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Improving the Stability and Antioxidant Activity of Curcumin in Stirred Yogurt Using Chitosan-Coated Milk Fat-Based Nanostructured Lipid Carriers

Arash Abaee[1], Mohammad Ghorbani[2*], Alireza Sadeghi Mahoonak[3], Sara Naji-Tabasi[4]

1- Ph.D. Student in Food Science and Technology, Department of Food Science and Technology, Faculty of Food Sciences and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

2- Professor, Department of Food Science and Technology, Faculty of Food Sciences and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

3- Professor, Department of Food Science and Technology, Faculty of Food Sciences and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

4- Department of Food Nanotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran

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*Corresponding Author E-

ABSTRACT

Abstract: The incorporation of bioactive compounds such as curcumin into food products is a promising strategy for developing functional foods with health-promoting properties; however, the stability and bioavailability of these compounds remain challenging. In this study, milk fat–based lipid nanocarriers were synthesized for curcumin encapsulation and subsequently coated with chitosan. These nanocarriers exhibited high curcumin-loading capacity, achieving an encapsulation efficiency of 91.83%. Chitosan coating led to an increase in particle size (from 306 nm to 497 nm) and a shift in zeta potential (from –26.96 mV to +6.74 mV). FT-IR analysis confirmed the formation of a stable lipid–curcumin/chitosan/milk protein multilayer structure and indicated that curcumin was incorporated into the lipid matrix in a non-crystalline (amorphous) form. Chitosan coating significantly enhanced the thermal stability of curcumin: after 4 hours at 90 °C, 50.15% of curcumin remained in coated samples, compared to only 5.1% in uncoated ones. Following freeze-drying, the nanocarriers were incorporated into stirred yogurt to enrich its curcumin content. Physicochemical and organoleptic evaluations revealed that, although the lipid nanocarrier formulation enhanced antioxidant activity (as assessed by the DPPH assay), its stability over time was maintained only in the presence of the chitosan coating. Moreover, chitosan coating effectively mitigated the negative sensory impacts of curcumin enrichment on yogurt acceptability.

1-Introduction

The addition of bioactive compounds and nutraceuticals such as vitamins, probiotics, prebiotics, bioactive peptides, and antioxidants to foods leads to the production of functional foods that are physiologically beneficial and reduce the risk of disease. To date, various delivery systems have been designed for the microencapsulation of bioactive compounds. These systems include simple solutions, emulsions, suspensions, aggregated colloids, solid matrices, and so on. The use of delivery systems has led to the production of products with biological properties such as antioxidant, antibacterial, anti-inflammatory, anticancer, and antidiabetic activities [1].

Turmeric, with the scientific name *Curcuma longa* Linn, is a medicinal plant that is widely used in the treatment of infectious and non-infectious diseases [2]. Turmeric rhizome extract is mainly composed of three phenolic compounds called curcuminoids, such as curcumin or curcumin-I, curcumin-II (demethoxycurcumin), and curcumin-III (bisdemethoxycurcumin) [3]. In recent years, curcumin has received special attention due to its wide range of pharmacological functions and biological properties. Curcumin can be used as a flavoring agent, food preservative, natural colorant (bright yellow color), and as an antioxidant in foods [4]. Many studies have reported the application of curcumin in the prevention and treatment of inflammatory diseases, including skin wound healing, incisional and topical wounds, Parkinson's disease, Alzheimer's disease, and some types of cancer [5]. However, the poor water dispersibility, rapid metabolism, and low stability of curcumin reduce its bioavailability and also limit the applications of this bioactive compound in food products [6]. To overcome these challenges and improve the bioavailability of curcumin upon consumption, many studies have attempted to encapsulate curcumin using delivery systems such as hydrogels, nanoparticles, and liposomes [7].

Solid lipid nanoparticles (SLN) are colloidal systems designed for the encapsulation, protection, and delivery of lipophilic compounds such as bioactive lipids and drugs. Fats that are solid at room temperature as well as body temperature are used in the structure of solid lipid nanoparticles. To form these systems, the liquid oil phase (containing the melted carrier lipid and bioactive compounds) and the aqueous phase containing the surfactant are homogenized at a temperature above the melting point of the carrier fat to form an oil-in-water emulsion [8]. In the next step, the formed emulsion is cooled below the freezing point of the carrier fat to obtain solid fat particles. The use of solid lipid for drug delivery, compared to liquid fat, controls the release rate of the encapsulated compound and increases its stability against environmental conditions [9]. Today, solid lipid nanoparticles are widely used in the pharmaceutical industry. These systems have a very high potential for the encapsulation, delivery, and preservation of bioactive food compounds. If these delivery systems are used in the food industry, the solid lipid nanoparticles must be composed of compounds that are Generally Recognized As Safe (GRAS) and a production process must be used that is economically viable and leads to large-scale production. However, in general, the use of solid lipid nanoparticles in the delivery of bioactive compounds has some disadvantages, including the fact that the drug loading capacity in solid lipid nanoparticles is limited due to the crystalline structure of the solid lipid, the possibility of polymorphism occurring, the possibility of particle growth and gel formation in solid lipid nanoparticle dispersions during storage, and the encapsulated compound may be expelled

due to the formation of a highly crystalline network [9]. Whereas, in another type of delivery system called nanostructured lipid carriers (NLC), a portion of the fat exists as a liquid and the crystal lattice is imperfect [9]. Therefore, most of the problems associated with solid lipid nanoparticles are resolved in nanostructured lipid carriers. Furthermore, the encapsulation efficiency and physical stability during storage are higher in nanostructured lipid carriers [10]. One of the main trends in the development of nanostructured lipid carriers is the use of edible oils and fats to replace synthetic matrices that are not suitable for use in the food industry [11]. Milk fat has a semi-crystalline state at room temperature and lower temperatures [12]. Therefore, due to the diversity in the fatty acid profile of milk and consequently having different melting points for each fatty acid, nanostructured lipid carriers can be produced from milk fat without adding liquid fat from other sources [13]. Milk is a natural delivery system for various nutrients such as carbohydrates, fats, and proteins to the human body, and the solubility of drugs in milk is higher compared to fat-free aqueous systems [14].

Chitosan, due to its non-toxic, biodegradable, environmentally friendly properties, and positive charge, is considered a suitable natural polysaccharide for applications in the food, biomedical, and pharmaceutical industries [15]. This natural linear polymer consists of glucosamine and N-acetyl-D-glucosamine linked by β−(1→4) glycosidic bonds and is obtained from the partial deacetylation of chitin [16]. The presence of hydroxyl and amine groups on the surface of chitosan undergoes protonation at low pH; consequently, the positive charge of the molecule helps to create electrostatic interactions with the negatively charged components of cells. Coating with chitosan may add a positive charge to the surface of nanoparticles and hence modify the physicochemical properties of these nanoparticles [17]. Improved physicochemical stability, increased release of compounds, expanded cellular interactions, and improved bioavailability and efficacy of compounds have been reported as benefits of coating nanoparticles with chitosan [18, 19, 20].

