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Chitosan-cinnamon essential oil nano-formulation: Application as a novel additive for controlled release and shelf life extension of beef patties

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ABSTRACT

In this study cinnamon essential oil-incorporated chitosan nanoparticles (CEO-CSNPs) were produced by an emulsion-gelation method. Size, zeta potential and polydispersity index of the formed nanoparticles were 235.6 nm, 25.1 mV and 0.33, respectively. In vitro release evaluation showed an initial burst effect, followed by a slow CEO release during 104 h (at pH~5). Different formulations of patties including control, samples containing free and encapsulated CEO and ascorbic acid were analyzed for physicochemical characteristics, microbial growth and sensorial attributes during 8 days of storage at 4 °C. Both free and encapsulated CEO decreased the microbial population of patties compared to the control ($p < 0.05$) throughout the experiment. On the 8th day, the best formulations in TBARS test were AA-0.05 (0.05% of ascorbic acid) and 0.1-EN-CEO (0.1% of encapsulated CEO). During storage, the color parameters of the patties containing encapsulated CEO changed slightly whereas color and metmyoglobin content significantly decreased in samples containing free CEO and control. Free CEO had an unfavorable impact on color and odor but the incorporation of encapsulated CEO improved the consumer acceptability. The principle component analysis clearly distinguished 6 formulation groups based on physicochemical characteristics.

1. Introduction

The consumer demand for meat and meat products, especially for minimally processed, easily prepared, and ready-to-eat products along with consumer's negative perception of synthetic preservatives, have rapidly increased in recent years. Meat products could be decayed by two principal causes: microbial growth and chemical deterioration. Therefore, there is a tendency to use natural antioxidant and antimicrobial agents instead of conventional chemical food additives that improve meat quality without any side effects on the product or the environment [1]. Although food industry primarily uses spices and herbs to impart flavor, aroma, and pungency to food, they represent an interesting source of natural molecules for food preservation [2]. Among a lot of natural compounds that have been examined, essential oils (EOs) are being evaluated as natural antioxidants or antimicrobial agents to preserve and improve the overall quality of meat and meat products [3–6].

Cinnamomum zeylanicum L., generally known as cinnamon, is identified to possess excellent antioxidant [7] and antimicrobial activity [8]. It has already been granted Generally Recognized as Safe-GRAS status by the Food and Drug Administration 21 CFR (Code of Federal Regulation) part 172.515 [8]. Nevertheless, the use of EOs as preservatives in food industry still faces limitations due to their interactions with food components and structures, low solubility in aqueous phase, high volatile character, and negative effects on original sensory quality of foodstuffs especially at high concentration, sensitivity to oxygen, light and heat during processing, utilization and storage [2].

Encapsulation of EOs is one of the most efficient techniques for enhancing the solubility and stability of EOs in adverse environmental conditions, masking their unwanted taste and the odor and controlling active compounds release [9]. At present, there has been an enormous attention to the development of nano-sized formulations of bioactive compounds since their subcellular size offers significant advantage over conventional encapsulation system in term of relatively higher intracellular uptake along with high specificity, high drug carrying capacity, release from the large surface area, leading to an extended biological activity [10]. Due to their unique futures of chitosan including biocompatibility, low toxicity

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and biodegradability, one of the nano-sized vehicles used for EOs encapsulation is chitosan nanoparticles (CSNPs) [11]. In addition, it has a broad spectrum of biological activities including antibacterial, antifungal, antioxidant, insecticidal and muchoadhesive properties [12,13]. Being versatile in nature, CS has a great potential for encapsulation of both hydrophobic and hydrophilic bioactive compounds widely used in foodstuff or pharmaceutical application [8,9,11,14]. There are some reports on the coating of meat products by edible films containing EOs in the literature [15–17] but, there are few studies about the preservative effects of encapsulated EOs in meat products. We mainly aimed in this study at a comparison between free and encapsulated CEO performances in beef patties to retard their deterioration during refrigerated storage and distinguish the best formulation in terms of lipid, microbial and color stability. The results from physicochemical evaluation were then processed by employing principle component analysis (PCA) to present a preliminary classification of different patties formulas.

2. Materials and methods

2.1. Materials

Chitosan (75–85% degree of deacetylation and Mw = 760 kDa), penta sodium tripolyphosphate (TPP) and cumene hydroperoxide were obtained from Sigma–Aldrich Chemical Co. (St. Louis, USA). Acetic acid glacial and trichloroacetic acid (TCA) were supplied from Rankem (New Delhi, India). Cinnamon essential oil was purchased from Zardband Pharmaceuticals Co. (Yasooj, Iran). Malondialdehyde and 2-thiobarbituric acid (TBA) were procured from Merck (Darmstadt, Germany). All media for microbiological analysis were purchased from Micromedia (Micromedia PTY-LTD, Hungary) and egg-yolk tellurite emulsion obtained from Liofilchem (Liofilchem® srl, Italy). Solvent and other chemical materials were analytical grade and used without any further purification.

2.2. Preparation and characterization of nanoparticles

The cinnamon essential oil incorporated chitosan nanoparticles (CEO-CSNPs) were prepared by a two-step process: oil-in-water (o/w) emulsification and ionic gelation. Chitosan (CS, 3.2 mg/ml) was dissolved in acetic acid solution using an ultrasonic water bath for 1 h (ULTRASONSH, Selecta, Spain). After removing undissolved CS particles through 1-μm pore size filter, the pH of the solutions adjusted to 4.0. Tween-80 (80 mg) was added as a surfactant to the solution (50 ml) and stirred at 60 °C for 30 min to obtain a homogeneous mixture. Ethanolic solution of CEO was prepared by dissolving 128 mg of EO in 4 ml ethanol to obtain CS: CEO weight ratio of 1:0.8. After cooling CS solution, CEO was gradually dropped into the aqueous phase during stirring at 1200 rpm for 20 min. In the second step, thirty ml of the TPP solution (1.87 mg/ml, pH = 4.0) was added dropwise into the former solution during stirring at 900 rpm for 60 min [9].

