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The Effect of Bixin/Crocetin Nanoparticles on Quality and Shelf Life Improvement of Cheddar Cheese

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Key words: Alginate, bixin, cheddar cheese, chitosan, crocetin, nanoparticles

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Abstract: This research aimed to the characterization of bixin/crocetin nanoparticles and investigation its effects on cheddar cheese quality and shelf life. In this study, chitosan/alginate nanoparticles containing bixin (12, 14 and 16 μg mL^{-1}) and crocetin (0.01, 0.03 and 0.05%) were prepared by ionic gelatinization method. Nanoparticles characterized by size, release, loading, entrapment efficacy and morphology. Cheese samples treated with nanoparticles and evaluated for DPPH assay, moisture content, acidity, pH, TBA, PV, colorimetric index and TMC in 1st, 20th, 40th and 60th days of storage. The results analyzed by SPSS Software Version 22. The results showed that mean size nanoparticles were 87-149 nm, release (%) were 32-61%. Morphological shapes of nanoparticles was spherical, soft with smooth surfaces. Also, acidity index of cheese treatments increased and pH and inhibitory level of free radicals decreased significantly ($p \leq 0.05$). Lightness index (L*) reduced during storage, yellowness (b*) and redness (a*) increased ($p \leq 0.05$). Peroxide value and TBA significantly diminished with increasing amount of Bixin/crocetin nanoparticles ($p \leq 0.05$). Over time, the index of hardness and cohesiveness increased and adhesiveness, chewiness, elasticity and chewing ability fall down during storage ($p \leq 0.05$). TMC soured meaningfully during storage but with the increase in the use of bixin and chromite nanoparticles, the TMC declined significantly. The results of microscopic evaluation confirmed the presence of nanoparticles within the cheeses network. Finally, sample with 16 μg mL^{-1} Bixin/crocetin was selected as the optimum treatment.

INTRODUCTION

Today conscious of food products consumers from the side-effects of artificial food colorantslead to increasing demand for natural colors in food industries. The application of natural antioxidants in food stuffs is one of the most effective way to declining the oxidation rate of lipids and increasing shelf life and keeping quality

and nutritional value of high fat food stuffs. There are a lot of pigments, especially carotenoids, anthocyanins and chlorophyll. One of the most widely used pigments is the group of carotenoids responsible for the color of yellow, orange and red which found in many foods. These compounds have biological function in human and animal body against major disorders such as cancer, cardiovascular disease, cataracts, atherosclerosis and other diseases associated with age[1]. In addition, carotenoids playsimportant role in decomposing oxygen radicals and reducing oxidative stress. Saffron is a spice derived from the flower of *Crocus sativus*, commonly known as the "saffron crocus"[2]. Crocin and Crocetin in saffron are the most important carotenoids and responsible for the color of saffron[3, 4]. But the main colorant ingredient is Crocin with chemical formula $C_{44}H_{64}O_{24}$ which is a derivative of glycolytic Crocetin[5]. Crocin is one of the few carotenoids in the nature that can easily be dissolved in water. This solubility is one of the reasons why it is widely used as a dye in food and medicine than other carotenoids[6]. Crocin can be metabolized in body and convert to Crocetin. Crocin has several therapeutic properties including: strong antioxidant and anti-inflammatory agent[7]. Annatto is obtained from the pericarp of tropical beans "*BixaOrlana"* which has extensive application in food, pharmaceutical and cosmetic industries. Annatto is a natural colorant which use in cheese, ice cream, butter and meat. In Annato seeds, carotenoids have been identified but the main pigment is "Bixin|" which it composed >80% of the total carotenoids in annatto. Bixin is a mono-methyl ester and neurobixin is also a soluble form that is structurally a dicarboxylic acid[8]. Apart from imparting color, anthocyanin pigments are efficient scavengers of free radicals other than antioxidants and the potential of anthocyanins depend on the chemical structure of the molecule. Bixin, like other carotenoids has an inhibitory effect of oxygen and nitrogen. Bixin is unstable in the presence of oxygen, heat and light. However, in some studies mentioned that its non-stability against air, ozone, oxygen and high temperatures[9]. There are several technique for keeping antioxidant capacity like as technique of encapsulation[10]. Encapsulation is a technique to pack materials (like natural colorants) in the form of micro-and nanoparticles[11]. Nanoparticles are nano-object with three external nanoscale dimensions[12]. Cheddar cheese was first produced in the village of "Somerset in England in the 16th century and today is one of the most popular cheeses produced in the world. Cheeses are a very rich source of proteins, vitamins and minerals such as calcium. During shelf life of cheddar cheese, oils and fats are oxidized in presence high temperatures and its nutritional value fall down. Yalameh *et al*.[13] antibacterial effect of Annatto on *Streptococcus pyogenes*, *Escherichia coli*, *Enterococcus faecalis* and *Bacillus subtilis* evaluated and they declared that antibacterial and antioxidant effects of annatto diminished during preservation. On another side, they investigated annatto antibacterial effect of Anato on *Salmonella enteritidis* in mayonnaise sauce which was in agreement with their previous research. Licon *et al*.[9] investigated volatile compounds in cheese made with lobster echium with *Crocus sativus L*. saffron spices. The results of this study showed that saffron compounds (crocin and crocin) can act as an effective ingredient in preventing oxidative cheeses. Due to the side effects of industrial preservatives in this study, the combination of two natural preservatives in form of nanoparticles used to increase the shelf-life of cheddar cheese.