In this study, milk fat was used as a lipid source to produce curcumin-loaded nanostructured lipid carriers, and a portion of these nanocarriers was coated with chitosan to improve stability. Then, by employing the freeze-drying process in the presence of milk proteins, a highly stable nutraceutical powder with direct application in dairy matrices was prepared. The distinction of this study compared to previous research is the simultaneous use of milk fat and protein, chitosan coating, conversion of nanocarriers into a nutraceutical powder, its use in the fortification of stirred yogurt, and the simultaneous evaluation of the physicochemical, antioxidant, and sensory properties of the product during storage.

2- Materials and methods

2.1. Materials

The raw materials used in this study included curcumin, n-hexane (HPLC grade, purity ≥99%), potassium hydroxide (purity ≥85%), sodium hydrogen sulfate (purity ≥99%), Tween 80, sodium azide (purity ≥99%), potassium bromide (purity ≥99%), Folin's reagent, and 2-propanol (purity ≥99.5%), all of which were purchased from Merck (Germany). Additionally, ethanol and methanol (purity ≥99.8%), acetic acid (purity ≥99%), and DPPH solution were purchased from Sigma-Aldrich (USA). Skim milk with 0.05% fat, 3.2%protein, 4.5% lactose, and a pH of 6.6, along with butter oil with 99.5% fat, were supplied by Pegah Iran Co. Yogurt starter containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains was obtained from Chr. Hansen (Denmark).

2.2. Preparation of nanostructured lipid carriers

To prepare the primary emulsion, curcumin was first dissolved in melted butter oil at 65 °C (oil phase), and then the aqueous phase (distilled water and Tween 80) was slowly added to the oil phase at 65 °C while stirring at 500 rpm (magnetic stirrer, Med Pipe, Iran). Next, this solution was homogenized using a homogenizer (Heidolph, Germany, model D911126) for 5 minutes at 8000 rpm. The emulsion consisted of curcumin, lecithin, and butter oil at weight ratios of 0.1, 2, and 12.9 as the oil phase, and distilled water and Tween 80 at weight ratios of 80 and 5% as the aqueous phase. In the next step, the primary emulsion was subjected to an ultrasound probe (Iranian Ultrasonic Technology Development Co., 400 W - 200 kHz) with an amplitude of 100 μm and 100% power for 10 minutes. After cooling to 25 °C, nanostructured lipid carriers were formed [21]. The final concentration of curcumin in the nanostructured lipid carriers was 1 mg/mL. The produced samples were kept in 50 mL Falcon tubes and stored in a refrigerator at 8 °C.

2.3. Coating nanoparticles with chitosan

Coating with chitosan was performed according to the method of Li et al. (2016) with slight modifications. Chitosan was added to a 2% acetic acid solution so that the weight percentage of chitosan in the solution was 1%. The chitosan solution was stirred for 8 hours using a magnetic stirrer and then filtered using filter paper to remove impurities. Coating with chitosan was performed by adding 10 mL of the nanoparticle solution to 10 mL of the chitosan solution under stirring conditions at 200 rpm for 12 hours at 25 ∘C. Then, ultrasound treatment with a power of 75 W for 5 minutes was applied to reduce the particle size and break any chitosan bridges that might have formed between the particles [18].

2.4. Freeze-drying of nanostructured lipid carriers

Drying of the nanostructured lipid carriers was carried out using a laboratory freeze-dryer (CHRIST Alpha 1-2 LDplus, Germany) at −40 °C and a vacuum pressure of approximately 0.1 mbar for 24 hours. Immediately after drying, the obtained powder samples were stored in dark glass vials with lids at refrigerator temperature (4 °C), away from light and moisture. The dried samples produced in this part of the study are presented in the table below:

Table 1 Treatments used in this study

Row	Treatment	Chitosan coating	Matrix	Curcumin
1	NLC	No	-	No
2	CS-NLC	Yes	-	No
3	Cur-NLC	No	-	Yes
4	CS-Cur-NLC	Yes	-	Yes
5	Milk-Cur-NLC	No	Milk	Yes
6	Milk-CS-Cur-NLC	Yes	Milk	Yes

2.5. Fourier-transform infrared (FT-IR) spectroscopy

Interactions and structures were investigated using the FT-IR method (Bruker, Germany, Tensor II model). For spectroscopic analysis preparation, 1 mg of

the dried sample was mixed with 100 mg of potassium bromide and ground in a mortar until a completely homogeneous mixture was obtained. The mixture was then poured into a pellet press and prepared as a pellet. The resulting spectra of the samples were examined in the wavenumber range of 400 to 4000 cm^{-1} [22].

2.6. Antioxidant activity assay by DPPH free radical scavenging method

The antioxidant activity of the free aqueous curcumin solution (unencapsulated) and curcumin-loaded nanostructured lipid carriers was measured using the DPPH radical scavenging assay according to the method of Mohammadian et al. (2019), with necessary modifications [23]. Briefly, 2 mL of the free aqueous curcumin solution (unencapsulated) or curcumin-loaded nanostructured lipid carriers with an equivalent concentration of curcumin was mixed with 2 mL of ethanolic DPPH solution with a concentration of 0.2 mM. The control sample was prepared by mixing 2 mL of DPPH solution and 2 mL of distilled water. The resulting solution was kept in the dark for 30 minutes at 25 °C and then centrifuged at 1500×g for 10 minutes. The decolorization of DPPH was measured by recording the absorbance at a wavelength of 517 nm using a spectrophotometer. The DPPH radical scavenging activity was calculated using the following equation:

DPPH(%)=[(Ac−As)Ac]×100

where AcA_cAc is the absorbance of the control sample and AsA_sAs is the absorbance of the main sample.

2.7. Determination of droplet size and zeta potential

Particle size was determined by a DLS instrument (Quadix model, Applied Photophysics, UK) and zeta potential by a Zetameter instrument (Particle Metrix Zeta Analyzer model, Metrix GmbH, Germany) at 25 °C. The samples were diluted 10-fold with deionized water to prevent multiple scattering phenomena due to interparticle interactions [24, 25].

2.8. Determination of curcumin encapsulation efficiency

First, a calibration curve was plotted using different amounts of curcumin in the range of 0.1 to 10 μg/mL10 (Y=0.138X+0.0049 R^2=0.9999). The encapsulation efficiency (EE) was determined based on the ratio of encapsulated curcumin to the initial content used. Accordingly, 1 mL of encapsulated curcumin was centrifuged at 4000 rpm for 20 minutes, and the supernatant was added to acetone at a ratio of 1:9. Finally, the absorbance was read at a wavelength of 426 nm using a UV-vis spectrophotometer [26]. The encapsulation efficiency was calculated using the following formula:

EE(%)=[Encapsulated curcumin (mg)/Tota l curcumin content (mg)]×100

2.9. Evaluation of the thermal stability of curcumin

An ethanolic solution of curcumin as a control sample was prepared by dissolving 10 mg of curcumin in 10 mL of ethanol to obtain a final concentration of 1 mg/mL. The CUR-NLC and CS-CUR-NLC treatments also contained an equivalent amount of curcumin (1 mg/mL). For analysis, 1 g of each control, CUR-NLC, and CS-CUR-NLC solution was diluted 10-fold with distilled water. Then, the solutions were heated in a water bath (Memmert, Germany, UFE500 model) at 90 °C for 4 hours. At intervals of 0, 0.5, 1, 2, 3, and 4 hours, the amount of curcumin was measured by recording the absorbance using a spectrophotometer (UV-1800 model, Shimadzu, Japan) at a wavelength of 420 nm [3].