An atomic force microscope (AFM, Autoprobe CP Research, Veeco, USA) was used for morphological characterization of CEO-CSNPs. A portion of nanoparticle suspension was diluted 50 times with distilled water and then placed in an ultrasonic bath for 1 min (Selecta ULTRASONS, Spain). Fifty microliters of the dilution was deposited on a cleaned glass surface in a vacuum oven at 30 °C (Memmert, Germany).

The particle size, polydispersity index (PDI) and zeta potential of freshly prepared CEO-CSNPs were measured after 30 s sonication using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne laser operating at 4.0 mW and 633 nm with a fixed scattering angle of 90°. The encapsulation efficiency (EE) and loading capacity (LC) of CEO-CSNPs were evaluated according to Keawchaoon and Yoksan [18]. These parameters were calculated as follows:

$$\%EE = (\text{Total amount of CEO} - \text{Amount of free CEO}) \times 100/\text{Initial amount of CEO} \quad (1)$$

$$\%LC = (\text{Amount of loaded CEO}) * 100/\text{Weight of sample} \quad (2)$$

CEO-loaded nanoparticles were separated from aqueous suspension medium through centrifugation at 24,000 rpm and 4 °C for 45 min. Then, the loaded particles were suspended in 5 ml acetate buffer (pH∼5) and shook (IKA, Germany) at 200 rpm for 104 h. At various times, samples were centrifuged at 9000 rpm for 5 min. The specific volume of supernatant (1 ml) was taken and re-placed with an equivalent volume of fresh medium. Amount of released CEO was measured at 285 nm, by UV–vis spectrophotometer. The cumulative release percent was plotted as a function of release time (during 104 h). All experiments were performed in triplicates. The cumulative percent of released CEO was calculated as follows:

$$\text{Cumulative release}\,(\%) = \sum_{t=0}^{t} \frac{M_t}{M_0} \times 100 \quad (3)$$

where M_t is the amount of CEO released at each sampling time (t), M_0 is the initial weight of the CEO that was loaded in CEO-CSNPs. All experiments were performed in triplicate.

2.3. Preparation of beef patties

Fresh beef meat (60% brisket and 40% flank) was collected from a local market and transported to the laboratory under refrigerated conditions within 30 min. The visible connective tissue was trimmed. After initial washing with tap water and rinsing, the meat was cut into approximately 5 × 5 cm pieces using a stainless steel knife, packed in low density polyethylene bags and transferred to −18 °C. On the day of the patty production, the meat was refrigerated at 4 °C for 4 h before utilization. Brisket and flank meat were initially ground using an electric meat mincer (KENWOOD, Germany) through a plate with 4 mm orifices then aseptically hand mixed for 2 min. The minced beef mixture was divided into 6 treatments to prepare following formulation: Control (without CEO), 0.05-UN-CEO (0.05% unencapsulated CEO), 0.1-UN-CEO (0.1% unencapsulated CEO), 0.05-EN-CEO (0.05% encapsulated CEO), 0.1-EN-CEO (0.1% unencapsulated CEO) and 0.05-AA (0.05% ascorbic acid) (all formulations had 10% water and 1.5% salt). Ascorbic acid was used as the positive control. In order to use CEO-CSNPs, nanoparticles were initially separated from aqueous phase by centrifuge at 24,000 rpm for 45 min at 4 °C and then the calculated amount of NPs containing 0.05 and 0.1% CEO was re-dispersed in water (10% w/w of meat). The minced meat was mixed manually with other ingredients in a steel bowl. The minced beef from each treatment was hand-formed into an individual round flat patties (25 ± 0.5 g) using sterile glass petri plates (3 cm diameter). Patties were packaged individually in sterile polyethylene bags (Zipak, Iran), held at 4 °C for 8 days and sampled on days 0, 2, 4, 6 and 8 for color evaluation, microbial enumeration, and chemical analysis.

2.4. Microbial evaluation

The microbiological analyses of patties included the enumeration of *S. aureus*, total mesophilic aerobic viable count (TMVC), *Enterobacteriaceae*, yeasts and molds (Y & M) and lactic acid bacteria (LAB). Analyses were carried out at 2-day intervals up to the 8th day of storage at 4 °C. At each sampling time, 10 g of patty samples was

homogenized in a stomacher bag for 2 min in 90 ml of sterile physiological serum. Resulted suspensions were serially diluted (1:10) in sterile physiological serum and microbial enumerations were performed according to our previous study [9]. The total mesophilic viable count (TMVC) was determined on plate count agar (PCA) incubated at35 °C for 24 h. *Staphylococcus aureus* was enumerated on Baird Parker Agar (BPA) supplemented with egg yolk tellurite emulsion as a medium after 48 h of incubation at 37 °C. *Enterobacteriaceae* were counted in pour plates of Violet Red Bile Glucose Agar (VRBGA) after incubation at 37 °C for 24 h. The enumeration of lactic acid bacteria (LAB) was conducted on de Man, Rogosa, and Sharpe (MRS) medium after 48 h incubation at 35 °C. Molds and yeasts were counted on Yeast Extract Glucose Chloramphenicol (YGC) medium with 5-day incubation at 25 °C. The results were converted to log10 colony forming units (cfu) per gram of sample.

2.5. Physicochemical analysis

2.5.1. pH and color measurement

The pH of patties was determined using a Metrohm pH meter (827 pH Lab, Switzerland) after homogenizing 1 g of sample in 10 ml of distilled water for 10 s at 13,000 rpm with an ULTRA-TURRAX T25 (IKA, Germany).