MATERIALS AND METHODS

Bixin≥90.0% (HPLC), Chitosan with a low molecular weight (190-50 kDa) with a degree of acetylation of 85-75%, sodium alginate (molecular weight = 120-80 kDa), mannoronic/glucuronic with a ratio (M/G = 1/56) and crocetin aldehyde purchased from Sigma-Aldrich company.

Preparation of crocetin loaded nanoparticles: Nanoparticles were prepared according to Tachaprutinum *et al*.[14] method with little modification. The concentration of 0.02% chitosan solution dissolved in glacial acetic acid 1% w/w and sodium alginate dissolved in deionized water for 30 min at 50°C. After that, Crocetin (0.01, 0.03 and 0.05%) was added to the ingestion of alginate solution with 0.06 w/v and lasted for 30 min. After gelation sodium alginate/Crocetin solution was added drop wise to chitosan solution at 1000 rpm for 1 h. The resulting nanoparticles were sonicated in 100 kHz for 3 min with a sonication device (probe type sonicator, Misonix Sonicator, S4000, USA). Then nanoparticles were centrifuged in 31200 g for 40 min and were stored in a freezer dryer (Operon, Korea) for further investigation and characterization[14].

Preparation of bixin loaded nanoparticles: Bixin nanoparticles were prepared by the interference of polymers according to Venturini, etc. Polymers (chitosan and alginate) (250 mg), triglyceride (400 μL), Span 60 (95 mg) and Bixin in a mixture of acetone (60 mL) and ethanol (7.5 mL) were stirred at 40°C. After mixing of the polymers, triglycerides and 60 Span, the organic phase was added to the aqueous phase (130 mL) containing tween 80 (195 mg) and remained for 10 min and the solution was diluted to a final volume of 25 mL. Solvents evaporated under vacuum[7].

Bixin and crocetin nanoparticle characterization: The morphology of nanoparticles was investigated using electron microscopy (SEM, KYKY-EM 3200, China) at 25 kV. Nanoparticles were diluted ten times with ionized water and dried overnight and then the samples were coated with vacuum particles and examined by electron microscopy in terms of appearance, uniformity and particle size[14].

Evaluation size and zeta potential of crocetin/bixin nanoparticles: The size and zeta potential of the nanoparticles obtained from each treatment and the frequency of each of them was determined using a particle size analyzer (SALD-2101 SHIMADZU japan). For this purpose, nanoparticles were dispersed in distilled water (Milli Q Millipore USA) with a conductivity coefficient of 0.054 μs and the results were reported based on the average diameter of the particle±standard error[15].

Entrapment efficacy bixin and crocetin loaded nanoparticles: The EE was calculated by comparing the difference in absorbance of total compound and free compound. Total compound assigned to compound solution only absorbance of total compound and free compound that was encapsulated. Both of the solutions must contain the same concentration of compound[3]. The EE% shows of the percentage of compound well enclosed in the nanoparticles; it was calculated using the following Eq. 1:

$$\text{EE}\ (\%) = \frac{\text{Total compound-free compound}}{\text{Total compound}} \times 100 \qquad (1)$$

The absorbance was measured using UV-VIS spectrophotometer Genesys 10-S (Thermo Fisher Scientific, Waltham, MA, USA) at wavelengths of 540 nm of Bixin and 440 nm for crocetin, respectively. Triplicate test (N = 3) analysis of single and dual compounds loaded were studied[16].

