Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Optimizing the preparation of chitosan-caffeic acid nanogel containing Shirazi thyme essential oil and nisin and investigating the effect of optimized nanogel on the quality of Iranian white cheese.

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ARTICLE INFO

Article History:
Received:2023/7/26
Accepted: 2024/2/3

Keywords:

Stepwise-response surface model;

Optimization; Co-encapsulation;

Chitosan-caffeic acid nanogel;

Iranian white cheese;

Shirazi thyme

DOI: **10.22034/FSCT.21.148.1.**

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ABSTRACT

This study aimed to optimize the formulation of chitosan-caffeic acid nanogel containing Shirazi thyme (*Zataria multiflora*) essential oil (ZEO) and nisin. The independent variables (the concentration of chitosan nanogel, Shirazi-thyme, and nisin) were optimized based on the highest zeta potential and encapsulation efficiency, besides the lowest particle size and IC_{50}(DPPH) values. The results of The Box-Behnken experimental design and Stepwise-response surface model showed the optimal nanogel formulation was as follows: chitosan concentration= 0.4 g; ZEO= 157.1 ppm and nisin= 10.1 ppm. The particle size, zeta-potential, antioxidant activity, and encapsulation efficiency of the optimal chitosan-ZEO-nisin nanogel were 411.39±18.11 nm, 32.90±1.10 mV, 0.79±0.06 mg.mL^{-1}, 71.06-82.69% respectively. Moreover, the addition of optimized nanogel to the Iranian white cheese formulation showed that the treated cheese samples with ZEO and nisin (free or encapsulated in chitosan nanogel) improved the microbial quality of chess. The antimicrobial activity of the ZEO and nisin encapsulated in chitosan-caffeic acid nanogel was higher than a free form of ZEO-nisin. The Coliforms population of cheeses treated with sodium nitrate and chitosan nanogel containing ZEO-nisin was acceptable during 60 days of storage. During the storage period, the most changes in the color and texture (hardness) of the cheese samples were related to the control sample, and the least change was obtained for samples treated with sodium-nitrate and chitosan nanogel ($P < 0.05$). Also, the sensory quality of the sample containing ZEO and nisin was acceptable for the sensory evaluator. The sample containing chitosan nanogel received an acceptable sensory score (>3) during 60 days of storage. In general, the potential of the nanogel in increasing the shelf-life of Iranian white cheese was comparable with sodium nitrate.

[DOI: 10.22034/FSCT.21.148.1]

1. Introduction

Cheese is a nutritious dairy product that is a valuable source of protein, vitamins and minerals (especially calcium and phosphorus). However, cheeses are prone to microbial and chemical spoilage due to their high content of moisture, protein and fat [1]. Adding preservatives has long been one of the easiest ways to increase the shelf life of cheese. Several antimicrobial substances such as niacin, natamycin, lysozyme, hydrogen peroxide, sodium and potassium nitrate, and sorbates are among the most common cheese preservatives. However, the use of synthetic antimicrobials as food preservatives has been gradually recognized as harmful by restrictive food safety regulations and over-recommended consumption due to their toxicity [2]. Among the common preservatives in the dairy industry, nisin is a cationic bacteriocin with antimicrobial effects on various groups of gram-positive bacteria, which are commonly recognized as GRAS.[1] is known [3]. However, studies conducted on rodents have shown the genotoxic effects of nisin and natamycin at concentrations higher than recommended [4]. Therefore, the search for alternative antimicrobial additives is increasing due to increasing consumer concern about synthetic preservatives. On the other hand, since nisin is an antimicrobial compound against gram-positive bacteria, the use of other antimicrobial compounds along with nisin can enhance their effectiveness.

The use of essential oils as natural preservatives in the food industry has been one of the topics of interest to science and food industry researchers in recent years. Phenolic compounds are mainly responsible for the antimicrobial activity of essential oils. In Shirazi thyme, carvacrol and thymol are the most abundant bioactive compounds [5, 6]. The antimicrobial effect of Shirazi thyme essential oil on a wide range of microorganisms such as Gram-positive and Gram-negative bacteria, viruses and fungi has been reported in previous studies [5-7].

The effective compounds of nisin and essential oils can react with food or lose their effectiveness under the influence of environmental conditions. Also, essential oils are generally compounds with a strong smell that can affect the sensory acceptance of the product. To overcome these limitations, encapsulation of bioactive materials is a suitable solution. Nanogels are networks that trap bioactive compounds to increase their efficacy at low concentrations, improve their stability and release [8]. Chitosan is an antimicrobial, environmentally friendly, and relatively inexpensive cationic polysaccharide that is widely used due to its antimicrobial potential (especially against*coliforms* And*Pseudomonas*) can increase the shelf life of cheese. In addition, chitosan does not affect the growth of lactic acid bacteria in dairy products. Also, chitosan can act as a carrier for bioactive compounds due to its significant film/coating capacity [9]. Chemical changes in the structure of chitosan can improve its usability. This can be done through its reactive groups (amino and hydroxyl). Among the compounds used to modify the structure of chitosan, organic acids such as cinnamic acid, gallic acid, caffeic acid, ρ-coumaric and some of their derivatives are suitable options. Because besides being abundant in nature, they include many biological activities. These organic acids can increase the affinity of chitosan with lipophilic substances, such as essential oils, and thus create substances with better antioxidant, antibacterial and antifungal properties [10, 11].

In order to achieve the best chitosan-caffeic acid microparticle containing Shirazi-Nicin thyme essential oil, it seems necessary to optimize the concentration of compounds. Response surface method[2] (RSM) is a nonlinear multidimensional model to understand the nonlinear relationship between different variables [12]. The review of the research background showed that so far no study has been observed on the simultaneous encapsulation of Shirazi thyme essential oil and nisin in chitosan-caffeic acid nanogel. Therefore, in the present study, the ratio of chitosan-caffeic acid nanogel, Shirazi thyme essential oil (ZEO) and nisin was optimized in order to achieve a particle with minimum particle size, highest encapsulation efficiency, free radical inhibition and zeta potential. In the second phase, the effect of the optimal combination of Shirazi thyme essential oil and nisin in free and encapsulated form on the physicochemical, sensory and microbial properties of Iranian white cheese during storage in the refrigerator was investigated.