The color was instrumentally determined on the surface of the beef patties by the CIE $L^*a^*b^*$ system using Colorflex Hunter-Lab (Virginia, USA). Color features including the L^* (lightness), a^* (redness) and b^* (yellowness) were directly recorded by the colorimeter. The standardization of the instrument was done using a black and white calibration plate (L^* = 92.23, a^* = −1.29, and b^* = 1.19). In addition, hue angles (H) were calculated as follows:

$$H = \text{arctg}\left(b^*/a^*\right) \quad (3)$$

2.5.2. Oxymyoglobin (OxyMb) and metmyoglobin (MetMb) percentage

Analysis of meat pigments of patties was conducted according to the modified method of Carlez et al. [19]. The oxymyoglobin (OxyMb) and metmyoglobin (MetMb) contents were calculated as follows [20]:

$$\text{OxyMb}(\%) = (0.882R_1 - 1.267R_2 + 0.0809R_3 - 0.361) \times 100 \quad (5)$$

$$\text{MetMb}(\%) = (-2.541R_1 + 0.777R_2 + 0.800R_3 + 1.098) \times 100 \quad (6)$$

where R_1, R_2 and R_3 are the absorbance ratio of A_{572}/A_{525}, A_{565}/A_{525} and A_{545}/A_{525}, respectively.

2.5.3. Lipid oxidation

The peroxide value was measured using the procedure reported by Richards and Hultin [21]. A standard curve was constructed using cumene hydroperoxide at a concentration range of 0–20 μmol/kg for quantification and the results were expressed as μmol hydroperoxide per kg of sample. Thiobarbituric acid reactive substance (TBARS) values were evaluated calorimetrically using 2-TBA procedure [22]. A standard curve was plotted using 1, 1, 3, 3-tetramethoxypropane (MDA) at concentrations ranging from 0 to 10 μM and TBARS values were expressed as mg of MDA equivalent per kg of beef patties.

2.6. Evaluation of sensory attributes of patties

The samples were evaluated by a 10-member semi trained panelists from scientists of the department of food science and technology, Tarbiat Modares University. All raw and cooked patties were coded with 3-digit random codes and offered to the panelist in a random order. The panelists scored the sensory attributes (color and odor of CEO and overall acceptability of raw patties) by using a 5-point descriptive scale and were asked to fill in a questionnaire in which 1 was the worst (extremely unacceptable) and 5 was the best (extremely acceptable). The sensory evaluation was performed on days 0, 4 and 8 of refrigerated storage. Shelf-life criteria assumed that a rejection would occur when the sensory attributes declined below 3.

2.7. Statistical analysis

ANOVA was performed using Minitab 16 (Minitab Inc., State College, PA, USA) program which included the formulation, storage time and their interactions. Analytical data was obtained from analyses of 3 samples for each individual treatment in chemical assays and microbial analysis. Means and standard errors of the samples were also calculated. Statistical significance of differences among means was assessed through Tukey's test at a confidence level of p<0.05. The principal component analysis (PCA) was applied as unsupervised exploratory data analysis methods using Minitab 16 to highlight the information of data matrix integrated by all chemical and instrumental parameters measured on samples on day 8 of the storage.

3. Results and discussion

3.1. Characterization of CEO-CSNPs

Dynamic light scattering (DLS) technique was used to determine the particle size, zeta potential and polydispersity index (PDI) of the synthesized particles that were 235.6 nm, 25.1 mV and 0.33, respectively (at pH = 4.0). The size of CEO-CSNPs by AFM is smaller than the one measured by DLS in aqueous solution. This may be arisen from the dry state of the samples in AFM measurement. From UV–vis spectrophotometric results, EE of CEO was 37.87%. This result was higher than the finding regarding the encapsulation of eugenol into CSNPs, which has been reported by Woranuch and Yoksan [14]. The morphology of the CEO-CSNPs was determined by AFM. The AFM analysis clearly showed the spherical shape and nanosized structure of individual particles (Fig. 1a).

We selected acetate buffer (pH∼5) as a medium for the release of CEO aiming to simulate the release mechanisms of CEO when CEO-CSNPs incorporated to a real food system with equal pH such as beef patties. The release curve of CEO presented an initial burst stage followed by a more gradual increase in the cumulative release and finally reached plateau without additional CEO release (Fig. 1b). In the first phase, the rate of CEO released from the CSNPs was the fastest in the first 8 h and the cumulative release of CEO at this stage was 38.4%. This behavior is coupled to the instantaneous diffusion of unencapsulated CEO molecules on the nanocarriers surface or entrapped near the surface layer. The concentration gradient and the high dissolution rate of the polymer near the surface can be effective as well. The second stage displays the gradual decrease of the release rate until reaching a constant value after 56 h. At this stage, the released amount varied from 38.38 to 61.7% due to CEO diffusion in the CSNPs along with polymer matrix degradation. No further CEO was released for up to 104 h. The data confirmed the potential for CSNPs loaded with CEO to provide a sustained release of EOs during storage of beef patties.