Manufacture of cheddar cheese at laboratory level: One batch of cheddar cheeses were made in Kaleh dairy factorypilot plant (IRAN). At first, 3.5% fat was pasteurized for 30 min at 65°C using a BenmariZenith Lab. The cheese milk was warmed to 33°C before inoculation with freeze-dried Direct Vat Set (DVS) mesophilic/thermophilic blend lactic starter culture (0.05 g kg^{-1} of milk, Chr. Hansen) which contained strains of *Streptococcus thermophilus, Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *Lactis.* After stabilizing the temperature at 32°C, chitosan/alginate nanoparticles as lyophilized powder containing bixin (12, 14 and 16 μg mL^{-1}) and crocetin (0.01, 0.03 and 0.05%) were added and then the rennet (microbial rennet (750 IMCU.mL^{-1}, Chr. Hansen, Bayswater, Australia) from R. miehei113 g rennet/1,000 l b (450 kg) of mix) was added to the milk at a rate of 2.5% once the pH was ~6.5. and was slowly stirred for 2 min until the rennet was completely dispersed then milk included starter culture and rennet kept in 32°C for 45 min. After 45 min, the clots were resting. The clots cut off gently to 1 cubic centimeter and cut into clots for 15 min to settle. The cooking temperature of container increased from 30-39°C by Ben-Marie for 30 min and kept at a temperature of 39°C with this maximum stirring speed of 40 rpm, until the pH was ~6.2, for 15 min. The mixture of clots and whey was separated by lacing and the cheddaring operation continued to pH = 5.42 and was salted to the clot 20%/w/w. The cheesecloth pressed at ~440 kPa overnight at room temperature for 12 h under a pressurized pressurization force of 10 kg and then packed in vacuum bags and stored in a refrigerator with temperature of 8°C for 60 days. The physicochemical and microbial tests on cheeses were performed on 0, 20, 40 and 60 days[17].

Cheddar cheese tests

Evaluation of free radical inhibitory activity: One of the methods for measuring antioxidant activity is through the evaluation of DPPH-free radical inhibitory activity. At this stage, 4 mL of DPPH mM solution was adde to 0.2 mL of the sample and then absorbed at 517 nm and the antioxidant activity was determined by the percentage of DPPH inhibition by the following Eq. 2:

$$\text{Antioxidant activity (\%)} = \frac{\text{Absorption of control}}{\text{Absorption of sample-Absorption of control}} \times 100$$

Measurement of cheddar cheese physicochemical parameters: In order to measure the titratable acidity of cheddar cheese was also calculated by titration based on lactic acid. Moisture content of cheese samples were evaluated by AACC 2000 Standard No. 16-44. Peroxide value determined according to AOCS method number 53-Cd8. Thiobarbituric acid was measured according to the AOCS method -19 Cd 90 Colorimetric evaluation of cheese samples were done by Minolta CR-400 Model colorimeter with standard black and white reference sheets for testing. White samples with a height of 1.5 cm were fitted to standard sheets of Y = 26.81, X = 83.32 and Z = 98.03 set. CIE system was used. L* (Lightness) from black (0) to 100 (white): a* (green to red) b* (blue to yellow) was calculated from -120 till+120[17].

Total Microbial Count (TMC) assessment: To prepare a homogeneous suspension, 25 g cheddar cheese and 225 mL sodium citrate solution (1% w/w) mixed in Inter-science container made by the Sigma Company and then transferred to the 400 circulator machine for a period of 5 min was completely homogenized and filtered to remove the suspended particles, so that, dilutions of 0.1, 0.01 and 0.001 were made with sterile peptone water, then the tubes containing dilutions prepared 1/9 mL was transferred to the surface of the MRS agar media by sampler (surface cultivation). After incubation in 37°C for 24 h colonies were counted, TMC performed in 0, 20, 40 and 60 days after production and in three replications[18].

Statistical analysis: A completely randomized design was used for analysis by SPSS Software Version 22. To test the existence of a significant difference between the means, the Duncan test was used at the 5% level.

RESULTS AND DISCUSSION

Morphological evaluation of crocetin and bixin nanoparticles: Figure 1 showed the morphology of Crocetin/Bixin nanoparticles. As presented in Fig. 1, nanoparticles had a spherical shape with a smooth appearance and their size was below 100 nanometers and they observed separately. Bixin/Crocetin nanoparticles had a homogeneous morphology structure with a spherical shape without agglomeration. The spherical shape of the nanoparticles is outcomes of the electrostatic balance of between polymers chitosan/alginate in the storage of Bixin and Crocetin which also has the ability to form an egg network. And acting as a core for entrapment Bixin and Crocetin, also provide connections in the outer portion of nanoparticles[19]. Zohri *et al*.[20] designed chitosan/alginate loaded nisin and the morphology of their nanoparticle had similarity with recent research outcomes. Lobato *et al*.[7] investigated characterization and stability evaluation of bixin nanocapsules and achieved to same results.

