likely due to variations in crystal number and shape within the continuous oil phase, ultimately enhancing foaming ability (Himawan *et al.*, 2006). These findings align with the results of Liu & Binks (2021), who investigated the production of oleofoam using olive and peanut oils, and found that the lowest temperature yielded the highest foamability. Similarly, Lei *et al*. (2020) achieved an overrun of approximately 70% for a whipped 10 wt% DAG-based oleogel. They accomplished this by rapidly cooling the sample from 80 ± 2°C to 5 ± 2°C, followed by gradual heating to 25°C.

The whipping time also influences the foamability and overrun of the oleofoam. The foam volume significantly increased in the first 5 minutes of the total aeration time (30 minutes) due to the high adsorption of MG crystals at the oil-air interface, allowing for the incorporation of more air. However, an excessively long whipping time for the sample prepared at 5°C resulted in decreased overrun. This occurrence can be attributed to the shearing forces encountered during the mixing process at high viscosity. These shearing forces have the potential to rupture the bubble film formed by the MG, leading to the collapse of the bubbles. The components of the MG film are crucial for providing structural stability and support the air bubbles within the matrix. Damage to these film components can ultimately result in the collapse of the foam. Similar results have been reported for the whipping of sunflower oleofoams prepared with different concentrations of mono and diglyceride (Saremnejad *et al*., 2019) and high-oleic acid sunflower oleofoams prepared with myristic acid at different concentrations (Liu & Binks, 2021).

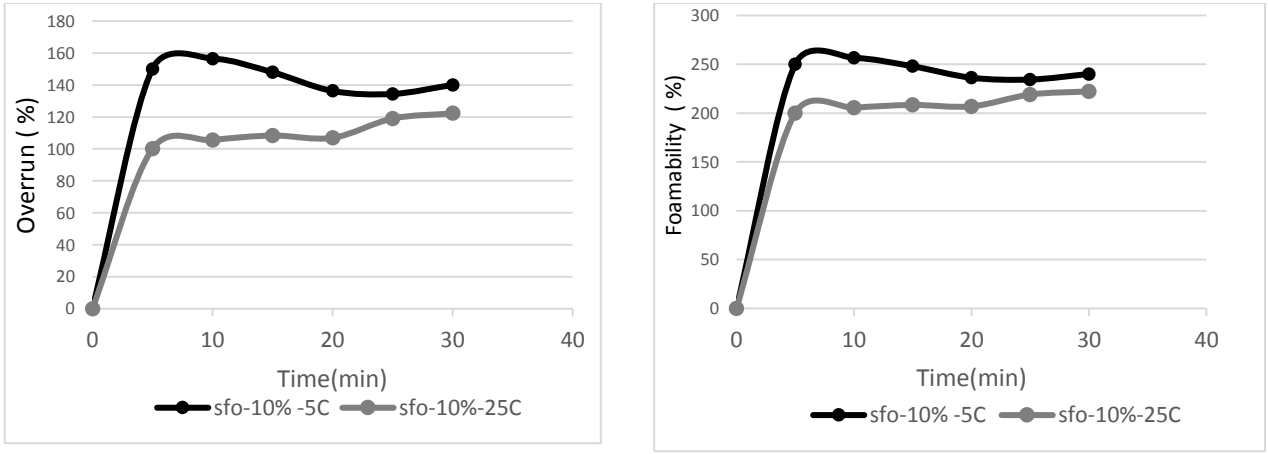

Fig. 1. Variation of the overrun and formability of the oleofoam based on monoglyceride (MG) measured each 5 min during the aeration

Optical microscope oleofoam observation

In the oleofoam micrograph (Fig. 2a and b), most bubbles are spherical due to the adsorption of surfactant crystals that hindered shape relaxation, in line with the findings of (Saremnejad *et al*., 2019; Liu & Binks, 2021). The two systems exhibited different size distributions due to variations in their preparing temperatures. As the temperature decreased, a narrower distribution and smaller bubble sizes were observed. The average bubble size in the sample prepared at room temperature was 20 µm, which decreased to 10 µm in the samples prepared at low temperature, which might be

attributed to several factors. Firstly, at lower temperatures, the viscosity of the oleofoam increases (Wildmoser et al., 2004). This higher viscosity restricts the mobility of the air bubbles, preventing their coalescence and leading to a narrower size distribution. The increased viscosity also hinders the growth of bubbles, resulting in smaller bubble sizes. Secondly, the higher solid fat content at lower temperatures promotes the stabilization of air cells within the oleofoam matrix. The solid fat acts as a structuring agent and are thus surface-active (Binks & Marinopoulos, 2017) providing stability and support to the air bubbles. This structural stability helps to maintain the integrity of the bubbles, preventing them from merging or collapsing and contributing to the smaller bubble size and narrower distribution. Lastly, the slower diffusion of gas at lower temperatures may also contribute to the formation of smaller bubbles. The reduced diffusion rate limits the expansion of the bubbles, resulting in smaller sizes. Overall, the combination of higher viscosity, solid fat content, and slower gas diffusion at low temperatures contributes to the observed narrow distribution and smaller size of bubbles in the oleofoam, the effect of temperature production on particle size or distributions has not been studied yet.

Fig. 3 Indicates microscopic images of oleofoam samples at different temperatures. It shows an apparent decrease in bubble size as the temperature decreases which caused more stable oleofoam due to the fact that at lower temperatures, the interfacial tension between the foam bubbles and the surrounding medium increases, making it more difficult for the droplets to coalesce and merge. As a result, the overall particle size of the emulsion tends to decrease.

Oleofoam stability

For production of double emulsion, the oleofoam must exhibit high stability against oil loss. The foam stability as a function of production temperature at 25°C is shown in Fig. 4. Samples prepared at 25°C showed oil drainage within less than 24 hours, which continued for 28 days. The most stable foam was obtained from samples prepared at 5°C. This can be attributed to the size and homogeneity of the initial bubble size distribution, which play a crucial role in the stability of oleofoam.