2- Materials and method

1-2- Materials

About 5 kg of aerial parts of Shirazi thyme (*Zataria multiflora* Boiss.) was collected from Jahrom city (Fars province, Iran) in Shahrivar 1401. Samples in the shade (25 ºC±5) They were dried to 10% moisture. The dried sample was ground (KRUPS GVX231 Expert Burr Grinder, Distrito Federal, Mexico), passed through a 40 sieve and stored in a dark bottle.

Chitosan with low molecular mass (CS) (degree of acetylation 75-85٪ and Da 50000-190000), caffeic acid (CA: 98%),١-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide[3] (EDC: 97% as binding agent[4]) and the microbial culture medium was obtained from Merck, Germany. 1-

1-Generally Recognized As Safe
2-Response surface method
3-1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
4-Coupling agent

[DOI: 10.22034/FSCT.21.148.1]

Hydroxybenzotyrazole[5] (HOBt: 98%) of the companyFluka (Bush SG, Switzerland) was prepared. Cheese starter is also included*Lactococcus lactis* subsp. *of milk*، *Lactococcus lactis* subsp. *cream* was obtained from Danisco Deutschland (GmbH Alemanha, Germany). Other chemicals were laboratory grade and were obtained from Dr. Majalli (Tehran, Iran), Merck (Darmstadt, Germany) and Sigma Chemicals (Sigma-Aldrich Chemical Co St. Louis, USA).

2-2- Shirazi thyme essential oil extraction*Zataria multiflora* (ZEO)

The essential oil of Shirazi thyme plant was extracted with the help of Cloninger in a period of 3 hours. The most important compounds of this essential oil include thymol (46،11%), carvacrol (18،32%), para-simine (8،5%) and gammaterpenine (8،33 %)[6] were detected by GC-MS (Perkin Elmer (PE) Auto System XL, USA). essential oil with the help of Na_2SO_4 It was drained and stored in a dark and cool environment [13].

3-2- Preparation of nisin

Stock solution of nisin by dissolving 1 gram of nisin (Sigma-Aldrich, USA) in 100 mL of hydrochloric acid solution (6،1 = pH, N 0،02) was obtained ($IU.g^{-1}10^4$). This solution with 0 µm filter،45 were filtered. Lower concentrations were prepared by adding distilled water [10].

2-4- Preparation of chitosan-caffeic acid nanogel

Caffeic acid (CA) was attached to chitosan by forming amide bonds through EDC and HOBt. Initially 0،5 chitosan in 50 mL of 2% (v/v) acetic acid solution with HOBt (1،6 grams, 7 mmol،83) under magnetic stirring (500 rpm, 25˚C±5, 24 hours) resolved. EDC solution (1،51 grams; 7 mmol،83) and caffeic acid (1،41 grams; 7 mmol،83) was dissolved in 2 mL of ethanol. This solution was slowly added to the chitosan solution with a sampler and under magnetic stirring at 250 rpm and the stirring continued for 24 hours. The solution was subjected to ultrasound (vCLEAN1-L3 Ultrasonic Cleaner, Tehran, Iran) with a frequency of 40 kHz for 5 minutes at room temperature. The pH of the solution using 1 M sodium hydroxide to precipitate zinc nanogel is 8،5-9،0 was set. This mixture was centrifuged at 6000 rpm for 15 minutes. The precipitated nanogel was washed with distilled water and ethanol to remove impurities. The nanogel was dried at -80°C for 48 hours with a freeze dryer (manufactured by Shiraz University, Iran) [11].

5-2- Encapsulation of Shirazi thyme essential oil and nisin in chitosan-caffeic acid nanogel structure

Shirazi thyme essential oil and nisin were simultaneously encapsulated by CS-CA nanogel. Different concentrations of CS-CA nanogel (0،1، 0،25 and 0،4 g) in 10 ml of acidic solution (4،0-3،pH = 5) was dissolved under magnetic stirring at room temperature. Different concentrations of Shirazi thyme essential oil (ppm 250, 150 and 50) were prepared in ethanol (w/v 1:1). Essential oil solution was combined with CS-CA nanogel solution. Different concentrations of nisin (ppm 2, 7 and 12) were also added drop by drop to the resulting solution. The resulting mixtures were placed in an ultrasonic bath (vCLEAN1-L3 Ultrasonic Cleaner, Tehran, Iran) at a frequency of 40 kHz for 5 minutes at room temperature. For the precipitation of chitosan-containing ZEO and nisin nanogel, pH between 8-9،5 Adjust and mix the result for 24 hours at a temperature of C˚4 was maintained. In the resulting two-phase mixture, the supernatant was discarded and chitosan nanogels containing Shirazi thyme essential oil and nisin (T_{czn}) were dried using a freeze dryer [11]. Box-Benken experimental design[7] To investigate the effect of independent variables (chitosan nanoparticle concentration, Shirazi thyme essence and nisin) on dependent variables (encapsulation efficiency, DPPH free radical absorption ability, particle size and zeta potential). The experimental design consisted of 15 runs (Table 1). The independent variables were optimized to achieve the highest encapsulation efficiency and zeta potential, as well as the lowest amount of antioxidant to inhibit DPPH free radical and particle size. The experimental data were fitted in a stepwise regression model to avoid the multicollinearity problem. Multicollinearity occurs when two or more independent variables are correlated in a multiple regression equation and cause an increase in the standard error of the coefficients [14].

6-2- Properties of nanogel

2-6-1- Antioxidant activity

Antioxidant activity (DPPH free radical scavenging capacity[8](by spectrophotometric method)Shimadzu 2501 UV spectrophotometer, Tokyo, Japan) Calculated. IC $value_{50}$ (50٪ inhibitory concentration) was calculated by linear regression analysis of RSC values [15].

2-6-2- The effectiveness of the inner cover[9] Chitosan nanogels

Encapsulation efficiency for nisin (EE_{Nisin}) and Shirazi thyme essential oil (EE_{ZEO}) respectively by agar diffusion test based on (ATCC 33090)*Harmless listeria*as indicator microorganism [16] and gravimetric analysis[10] [17] was calculated.

2-6-3- Particle size and zeta potential

Particle size and zeta potential of nanogel optimized by dynamic light scattering[11] (DLS instrument, SZ-100 HORIBA, Japan) was measured.