3.2. Antimicrobial activity of free and encapsulated CEO in beef patties

Microbial growth inhibition in beef patties by different formulations of CEO is summarized in Fig. 2. During the experiment, significant effects (p < 0.05) of formulation, storage time, and their interaction were seen for all microbial counts. When unencapsulated CEO was assayed at 0.05 and 0.1%, a decrease of 1.19 and 2 log

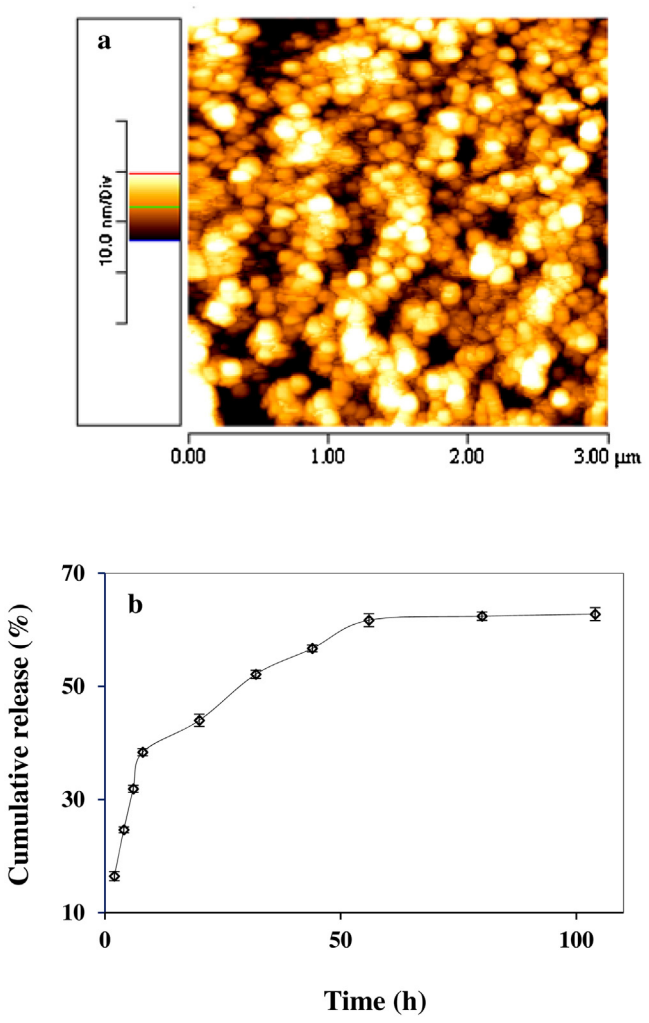

Fig. 1. a) AFM image (3D) of cinnamon essential oil loaded (CEO) chitosan nanoparticles (CSNPs) prepared using an initial weight ratio of chitosan to CEO of 1:0.8. b) Release behavior of CEO-CSNPs at pH = 5.0.

cycles in the TMVC in comparison to control sample was observed after 8 days. On the 8th day, 0.05-EN-CEO and 0.1-EN-CEO showed the most noticeable fall in TMVC compared to the control by 2.23 and 2.86 log cycles reduction, respectively (Fig. 2a). The TMVC of all CEO treated samples did not exceed the sanitary specifications, considered as the maximum allowable number (7 log cfu/g) for fresh meat as defined by ICMSF [23]. It is obvious that unencapsulated and encapsulated CEO had a pronounced impact on the reduction of TMVC, prolonging the microbiological shelf life of patties by 2–4 days, respectively.

During the storage, in all five groups of patties, there was a rise in the LAB count but the incidence and development of the LAB in beef patties treated by CEO was significantly lower than the control (Fig. 2b). In 0.1-EN-CEO treated samples, the population of LAB was kept below 4 log cfu/g up to 8th day of the storage. Similar to our results, Michalczyk et al. [5] also reported the weakest antimicrobial effects of coriander and hyssop essential oils on LAB (up to 1 log cycle) population at the beginning of storage.

No significant differences were observed among the treatments in the beginning of storage but encapsulated and unencapsulated CEO showed a distinguished inhibition pattern of *S. aureus* in the following days. As seen in Fig. 2c, the counts of *S. aureus* at the end of storage were significantly higher than the initial counts of control, 0.05-UN-CEO and 0.1-UN-CEO ($p < 0.05$). On the contrary, a gradual decrease of *S. aureus* count was observed after day 2 of storage for 0.05-EN-CEO and 0.1-EN-CEO. The control reached 6.85 log cfu/g on day 8, while 0.05-UN-CEO, 0.1-UN-CEO, 0.05-EN-CEO and 0.1-EN-CEO showed 2.12, 2.62, 3.99 and 4.59 log cfu/g decrease in *S. aureus* counts in comparison to the control sample, respectively. These results may be explained by the antibacterial activity of CEO on *S. aureus* by morphological destruction, destroying the integrity of membrane, and leakage of small electrolytes, protein, and nucleic acids [24]. Djenane et al. [25] reported that incorporation of 0.24 and 0.1% of *Satureja hortensis* and *Eucalyptus globulus* essential oils into minced beef resulted in 2.2 and 3.2 log reduction of *S. aureus* as compared to the control after 1 week of storage.

The *Enterobacteriaceae* amounts of all beef patties were similar at the beginning of the storage (3.2-3.24 log cfu/g), which was an indicative of good hygienic practices in the production of patties. *Enterobacteriaceae* counts of all control samples, 0.05-UN-CEO and 0.1-UN-CEO treatments increased during storage, but the increases in unencapsulated CEO treated formulation were lower than the control (Fig. 2d). These results demonstrate the inhibitory effect of CEO on these gram negative bacteria by 1.15–1.79 log cycles reduction compared to control at the end of storage. Beef patties treated with encapsulated CEO did not show any increase, even reaching final values of 3.35 and 2.72 log cfu/g, which were 3.23 and 3.86 log cfu/g lower than control. Therefore, encapsulated CEO showed a good aptitude in reducing *Enterobacteriaceae* growth during storage, with prolonged the antimicrobial effects. Our results are in agreement with those of Hu et al. [8] who reported a reduction of 0.1–1 log cfu/g in *Enterobacteriaceae* chilled pork meat wrapped with the active low density polyethylene films coated with encapsulated cinnamon oil during 15 days of storage.

Yeast and mold (Y & M) counts of different treatments are shown in Fig. 2e. Y & M counts of control patties increased significantly from an initial value of 2.59 log cfu/g to 5.92 at the end of storage ($p < 0.05$). Both free and encapsulated CEO caused a reduction of Y & M counts, but the inhibitory effect was most obvious for encapsulated CEO during first 4 days of storage. The use of UN-CEO and EN-CEO resulted in a reduction of Y & M on 8th day by 1.62–1.92 and 2.96–3.16 log cycles compared to control, respectively.