2.10. Production of fortified stirred yogurt

To produce full-fat yogurt (5±0.1 g fat per 100 g), milk with 5% fat was pasteurized at 90 °C for 30 minutes. It was then cooled to 45 °C and inoculated with yogurt starters (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*). The samples were incubated at 45 °C for 4 hours. Subsequently, 5 g of curcumin-loaded NLC lyophilized powder (equivalent to 1 mg of pure curcumin) was added to 500 g of yogurt and stirred at 50 rpm for 30 minutes. In the next step, the cooled yogurt was transferred to a cold room at 4 °C. The pH of the yogurt on the first day in the cold room was 4.6 and at the end of 21 days was 4.4 [17].

2.11. Sensory evaluation

Sensory evaluation of the samples was performed using a 5-point hedonic scale (1 = completely undesirable, 2 = undesirable, 3 = moderate, 4 = desirable, 5 = completely desirable). This test was conducted by 20 trained evaluators (12 females and 8 males, aged 20–45 years) in two sessions (morning and evening, with a 48-hour interval) under standard sensory laboratory conditions (indirect lighting, 22 ∘C temperature, random three-digit coding of samples, and the use of mineral water for mouth rinsing between samples). The sensory attributes of the evaluated samples included taste, creaminess, syneresis, aftertaste, bitterness, sweetness, sourness, color, odor, and astringency [27].

2.12. Measurement of water holding capacity in fortified stirred yogurt

The water holding capacity (WHC) of the yogurt was measured according to the method of Taghadosi et al. (2024) with necessary modifications [28]. 20 g of yogurt (W1) was transferred to a Falcon tube and centrifuged at 3000×g for 10 minutes at 4 °C. After centrifugation, the separated yogurt water was weighed (W2), and the water holding capacity of the samples was calculated using the following formula:

$$WHC(\%)=[(W1-W2)/W1]\times100$$

2.13. Antioxidant activity of curcumin-fortified stirred yogurt during storage

To monitor the trend of changes in antioxidant activity from the beginning of the period to the end of the optimal consumption life, the antioxidant activity of the stirred yogurt samples during the storage period (up to 28 days at 4 °C) was evaluated on days 1, 7, 14, 21, and 28 using the DPPH free radical scavenging method. To prepare the methanolic extract, 5 g of each yogurt sample was successively mixed twice with 20 mL of 80% methanol. The resulting mixture was centrifuged for 10 minutes at 4500×g at room temperature, and the supernatant was separated through Whatman Grade 1 filter paper. The obtained extracts were combined and centrifuged again for 15 minutes at 1500×g. Then, the final clear liquid, after re-filtration, was used as the working extract. For the DPPH assay, 2 mL of the methanolic extract of the sample was mixed with 2 mL of ethanolic DPPH solution with a concentration of 0.2 mM. The resulting mixture was kept in the dark for 30 minutes at 25 °C to complete the reaction. After this time, the solution was centrifuged for 10 minutes at 1500×g, and the absorbance of the final clear liquid was measured at a wavelength of 517 nm using a spectrophotometer (UV-1800 model, Shimadzu, Japan). The following formula was used to calculate the DPPH radical scavenging activity [28]:

$$DPPH(\%)=[(Ac-As)/Ac]\times100$$

where Ac is the absorbance of the control sample and As is the absorbance of the main sample.

2.14. Statistical analysis

A completely randomized design was used for data analysis and evaluation. To determine the significance of differences between data means after analysis of variance (ANOVA), Duncan's multiple range test at a 5% significance level was employed. All stages of statistical analysis of the results were performed using SPSS 16 software. Excel software was used to draw the graphs. All tests were conducted in triplicate.

3-Results and discussion

3.1. Fourier-transform infrared (FT-IR) spectroscopy

The FTIR spectra for different samples of curcumin-loaded nanostructured lipid carriers are shown in Figure 3. This analysis was performed to investigate the possible chemical interactions among curcumin, the components of the lipid nanocarrier, the chitosan coating, and the milk matrix proteins.

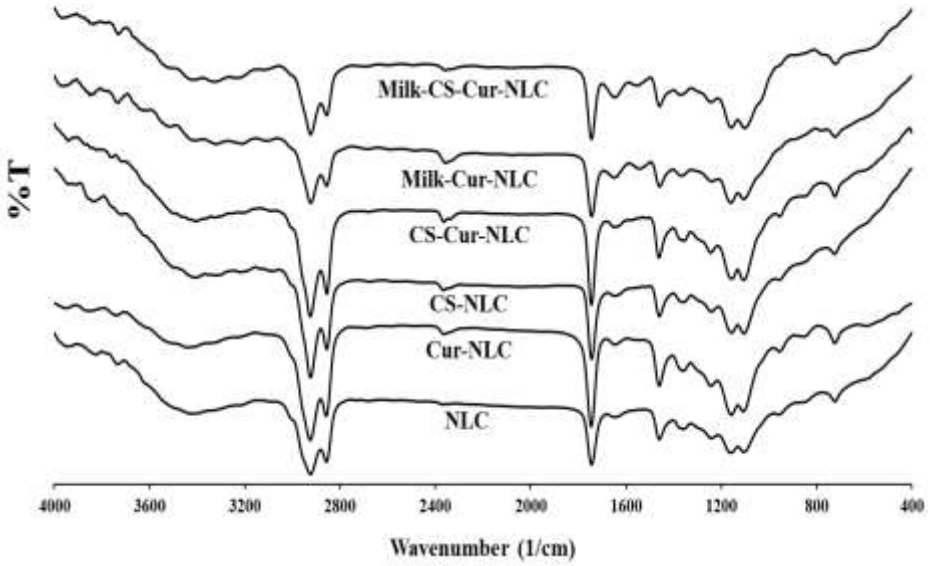


Figure 3 FTIR spectra of nanostructured lipid carriers (NLC), curcumin-loaded nanostructured lipid carriers (Cur-NLC), chitosan-coated nanostructured lipid carriers (CS-NLC), curcumin-loaded nanostructured lipid carriers coated with chitosan (CS-Cur-NLC), and nanostructured lipid carriers in milk matrix (Milk-Cur-NLC and Milk-CS-Cur-NLC)

In the NLC sample spectrum, a strong band in the range of 2920−2850 cm^{-1} was attributed to the stretching vibrations of methylene ($–CH_2$) and methyl ($–CH_3$) groups of the lipid fatty acid chains, indicating the presence of solid lipids in the nanocarrier structure. Furthermore, a distinct peak at 1740 cm^{-1} corresponding to the stretching vibration of the carbonyl (C=O) group of triglyceride esters was observed, which is a characteristic feature of the glyceride compounds used in the NLC. A comparison of the NLC and Cur-NLC spectra revealed slight changes in the intensity and position of the spectral bands. In Cur-NLC, the bands related to $–CH_2$ and $–CH_3$ stretching vibrations slightly shifted to higher wavenumbers and their intensity increased. These changes indicate hydrophobic interactions between curcumin molecules and the lipid matrix, leading to the disruption of a part of the ordered crystalline structure of the lipids [29]. Additionally, the intensity of the peak

at 1740 cm^{-1}1740 was higher in Cur-NLC, representing the presence of C=C and C=O bonds of curcumin [30].