5-1- hydroxy benzotriazole
6-Thymol (46.11); Carvacrol (18.32 %), *p*-Cymene (9.85 %) and γ-Terpinene (8.33 %)
7- Box-Behnken
8- 1,1-diphenyl-2-picrylhydrazyl
9-Encapsulation efficiency
10-Gravimetric analysis
11-Dynamic light scattering

[DOI: 10.22034/FSCT.21.148.1]

Table 1. Box-Behnken experimental design with process variables and experimental results of various chitosan nanogels containing Shirazi thyme and nisin

Runs	ZEO ppm	Nisin ppm	Chitosan (g)	Particle Size (nm)	Zeta Potential (mV)	IC_{50} ($mg.mL^{-1}$)	EE_{ZEO} (%)	EE_{Nisin} (%)
1	50	7	0.1	389	30.20	1.38	66.62	61.84
2	50	2	0.25	392	31.40	1.18	80.98	58.33
3	50	12	0.25	374	34.50	1.17	84.96	65.82
4	50	7	0.4	385	37.20	1.14	87.90	68.81
5	150	2	0.1	468	25.00	0.95	71.06	59.16
6	150	12	0.1	421	28.10	1.09	69.11	63.20
7	150	7	0.25	415	30.10	0.81	83.18	68.16
8	150	2	0.4	438	27.80	0.77	88.03	67.12
9	150	12	0.4	422	29.16	0.78	86.49	76.23
10	250	7	0.1	481	25.60	0.87	68.11	68.11
11	250	2	0.25	487	27.16	0.71	77.05	61.28
12	250	12	0.25	461	27.80	0.74	74.34	72.42
13	250	7	0.4	471	32.20	0.71	82.81	73.11
14	150	7	0.25	423	30.40	0.76	81.60	67.12
15	150	7	0.25	413	31.03	0.78	82.21	69.29

EE_{nisin}: encapsulation efficiency of nisin; EE_{ZEO}: encapsulation efficiency of *Zataria multiflora*

2-7- Model optimization and validation

Analysis of variance (ANOVA) was used to find significant independent variables. Adequacy of the model in terms of lack of fit test, coefficient of determination (R^2), adjusted coefficient of determination (R^2(adj)), predicted coefficient of determination (R^2 (pred)), standard error (S) was analyzed. A suitable model can be introduced based on the following principles: (a) high value of the coefficient of determination (weak model: 0،50 >R^2; Medium model: 0،70 ><$R^2$0،50; Good model: 0،90 ><$R^2$0،70 and excellent model: 0،90 R^2>); (b) low standard error (S) and (c) small difference between R^2(adj) and R^2(pred) [18]. All steps were performed in Minitab software (version 20, Minitab Inc., USA).

8-2- Preparation of cheese

Iranian white cheese using pasteurized cow's milk (2،5% fat) and based on initial culture %w/v 0،5 and 2 mL of microbial whey were produced [19]. The experimental treatments were as follows: T_{CO}: Cheeses produced without adding preservatives. T_{czn} - Cheeses produced by adding optimal chitosan nanogel (0،4 g) containing ZEO and nisin (ppm 157،1 Shirazi thyme essential oil and 10 ppm،1 niacin). T_{FZN}: Cheeses produced by adding 157.1 ppm Shirazi thyme essential oil and 10.1 ppm Nisino T_S: cheeses produced with the addition of sodium nitrate (35 ppm; based on INSO-11832) [20]. Cheeses prepared for 8 hours in salt water (20% w/w) at room temperature (25˚C±5) were placed. After this time, out of the salt water, the pieces of cheese with specific dimensions (10 x 5 x 5 cm) were placed in a sterile container containing 8% by weight of salt water and preservative. Cheese samples were packed and stored at 4°C for 60 days.

9-2- Characteristics of cheese

2-9-1- Microbial characteristics of cheese

Total live mesophyll count (TMC)[12], the amount of mold and yeast[13] (YMC) and coliforms were measured according to the methods proposed in the standard [21-23]. All microbiological results are expressed in logarithmic units of colony formation per gram (log_{10} CFU/g) were expressed.

2-9-2- Content of volatile nitrogen bases (TVB-N)

The TVB-N content of the samples was determined based on the steam distillation technique and by Kjeltec PDU-500, PECO, Shiraz, Iran. This index is expressed in milligrams of nitrogen per 100 grams of sample (mg $N/100g^{-1}$) sample was reported [24].

2-9-3- cheese texture

Hardness of cheeses with the help of texture analyzer (Brookfield Engineering Laboratories Inc.)Middleboro, MA. USA) was done [25].

2-9-4- Sensory characteristics of cheese

30 semi-trained sensory evaluators (17 women and 13 men) from the students and employees of the Department of Food Science and Technology of Islamic Azad University, Sabzevar branch (age between 24 and 51 years) were selected to evaluate the sensory characteristics of the product. The assessors were selected based on the following characteristics: consuming Iranian white cheese, not

12- Total viable mesophilic counts

13- Yeasts and molds count

[DOI: 10.22034/FSCT.21.148.1]

smoking, availability and interest in participating in the test. A five-point hedonic scale (5 excellent and 1 very bad) was used to confirm the sensory characteristics. Consumers evaluated the texture and appearance, color, taste and smell of the product. , evaluated taste, softness and smell/aroma [26]. The overall acceptability of the samples was also estimated based on the weighted average score of the other investigated characteristics (the coefficients for taste, smell, texture, and color were considered 4, 3, 2, and 1, respectively) [27].

2-10- Statistical analysis

All the tests performed on Iranian white cheese were performed in three replicates and the results were averaged and standard deviation (SD±Mean) was recorded. Statistical analysis was performed based on general linear model (GLM) analysis of variance. Significant difference between means with Tukey's test (0،05 $p <$) Checked out. Statistical calculations in softMinitab software (version 18, State College, USA) was performed.

3. Results and Discussion

1-3- Particle size and zeta potential

The particle size and zeta potential of nanogels containing Shirazi thyme essential oil are shown in Table 1. As it is known, the particle size of the nanogel containing Shirazi thyme essential oil and nisin is between 374 (run 3: Shirazi thyme essential oil: 150 ppm; nisin: 12 ppm and chitosan nanogel: 0 g،25) to 487 (run 11: essential oil of Shirazi thyme: 250 ppm; nisin: 2 ppm and chitosan nanogel: 0 g،25) nm was variable. These data are in agreement with the reported results for chitosan-dihydro-caffeic acid nanogel containing essential oil*Matricaria recutita* (81،3 ± 331،5 nm) is comparable [11]. The investigation of factors affecting particle size showed (Table 2), the concentration of nisin and Shirazi thyme essential oil has a significant effect on the particle size of the studied bioactive nanogels. By increasing the concentration of Shirazi thyme essential oil, the particle size increases, while increasing the level of nisin concentration decreases the particle size. Probably, the increase in the average size of the nanogel particles is due to the increase in the concentration of the essential oil due to the rearrangement of the nanogel structure and the formation of a hydrophobic area for greater interaction with the essential oil [11, 28]. The regression equation for particle size estimation could predict about 90% of the particle size changes of the designed nanogel.