There is a relationship between the chemical constitutes of EOs and the antimicrobial characteristics. The major components of CEO were *trans*-cinnamaldehyde (26.9%), α-pinene (24.42%), eucalyptol (14.56%) (data not shown). The antimicrobial action of *trans*-cinnamaldehyde is associated with an inhibitory effect on different enzymes such as bacterial ATP-ase or fungal cell wall synthesizing enzyme and perturbation of bacterial cell membrane [2,26]. Fig. 2 shows that 0.1-EN-CEO has highest growth inhibition at the end of storage time. The CEO-CSNPs made by us could effectively control the CEO compound release, while unencapsulated CEO at different concentrations was able to decline microbial population within a limited period. These results were in good agreement with those reported by Sadgrove et al. [27]. They mentioned that encapsulated cineole-rich essential oils can enhance antimicrobial activity in comparison with free ones. They concluded that encapsulation could reduce evaporation of EOs and facile its delivery of the bacterial cell wall. In addition, the antibacterial effect of EN-CEO treatments was expected, since preliminary antibacterial studies (minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)) done on some gram positive and gram negative bacteria in our laboratory revealed that CSNPs have no antibacterial effect in concentrations lower than 0.3%, while free and encapsulated CEO possessed antibacterial effects even at lower concentrations (results not shown). Finally, Keawchaoon and Yoksan [18] observed that chitosan nanoparticles with a concentration below 8.225 mg/ml could not inhibit the growth of *S. aureus*, *B. cereus* and *E. coli*.

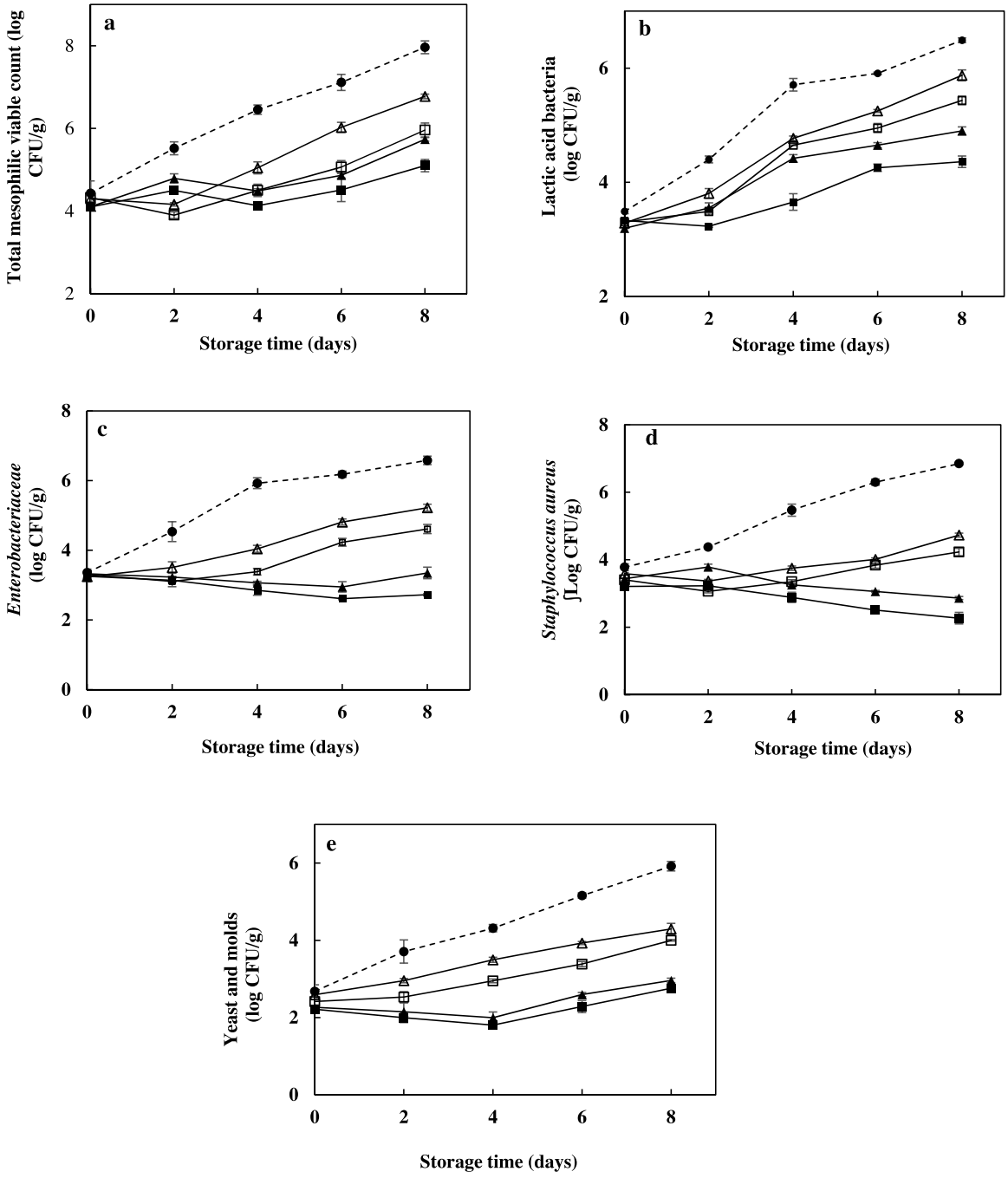

Fig. 2. Microbiological counts (log cfu/g).of the beef patties with different formulations during 8 days storage at 4 °C. (●) Control, (△) 0.05-UN-CEO, (□) 0.1-UN-CEO, (▲) 0.05-EN-CEO and (■) 0.1-EN-CEO. Control: sample without any cinnamon essential oil; 0.05-UN-CEO, 0.1-UN-CEO and 0.05-EN-CEO, 0.1-EN-CEO and AA-0.05 samples containing 0.05 and 0.1% unencapsulated cinnamon essential oil and 0.05 and 0.1% encapsulated cinnamon essential oil and 0.05% ascorbic acid, respectively.