Evaluation size and zeta potential of crocetin/bixin loaded nanoparticles: Table 1 presented that crocetin/ bixin nanoparticles containing 0.01 and 14 μg mL^{-1} bixin had the minimum size (34±0.01). In general, there was a significant increasing in the mean size of crocetin/bixin nanoparticles with increasing of their concentration in nanoparticle formulation, the maximum size nanoparticle was observed in treatment with 0.33 mg crocetin and 16 mg bixin. By increasing the crocetin and bixin concentration in formulation of nanoparticle, size increased which is related to the molecular weight of the nanoparticles. The molecular weight of the bixin molecule is 394.5 mol g^{-1} and molecular weight of the crocetin molecule is also 328.4 g mol^{-1} which increases with its concentration in formulation, led to larger size. Mi *et al*.[19] investigated the effect of different weight ratios of chitosan/tri-polyphosphate and concluded that in a weighing ratio >3.75, nanoparticles of larger size were formed. Generally, by increasing the ratio of chitosan to triple polyphosphate, the concentration of chitosan in the solution soured and the solution viscosity raised, thereby increasing the resistance of the liquid phase to the dispersion and produced nanoparticle with higher size. Shukla *et al*.[21] also investigated the factors affecting the formation and stability of chitosan nanoparticles and

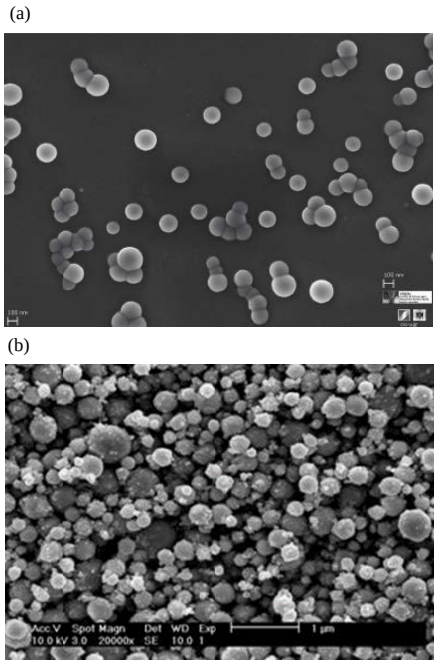

Fig. 1(a, b): Electron microscope images of crocetin nanoparticles

Table 1: Size, loading efficacy, release of bixin/crocetin nanoparticles

Treatment	Size (nm)	EE (%)	Zeta potential (mV)	Release (%)
T1	87±0.01^{a}	34±0.01^{a}	-35±0.01^{a}	32±0.01^{a}
T2	89±0.02^{ab}	42±0.02^{b}	-34±0.04^{a}	35±0.02^{ab}
T3	92±0.02^{b}	42±0.03^{b}	-32±0.01^{ab}	38±0.03^{b}
T4	95±0.02^{bc}	45±0.02^{bc}	-29±0.01^{b}	40±0.02^{b}
T5	98±0.02^{bc}	52±0.01^{c}	-28±0.03^{b}	43±0.01^{bc}
T6	102±0.01^{c}	61±0.03^{cd}	-25±0.02^{bc}	55±0.03c
T7	115±0.02^{cd}	69±0.01^{d}	-21±0.01^{c}	61±0.01^{cd}
T8	130±0.02^{d}	60±0.04^{cd}	-19±0.02^{cd}	45±0.04^{d}
T9	149±0.02^{e}	58±0.02^{cd}	-17±0.02d	44±0.02^{d}

T1 = Nanoparticles (0.01 crocetin and 14 μg mL^{-1} bixin); T2 = Nanoparticles (0.03 crocetin and 14 μg mL^{-1} bixin); T3 = Nanoparticles (0.05 crocetin and 14 μg mL^{-1} bixin); T4 = Nanoparticles (0.01 crocetin and 16 μg mL^{-1} bixin); T5 = Nanoparticles (0.03 crocetin and 16 μg mL^{-1} bixin) T6 = Nanoparticles (0.05 crocetin and 16 μg mL^{-1} bixin); T7 = Nanoparticles (0.01 crocetin and 18 μg mL^{-1} bixin) T7 = Nanoparticles (0.03 crocetin and 18 μg mL^{-1} bixin); T7 = Nanoparticles (0.05 crocetin and 18 μg mL^{-1} bixin)

found that by increasing concentration of the compounds in nanoparticle formulation, the size of nanoparticles increased which had similarity with the results of this study. Zeta potential out comes from the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (±) 30 mV have been shown to be stable in suspension as the surface charge prevents aggregation of the particles. As seen in results (Table 1) zeta potential declined in higher amount of bixin and crocetin in comparison with control. It sounds this outcome can be related to imbalance electrical charge between positive and negative charges can effect on surface zeta potential. Nanoparticles (Crocetin = 0.05 and Bixin = 18 μg mL^{-1}) had minimum zeta potential between treatments. Lobato *et al*.[7] in characterization and stability evaluation of bixin nanocapsules found that increasing loading of bixin decreased zeta potential of nanoparticles which was in agreement with recent research.