The zeta potential of different nanogels is also between 25 mV (run 5: Shirazi thyme essential oil: 150 ppm; nisin: 2 ppm and chitosan nanogel: 0%،1) to 37.2 (run 4: essential oil of Shirazi thyme: 50 ppm; nisin: 7 ppm and chitosan nanogel: 0%،4) was variable (Table 1). Zeta potential is related to physico-chemical stability and behavior of materials in electrically charged environments. Values of zeta potential greater than 30 mV (in one module) are more stable due to the repulsive module forces that prevent their particle aggregation [4, 10]. Shirazi-Nisin thyme chitosan nanogel had a positive charge, which is related to the presence of free amino groups on the surface of the nanogel. The result with the results obtained for chitosan-dihydrocaffeic acid nanogel containing essential oil *Matricari dry up* (mV35،20+) was comparable [11]. The regression model of zeta potential estimation showed that the concentration of Shirazi thyme essential oil; Nisin, chitosan and the second-order effects of thyme and nisin were significantly effective in predicting this index. The most positive effect was related to chitosan concentration (coefficient 2،179). Chitosan has a positive charge in the environment and it is normal to see an increase in the zeta potential with an increase in chitosan concentration [10]. But the effect of the concentration of thyme and the square of the concentration of nisin has significantly reduced the zeta potential, which can be due to the reduction of the positive charge in the environment [11]. The quadratic equation designed to predict this index had moderate power in predicting zeta potential.

3-2- DPPH free radical inhibition index

Antioxidant activity of chitosan-caffeic acid nanogels containing Shirazi thyme essential oil and nisin based on IC_{50} It is specified in Table 1. The value of this index is between 0 mg/mL،71 (Runs 11 and 13: Shirazi thyme essential oil: 250 ppm; nisin: 2 or 7 ppm and chitosan nanogel: 0 g،4 or 0،25) up to 1،38 (Run 1: Shirazi thyme essential oil: 50 ppm; nisin: 7 ppm and chitosan nanogel: 0 g،1) Was. Investigating the effect of independent variables on the antioxidant activity index, the results showed that with the increase in the concentration of Shirazi thyme essential oil and chitosan, the value of IC_{50} decreases and thus the antioxidant activity of the nanogel increases. The antioxidant activity of these nanogels can be caused by monoterpenes and phenolic compounds of Shirazi thyme essential oil. Also, caffeic acid present in nanogel can have antioxidant activity, so with increasing concentration of chitosan nanogel, the amount of antioxidant activity also increased [8, 29-31]. The step-by-step regression model defined to predict the antioxidant activity index was able to estimate more than 90% of the changes in this index (excellent performance).

3-3-Efficiency of lining

Encapsulation efficiency of thyme essential oil for designed bioactive nanogel particles between 64،62% (execution 1: thyme essential oil: 50 ppm; nisin: 7 ppm and chitosan nanogel: 0 g،10) to 88،03 (execution 8: Shirazi thyme essential oil: 150 ppm; nisin: 2 ppm and chitosan nanogel: 0 g،4) was variable (Table 1). These results are similar to those for chitosan-cinnamic acid containing nanogel*Aromatic syzygium*(4،0 ± 89،0 %), containing chitosan-cinnamic acid*Cinnamomum*

[DOI: 10.22034/FSCT.21.148.1]

ssp. (3،0 ± 74،0 %) [10] Vicitosan-dihydrocaffeic acid containing essential oil*Matricaria recutita* (61،23 percent) was comparable [11]. The difference between the results of different researches depends on the type and concentration of raw materials, the method and the conditions of encapsulation.

Table 2. Stepwise response surface regression: analysis of variance, coded coefficient, model equations and Models summary for evaluation of quality properties of various chitosan nanogels containing Shirazi thyme and nisin

Parameter	Model (p-value)	Lack of fit	Coded coefficient (p-value)	Model summary			
				S	R^2	R^2(adj)	R^2(pred)
Particle size	0.000	0.190^{ns}	Constant: 429.33 (0.000) Z: 45.00 (0.002) N: -13.38 (0.004)	10.63	92.86 %	91.67 %	89.96%
	Equation		Particle size= 380.56+0.45Z-2.675N				
Zeta potentiate	0.00	0.083^{ns}	Constant: 29.93 (0.000) Z: -2.567 (0.001) N: 1.029 (0.072) C: 2.179 (0.002) Z^2: 1.814 (0.037) N^2: -1.963 (0.027)	1.428	87.44 %	80.46 %	61.15%
	Equation		Zeta potential (Mv) = 28.94 - 0.0801 Z + 1.305 N + 14.53 C + 0.000181 Z^2- 0.0785 N^2				
IC_{50}	0.000	0.183^{ns}	Constant: 0.7937 (0.000) Z: -0.2317 (0.000) C: -0.111 (0.05) Z^2: 0.1489 (0.000) C^2: 0.0930 (0.001)	0.040	97.51 %	96.52 %	94.33%
	Equation		IC50=.9197 - 0.006783 Z - 2.808 C + 0.000015 Z^2+ 4.135 C^2				
EE_{ZEO}	0.000	0.134^{ns}	Constant: 81.118 (0.000) Z: -2.267 (0.008) C: 8.792 (0.000) Z^2: -2.626 (0.025) C^2: -3.290 (0.008) ZC: -1.645 (0.114)	1.88	95.85 %	93.54 %	82.61%
	Equation		EE_{ZEO}= 51.71 + 0.0835 Z + 148.2 C - 0.000263 Z^2 - 146.2 C^2- 0.1097 ZC				
EE_{Nisin}	0.000	0.244^{ns}	Constant: 68.05 (0.000) Z: 2.52 (0.003) N: 3.97 (0.000) C: 4.125 (0.000) N^2: -5.44 (0.022)	1.87	90.65 %	86.91 %	76.58%
	Equation		EE_{Nisin}=46.70 + 0.02524 Z + 2.260 N + 27.50 C - 0.1047 N^2				

EE_{nisin}: encapsulation efficiency of nisin; EE_{ZEO}: encapsulation efficiency of *Zataria multiflora*
Stepwise Selection of Terms: α to enter = 0.15, α to remove = 0.15
S: *the standard deviation of the distance between the data values and the fitted values; R2: c*oefficient of determination; R^2(adj): adjusted coefficient of determination; R^2(pred): predicted coefficient of determination

Among the investigated independent variables, the concentration of Shirazi thyme essential oil and chitosan nanogel (its simple and square effect) has been significantly effective on the encapsulation efficiency of Shirazi thyme essential oil. Increasing the concentration of chitosan significantly increases the encapsulation efficiency of Shirazi thyme essential oil, while increasing the concentration of Shirazi thyme essential oil decreases the chance of complete encapsulation of the essential oil (Table 2). By decreasing the concentration of the core material or increasing the concentration of the wall material, the amount of uncoated bioactive compounds decreases and the coating efficiency increases [8]. The stepwise regression model designed to estimate this index showed a good performance.