3.3. Lipid oxidation of samples

The efficacy of unencapsulated and encapsulated CEO as a natural antioxidant to inhibit primary and secondary lipid oxidation in beef patties was assessed by measurement of PV and TBARS values, respectively (Fig. 3a & b). The results indicated that lipid oxidation occurred since the production day, which was due to a prolonged exposure of the meat to oxygen during mincing and preparation. By comparing PV of samples, it is obvious that the control sample has a higher PV than the samples containing CEO throughout the storage ($p < 0.05$) (inserted graph in Fig. 3a). At the end of storage, 0.1-EN-CEO and 0.05-AA treatments had lower PVs than the others (61.7–63.3% reduction of PV) and therefore the greatest resistance to oxidation.

Fig. 3b shows the TBARS values of the beef patties during refrigerated storage. The initial TBARS value of control was 0.06 mg MDA/kg sample which intensively increased over time, finally reaching to 1.72 mg MDA/kg. According to Sheard et al. [28], a TBARS value of 0.5 mg MDA/kg sample was the threshold of off-odor perception by consumers. Despite the overall rise of TBARS, all samples (except 0.05-UN-CEO) did not have higher TBARS values than the reported threshold.

The antioxidant activity of *trans*-cinnamaldehyde has a key function for retarding MDA formation. Results are in accordance

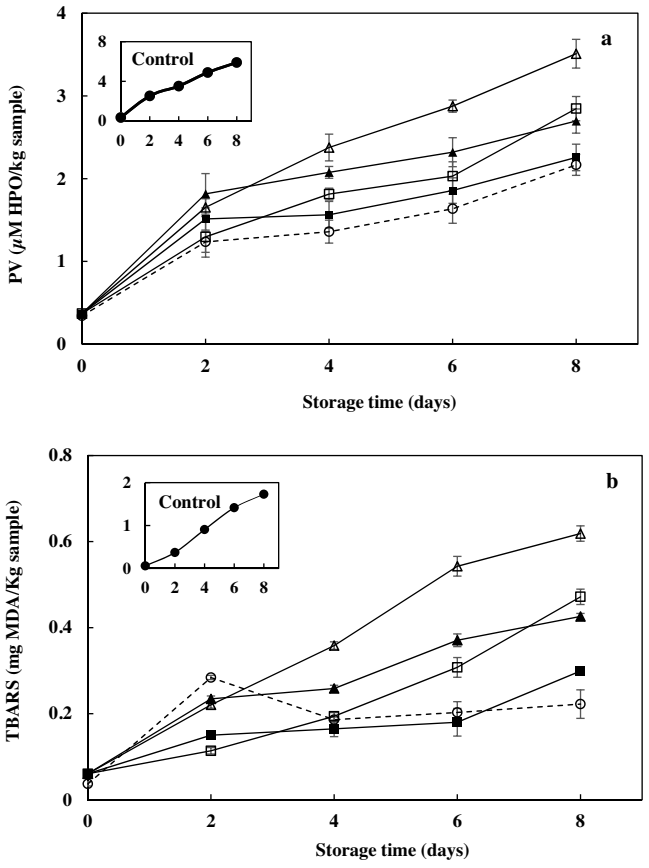

Fig. 3. Peroxide value (a) and TBARS (b) of the beef patties with different formulations during storage for 8 days at 4 °C. (●) Control, (○) 0.05-AA, (△) 0.05-UN-CEO, (□) 0.1-UN-CEO, (▲) 0.05-EN-CEO and (■) 0.1-EN-CEO. For abbreviations see Fig. 1.

with Amalaradjou et al. [3] who reported that secondary oxidation of lipid in ground beef patties was greatly suppressed by *trans*-cinnamaldehyde. Our results demonstrated that encapsulation of CEO could effectively retard lipid oxidation even at a lower concentration during storage. It could be concluded that encapsulation has the potency to preserve CEO from evaporation and decomposition during storage. Furthermore, this higher antioxidant activity of encapsulated CEO partly corresponds to the interaction of CS with fat's derivative and its ability to chelate the ferrous ions liberated from myoglobin. These results agreed with those reported by Jung et al. [29], as they mentioned that the application of ordinary plastic coated with encapsulated horseradish extract leads to the inhibition of malondialdehyde generation in pork and fish meat by 55.3% and 38.9%, respectively.

3.4. Physicochemical changes of beef patties

3.4.1. Color evaluation

In this study, color is evaluated in terms of L^*, a^*, b^* values, hue angle, OxyMb and MetMb (Fig. 4). Considering the main effects, the treatment, storage time and their interaction affected all color parameters ($p < 0.05$).

At the beginning, the patties treated by UN-CEO had significantly higher L^* than the other treatments ($p < 0.05$). As seen in Fig. 4a, with the exception of AA-0.05, a significant increase in L^* values was noted during storage ($p < 0.05$) for all formulations. At 8th, higher L* values were measured for the control and treatments containing unencapsulated CEO, while AA treated patties had the lowest L^* values with the mean of 34.9 ± 0.16. Similarly, a reduction in the L^* values was reported for rabbit burgers incorporated with 0.1% ascorbic acid [30]. By increasing storage time, the L^* values increased, but treatments containing encapsulated CEO were more stable during the storage (Fig. 4a).