In the chitosan-coated samples (CS-NLC and CS-Cur-NLC), a broad and strong band in the 3400 cm^{-1} region became significantly more prominent. This band corresponds to the stretching vibrations of the hydroxyl (–OH) and amine ($–NH_2$) groups of chitosan, the presence of which indicates the coating of the nanoparticles' surface with this polar polysaccharide. The broadness and high intensity of this band signify the formation of multiple hydrogen bonds between the functional groups of chitosan and the nanoparticle surface. These interactions play a key role in the stability of the coating layer and the controlled release of the drug. Consistent with the results of this study, Al-Ziyadi et al. (2024) reported that the peak appearing at 3306 cm^{-1} in the spectrum of chitosan-coated nanostructured lipid carriers corresponds to the stretching vibrations of the OH and NH_2 bands of chitosan [31].

In the samples prepared in the milk matrix (Milk-Cur-NLC and Milk-CS-Cur-NLC), notable changes were observed in the protein-related regions. In particular, the peaks at 1650 cm^{-1} corresponding to the Amide I band (arising from the C=O stretching of the peptide group) and 1540 cm^{-1} corresponding to the Amide II band (including N–H bending and C–N stretching) became prominent, indicating the presence of milk proteins (mainly casein and whey proteins) in the final structure of the formulation. These changes suggest surface interactions between milk proteins and lipid nanoparticles, which can lead to the formation of a stable protein layer on the surface of the particles [32]. This layer not only prevents nanoparticle aggregation but also improves the colloidal stability of the system during the freeze-drying and re-dissolution processes. The presence of chitosan in this system caused this peak to shift (increase) to 1557 cm^{-1}. This shift may be due to the formation of hydrogen bonds between milk proteins and chitosan, which can increase the bending vibration frequency of N–H groups [33]. Moreover, the decreased intensity of the bands related to the lipid carbonyl groups at 1740 cm^{-1} and the changes in the $–CH_2$ and $–CH_3$ bands in the milk-containing samples indicate the partial coverage of the lipid surface by milk proteins. This phenomenon can be interpreted as evidence of the formation of lipid-protein complexes on the nanoparticle surface, which is effective in improving the stability and performance of the curcumin delivery system.

Overall, the main peaks of the samples appeared in approximately similar positions. However, slight changes in the intensity and location of the peaks were observed, reflecting chemical changes caused by intermolecular interactions among the formulation components. The FT-IR results confirmed the existence of hydrophobic (curcumin–lipid), hydrogen (chitosan–lipid/protein), and electrostatic (chitosan–milk protein) interactions, which led to the formation of a stable multilayer lipid-curcumin/chitosan/milk protein structure.

3.2. Antioxidant activity

Curcumin is well known for its strong antioxidant activity; however, its bioactivity is low due to its poor water solubility. The DPPH free radical scavenging activity is based on the kinetics of electron transfer or hydrogen atom transfer reactions. The extremely low solubility of curcumin in water leads to the formation of numerous curcumin aggregates, which reduces the amount of available curcumin for scavenging free radicals and lowers its reducing power. In contrast, aqueous solutions of lipid carriers demonstrated a high capacity for free radical scavenging. In this regard, the encapsulation of curcumin in lipid carriers prevented its degradation and increased its

antioxidant activity compared to free curcumin. Furthermore, chitosan coating can enhance curcumin's antioxidant capacity by increasing its stability. Overall, the DPPH free radical scavenging activities of Cur, Cur-NLC, and CS-Cur-NLC systems were 35%, 65%, and 64.2%, respectively (Figure 5).

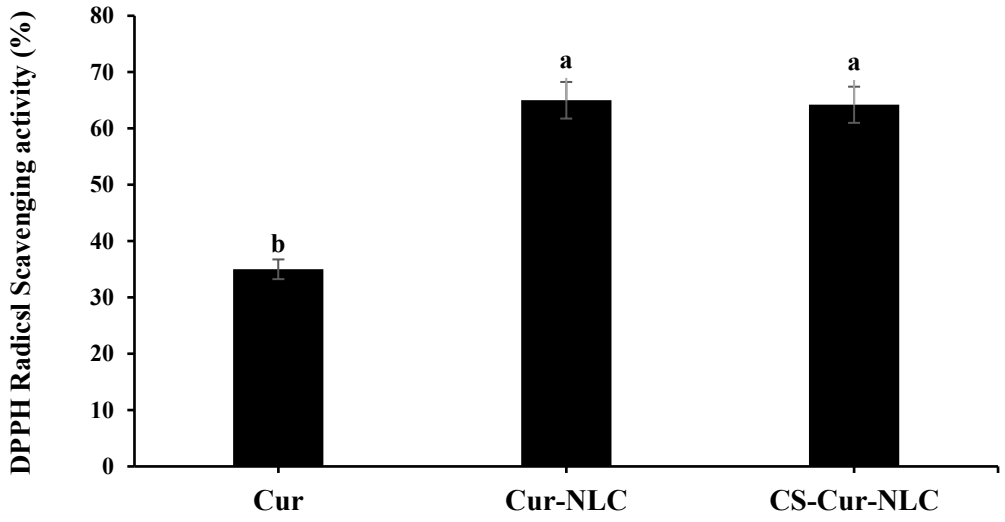


Figure 5 Antioxidant activity of free curcumin (Cur), curcumin-loaded nanostructured lipid carriers (Cur-NLC), and chitosan-coated curcumin-loaded nanostructured lipid carriers (CS-Cur-NLC), expressed as DPPH free radical scavenging activity

These results indicate that the encapsulation of curcumin in lipid carriers provides a more suitable molecular space for curcumin to react with free radicals and accelerates the electron/hydrogen transfer reaction.

Previous studies indicate that curcumin exhibits very low antioxidant activity in aqueous environments, and its microencapsulation in a lipophilic polymer matrix is required to significantly enhance its bioactivity [34, 35]. Chang et al. (2017) reported that the microencapsulation of curcumin in protein-polysaccharide complex nanoparticles dramatically improved its free radical scavenging activity compared to free curcumin dissolved in ethanol or dispersed in water. This can be explained by the improved dispersion of microencapsulated curcumin within the nanoparticles, which facilitates the reaction kinetics with free radicals [36].