The encapsulation efficiency of nisin by chitosan-caffeic acid nanogels is also between 58،31% (execution 2: essential oil of Shirazi thyme: 50 ppm; nisin: 2 ppm and chitosan nanogel: 0%،25) to 76،39 (run 9: Shirazi thyme essential oil: 150 ppm; nisin: 12 ppm and chitosan nanogel: 0%،4) was (Table 1).The efficiency of nisin encapsulation in bacterial cellulose nanocrystals is between 93%،3 – 80،5 [32], about 65 in pectin-chitosan polyelectrolyte،9% [33] and about 67 in chitosan nanoparticles،32% [34] was reported. Examining the results related to the modeling variable of this index also showed that the concentration of chitosan, thyme and nisin have a significant direct effect on the efficiency of nisin encapsulation. The designed regression equation was able to estimate more than 76% of the changes in the percentage of nisin encapsulation efficiency

[DOI: 10.22034/FSCT.21.148.1]

with the help of independent variables, which indicates the moderate-good performance of the model.

3-4- Optimizing and verifying the model

The independent variables were numerically optimized and the results showed that at a concentration of g 0،4 nanogels, 157 ppm،1 Shirazi thyme essential oil and 10 ppm،1 Nisin formed the best bioactive nanogel (Table 3). Examining the characteristics of the optimal nanogel containing thyme and nisin essence showed that, based on the one-sample t-test, there is no significant difference between the predicted and experimental values, and the changes between the values of the investigated characteristics were less than 10%. Therefore, the appropriateness of stepwise regressions in estimating responses can be confirmed.

Table 3. The t-test conducted to compare the predicted and actual experimental response values and optimized condition for preparation chitosan nanogels containing Shirazi thyme and nisin

Response	Goal	Fitted value	SE Fit	experimental value	% Variation	Mean difference	Significance Two-tailed (p-value)
PS	Minimum	424.27	3.60	411.39 ±18.11	-0.68	12.88	-1.23 (0.343)^{Ns}
ZP	Maximum	31.82	0.81	32.90±1.10	+3.28	1.08	1.70 (0.231)^{Ns}
IC_{50}	Minimum	0.76	0.02	0.79±0.06	+3.79	0.03	0.87 (0.478)^{Ns}
EE_{Nisin}	Maximum	73.81	0.93	71.06±2.66	-3.87	2.75	-1.79 (0.215)^{Ns}
EE_{ZEO}	Maximum	87.33	1.06	82.69±3.12	-5.61	4.64	-2.58 (0.123)^{Ns}
Multiple Response Prediction (optimized condition)							
Chitosan nanogel		ZEO		Nisin		Composite Desirability	
0.4 g		157.1 ppm		10.1 ppm		0.69	

Ns: not significant difference (P > 0.05); *: Significant difference (P < 0.05)
PS: Particle size (nm); ZP: Zeta potential (mV); EE_{nisin}: encapsulation efficiency of nisin (%); EE_{ZEO}: encapsulation efficiency of *Zataria multiflora* (%)
Stepwise Selection of Terms: α to enter = 0.15, α to remove = 0.15

5-3- The effect of chitosan nanogel containing Shirazi thyme essential oil and nisin on the properties of Iranian white cheese.

3-5-1- Microbial characteristics

As can be seen in Table 4, the level of mold and yeast (YMC) of cheese samples on the first day of storage was Log CFU/g 2،29 -2،It was 70. According to the INSO-2406 standard, the threshold limit of YMC level for primary cheese is less than 3 Log CFU/g [35]. Therefore, the amount of YMC of all samples was acceptable. Comparison of different cheese samples showed that the level of mold and yeast in samples of T_{czn} (Log CFU/g2،29) and T_S (Log CFU/g 2،34) significantly less than the control sample (Log $CFU.g^{-1}$2،70) was (0،05 p <). Also, the difference between T_{FZN} It was not significant with other treated samples and with the control sample at the 5% level (0،05 p >). On the 60th day of storage, although the YMC level of the samples was lower than on the first day of storage, it was significantly higher than on the 30th day of storage. At the end of the storage time, the difference between the treated samples and the control sample was significant (0،05 p <). In Iranian white cheese ripened in salt water, the acceptable limit for the level of mold and yeast is Log CFU/g2 [35]. According to this standard, on the 30th day of storage, all samples are within the standard range, but on the 60th day of storage, only sample T_{czn} It was within the acceptable range. Thymol and carvacrol are the main components of Shirazi thyme essential oil. These compounds against yeasts such as*Saccharomyces cerevisiae* and filamentous fungi such as*Aspergillus* show inhibitory activity [5, 36]. However, studies have shown that nisin has little or no effect on filamentous fungi, yeasts, and gram-negative bacteria. However, the microbial synergistic effect of nisin and plant essential oils has been reported in previous studies [37, 38]. The higher antimicrobial effect of the encapsulated sample (T_{czn}) compared to the free sample (T_{FZN}) can also be related to the protective effect of the encapsulation process on bioactive compounds as well as the antimicrobial effect of chitosan and caffeic acid [8].

Coliforms: During the storage period, the coliform level of all samples showed a decreasing trend (0،05 p <). After 60 days of storage, coliform in the sample of T_{czn} and T_S Not identified. The acceptable limit of coliform for raw cheese and ripened cheese was 3 and 1 Log CFU/g, respectively [35]. Therefore, only samples of T_{czn} and T_S At the end of maintenance, they were in the standard range. In this way, chitosan nanogel containing Shirazi thyme and nisin can be a good substitute for chemical preservatives such as sodium nitrate.