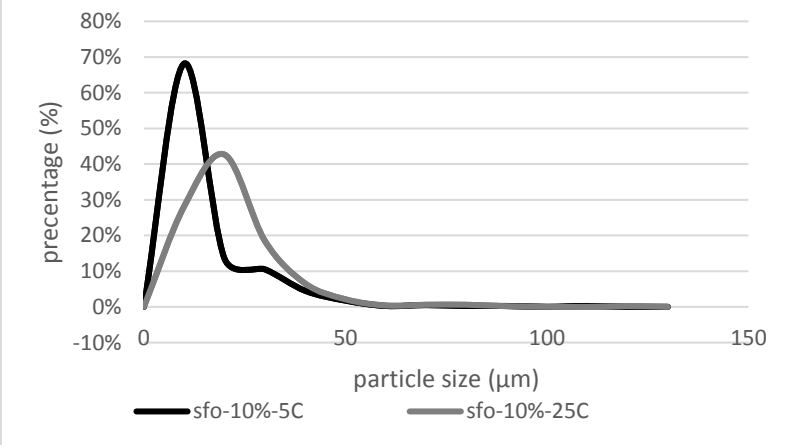

Fig. 2. Particle size distribution profiles of the 10% monoglyceride (MG) oleofoam

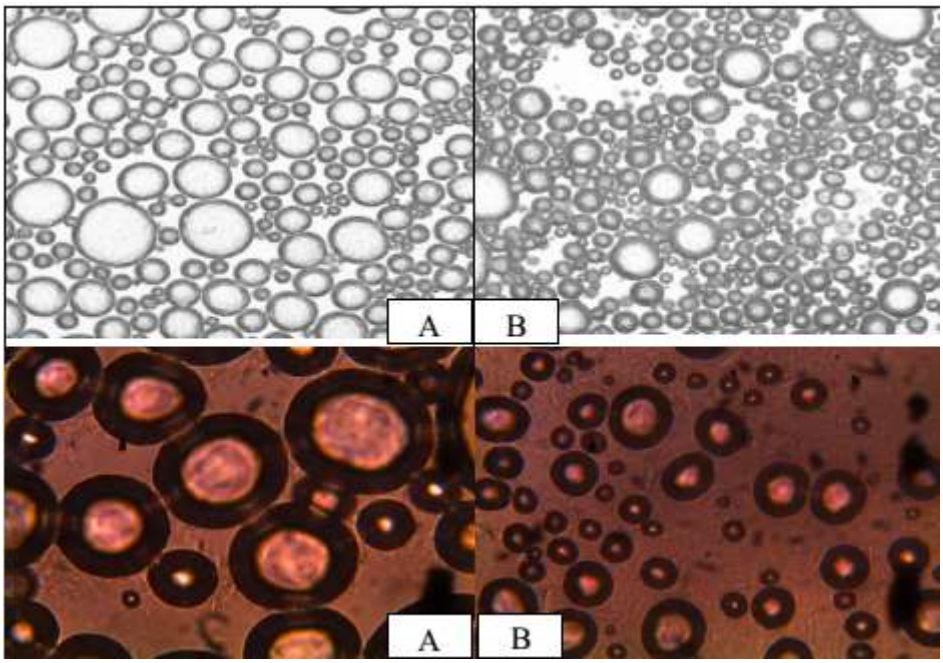

Fig. 3. Optical micrograph about the effect of temperature on particle size in sunflower oleofoams (a) at 25°C (b) at 5°C scale bars = 60µm (upper pictures) and (lower); polarized optical microscope image (a) at 25°C (b) at 5°C scale bars = 5µm

Small bubbles can be perfectly surfaced with a dense layer of MG crystals to protect them from coalescence and Oswald ripening (Callau *et al*., 2020). Additionally, low temperatures enhance the formation of small bubbles, and this finding is consistent with study of *Callau et al*. 2020, which showed that fast cooling can decrease the size of bubbles and increase their stability against oil loss. Also, Binks *et al*. (2016) discovered that the formation of stable foams was restricted to lower temperatures (22 °C and 30 °C) when utilizing crystal dispersions. Conversely, when employing molecular solutions, no foam formation was achievable at elevated temperatures (35 °C and 40 °C).

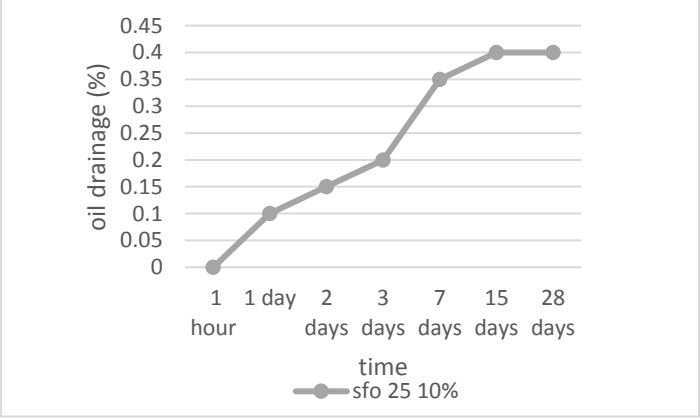

Fig. 4. Oil loss of oleofoam produced at 25 C during the aging time

A/O/W double emulsion formation

Generally, the oleofoam sample prepared at low temperatures showed higher overrun value, smaller bubble size, and oil drainage resistance; thus, according to these results, it was selected for preparing the A/O/W double emulsion for further studies.

Air encapsulation efficiency after A/O/W double emulsion formation

The results shown in Fig. 5 indicate that in the emulsion system, the level of sodium caseinate and kappa carrageenan significantly affects the amount of air encapsulated in the emulsion. In contrast, the level of oleofoam was not effective in this system. However, encapsulated air bubbles in all emulsions ranged from 87.8 to 117.3%. In comparison with other studies, it was relatively higher due to the high amount of air (~140 ٪) entering the system from the oleofoam. The amount of encapsulated air increased with an increase in the level of sodium caseinate. For example, in 20% oleofoam concentration with the presence of 8.0% kappa carrageenan, the level of air encapsulation increased from approximately 105% to ~111%, and in 25% oleofoam concentration, it increased from approximately 110% to ~117%. This is because the droplets of the external oil in the A/O/W emulsion were subsequently stabilized by using sodium caseinate as an emulsifying agent, and an increase in concentration resulted in an increase in the number of encapsulated globules (Goibier *et al.*, 2019). Generally, this natural surfactant covers the surface of oil droplets and prevents their coalescence in the continuous aqueous phase (Thanh Diep *et al.*, 2018). Lu and colleagues examined the effects of sodium caseinate and acetylated mung bean starch on encapsulating lutein. They reported that increasing the amount of sodium caseinate resulted in increased lutein in the microcapsules (Lu *et al.*, 2021). Goibier and colleagues used sodium caseinate (12%) as an emulsifier to form stable emulsions (Goibier *et al.*, 2019).