In studies by Huang et al. (2016) and Hu et al. (2020), higher antioxidant activity was reported for curcumin in nanoparticles compared to its free form; this was attributed to the low aqueous solubility of curcumin, but its incorporation into nanoparticles increases the amount of curcumin available to interact with free radicals [37, 38]. In this regard, Karimi et al. (2018) reported that the encapsulation of turmeric extract in nanostructured lipid carriers significantly increased its antioxidant activity based on the DPPH method, which was attributed to the presence of curcuminoids and hydroxyl groups in the structure of the turmeric extract. The antioxidant role of its double bonds and carbonyl groups with para-

hydroxy groups was also highlighted [39]. The high antioxidant properties of curcumin are a useful tool in increasing the shelf life of foods and reducing lipid oxidation and the degradation of bioactive components.

3.3. Particle size and zeta potential

Figure 2 shows the particle sizes of nanostructured lipid carriers (NLC), curcumin-loaded nanostructured lipid carriers (Cur-NLC), chitosan-coated nanostructured lipid carriers (CS-NLC), and curcumin-loaded, chitosan-coated nanostructured lipid carriers (CS-Cur-NLC). The particle size is represented by D_{50}, which is the median diameter of the nanoparticles.

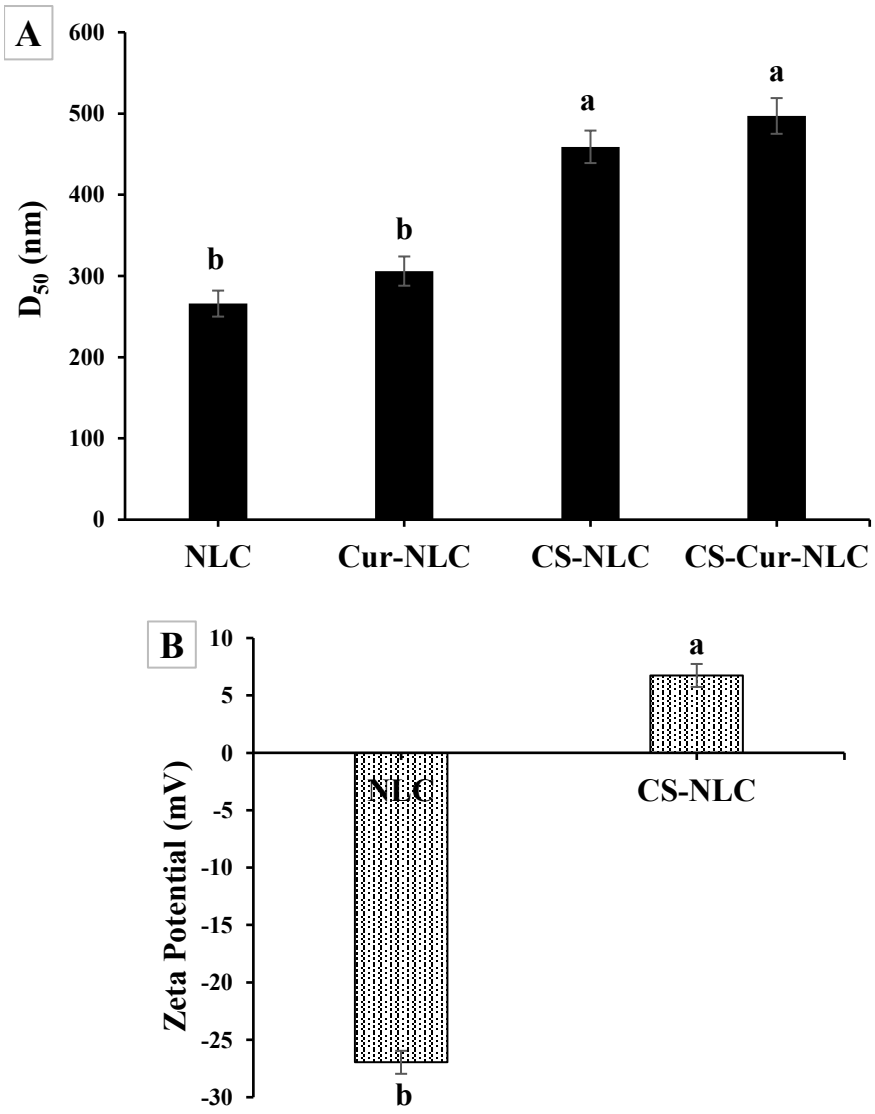


Figure 2. A) Particle size of nanostructured lipid carriers (NLC), curcumin-loaded nanostructured lipid carriers (Cur-NLC), chitosan-coated nanostructured lipid carriers (CS-NLC), and curcumin-loaded nanostructured lipid carriers coated with chitosan (CS-Cur-NLC) B) Zeta potential of nanostructured lipid carriers (NLC) and chitosan-coated nanostructured lipid carriers (CS-NLC)

The particle sizes of the NLC and Cur-NLC treatments were 266 and 306 nm, respectively. The results indicate that the addition of curcumin increases the particle size, which may be due to the extra volume occupied by the curcumin molecules within the lipid matrix.

The particle size for CS-NLC was found to be 459 nm. Coating the nanostructured lipid carriers with chitosan led to a significant increase in the average particle size compared to NLC ($p<0.05$). This increase can be attributed to the additional chitosan layer around the nanoparticles. The particle size for CS-Cur-NLC was reported as 497 nm, which was the largest particle size among the four types of nanostructured lipid carriers, indicating that both curcumin loading and chitosan coating play a role in the size increase. In fact, when both curcumin loading and chitosan coating were applied, the particle size increased further, which could be due to the combined effects of the internal curcumin loading and the external chitosan layer.

It is worth noting that coating the nanostructured lipid carriers with chitosan caused the zeta potential of the particles to shift from negative values (−26.96 mV) to positive values (+6.74 m), which is evidence of the successful coating of the nanoparticles with chitosan.

In agreement with the findings of this research, Al-Ziyadi et al. (2024) reported that chitosan-coated nanostructured lipid carriers had a particle size of 198 nm and a surface charge of +8.41 mV, which prevents their aggregation in aqueous media and reflects their acceptable stability [31]. Furthermore, Bashiri et al. (2020) showed that coating NLC with chitosan increases the particle size, and this was attributed to the cationic nature of chitosan and the resulting electrostatic interactions between its chains and the negatively charged components on the surface of the NLC particles [24]. An increase in chitosan concentration can lead to the formation of a thick multilayer surface around the NLC particles and subsequently increase their particle size. Moreover, the chitosan coating shifted the zeta potential from −17.56 mV to +21.4 mV. The negative surface charge of NLC was attributed to the free hydroxyl groups of phenolic compounds in the essential oil or free fatty acids (carboxylic acid) and the phosphate group of phospholipids, which are naturally present in most edible oils and lipids. This negatively charged NLC surface can electrostatically interact with the positively charged amine groups (−NH3+) of chitosan, which in turn alters the surface charge of the chitosan-coated samples from negative to positive values [24]. Luo et al. (2015) reported that when the melted lipid phase was homogenized in the aqueous phase containing chitosan, the particle size of the lipid nanoparticles increased from 252.5 nm to 269 nm, and the zeta potential changed from a negative (−15.9 mV) to a positive value (+26.1 mV), confirming the successful coating of the particles with chitosan [31]. In another study, it was found that coating lipid nanoparticles with chitosan increased the particle size from 140 nm to 235 nm; in addition, the zeta potential of the lipid nanoparticles increased from −35 mV to +29 mV [20]. Similarly, coating lipid nanoparticles with chitosan caused the particle size to increase from 243 nm to 470 nm and the zeta potential to shift from −25.1 mV−25.1 mV to +34.2 mV. The increase in particle size and the inversion of surface charge confirm the presence of the chitosan coating around the surface of the nanostructured lipid

carriers. Chitosan-coated lipid nanoparticles with a positive surface charge can interact with the negatively charged membrane of intestinal cells [40].