According to Table 4, the amount of total mesophilic bacteria showed no significant change during 30 days of storage. However, the increasing trend of TMC in samples of T_{CO} and T_{FZN}It was observed, while in the other two samples, the TMC level decreased during 30 days of storage. On the 60th day of storage, the TMC level in all samples showed an increasing trend, but this increase in the control sample was more than twice that of the T sample.$_{czn}$ Was. Lipophilic bioactive compounds (such as carvacrol and thymol) of essential oils alter the

[DOI: 10.22034/FSCT.21.148.1]

permeability of the cell wall, causing the loss of ions and cytoplasmic components and affecting ATP synthesis [5].

3-5-2- Index of volatile nitrogen bases (TVB-N)

As shown in Table 4, the index of volatile nitrogen bases on the first day of storage is about 0.100 mg N/g.،was 25, which increased significantly over time in all samples. On the 60th day, the maintenance of this index in the control sample was significantly higher than the other samples. The comparison between the three treated cheeses also showed that the amount of TVB-N index in two samples of T_S and T_{czn} significantly less than the sample of T_{FZN} is (0،05 p <). Compound volatile nitrogen bases from trimethylamine[14] (TMA), dimethylamine[15] (DMA), ammonia and other volatile nitrogen compounds. Dimethylamine is generally produced by endogenous enzymes and trimethylamine by bacterial enzymes. Therefore, the results of TVB-N content are related to the microbial load of the product. Since it is time-consuming to measure the microbial load on an industrial scale, the amount of TVB-N can be an indicator for rapid estimation of the microbial quality of protein products [18]. The effect of nisin essential oil (in free and encapsulated form) and sodium nitrate is related to their antimicrobial properties (Section 1-5-3).

3.5.3 Texture hardness

The hardness of cheese samples during 60 days of storage is also shown in Table 4. On the first day of storage, the hardness of the cheeses did not differ significantly from each other. During 60 days of storage, the hardness of the samples decreased significantly. However, the tissue changes of the treated samples were more compared to the control treatment. On the 60th day of storage, the hardness of T. cheese samples$_{czn}$ and T_S It was significantly more than the other two samples. Less textural changes in the treated cheese samples were probably due to the lower microbial load of these samples. Microorganisms cause cheese softness by destroying the protein tissue of cheese. Esparvarini et al. (2022) also found edible coatings containing cumin essential oil to be effective in controlling cheese textural changes [39].

3-5-4- Color components of cheese

On the first day of maintenance, there was a significant difference between the color indices (L*، a* And$b$*) samples were not seen (Table 4). After 30 days of storage, the brightness of the samples decreased significantly, and this change was greater in the control sample (0،05 p <). Indicator$a$* increased in all samples. This increase in the control sample and T_S It was significant (0،05 p <). Indicator$b$* also showed a significant increase (except in T_{czn}). On the 60th day of index maintenance$L$* The control sample had an increase compared to day 30, in other samples the trend of this change was decreasing (0،05 p <). The maximum amount of index$L$* On the last day of storage in the samples of T_S and T_{czn} Was. The lowest level of this index is also in the sample of T_{FZN} Was. Indicator$a$* And$b$* All samples had an increase compared to day 30. This increase was significant in the control sample.

Since the color components can affect each other, the estimation of the color change index compared to the control sample can provide a better comparison between different treatments and times. Examining the ΔE index showed that although there was a significant difference between the color of the control sample and other samples on the first day of storage, this difference was less than 3 and was not visible to the eye. This color difference can be caused by the color of additives. Over time, the ΔE index showed a significant increase in all samples. The most color changes related to the control sample and the least related to T_S and T_{czn} Was. More color changes in the control sample can be attributed to oxidation, enzyme activity and microorganisms [39].

Table 4 Effect of the combination of nisin and Shirazi thyme (*Zataria multiflora*) essential oil on physicochemical and microbial properties of Iranian white cheese during 60 days of cold storage.

Storage time (days)	Treatment			
	T_{Co}	T_{FZN}	T_{CZN}	T_S
	Yeasts and molds count (Log CFU/g)			
0	2.70±0.05^{A}	2.58±0.02^{AB}	2.29±0.05^{BCD}	2.34±0.12^{BCD}
30	1.80±0.04^{FG}	1.69±0.09 ^{GH}	1.32±0.28 ^{I}	1.46±0.15 ^{HI}
60	2.47±0.01^{ABC}	2.17±0.10 ^{CDE}	1.84±0.06 ^{EFG}	2.04±0.04 ^{DEF}
	Coliforms (Log CFU/g)			
0	3.14±0.03^{A}	3.01±0.04^{A}	2.81±0.05^{B}	2.63±0.06^{BC}
30	2.66± 0.03^{BC}	2.59±0.04^{C}	2.07±0.10^{E}	2.40±0.05^{D}
60	2.35±0.10D	2.12±0.10^{E}	0.00 ^{F}	0.00^{F}
	Total viable mesophilic counts (Log CFU/g)			
0	1.84±0.06^{CDE}	1.76±0.09^{DEF}	1.60±0.12^{EF}	1.59±0.26^{EF}
30	2.10±0.07^{CD}	1.86±0.03^{CDE}	1.32±0.25^{F}	1.46±0.15^{EF}
60	4.25±0.07^{A}	2.79±0.10^{B}	2.10±0.17^{CD}	2.26±0.24^{C}

14- Trimethylamine

15- Dimethylamine

[DOI: 10.22034/FSCT.21.148.1]