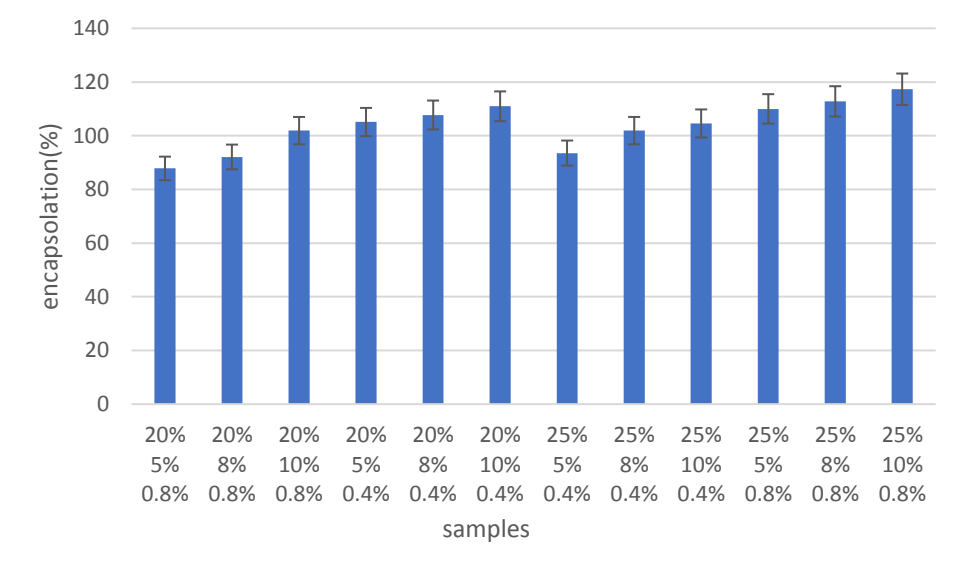

Fig. 5. Air encapsulation efficiency of (A/O/W) double emulsions

The results indicate that at different concentrations of kappa-carrageenan (0.4%, 0.8%), the amount of encapsulated particles containing air bubbles increases with an increase in sodium caseinate (i.e., it has a synergistic effect) because of the protein-polysaccharide interaction, which formed a more bulky polymeric layer at the interface. Furthermore, Kappa-carrageenan as a thickening agent can serve as a suitable supporting additive to limit the phenomenon of flocculation and coalescence since it can increase the viscosity and cause better steric stabilization. Similarly, O'Regan & Mulvihill (2010) reported the combined effect of introducing polysaccharides into W/O/W emulsions. Specifically, they incorporated vitamin B12 into W/O/W emulsions utilizing

gelatin to solidify the inner water phase, medium chain triglyceride oil as the oil phase, PGPR as the lipophilic emulsifier, and either sodium caseinate or sodium caseinate-maltodextrin conjugate as the hydrophilic emulsifier. Their investigation revealed that the encapsulation efficiency of the double emulsion stabilized with sodium caseinate-maltodextrin surpassed that of the sodium caseinate-stabilized emulsion.

Particle size

The size of particles present in emulsions has a crucial role in their stability, accessibility, biological activity, and physical and sensory properties. Smaller particle sizes lead to more desirable properties. The study results presented in Table 1**,** demonstrate that the percentage of sodium caseinates and kappa carrageenan significantly affects the size of oleofoam droplets in the A/O/W double emulsion. However, the percentage of oleofoam did not significantly affect the final particle size.

The largest particle size (56.659 ± 4.20) micrometers was observed in the emulsion with the lowest percentage of sodium caseinates and kappa carrageenan. The smallest particle size (1.73 ± 0.1) micrometers was observed in the sample containing 20% oleofoam, 10% sodium caseinates, and 0.8 % kappa carrageenan. This A/O/W double emulsion can be considered a fine multiple emulsion because the particle size is between (1-3 µm) (Aserin, 2007).

Table 1- Effect of oleofoam, SC, and KC percentage on the droplet size of A/O/W double emulsion

Samples	D 3,4(µm)
sfo20%, SC 5%, KC 0.4%	50.904 ± 5.62a
sfo20%, SC 8%, KC 0.4%	35.434 ± 2.60b
sfo20%, SC 10 %, KC 0.4%	24.756 ±2.30c
sfo20%, SC 5%, KC 0.8 %	12.705 ±1.30d
sfo20%, SC 8%, KC 0.8%	6.606 ±0.30e
sfo20%, SC 10%, KC 0.8%	1.729 ±0.01f
sfo25%, SC5%, KC 0.4%	56.659 ±4.20a
sfo25%, SC8%, KC 0.4%	33.940 ±2.03b
sfo25%, SC10%, KC 0.4%	23.559 ±0.66c
sfo25%, SC5%, KC 0.8%	12.586 ±0.89d
sfo25%, SC8%, KC 0.8%	7.3703 ±0.74e
sfo25%, SC 10%, KC 0.8%	1.886 ±0.17f

[a-f] Different case letters indicate statistically significant ($P < 0.05$) differences among values.
Data represent the mean of duplicate determinations ± standard deviation.

The diameter of the particles significantly decreased with an increase in the percentage of sodium caseinates. For instance, in the sample containing 20%oleofoam and 0.4 % kappa carrageenan, the diameter of particles decreased with the increasing of sodium caseinates from 50.904 ± 5.62 to 24.756 ± 2.03 micrometers. This reduction in particle size distribution is attributed to the increased protective effect of the sodium caseinates film coating at the oil-water interface. This finding is consistent with the results reported by *Lin et al*. (2020); it was found that increasing the percentage of sodium caseinates from 10 to 12% in a water-in-oil-in-water double emulsion containing polyether-modified siloxanes (PMS1) emulsifiers resulted in a decrease in droplet size from 12.2 to 7.7 micrometers. Similarly, Dwyer et al. (2013) observed that increasing the concentration of sodium caseinates from 0.25 to 3 % reduced the mean particle size of oil-in-water emulsion (from 1179 to 325 nm).

As seen in Table 1, an increase in the concentration of kappa carrageenan leads to a reduction in the size of oleofoam droplets in A/O/W double emulsion. Specifically, in an oleofoam containing 5% sodium caseinates, an increase in kappa-carrageenan concentration from 0.4 % to 0.8 % resulted in a significant

decrease in droplet size from 50. 904 ± 5.62 to 12. 705 ±1.30 micrometers. This reduction in droplet size can be attributed to the increased viscosity of the solution and decreased interfacial tension of the emulsion with an increase in kappa-carrageenan concentration. Thanh Diep *et al*. (2018) reported that an appropriate combination of 0.5

% sodium caseinates (SC) and 1% kappa carrageenan (KC) were found to stabilize an oil-in-water (O/W) emulsion with a 4:6 ratio and decrease the particle size to 2.089 ± 0.332 (µm). The interaction between sodium caseinates and kappa carrageenan has a synergistic effect on reducing the droplet size of oleofoam globules in the emulsions due to electrostatic interactions between the negatively charged sulfate groups present in kappa carrageenan and the positively charged amine groups in the sodium caseinates molecule. This results in weak adsorption of polysaccharides onto protein-coated oil droplets, causing the polysaccharides to remain in the continuous phase and increase the viscosity of the emulsion. The competition between these two processes determines the stability of the emulsion. Similarly, Perrechil & Cunha, (2013) showed that an increase in the concentration of sodium caseinates and kappa carrageenan leads to a decrease in the average droplet size of the emulsion.