3.4. Encapsulation efficiency

The encapsulation efficiency of curcumin in the nanostructured lipid carriers was 91.83±0.50%, indicating the high efficiency of the nanoparticles in encapsulating curcumin. In this regard, a curcumin encapsulation efficiency of more than 91% has been reported in nanostructured lipid carriers [41]. Additionally, the encapsulation efficiency of curcumin in chitosan nanoparticles has been reported in the range of 80.40% to 88.40%, depending on the concentration of chitosan and tripolyphosphate [42]. In another study, the encapsulation efficiencies of curcumin in control lipid nanoparticles, chitosan-coated lipid nanoparticles, and trimethyl chitosan-coated lipid nanoparticles were reported to be 93.13%, 93.12% and 93.12%, respectively [43]. It seems that the high hydrophobicity of curcumin leads to its high encapsulation efficiency within the lipid core. In the study by Taghadosi et al. (2024), it was found that curcumin nanocapsules stabilized with Persian gum had a negative surface charge (−30.78 mV) and a high encapsulation efficiency (96.84%). Maintaining a high concentration of curcumin in these structures was attributed to the possible formation of hydrogen bonds between the carboxyl groups of the hydrocolloid and the hydroxyl groups of curcumin, as well as hydrophobic interactions between the lipophilic regions of the gum and the aromatic rings of the curcumin structure [28].

3.5. Thermal stability of curcumin

Figure 4 shows the thermal stability of curcumin in the two systems of curcumin-loaded nanostructured lipid carriers (Cur-NLC) and chitosan-coated curcumin-loaded nanostructured lipid carriers (CS-Cur-NLC) over a heating time of 4 hours at 90°C According to the results, increasing the heating time led to a significant decrease in the amount of curcumin in both systems. After 2 hours of heating, the residual amount of curcumin in the Cur-NLC and CS-Cur-NLC systems was 8.3% and 22.3%, respectively. With continued heating, after 4 hours at 90°C, only 1.5% of curcumin was retained in the Cur-NLC system, whereas CS-Cur-NLC was able to retain 15.50% of curcumin, demonstrating the remarkable efficiency of the chitosan coating in improving the thermal stability of curcumin.

In line with the results of this study, researchers reported that although increasing the storage temperature accelerates the degradation of curcumin in lipid carriers, the presence of higher concentrations of chitosan on the surface of the nanoparticles led to the retention of a higher concentration of curcumin within the nanoparticles. By forming a protective layer on the surface of the nanoparticles, chitosan can prevent the direct contact of curcumin with the thermal environment and its subsequent oxidation and hydrolysis under thermal conditions. Furthermore, by creating a positive charge on the surface of the nanoparticles, chitosan can enhance colloidal stability and prevent particle aggregation under the influence of heat [3].

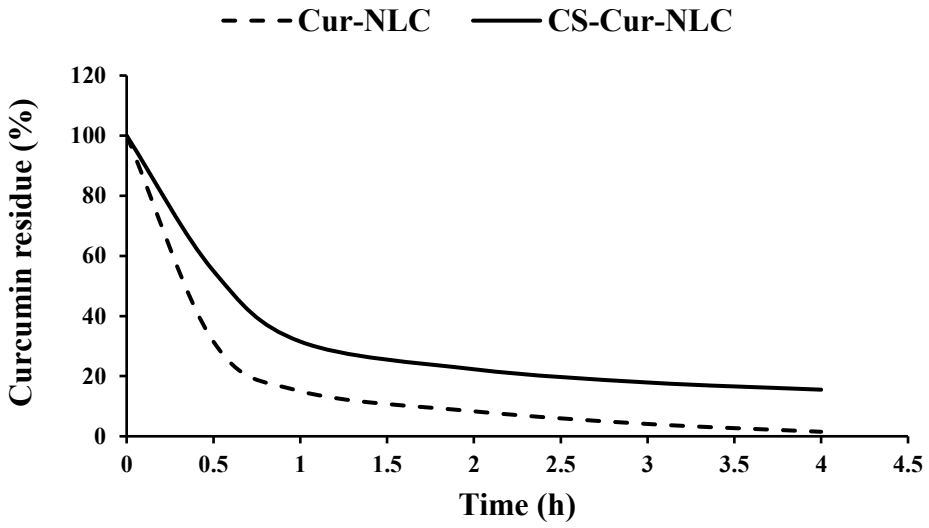


Figure 4 Thermal stability (residual mass) of curcumin in nanostructured lipid carriers (Cur-NLC) and chitosan-coated nanostructured lipid carriers (CS-Cur-NLC)

3.6. Sensory evaluation of fortified stirred yogurt

Figure 6 shows the sensory properties of the control yogurt, Yog-Milk-Cur-NLC, and Yog-Milk-CS-Cur-NLC based on a 5-point hedonic test. The control yogurt (4) and the Yog-Milk-CS-Cur-NLC treatment (5) received the lowest and highest creaminess scores, respectively. The chitosan coating may cause less disruption to the texture of the final product by establishing stability in the nanoparticles. In this regard, the Yog-Milk-CS-Cur-NLC sample exhibited the highest sensory score for syneresis (the lowest amount of syneresis), which was consistent with the water-holding capacity findings of the samples. Accordingly, the water-holding capacity in the control, Yog-Milk-Cur-NLC, and Yog-Milk-CS-Cur-NLC samples was obtained as 62±0.60%, and 65±0.50%, respectively. This condition might be due to the water-absorbing property of chitosan and the interaction of chitosan with the yogurt protein network structure, thereby increasing its strength. Taghadosi et al. (2024) showed that, in general, adding curcumin nanocapsules prepared with Persian gum to the yogurt formulation increased the total solids content, consequently leading to an increase in water-holding capacity [28]. Akgün et al. (2020) also observed a reduction in syneresis in yogurt formulated with chitosan-coated cherry extract liposomes [44]. Furthermore, it has been reported that the addition of hydrocolloids, such as chitosan, in yogurt formulation can reduce syneresis by increasing water-holding capacity and viscosity [45].