Evaluation of free radical inhibitory activity (DPPH test): According to Fig. 2, it was observed that there was a significant discrepancy between the mean of free radical inhibitory index of cheddar cheese treatments due to differences in bixin and crocetin concentration in nanoparticles which used in cheddar cheese formulation ($p \leq 0.05$). As illustrated in Fig. 2, DPPH% was higher with increasing of crocetin and bixin concentration in the formulation of used nanoparticles in cheddar cheeses ($p \leq 0.05$). The highest percentage of inhibition of free radicals was observed for cheese treatment with crocetin (0.05 μg mL^{-1}) and bixin (18 μg mL^{-1}) and lowest for control cheese ($p \leq 0.05$). The highest free radical inhibitory effect on the first day of cheddar cheese production and the lowest free radical inhibition was maintained until the sixth day of storage ($p \leq 0.05$).A significant reduction was observed in mean of inhibitory activity of free radicals (DPPH) of cheddar cheese treatments during storage time (60 days) ($p \leq 0.05$). During storage, the highest reduction in the inhibition percentage of free radicals of cheddar cheese was observed in treatment with 1% crocetin and 14 mg bixin. Also, at the end of the sixth day, the highest free radical inhibitory index belonged to treatment with crocetin 0.05 μg mL^{-1} and bixin 18 μg mL^{-1} (T7) and even minimum amount of DPPH was observed in control cheese (T) ($p \leq 0.05$). Determining DPPH free radicals is one of the valid, accurate, easy and affordable methods with high repeatability which is used to evaluation of antioxidant activity of various compounds in laboratory conditions. Increasing the concentration of antioxidant compounds directly increases the capacity of various antioxidant compounds to inhibition free radicals. In this research, in treatment with higher crocetin and bixin, DPPH gained in

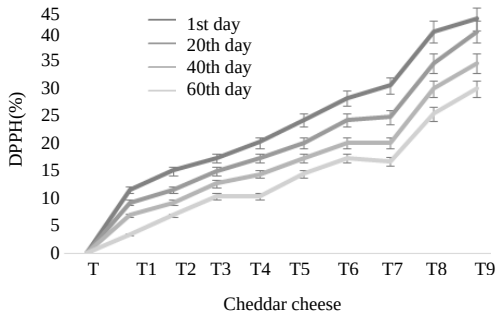

Fig. 2: Inhibitory free radicals index of cheddar cheese during 60 th day storage

comparison with control and low amount of crocetin and Bixin, due to the increasing of hydroxyl groups present in the reaction medium and hydrogen transfers probability to free radical and inhibition power of nanoparticles in cheese accreted. The results have demonstrated that bixin and neurobixin as well as active compounds of saffron such as crocin, crocetin and safranal are capable of inhibiting free radicals[22]. It sounds during storage, release of bixin and crocetin diminished due to cluster and agglomeration forming between nanoparticles (Table 1), furthermore bixin and Crocetin reacts with proteins and fat globules of cheese which led to inactivation of functional groups and it declined DPPH. Rahaiee *et al*.[10] also developed crocin loaded alginate chitosan nanoparticles and found that the free radical inhibitory effect was directly related to the concentration of crocin used in nanoparticle formulation and with increasing concentration of crocin, the amount of radical inhibitory power significantly surged that was in agreement with results of recent research.

Evaluation cheddar cheese acidity: Figure 3 demonstrated that acidity of cheddar cheese including bixin and crocetin nanoparticle decreased gradually during 60 day storage in comparison with control. It seems because of the outer coating of nanoparticles composed of chitosan and it solvesin acidic media, hence, the amino groups of chitosan molecules can react with the functional groups of lactic acid present in the cheese and reduce the preservation titratable lactic acidgained ($p \leq 0.05$). Among the treatments of cheddar cheese, the acidity level in the control was higher than other treatments, autoxidation and lipolysis is one of affective factors for increasing acidity of cheese media. The presence of crocetin and bixin in cheddar cheese during storage can prevent from the microbial growth rate and oxidative reactions to a large extent and the acidity of the cheddar cheese treatment experienced gradually in comparison with control. Rashidinejad *et al*.[22] found similar results in the study of the effects of catechins of