A/O/W emulsion stability

In order to replace animal fat in food formulations, an A/O/W double emulsion was created. However, it was crucial to consider the thermal stability of this emulsion due to the need for pasteurization of the blended ingredients. This pasteurization process plays a significant role in preserving the quality of the final product. No significant separation was observed at room temperature and 4 °C during 30 days of storage, indicating excellent stability at room and low temperatures.

The results showed in Figure 6 indicate that the percentage of oleofoam used in the preparation of oil-in-water-in-oil (A/O/W) emulsion may be the most essential factor in determining its stability. Specifically, the analysis showed that the foam percentage significantly affects emulsion stability, with samples containing higher percentages of oleofoam and lower levels of sodium caseinates and kappa carrageenan exhibiting the highest thermal instability. The observed thermal instability is attributed to the inadequate levels of sodium caseinates and kappa carrageenan, which lead to the accumulation of oleofoam droplets. The droplets contain air bubbles are irregularly shaped and relatively large. This irregular shape is inherent to the foam's firmness and, during heating, causes the oil droplets containing the air bubbles to coalesce rapidly due to insufficient coverage. The monoglyceride crystals at the oil-air interface have reached their melting point, causing the oil to separate from the system. However, with an increase in the levels of sodium caseinates and kappa carrageenan, the coverage of the air-containing oil droplets increases and becomes more robust as the size of the globules is smaller. This prevents the oil from being released outside the system. Similarly, Lei *et al.* (2019) reported on the effect of heating on the thermal stability of oleofoam, stating that when the temperature reaches 60°C, the entire oleofoam is converted to a liquid state due to the melting of monoglyceride crystals at the oil and air interface in the continuous phase. It should be noted that in oleofoam, where the only emulsifying agent is fat crystals, the degree of instability is much higher than the results presented in this study.

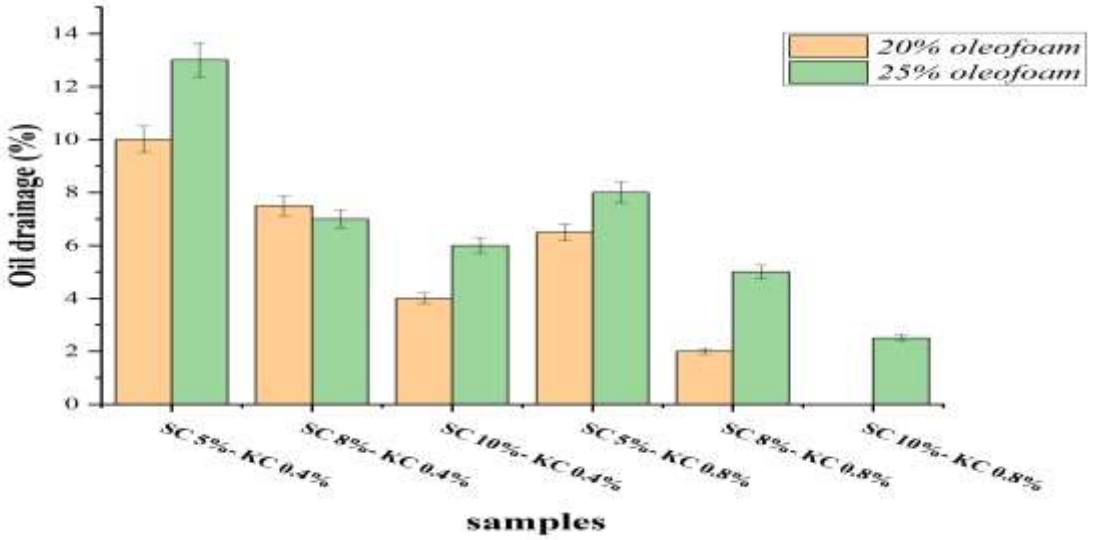

Fig. 6. Thermal stability (%) of A/O/W double emulsions

The percentage of sodium caseinate significantly affected thermal stability, as an increase in its percentage led to an increase in thermal stability. Increasing the percentage of sodium caseinate from 5% to 10% in samples containing 20% oleofoam and 0.8% kappa carrageenan increased stability and decreased oil separation from 6%to zero. The enhancement of stability was observed with an increase in sodium caseinate percentage, attributed to the reduction in size of air-containing oil droplets at higher concentrations of sodium caseinate. Additionally, a greater coverage of these oil droplets was evident at elevated levels of sodium caseinate. According to literature, the emulsions stabilized by caseinate tend to exhibit superior thermal stability due to the unique structure of this protein, which is resistant to conformational changes caused by heating. The combination of electrostatic and steric mechanisms of stabilization offered by sodium caseinate effectively protects emulsion droplets at neutral pH conditions (Sharma *et al*., 2017). Li *et al*. (2017) examined the thermal stability of multiple emulsions containing different protein and polysaccharide combinations at 60 C. They found that adding protein compounds improved the stability of the emulsion, but the amount added was observed to be a more significant factor. As a result, increasing the percentage of sodium caseinate from 0.2% to 1% increased the emulsion compounds' stability. These findings agree with the present study, indicating that the percentage of sodium caseinate plays a crucial role in improving the thermal stability.

Adding kappa-carrageenan increased the emulsion's stability, as emulsions with higher concentrations of kappa-carrageenan were found to be more stable because kappa-carrageenan increases the viscosity of the emulsion and forms small-sized globules. The results showed that with an increase in the percentage of kappa-carrageenan from 0.4% to 0.8%, the stability of the emulsion containing 20% oleofoam and 10% sodium caseinate increased from 95%to 100%. According to the report by Zhao *et al*. (2015), a three-dimensional network structure was formed mainly from carrageenan in the carrageenan-protein system. Similarly, Perrechil & Cunha (2013) reported the successful production of multiple emulsions using surface complexes of polysaccharide (kappa-carrageenan) and protein (sodium caseinate). They demonstrated that high concentrations of kappa-carrageenan led to the production of stable emulsions under neutral and acidic conditions. These findings suggest that kappa-carrageenan can effectively stabilize multiple emulsions.