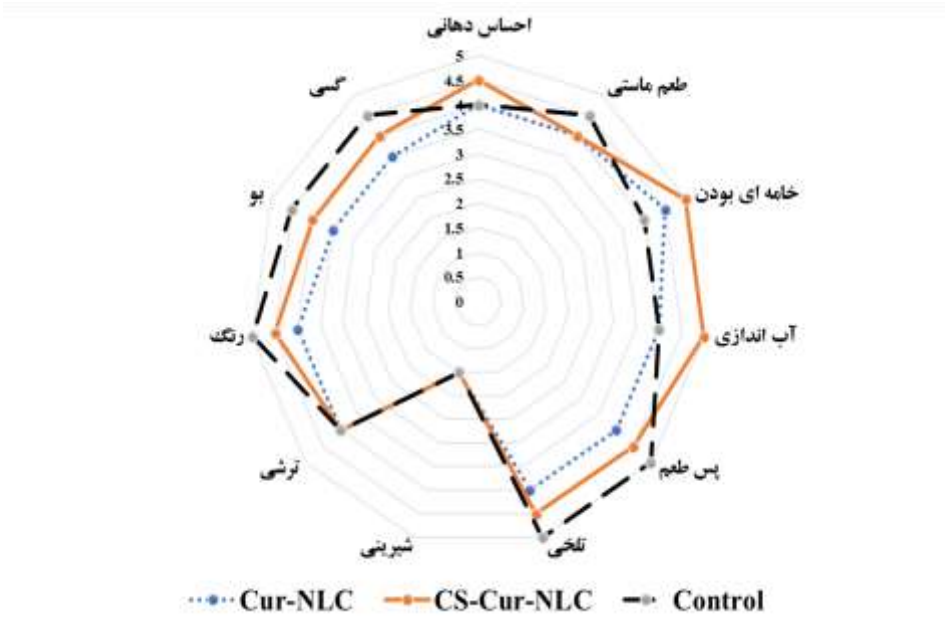


Figure 6: Sensory evaluation (sensory scores) of control yogurt samples (Control), yogurt enriched with lipid nanocarriers (Yog-Milk-Cur-NLC), and yogurt enriched with chitosan-coated lipid nanocarriers (Yog-Milk-CS-Cur-NLC)

Although the addition of nanostructured lipid carriers did not alter the sensory scores for sweetness and sourness of the final product, the fortified samples received lower sensory scores for bitterness, aftertaste, color, odor, and astringency compared to the control sample, which is attributed to the presence of the polyphenolic compound curcumin. However, coating the nanoparticles with chitosan reduced the negative sensory effects of curcumin. In fact, the chitosan coating helps improve sensory properties, including reducing bitterness and aftertaste, as well as relatively improving the texture. In this regard, it has been reported that coating carrier systems with chitosan leads to a decrease in the bitter taste of drugs due to their slower release in the oral phase [46]. Although the samples containing nanoparticles exhibited higher antioxidant properties, this might negatively affect consumer sensory acceptance. For the production of functional yogurt, optimizing the size and concentration of nanoparticles or utilizing complementary flavorings could enhance sensory acceptance.



Figure 7: Visual appearance images of control yogurt samples (Control), yogurt enriched with lipid nanocarriers (Yog-Milk-Cur-NLC), and yogurt enriched with chitosan-coated lipid nanocarriers (Yog-Milk-CS-Cur-NLC).

Similar results have been reported in the literature. In this regard, Taghadosi et al. (2024) reported that when curcumin-loaded nanocapsules were added to the yogurt mixture, the sensory scores for the texture and odor of the samples did not change significantly. However, the acceptance scores for color, taste, and overall acceptability were all lower. In terms of sensory characteristics, all samples were considered satisfactory and received good sensory scores, although the sensory scores of some attributes had decreased. In their study, Persian gum was used to encapsulate curcumin, which led to a reduction in the taste, color, and odor intensity of this compound [28]. In another study, Liu et al. (2018) reported a decrease in the taste score of yogurt upon curcumin incorporation, but no significant changes were observed in the odor and texture scores of the samples, and the fortified sample was still considered generally acceptable [47].

3.7. Antioxidant activity of stirred yogurt fortified with curcumin during the storage period

Curcumin, as a polyphenolic compound, is well known for its significant antioxidant properties. Figure 8 shows the changes in the antioxidant activity of the control

yogurt, yogurt fortified with curcumin-loaded nanostructured lipid carriers (Yog-Milk-Cur-NLC), and yogurt containing chitosan-coated curcumin nanostructured lipid carriers (Yog-Milk-CS-Cur-NLC) during the storage time (1, 7, 14, 21, and 28 days), based on DPPH free radical scavenging activity assays.

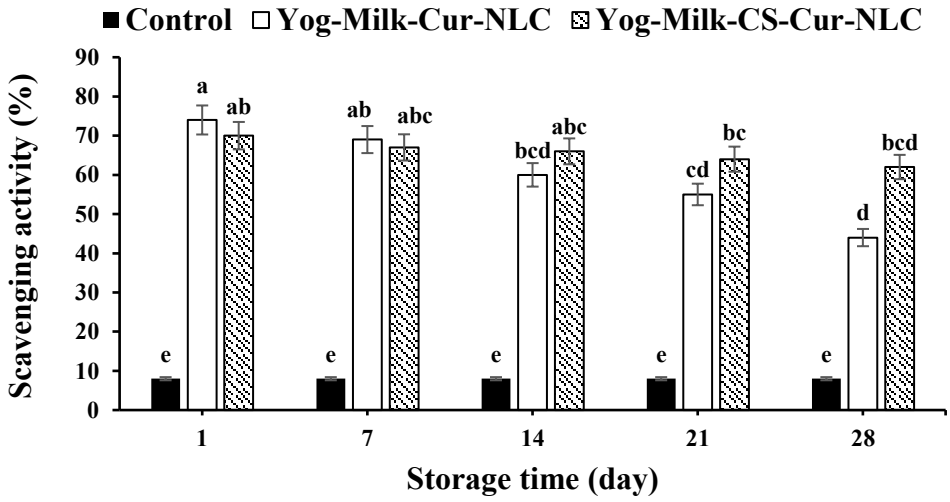


Figure 8 DPPH free radical scavenging activity of control yogurt samples (Control), yogurt enriched with lipid nanocarriers (Yog-Milk-Cur-NLC), and yogurt enriched with chitosan-coated lipid nanocarriers (Yog-Milk-CS-Cur-NLC).

The results indicated that the control sample, despite the absence of curcumin, exhibited a basal antioxidant activity of approximately 8% throughout all storage days, which remained without a significant reduction. This activity can be attributed to the natural bioactive compounds present in milk and yogurt, including antioxidant peptides derived from the activity of lactic acid bacteria, antioxidant vitamins such as vitamin E, antioxidant defense enzymes, and secondary metabolites produced during fermentation [48].