As shown in Fig. 4b, the b^* values of all formulations gradually increased during the storage. The b^* values of the beef patties containing unencapsulated CEO were close to those obtained from control after 8 days ($p < 0.05$). The rate at which the b^* value raised in beef patties was dampened by the encapsulation of CEO in CSNPs. At the end of the experiment, beef patties containing AA and 0.1% of EN-CEO had the lowest b^* value in comparison to samples containing unencapsulated CEO. It seems that the inhibition of MetMb formation is caused by OxyMb oxidation by AA or encapsulated CEO. In the same way, Hu et al. [8] showed that adding encapsulated cinnamon essential oil keep b^* values lower than the control sample in pork meat.

A continuous and significant a^* value reduction was seen in both CEO and AA treated beef patties ($p < 0.05$). On the second day, the red color of 0.05-UN-CEO and 0.1-UN-CEO strongly turned into yellow and continued up to the end of the storage (Supplementary Fig. S1). In control, 0.05-UN-CEO, 0.1-UN-CEO, 0.05-EN-CEO, 0.1-EN-CEO and AA-0.05 formulation, the a^* values decreased by about 42.3, 57.9, 61.9, 36, 13.3 and 5.96% after 8 days of storage. Even if a^* values of the 0.1-EN-CEO, AA and treated patties tended to decline by the second day, these formulations tended to stabilize red color in the remaining time (Fig. 4c). These observations were partially related to the gradual release of CEO from CSNPs during experiments and retarding oxidation of meat pigment after AA addition [31].

The discoloration of the patties was confirmed by calculation of the hue angle (H^*). Results demonstrated that 0.1-UN-CEO treatment had remarkably higher H^* values ($p < 0.05$) (Fig. 4d), indicating these samples had lower a^* and higher b^* values than other formulations during the storage. This was consistent with the results of Naveena et al. [6] who reported that metmyoglobin formation could be accelerated in spent hen meat, by the adding of *trans*-cinnamaldehyde. The addition of AA and 0.1% encapsulated CEO significantly lowered the H^* values with respect to control ($p < 0.05$), and the influence became stronger after 2 days of storage. The color deterioration during chilled storage of beef patties can be described by the degradation of hem molecule [32] or adverse effect of lipid oxidation on color [33]. Improved color characteristics have also been reported for pork fillet preserved with microencapsulated horseradish extract [29].

3.4.2. pH

The initial pH values of beef patties were varied from 5.62 to 5.65 (Table 1). During storage, pH values increased slightly and the rise became more obvious for control, reaching to 5.84 on 8th day of the storage. This could be caused by the accumulation of volatile bases (e.g. ammonia and trimethylamine) generated during microbial or enzymatic degradation of amino acids [30]. Furthermore, a more preservative impact was noted for patties incorporated with encapsulated CEO which maintained the natural pH of meat especially during days 4–8 of the storage. Throughout the experiment, pH values of AA-0.05 treated samples were significantly lower than other treatments ($p < 0.05$). It may be partially due to the poor antimicrobial activity of AA to inhibit LAB growth.

3.4.3. Oxymyoglobin and metmyoglobin contents

The OxyMb and MetMb contents (%) of beef patties are shown in Table 1. A decrease in OxyMb content by 27.6–45% over 8 days was observed for all formulations. Significant differences ($p < 0.05$) were found among CEO treated samples and the control sample for all testing days with the exception of the production day, which had an OxyMb content in the range of 60.8–63.28%. During the experiment, as free CEO concentration increased, OxyMb content

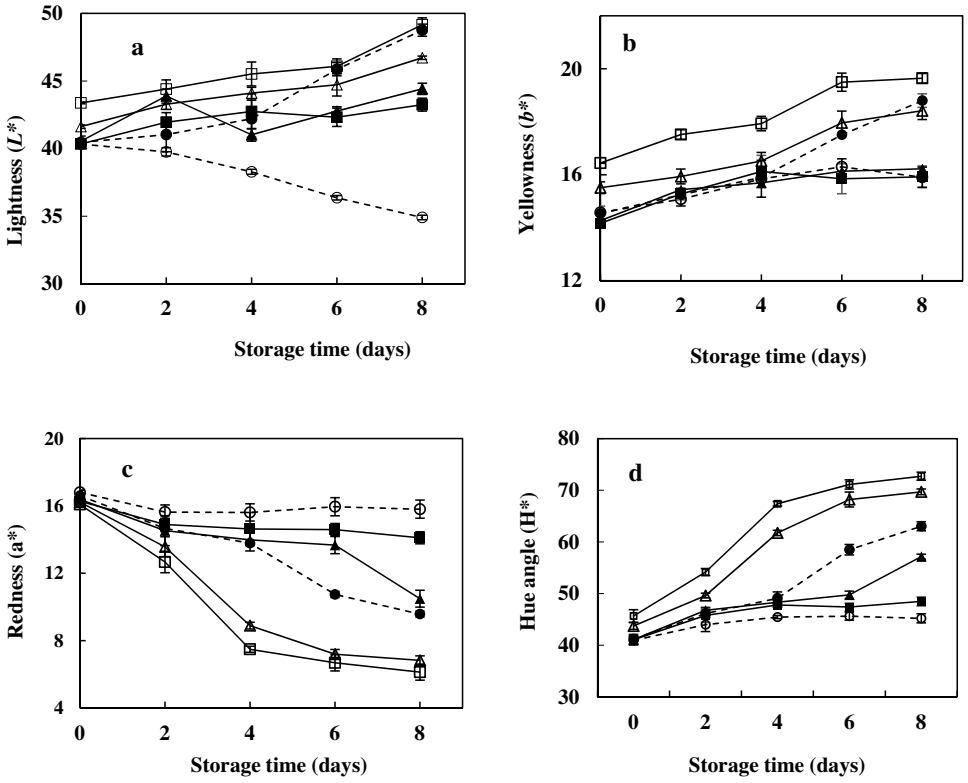

Fig. 4. Color evaluation of the beef patties with different formulations during 8 days storage at 4 °C. Lightness (a), yellowness (b), redness (c) and hue angle (d). (●) Control, (○) 0.05-AA, (△) 0.05-UN-CEO, (□) 0.1-UN-CEO, (▲) 0.05-EN-CEO and (■) 0.1-EN-CEO. For abbreviations see Fig. 1.