Statistical results indicate a synergetic effect between kappa-carrageenan and sodium

caseinate, whereby an increase in the concentration of both compounds positively impacts stability. This is due to the surface molecular interaction between casein and kappa-carrageenan, as reported by Tang *et al*. (2019), who found that adding kappa-carrageenan improved the thermal stability properties of casein.

Optical microscopy

The particle size of a prepared A/O/W emulsion was examined to confirm the proper encapsulation of the oleofoam within the internal coating material. Microstructures of the samples were visualized immediately after emulsion fabrication for this purpose and shown in Fig. 7a and b. The micrographs showed that large irregular particles were observed in the double emulsion with a lower percentage of sodium caseinate and kappa carrageenan (Fig. 8a and b) due to the intrinsic firmness of the oleofoam and low viscosity, which did not allow total shape relaxation. Conversely, with an increase the percentage of sodium caseinate and kappa carrageenan, smaller and uniformly size particles were observed in the double emulsion (Fig. 8a and b), indicating that this protein can act as a robust emulsifier at the interface of oil and water by reducing the interfacial tension. Moreover, sodium caseinate can generate electrostatic and steric repulsive forces between oil droplets, thereby hindering their proximity. According to Stokes' law, smaller oil droplets experience less resistance as they are more efficiently dispersed in the continuous phase, leading to slower coalescence and the formation of larger droplets or delayed flocculation (Mollakhalili Meybodi *et al.*, 2014). Sodium caseinate and kappa carrageenan can form a protective layer around the oil droplets, preventing them from coming into close contact and minimizing the potential for aggregation. This steric stabilization is mainly attributed to the large size and complex structure of the protein, which creates a physical barrier between the droplets. Electrostatic regulation involves the repulsive forces generated between charged particles. Sodium caseinate, as a protein, carries a net negative charge due to the presence of ionic groups, such as carboxyl and phosphate groups. Kappa carrageenan, on the other hand, is a sulfated polysaccharide and also carries a negative charge. The negative charges on both sodium caseinate and kappa carrageenan molecules create electrostatic repulsion between the oil droplets, preventing their close proximity and reducing the likelihood of coalescence. Generally, smaller droplet size offers more interfacial surface area, which increases the emulsifier's absorption capacity and increases the emulsion stability.

In the current study, smaller oil droplet size (Fig. 7a and 8a) was achieved using a relatively low concentration of emulsifier (sodium caseinate) and thickener (kappa carrageenan) compared to previous studies conducted by Goibier *et al.* (2019) and Burn *et al*. (2015). Goibier *et al*. (2019) utilized oleofoam-containing oil and dehydrated milk fat with an aeration rate of 30% and sodium caseinate (12%) as an emulsifier to prepare A/O/W emulsion. The emulsion was stabilized using a 10% hydroxyethyl cellulose solution. The droplet size obtained at the highest shear speed (7350/s) was approximately 10 micrometers. Similarly, Burn *et al*. (2015) prepared two A/O/W emulsions with 50 vol. % oleofoam, 5 wt% sodium caseinate (emulsifier), and 1 wt% hydroxyethyl cellulose (thickener). The average diameters of the air bubbles and oil droplets were 10 and 100 micrometer, respectively.

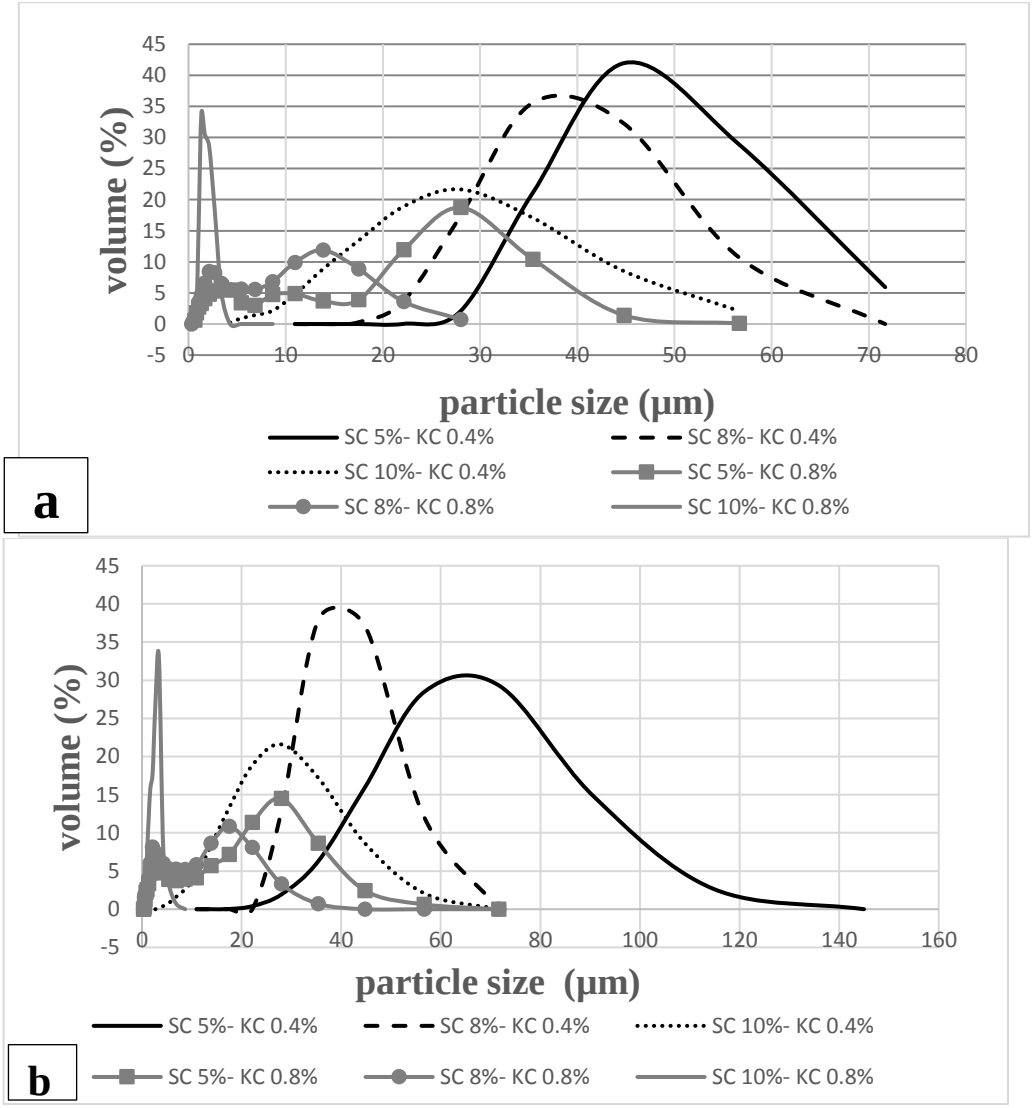

Fig. 7. Droplet size distribution of oil containing air in A/O/W double emulsion with a concentration of 20% foam(a),Particle size distribution of oil droplets containing air in A/O/W ouble emulsion with a concentration of 25% foam(b)

Rheological properties

Flow curves were obtained by an up-down-up steps program with the shear rate varying between 10 and 400 s-1 (Fig. 9a and b). Flow curves exhibited different behaviors depending on the emulsion fractions (percentage of oleofoam, sodium caseinate, and kappa carrageenan).