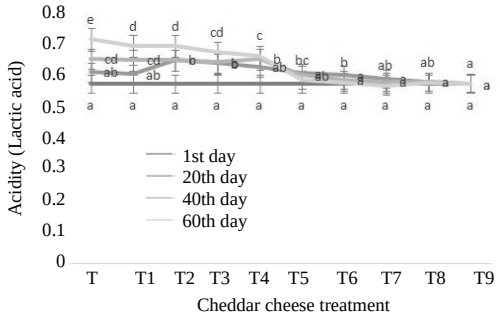

Fig. 3: Acidity changes of cheddar cheese during 60th day storage

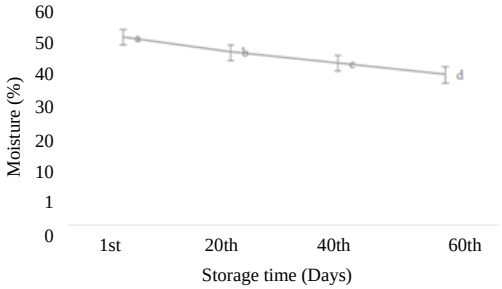

Fig. 4: Moisture changes of cheddar cheese during 60th day storage

greentea and its antioxidant compounds on low fat cheeses. They found that antioxidant compounds during the maintenance period prevented the increasing of acidity in low-fat cheese which was in agreement with the results of this study.

Evaluation cheddar cheese moisture percentage: As demonstrated in Fig. 4 there are significant changes in moisture content between treatments. Since, crocetin and bixin are not hygroscopic and adsorbent, they are not able to maintain moisture during storage. Chitosan does not have the ability to dissolve in an aqueous medium and it can solve in an acidic environment, therefore, it cannot play an important role in the absorption and maintenance of water in cheddar cheese treatments. However, during the storage period, moisture exchange with because of increasing exchange with ambient air humidity by increasing surface evaporation, environmental changes can cause surface evaporation of water molecules in cheddar cheese treatments and decrease the moisture content of cheese during the 60-day storage period. Ritota *et al.*[23] also reached similar results in the study of the effect of crocin in cheese. The use of effective crocin had no significant effect on the moisture content of cheese treatments ($p>0.05$) and this agreement was not conclusive.

Evaluation peroxide index in cheddar cheese: Figure 5 displayed that peroxide in cheddar cheese formulated with

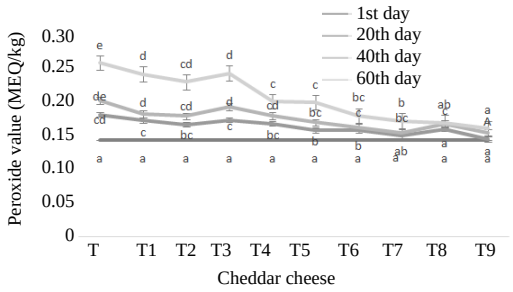

Fig. 5: Peroxide index changes of cheddar cheese during 60th day storage

nanoparticles with levels of 0.01, 0.03 and 0.05% of crocetin and bixin was 14 μg mL^{-1} increased with slow slope enlarge meaningfully ($p\leq0.05$). However, there were no significant differences between cheddar cheese treatments with same crocetin and bixin levels ($p\leq0.05$). During preservation of cheese peroxide value of cheese in all samples expanded. Cheddar cheese with 0.05 μg mL^{-1} crocetin and bixin 18 μg mL^{-1} experienced minimum growth and control reached to maximum level during 60 days. Various factors such as light, metal ions and oxygen can enhance the peroxide index. So that, the increase of peroxide value diminished the oxidative stability[8]. Carotenoids including bixin and neurobixin have antioxidant properties and the elimination of free radicals, single oxygen suppressants, cleans up of active oxygen species and nitrogen species and therefore have high anti-mutated and anti-cancer suppressant potentials[24]. Application of nanoparticles with high bixin and crocetin decreased peroxide of cheese during storage to inhibit free radicals due to oxidation as well as increasing the expansion of secondary processes of autoxidation and the production of secondary compounds. On the sixtieth day, maintenance of the amount of peroxide compounds due to conversion to malondialdehyde and the increase in the index of thiobarbituric acid is also reduced. On the sixtieth day, peroxide index diminished extent due to conversion to malondialdehyde and the increase of the thiobarbituric acid index is also observed simultaneously (Fig. 5). It sounds that oxidation of fats in cheddar cheese related to production secondary compounds like as aldehydes, ketones, alcohols and fatty acids. Increasing the peroxide value during storage is due to acceleration of oxidation during time. Oxidation is one of the most important processes in the production of free radicals in food, chemical systems and even biological systems. Saffron including compounds of crocin, crocetin and safranal which have the capacity to destruction of free radicals and inhibit them, so enhancing dosage of crocetinin formulation of cheddar cheese declines free radicals and inhibits the peroxide value. Ritota *et al.*[23] also found

same results in their research entitled the effect of crocin in cheese that the use of the active ingredient of crocin by increasing free radical inhibition and it can have a significant effect on the production of secondary compounds of cheese which was in agreement with recent research.