TVB-N (mg N/100g)				
0	0.25±0.03^{F}	0.26±0.01^{F}	0.25±0.01^{F}	0.26±0.01^{F}
30	0.68±0.02^{D}	0.59±0.03^{E}	0.53±0.03^{E}	0.53±0.01^{E}
60	1.08±0.02^{A}	0.90±0.02^{B}	0.83±0.04^{C}	0.81±0.03^{C}
Hardness (N)				
0	6.56±0.46^{A}	6.50±0.35^{A}	6.54±0.28^{A}	6.49±0.32^{A}
30	2.72±0.19^{C}	3.29±0.19^{B}	3.80±0.22^{B}	3.82±0.14^{B}
60	1.27±0.10^{D}	1.52±0.09^{D}	2.79±0.06^{C}	2.62±0.13^{C}
L*				
0	81.96±0.51^{A}	82.03±0.45^{A}	82.30±0.32^{A}	81.80±0.58^{A}
30	70.46±0.22^{F}	78.64±1.05^{BC}	79.59±0.08^{B}	79.59±0.08^{B}
60	76.46±0.32^{D}	72.09±0.36^{E}	78.05±0.61^{C}	78.70±0.10^{BC}
a*				
0	5.94±0.21^{F}	6.47±0.46^{EF}	6.78±0.26^{DEF}	6.09±0.16^{F}
30	7.51±0.02^{BCD}	7.25.11^{BCDE}	7.40±0.27^{BCD}	7.05±0.27^{CDE}
60	8.85±0.31^{A}	8.04±0.47^{AB}	7.86±0.11^{BC}	7.42±0.42^{BCD}
b*				
0	11.84±0.43^{F}	12.32±0.98^{F}	12.94±0.55^{EF}	11.86±0.21^{F}
30	15.43±0.29^{BC}	14.67±0.10^{BCD}	13.91±0.20^{DE}	13.90±0.20^{DE}
60	17.80±0.10^{A}	15.77±0.49^{B}	14.33±0.27^{CD}	14.40±0.30^{CD}
ΔE				
0	0.00±0.00^{I}	1.40±0.09^{GH}	1.76±0.20^{G}	0.64±0.52^{HI}
30	11.57±0.29^{A}	4.15±0.62^{D}	3.09±0.07^{EF}	2.92±0.29^{F}
60	8.20±0.35^{C}	10.28±0.41^{B}	4.59±0.30^{D}	3.97±0.33^{DE}

*Each value represents the mean ± SD of triplicate experiments
Different capital letter for each factor indicated significant differences between various times or treatments (P< 0.05).
T_{CO}: Cheeses produced without the addition of preservative; T_{czn}: Cheeses produced with the addition of optimal chitosan nanogel containing ZEO and nisin (based on 0.4 g chitosan, 157.1 ppm ZEO and nisin at 10.1 ppm); T_{FZN}: Cheeses produced with the addition of 157.1 ppm ZEO and nisin at 10.1 ppm and T_S: Cheeses produced with the addition of sodium nitrate (35 ppm; based on INSO-11832)

5-5-3- Sensory characteristics of Iranian white cheese

On the first day of storage, no significant difference was seen between different samples (Figure 1). On the 30th day of keeping the taste and smell of T_S and T_{czn} There was no significant difference compared to the first day. But from the score of T samples$_{FCN}$ And the control sample was significantly reduced. Until the 30th day of storage, the taste and smell scores for all samples were higher than 3. The taste score on the last day of storage in all samples was significantly lower than the 30th day. Previous studies have shown that the smell of herbal essential oils such as sage, thyme, rosemary, grapefruit, orange and lemon does not have a negative effect on the taste and smell of cheese [2]. On the 30th day, the tissue retention score of the treated samples was not significantly different from each other and from the first day. Only the score of the control sample on this day was significantly lower than the first day. On the 60th day of storage, it can be said that almost none of the samples obtained an acceptable score for texture. These results were in line with what was previously reported about cheese texture (hardness). On the 30th day of storage, the color difference in the samples of T_{czn} and T_S It was not different from the first day of storage. On this day, the difference between the color of the treated samples was not significant at the 5% level. But the color score of the control sample is significantly lower than the two T samples$_{czn}$ And $_{T}$S was On the last day of storage, there was no significant difference between the color score in different samples. The color difference of different samples on the 30th and 60th day of storage was not significant. These results are also consistent with what was mentioned about the color changes of cheese. The overall acceptance of samples on the first day of storage is higher than 4،6 and all samples were of excellent quality. On the 30th day of maintaining the T sample score$_{czn}$ There was no significant difference with the zero day of this sample. Comparison of different samples on day 30 showed that the score of two samples of T_{czn} and T_S It was higher than good (greater than 4) and the difference between these two samples and the control sample and T_{FZN} It was significant at the 5% level. On the last day of storage, the overall acceptance score of all samples was significantly reduced. At the end of the storage time, only the TCZN sample was able to score above 3. The difference between this sample and other samples was also significant at the 5% level. The lowest score is related to the control sample (1،61) was In general, it can be said that chitosan nanogel containing Shirazi thyme essential oil and nisin can be a good substitute for the chemical preservative sodium nitrate and does not have a negative effect on the sensory characteristics of Iranian white cheese.

[DOI: 10.22034/FSCT.21.148.1]