The results obtained for the relation between viscosities of oleofoams and shear rates showed that the apparent viscosity of the A/O/W emulsion oil decreases with increasing shear rate. This behavior is consistent with the shear thinning property of emulsions, which is usually observed in complex fluids such as colloidal suspensions. The emulsion had a high viscosity at low shear rates, indicating that the oil droplets were tightly packed together. However, with increasing shear rate, the droplets were forced to align and slide past each other, leading to a decrease in viscosity, which may be related to the deformation of oleofoam particles, destruction of air bubbles, and weak interactions between them under the influence of shear forces. This drop increases with increasing shear rate, indicating the dependence of viscosity on shear rate.

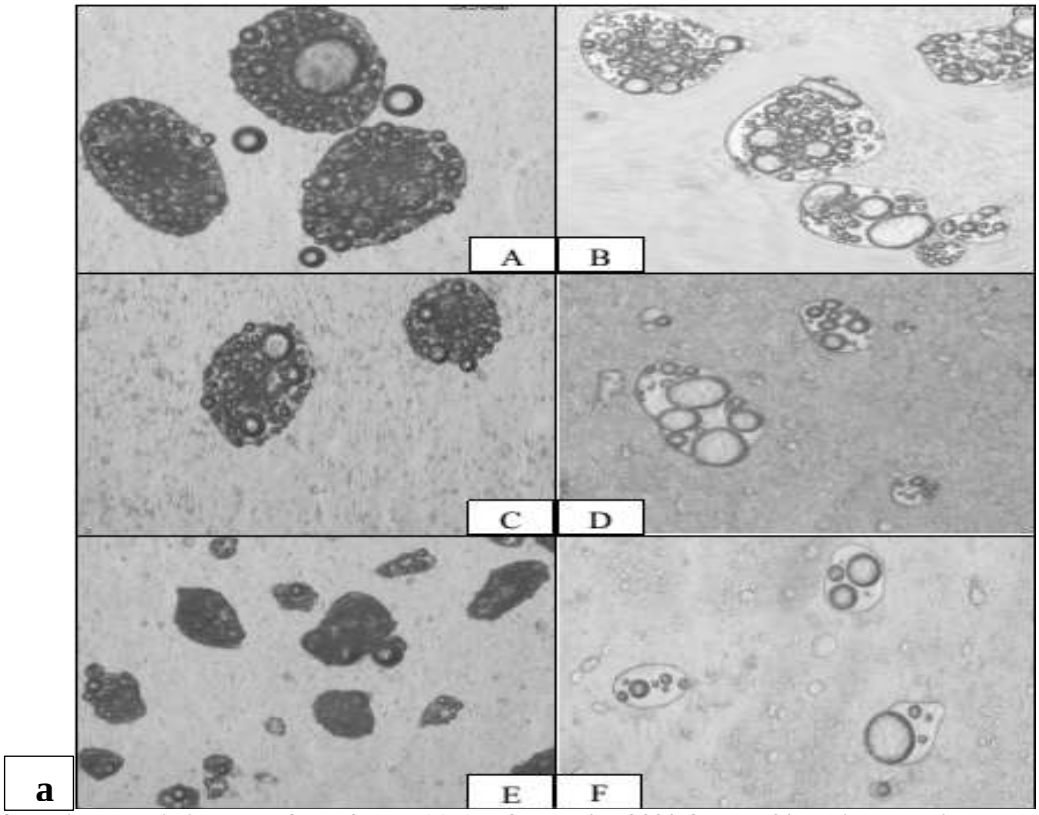

Fig. 8a. Microscopic images of oleofoams (a) oleofoam with 20% foam, 5% sodium caseinate, and 0.4% kappa carrageenan, (b) oleofoam with 25% foam, 5% sodium caseinate, and 0.4% kappa carrageenan, (c) oleofoam with 20% foam, 8 % sodium caseinate, and 0.4% kappa carrageenan, (d) oleofoam with 25% foam, 8% sodium caseinate, and 0.4% kappa carrageenan, (e) oleofoam with 20% foam, 10% sodium caseinate, and 0.4% kappa carrageenan, (f) oleofoam with 25% foam, 10% sodium caseinate, and 0.4% kappa carrageenan(b)

The flow curves showed the emulsions with high percentage of oleofoam (25%) were more viscous than those with 20%, which could be mostly related to high viscosity in oleofoam which was favorable for the gel-network formation and limited the movement of air bubbles (Fameau *et al.*, 2015). Increasing oleofoam fraction increases the monoglycerides percentage in the emulsion which traps oil droplets and increases resistance to shear. Liu *et al.* (2017), who investigated the effects of monoglycerides on the properties of protein-stabilized emulsions containing isolated whey protein and encapsulated β-carotene, reported that increasing the percentage of monoglycerides from zero to 2% led to an increase in emulsion viscosity, Davis *et al.* (2000) also reported for sodium caseinate-stabilized emulsions. Qiu *et al.* (2021) reported that the viscoelasticity of the A/O/W emulsion was higher than that of the aqueous phase, indicating foam mixing in the emulsion slightly increased the stiffness of the mixture.

In general as seen in Fig. 9a and b, with an increasing percentage of kappa carrageenan, the viscosity of the samples increased in both oleofoams concentrations. The highest viscosity was observed in samples containing 0.8% kappa carrageenan; for example, in the oleofoam containing 20% and 5% sodium caseinate with 0.4%kappa carrageenan (5.38E+00 Pas) was less than the sample containing 5% sodium caseinate with 0.8% kappa carrageenan (8.92E+00 Pas), which could be caused by the higher concentration of free polysaccharides in the aqueous phase.