Evaluation Thiobarbituric Acid index (TBA) changes in cheddar cheese: The results of this study showed that the amount of thiobarbituric acid index in the treatment of cheddar cheese formulated using nanoparticles with levels of 0.01, 0.03 and 0.05% of crocetin and bixin at 14 μg mL^{-1} was minimum level during 60 day. However, there was no significant difference between the thiobarbituric acid indexes of cheddar cheese treatments with equal amounts of bixin ($p>0.05$). With increasing the amount of bixin consumption by 16 μg mL^{-1}, the index of thiobarbituric acid index of cheddar cheese treatments significantly decreased ($p\leq0.05$) in comparison to cheddar cheese preparations with values of 14 μg mL^{-1} ($p\leq0.05$). Figure 4 demonstrated that thiobarbituric experienced significant increasing in control treatment during the 60 days storage period ($p\leq0.05$). Usually measurements of secondary oxidation changes in food are preferable because hydro peroxides are volatile, colorless and non-flavored while secondary products such as ketones, aldehydes, hydrocarbons and alcohols are more stable and odorous. The results of this study showed that TBA index of cheddar cheese increased during storage which could be due to the diminishing in pH during the storage come outs from microbial oxidation of bacteria. Sharma *et al*.[25] studied the production of white cheese. They found that the use of antioxidant compounds in cheese can inhibit TBA during storage which was in agreement with recent research findings.

Evaluation cheddar cheese colorimetry indexes: Figure 6 demonstrated that bixin/crocetin nanoparticles had no meaningful effects on the Lightness index (L*) of cheddar cheese but it declined during storage meaningfully which was due to oxidation changes during storage, so that, minimum level of Lightness (L*) was observed in 60h day. Using crocetin and bixin inhibits fat oxidation can yellowness index (b*) also it increased gradually in comparison with control ($p\leq0.05$). In addition, chitosan used as carrier for crocetin and bixin has antimicrobial effects and significantly inhibits the microbial growth of cheddar flora which affects the yellowness index (b*). Crocetin and bixin have red pigments incremented yellowing index (b*) of cheddar cheeses but during preservation, yellowing index (b*) gained and redness (a*) declined significantly ($p\leq0.05$). Ritota *et al*.[23] used active ingredient of crocin reduced the redness index (a*) and jaundice index (b*) significantly which was in agreement with results of this research.