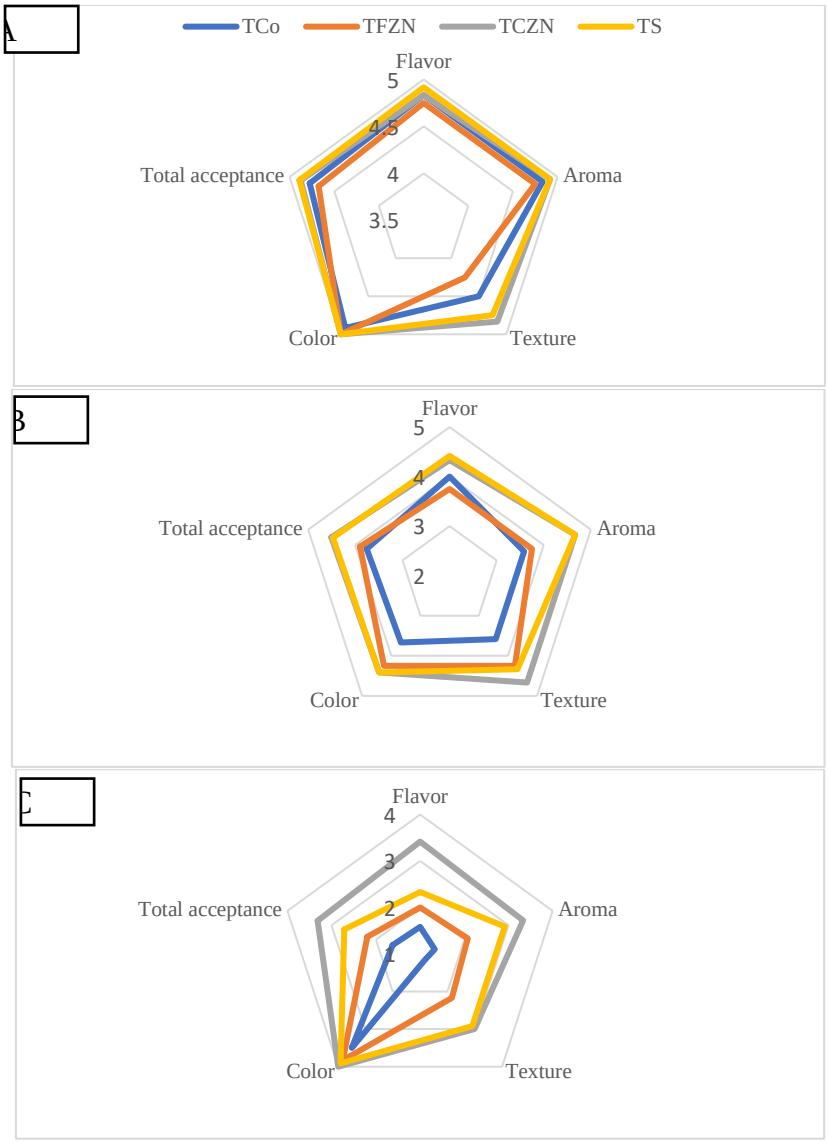

Fig 1. Effect of the combination of nisin and Shirazi thyme (*Zataria multiflora*) essential oil on the sensory properties of Iranian white cheese on A) 1st day; B) 30th day and C) 60th day of cold storage;

T_{CO}: Cheeses produced without the addition of preservative; T_{czn}: Cheeses produced with the addition of optimal chitosan nanogel containing ZEO and nisin (based on 0.4 g chitosan, 157.1 ppm ZEO and nisin at 10.1 ppm); T_{FZN}: Cheeses produced with the addition of 157.1 ppm ZEO and nisin at 10.1 ppm and T_S: Cheeses produced with the addition of sodium nitrate (35 ppm; based on INSO-11832)

4 - Conclusion

Box-Benken experimental design of stepwise response surface model was able to properly optimize the concentration of chitosan nanogel, Shirazi thyme essence and nisin. The optimal nanogel contains 0،4 grams of chitosan nanogel, 157 ppm،1 Shirazi thyme essential oil and 10 ppm،1 was Nicene. This nanogel has a particle size of about 411،39 nm, zeta potential 32 mV،90, free radical scavenging power based on the IC50 index of about 0 mg/mL،79, nisin encapsulation efficiency 71،06% and the efficacy of Shirazi 82 thyme essential oil،showed 69 percent. The use of essential oil of Shirazi thyme and nisin in an optimized amount in the form of nanogel and free could control the microbial growth in cheese. It also reduces the process of texture and color changes in cheese. The addition of thyme-shirazi-nisin essential oil had no negative effect on the sensory quality of Iranian white cheese, and the effect of nanogel in increasing the shelf life of white cheese was competitive with sodium nitrate. It seems that simultaneous encapsulation of plant essential oils and bacteriocins in chitosan-caffeic acid nanogel can be a natural alternative to chemical preservatives.

5- References

[DOI: 10.22034/FSCT.21.148.1]

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[DOI: 10.22034/FSCT.21.148.1]

مجله علوم و صنایع غذایی ایران

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مقاله علمی_پژوهشی

بهینه‌یابی تولید نانوژل کیتوزان- اسیدکافئیک حاوی اسانس آویشن شیرازی و نایسین و بررسی اثر نانوژل بهینه بر کیفیت پنیر سفید ایرانی

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۲/۵/۴

تاریخ پذیرش: ۱۴۰۲/۱۱/۱۴

کلمات کلیدی:

آویشن شیرازی؛

بهینه‌یابی؛

پنیر سفید ایرانی؛

رگرسیون گام به گام؛

نانوژل کیتوزان-اسیدکافئیک

DOI: 10.22034/FSCT.21.148.1.

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چکیده

این مطالعه با هدف بهینه‌یابی فرمولاسیون نانوژل کیتوزان- کافئیک اسید حاوی اسانس آویشن‌شیرازی (ZEO) و نایسین انجام شد. متغیرهای مستقل (غلظت نانوژل کیتوزان-اسیدکافئیک، آویشن شیرازی و نایسین) بر اساس بالاترین پتانسیل زتا و کارایی درون‌پوشانی، در کنار کمترین اندازه ذرات و مقادیر $IC_{50(DPPH)}$ (بیش‌ترین قدرت مهار رادیکال آزاد DPPH) بهینه شدند. فرمولاسیون نانوژل بهینه مطابق نتایج طرح آزمایشی باکس-بنکن و مدل سطح پاسخ گام‌به‌گام عبارت است از: غلظت کیتوزان: ۰/۴ گرم؛ غلظت اسانس آویشن‌شیرازی: ۱۵۷/۱ ppm و نایسین: ۱۰/۱ ppm. اندازه ذرات، پتانسیل زتا، $IC_{50(DPPH)}$ و راندمان کپسولاسیون نانوژل کیتوزان حاوی ZEO و نایسین به ترتیب ۴۱۱/۳۹±۱۸/۱۱ نانومتر، ۱/۱۰ mV ± ۳۲/۹۰، ۰/۷۹ ± ۰/۰۶ $mg.mL^{-1}$، ۸۲/۶۹-۷۱/۰۶ ٪ بود. افزودن ZEO و نایسین (آزاد یا محصور شده در نانوژل کیتوزان) به فرمولاسیون پنیر سفید ایرانی، کیفیت میکروبی و فیزیکوشیمیایی پنیر را بهبود بخشید. فعالیت ضد میکروبی نانوژل کیتوزان حاویZEO و نایسین در مقایسه با فرم آزاد آن بیش‌تر بود. جمعیت *کلی‌فرم* پنیرهای تیمار شده با نیترات سدیم و نانوژل کیتوزان حاویZEO-نایسین طی ۶۰ روز نگهداری در محدوده قابل قبول بود. در طول مدت نگهداری، بیشترین تغییرات رنگ و بافت (سختی) نمونه پنیر مربوط به نمونه شاهد و کمترین تغییر مربوط به نمونه‌های تیمار شده با نیترات سدیم و نانوژل کیتوزان-ZEO-نایسین بود ($p<0/05$). همچنین کیفیت حسی نمونه حاویZEO و نایسین برای ارزیاب حسی قابل قبول بود. نمونه حاوی نانوژل کیتوزان در مدت ۶۰ روز نگهداری نمره حسی قابل قبولی (بالای ۳) دریافت کرد. به طور کلی، نانوژل کیتوزان-ZEO-نایسین در افزایش ماندگاری پنیر سفید ایرانی جایگزین مناسبی برای نگهدارنده شیمیایی نیترات سدیم بود.

[DOI: 10.22034/FSCT.21.148.1]