The Yog-Milk-Cur-NLC treatment exhibited higher antioxidant activity compared to the other samples on the first and seventh days of storage. However, over time, its antioxidant activity decreased, and the CS-Cur-NLC sample demonstrated better antioxidant properties; such that the antioxidant activities of the Yog-Milk-Cur-NLC and Yog-Milk-CS-Cur-NLC samples on the 28th day of storage were 44% and 62%, respectively. This higher stability in the CS-Cur-NLC sample can be attributed to several factors: First, the chitosan coating acts as a protective layer against degrading agents such as light, oxygen, and low pH. Second, by inducing a positive surface charge, chitosan improves the colloidal stability of the nanostructures in the yogurt matrix and prevents their aggregation. Third, by controlling the gradual release of curcumin, this coating prevents its rapid degradation and preserves its activity over time. Furthermore, chitosan itself possesses intrinsic antioxidant activity and can create a synergistic effect with curcumin through electron donation and metal ion chelation [18]. These combined factors led to the superior performance of the Yog-Milk-CS-

Cur-NLC sample compared to the Yog-Milk-Cur-NLC sample. Therefore, the use of chitosan-coated nanoparticles can be an effective strategy for producing functional yogurts with stable antioxidant properties. The findings of this study are consistent with previous research demonstrating that coating lipid nanocarriers with biocompatible polymers such as chitosan not only improves the physical and chemical stability of sensitive compounds but also prevents the rapid decline of their biological activity during storage [39, 49]. Additionally, studies have shown that chitosan-coated systems perform better in acidic food environments compared to uncoated nanocarriers [36, 50]. The degradation of phenolic chemical compounds over time, particularly by lactic acid bacteria, is likely the cause of the decreased antioxidant activity in the fortified yogurt samples [51]. Furthermore, Taghadosi et al. (2024) reported that the antioxidant activity of fortified yogurt samples significantly increased compared to the control sample following the addition of curcumin-loaded nanocapsules at increasing levels (1% to 3%), and the decrease in antioxidant activity during the yogurt storage time was attributed to the degradation of curcumin by lactic acid bacteria [28].

Overall, the results of this research indicate that although the formulation of curcumin in lipid nanocarriers can significantly enhance the initial antioxidant activity, this activity rapidly decreases over time without a protective coating. In contrast, coating these nanostructures with chitosan leads to a considerable retention of antioxidant activity until the end of the storage period. Therefore, the Yog-Milk-CS-Cur-NLC system is introduced as an effective and stable strategy for the production of functional yogurts with high and sustained antioxidant activity, and it can be utilized in the food industry as a valuable technology to improve the nutritional quality and extend the shelf life of fortified dairy products.

4- Conclusion

The findings of this study demonstrated that utilizing milk fat-based nanostructured lipid carriers is an efficient approach for curcumin encapsulation and the fortification of dairy products. The high encapsulation efficiency (~91.8%), along with Fourier-transform infrared spectroscopy (FT-IR) evidence, confirmed the successful formation of a stable multi-layered lipid–curcumin/chitosan/milk protein structure. Chitosan coating played a key role in improving the thermal stability and preserving the antioxidant activity of curcumin during processing and storage; such that in the coated samples, the residual amount of curcumin after 4 hours at 90°C was more than 15 times higher than that of the uncoated samples. The application of these nanocarriers in stirred yogurt showed that although their presence might somewhat affect the sensory acceptance of the product, the chitosan coating is capable of effectively mitigating this negative effect while maintaining the functional stability of the bioactive compound. Overall, the development of coated lipid nanocarriers can pave the way for the production of functional foods rich in sensitive bioactive compounds. To expand the application of this system, it is recommended that its behavior on an industrial scale and in other food matrices be investigated in future studies.

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Data Availability

The data supporting the findings of this study are presented within the article, and

all reported results are based on the data available in the tables and figures of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest (financial, personal, or organizational) regarding this research and its results.

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Authors' Contributions

- **Arash Abaee:** Conducting experiments, data collection, statistical analysis, and writing the original draft.
- **Mohammad Ghorbani:** Corresponding author, study conceptualization, methodology design, supervision of all research stages, and final review of the article.
- **Alireza Sadeghi Mahoonak:** Consultation in experimental design, technical review, and specialized editing of the manuscript.
- **Sara Naji:** Collaboration in specialized parts of the research (nanotechnology), data analysis, and manuscript review.

All authors have read and approved the final version of the manuscript.

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مقاله علمی_پژوهشی

بهبود پایداری و فعالیت آنتی‌اکسیدانی کورکومین در ماست همزده با استفاده از نانوحامل‌های لیپیدی بر پایه چربی شیر پوشش‌دهی شده با کیتوزان

آرش آبایی؛ محمد قربانی*، علیرضا صادقی ماهونک، سارا ناجی طبسی

۱- دانشجوی دکتری صنایع غذایی، گروه علوم و صنایع غذایی، دانشکده علوم و صنایع غذایی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.

۲- استاد تمام، گروه علوم و صنایع غذایی، دانشکده علوم و صنایع غذایی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.

۳- استاد، گروه علوم و صنایع غذایی، دانشکده علوم و صنایع غذایی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.

۴- گروه نانوفناوری مواد غذایی، مؤسسه پژوهشی علوم و صنایع غذایی، مشهد، ایران.

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مسئول مکاتبات:*

چکیده

افزودن ترکیبات زیست‌فعال مانند کورکومین به مواد غذایی، راهکاری برای تولید غذاهای فراسودمند است که خواص سلامتی دارند، اما پایداری و زیست‌دسترسی این ترکیبات چالش‌برانگیز است. در این پژوهش، نانوحامل‌های لیپیدی بر پایه چربی شیر برای ریزپوشانی کورکومین سنتز و سپس با کیتوزان پوشش‌دهی شدند. این نانوحامل‌ها با بازده ریزپوشانی ۹۱/۸۳٪ توانایی بالایی در بارگذاری کورکومین نشان دادند. پوشش با کیتوزان منجر به افزایش اندازه ذرات (از ۳۰۶ به ۴۹۷ نانومتر) و تشکیل FT-IR تغییر پتانسیل زتا (از ۲۶/۹۶- به ۶/۷۴ میلی‌ولت) گردید. آنالیز ساختار چندلایه پایدار لیپید–کورکومین/کیتوزان/پروتئین شیر را تأیید کرد و نشان داد کورکومین در ماتریکس لیپیدی به‌صورت غیرکریستالی جاسازی شده است. پوشش کیتوزان پایداری حرارتی کورکومین را به‌طور چشمگیری بهبود بخشید؛ به‌طوری‌که پس از ٤ ساعت در ۹۰ درجه سلسیوس، ۱۵/۵۰٪ کورکومین در نمونه‌های پوشش‌دار باقی ماند، درحالی‌که این مقدار در نمونه‌های بدون پوشش تنها ۵/۱ ٪ بود. نانوحامل‌ها پس از خشک‌کردن انجمادی، برای غنی‌سازی ماست همزده استفاده شدند. ارزیابی فیزیکوشیمیایی و ارگانولپتیکی نشان داد که اگرچه فرمولاسیون را افزایش می‌دهد، اما DPPH با روش نانوحامل لیپیدی فعالیت آنتی‌اکسیدانی پایداری آن در طول زمان تنها در حضور پوشش کیتوزان حفظ شد. همچنین، پوشش کیتوزانی به‌خوبی اثرات منفی غنی‌سازی بر پذیرش حسی ماست را کاهش داد.