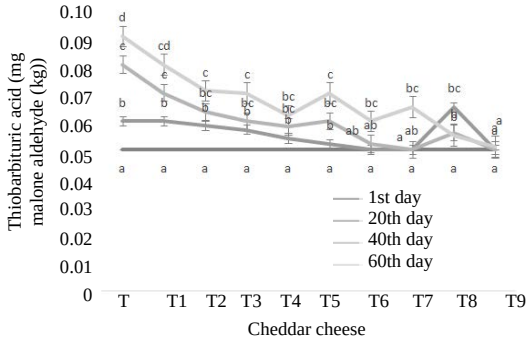

Fig. 6: Thiobarbituric acid changes of cheddar cheese during 60th day storage

Evaluation Microbial Total Count (TVC) in cheddar cheese: Figure 7 showed that during preservation of cheddar cheese, there was significant multifarious between sample and cheddar cheese with crocetin and bixin nanoparticles ($p\leq0.05$). Chitosan and alginate nanoparticles containing crocetin and bixin in cheese formulated inhibited the growth of total microbial count during preservation. Concerning the action of crostein/bixin nanoparticles in inhabitation of bacteria, it sounds that bixin and crocetin are distributed in cell wall and mitochondrial lipid portions of the bacteria which caused to pore formation. Following this, a large portion of the ions and other vital contents of the cell leak out which ultimately leads to bacterial death. Chitosan is a compound of glucosamine and n-acetyl glucosamine, linked together by glycosides 1 and 4. Today, antibacterial and antifungal properties of chitosan have been reported against a wide range of bacterial and fungal diseases[20]. However, chitosan has limitations for use due to its low solubility in water and acidity solubility. In recent years, several studies have been conducted on the production of water-soluble chitosan with antibacterial properties[26]. Chitosan with a wide range of antimicrobial activity exhibits a various inhibitory effect on fungi, a gram positive and negative bacterium. Antibacterial activity is a complex stage that differs between gram-positive and gram-negative bacteria due to cell surface specification. Chitosan has been reported to have a stronger bactericidal effect on gram-positive bacteria than gram-negative bacteria. This may be due to the external membrane in bacteria cell wall damping of the gram-negative bacteria. Chitosan is at a pH below 6 polycationic and reacts easily with negative compounds such as proteins, anionic polysaccharides, fatty acids and phospholipids. Chitosan, due to these polycationic properties, causes bacterial membrane degradation and has antibacterial properties[18]. Indeed, the antibacterial property of chitosan depends on the protonation of amino groups that can reacts with the negative charge of the cell

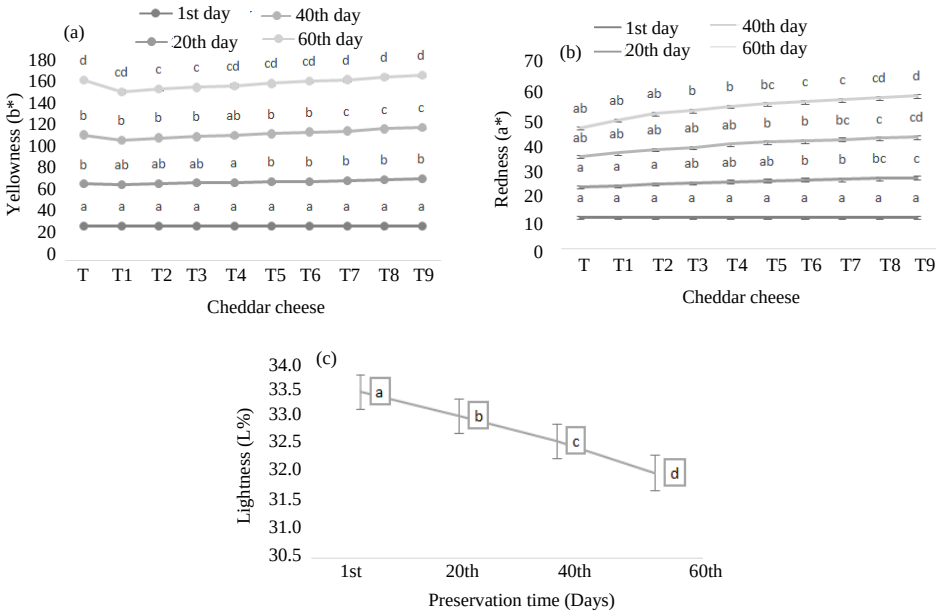

Fig. 7(a-c): Colorimetric changes of cheddar cheese during 60th day storage

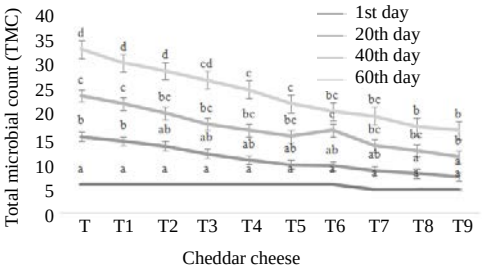

Fig. 8: Total microbial count changes of cheddar cheese during 60 days storage

surface and It can led to bacterial cell destruction. The molecular weight and degree of deacetylation of chitosan plays various roles in solubility of chitosan. By increasing the amount of crocetin and bixin entrapment efficacy diminished somewhat and the level of free crocetin and bixin gained on the surface of nanoparticles which at high concentrations can also be used to detect lethal effects through surface reaction with chitosan. Ritota *et al*.[23] also found similar results in the study of the effect of crocin in cheese. The use of crocin content of total microbial index diminished significantly which was consistent with the results of this study (Fig. 8).

CONCLUSION

Crocetin and bixin nanoparticles were prepared and formulated for use in nanoparticles. The nanoparticles had a spherical, soft and smooth surface. Also, acidity index of cheese increased and pH and DPPH decreased ($p \leq 0.05$). Lightness (L*) declined during preservation and yellowness (b*) and redness (a*) increased ($p \leq 0.05$). Peroxide and thiobarbituric acid index as well as significantly decreased with increasing dosage of crocetin/bixin in nanoparticles formulation ($p \leq 0.05$). Finally, treatment with 16 μg mL^{-1} bixin and crocetin was selected as the optimum treatment.

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