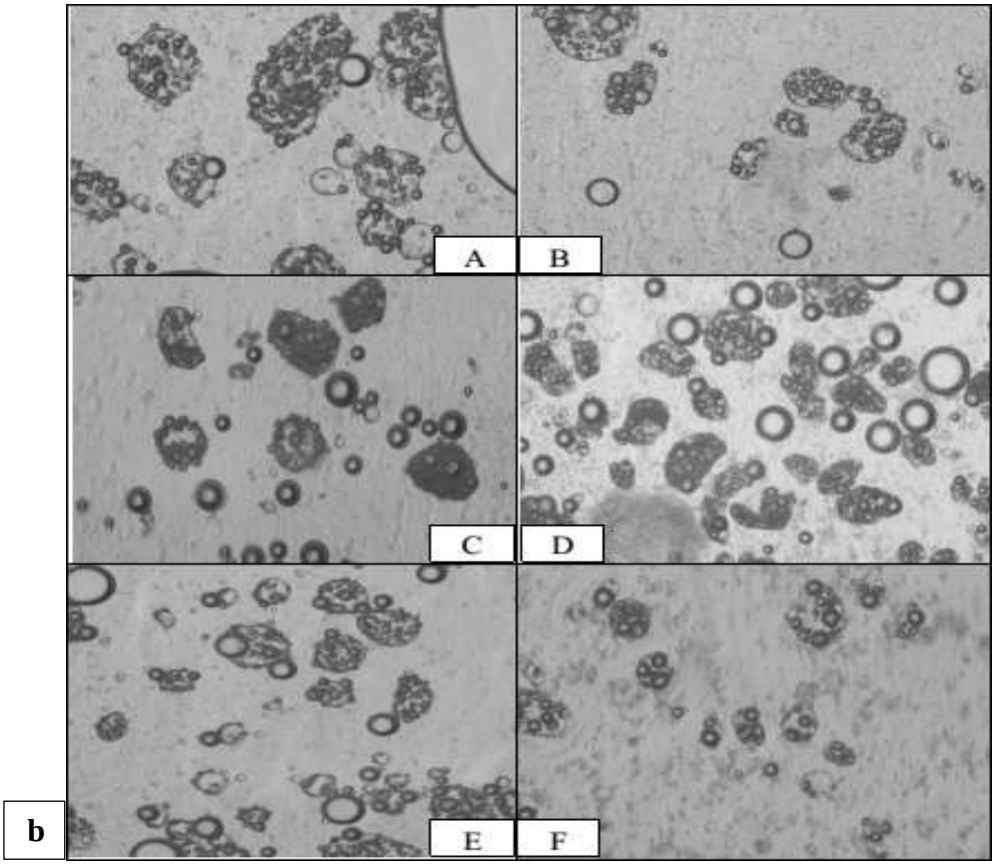

Fig. 8b. Microscopic images of oleofoams (A) oleofoam with 20% foam, 5% sodium caseinate, and 0.8% kappa carrageenan, (B) oleofoam with 25% foam, 5% sodium caseinate, and 0.8% kappa carrageenan, (C) oleofoam with 20% foam, 8 % sodium caseinate, and 0.8% kappa carrageenan, (D) oleofoam with 25% foam, 8% sodium caseinate, and 0.8% kappa carrageenan, (E) oleofoam with 20% foam, 10% sodium caseinate, and 0.8% kappa carrageenan, (F) oleofoam with 25% foam, 10% sodium caseinate, and 0.8% kappa carrageenan (b)

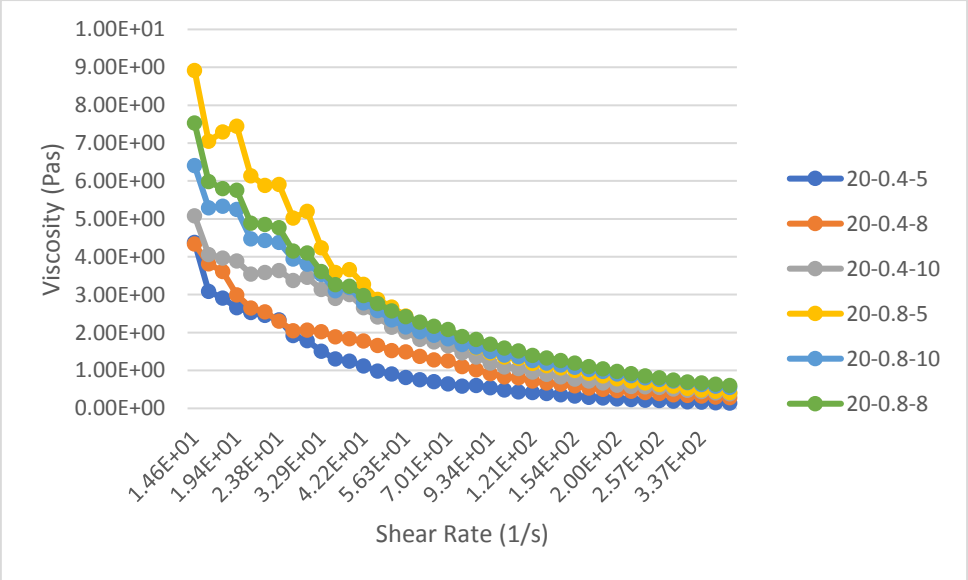

Fig. 9a. Flow curves of A/O/W double emulsion samples containing 20% oleofoam with different concentrations of sodium caseinate (5, 8, and 10%) and kappa carrageenan (0.4 and 0.8%)

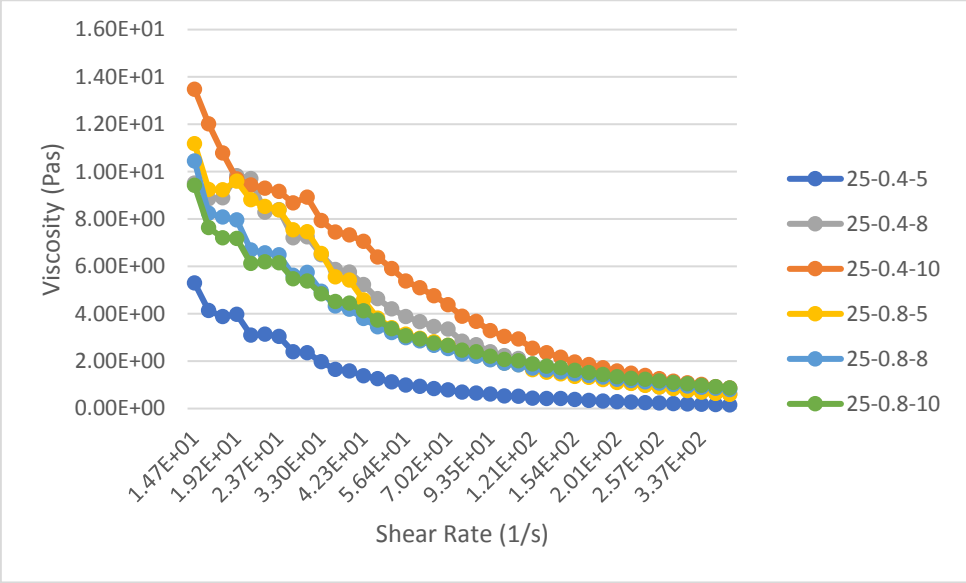

Fig. 9b. Flow curves of A/O/W double emulsion samples containing 25% oleofoam with different concentrations of sodium caseinate (5, 8, and 10%) and kappa carrageenan (0.4 and 0.8%)

These polysaccharide compounds have large hydrophilic groups and absorb much water, and increasing their percentage leads to an increase in the number of hydrophilic groups and more water absorption. In the presence of kappa carrageenan, the emulsion was significantly pseudoplastic, meaning that its apparent viscosity decreased with increasing shear rate and exhibited shear-thinning behavior.

Borsella *et al.* (2013) reported that increasing the percentage of kappa carrageenan at neutral pH in a multiple emulsion containing sodium caseinate led to an increase in viscosity. As seen in Fig. 9a and b, increasing the concentration of carrageenan has more significant effect on viscosity compared to the ratio of caseinate, and the highest viscosity is observed at higher concentrations of kappa carrageenan because sodium caseinate acts as an emulsifying agent and is responsible for the emulsification in this system. The same results reported by Perrechil *et al.* (2020) who investigated the effect of kappa carrageenan concentration on the rheological properties of beta-carotene encapsulation in multilayer emulsions containing SC at neutral and acidic pH.

Regarding the effect of sodium caseinate percentage on emulsion viscosity, the results have shown that there have been two behaviors based on particle size and percentage. In samples containing 0.4% kappa carrageenan, increasing the sodium caseinate percentage increases the viscosity because the globule size is large in this concentration, and they require a low amount of sodium caseinate for coverage, while the rest of the sodium caseinate absorbs water and increases the viscosity. However, at higher concentrations of 0.8 % kappa carrageenan, the emulsion globule size decreases with increasing sodium caseinate percentage. Which sodium caseinate on the rheological behavior of different suspensions containing 20% oleofoam was smaller for the systems containing 25% oleofoam. This demonstrates the mutual effect between particle size and emulsion viscosity.

The fitting of rheological models reflected only the transient behavior of the samples and not their intrinsic properties, as Wei *et al.* (2018) reported. The power law model could fit the curves of oleofoams containing high percentages of sodium caseinate and kappa carrageenan, whereas the curves of other samples could not be fitted to any rheological model. This suggests that these samples exhibit unstable behavior and undergo changes in rheology at specific applied shear rates.

Conclusion

This study aimed to evaluate the production of A/O/W double emulsion using a combination of proteins as emulsifiers and polysaccharides as thickeners with oleofoam. Initially, the oleofoam was prepared by incorporating air bubbles in a mixture of sunflower oil and MDG at two different production temperatures (5-25°C). The oleofoam prepared at 5°C exhibited higher overrun, smaller bubble size, and more stable foam than the one prepared at 25°C. Thus, it was selected for the preparation of A/O/W double emulsion. The results indicated that an increase in kappa carrageenan and sodium caseinate percentages increased the apparent viscosity, promoted the formation of smaller oleofoam droplets with a narrow distribution, and increased the encapsulated particles containing air bubbles and thermal stability in the A/O/W double emulsion. These findings suggest that A/O/W double emulsion technology could enable the food industry to develop products with enhanced nutritional components, better appeal, and longer shelf-life, they pave the way for developing innovative food products benefiting both the food industry and consumers alike.

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مقاله پژوهشی

جلد ۱۹، شماره ۶، بهمن-اسفند، ۱۴۰۲، ص. ۱۹۵-۱۷۷

بهینه‌سازی نسبت اولئوفوم و پروتئین-پلی ساکارید برای بهبود ویژگی‌های فیزیکوشیمیایی امولسیون دوگانه A/O/W: کاربردهای بالقوه در صنایع غذایی

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تاریخ دریافت: ۱۴۰۲/۰۸/۲۴
تاریخ پذیرش: ۱۴۰۲/۰۹/۲۰

چکیده

تهیه امولسیون دوگانه هوا در روغن در آب (A/O/W) شامل دو مرحله کلیدی است: تشکیل اولئوفوم و پراکندگی اولئوفوم در محلول آبی حاوی پروتئین به عنوان امولسیفایر و هیدروکلوئید به‌عنوان عامل غلیظ‌کننده. این مطالعه با هدف بررسی اثر سطح اولئوفوم و غلظت‌های مختلف نسبت پروتئین-پلی‌ساکارید بر پایداری حرارتی، بازده کپسولاسیون و خواص رئولوژیکی امولسیون دوگانه A/O/W انجام شد. یک اولئوفوم با استفاده از یک امولسیفایر چربی‌دوست مونوگلیسرید مقطر (MG) و روغن آفتابگردان در دمای ۵ درجه سانتی‌گراد با حداکثر پایداری به دست آمد. دو سطح اولئوفوم (۲۰ درصد و ۲۵ درصد وزنی) به محلول آبی حاوی غلظت‌های مختلف کازئینات سدیم (SC) ۵، ۸ و ۱۰ درصد وزنی و کاپا کاراگینان (KC)۰/۴ و ۰/۸ درصد وزنی اضافه شد. نتایج نشان می‌دهد که سطح اولئوفوم به‌طور قابل توجهی بر راندمان کپسولاسیون هوا و اندازه ذرات تأثیر نمی‌گذارد، درحالی‌که نسبت پروتئین-پلی ساکارید می‌تواند به‌طور قابل توجهی بر تمام خواص امولسیون دوگانه A/O/W تأثیر بگذارد. افزایش غلظت کازئینات سدیم و کاپا کاراگینان باعث بهبود پایداری حرارتی و راندمان کپسوله‌سازی شد درحالی‌که به‌طور همزمان اندازه ذرات را کاهش داد. همه امولسیون‌های A/O/W رفتار نازک شدن برشی را در میان طیف نرخ‌های برشی مورد مطالعه نشان دادند که نشان‌دهنده پتانسیل قابل توجهی برای کاربردهای غذایی است.

واژه‌های کلیدی: امولسیون دوگانه، اولئوفوم، نسبت پروتئین-پلی‌ساکارید، ویژگی‌های رئولوژی

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مندرجات

بررسی خصوصیات فیتوشیمیایی پوست ژنوتیپ‌های انار ایرانی و معرفی آن به‌عنوان افزودنی غذایی طبیعی ۱۰۹

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