Table 1
Changes in pH, oxymyoglobin (%) and metmyoglobin (%) of beef patties with different formulations during 8 days of refrigerated storage.

Chemical property	Treatments	Storage time (days)				
		0	2	4	6	8
pH	Control	5.64 = 0.01^{a}	5.68 = 0.01^{a}	5.69 = 0.00^{a}	5.71 = 0.00^{a}	5.84 = 0.01^{a}
	0.05-UN-CEO	5.65 = 0.00^{a}	5.65 = 0.00^{b}	5.69 = 0.00^{a}	5.72 = 0.00^{a}	5.74 = 0.00^{b}
	0.1-UN-CEO	5.65 = 0.00^{a}	5.68 = 0.00^{a}	5.68 = 0.00^{a}	5.69 = 0.01^{a}	5.71 = 0.02^{b}
	0.05-EN-CEO	5.65 = 0.00^{a}	5.62 = 0.00^{bc}	5.67 = 0.01^{a}	5.64 = 0.00^{b}	5.64 = 0.01^{c}
	0.1-EN-CEO	5.65 = 0.00^{a}	5.65 = 0.00^{ab}	5.63 = 0.00^{b}	5.66 = 0.00^{b}	5.66 = 0.00^{c}
	AA-0.05	5.63 = 0.00^{a}	5.60 = 0.00^{c}	5.56 = 0.00^{c}	5.55 = 0.00^{c}	5.55 = 0.01^{d}
OxyMb (%)	Control	63.20 = 1.61^{b}	44.23 = 0.54^{bc}	32.36 = 0.46^{de}	27.25 = 0.46^{de}	18.22 = 0.54^{e}
	0.05-UN-CEO	60.68 = 0.60^{b}	42.96 = 1.24^{c}	34.55 = 0.69^{d}	29.32 = 0.82^{d}	21.74 = 0.36^{d}
	0.1-UN-CEO	60.86 = 0.49^{b}	39.00 = 0.69^{d}	30.50 = 0.55^{e}	25.28 = 0.46^{e}	19.54 = 0.55^{de}
	0.05-EN-CEO	63.19 = 0.61^{b}	42.58 = 2.31^{c}	36.92 = 1.10^{c}	32.19 = 0.48^{c}	29.28 = 0.98^{c}
	0.1-EN-CEO	63.28 = 1.08^{b}	45.38 = 0.64^{b}	39.67 = 0.62^{b}	36.37 = 1.24^{b}	31.86 = 0.53^{b}
	AA-0.05	65.00 = 1.32^{a}	48.92 = 0.69^{a}	45.36 = 1.30^{a}	40.88 = 0.57^{a}	37.41 = 0.87^{a}
MetMb (%)	Control	17.34 = 0.93^{a}	30.24 = 0.67^{a}	47.93 = 0.52^{a}	58.30 = 1.28^{a}	70.58 = 0.83^{b}
	0.05-UN-CEO	17.56 = 0.91^{a}	27.66 = 0.91^{b}	43.12 = 0.85^{b}	53.63 = 2.30^{b}	71.67 = 1.55^{ab}
	0.1-UN-CEO	18.16 = 1.23^{a}	30.01 = 0.94^{a}	46.70 = 1.23^{a}	51.24 = 1.17^{c}	73.51 = 2.39^{a}
	0.05-EN-CEO	17.18 = 2.49^{a}	26.52 = 1.21^{bc}	36.70 = 0.44^{c}	44.45 = 0.94^{d}	59.88 = 1.18^{c}
	0.1-EN-CEO	17.37 = 1.45^{a}	24.61 = 0.83^{c}	33.03 = 2.17^{d}	43.66 = 1.33^{d}	51.13 = 1.39^{d}
	AA-0.05	16.27 = 1.12^{a}	22.01 = 0.62^{d}	29.15 = 1.52^{e}	37.93 = 2.19^{e}	42.14 = 1.44^{e}

For each property at each day, values in the same column with the same lowercase letter (a, b, c,. . .) are not significantly different ($p < 0.05$). Control: sample without any cinnamon essential oil; 0.05-UN-CEO, 0.1-UN-CEO and 0.05-EN-CEO, 0.1-EN-CEO and AA-0.05 samples containing 0.05 and 0.1% unencapsulated cinnamon essential oil and 0.05 and 0.1% encapsulated cinnamon essential oil and 0.05% ascorbic acid, respectively.

decreased with a corresponding increase in discoloration. By day 8, AA-0.05 and 0.1-EN-CEO formulation showed higher protection of OxyMb (37.4 and 31.8%, respectively) ($p < 0.05$), while patties containing 0.1-UN-CEO were similar to the control sample. So, we can conclude that unr antimicrobial activity of AA to inhibit LAB growth.

3.4.3. Oxymyoglobin and metmyoglobin contents

The OxyMb and MetMb contents (%) of beef patties are shown in Table 1. A decrease in OxyMb content by 27.6-45% over 8 days was observed for all formulations. Significant differences ($p < 0.05$) were found among CEO treated samples and the control sample for all testing days with the exception of the production day, which had an OxyMb content in the range of 60.8–63.28%. During the experiment, as free CEO concentration increased, OxyMb content decreased with a corresponding increase in discoloration. By day 8, AA-0.05 and 0.1-EN-CEO formulation showed higher protection of OxyMb (37.4 and 31.8%, respectively) ($p < 0.05$), while patties containing 0.1-UN-CEO were similar to the control sample. So, we can conclude

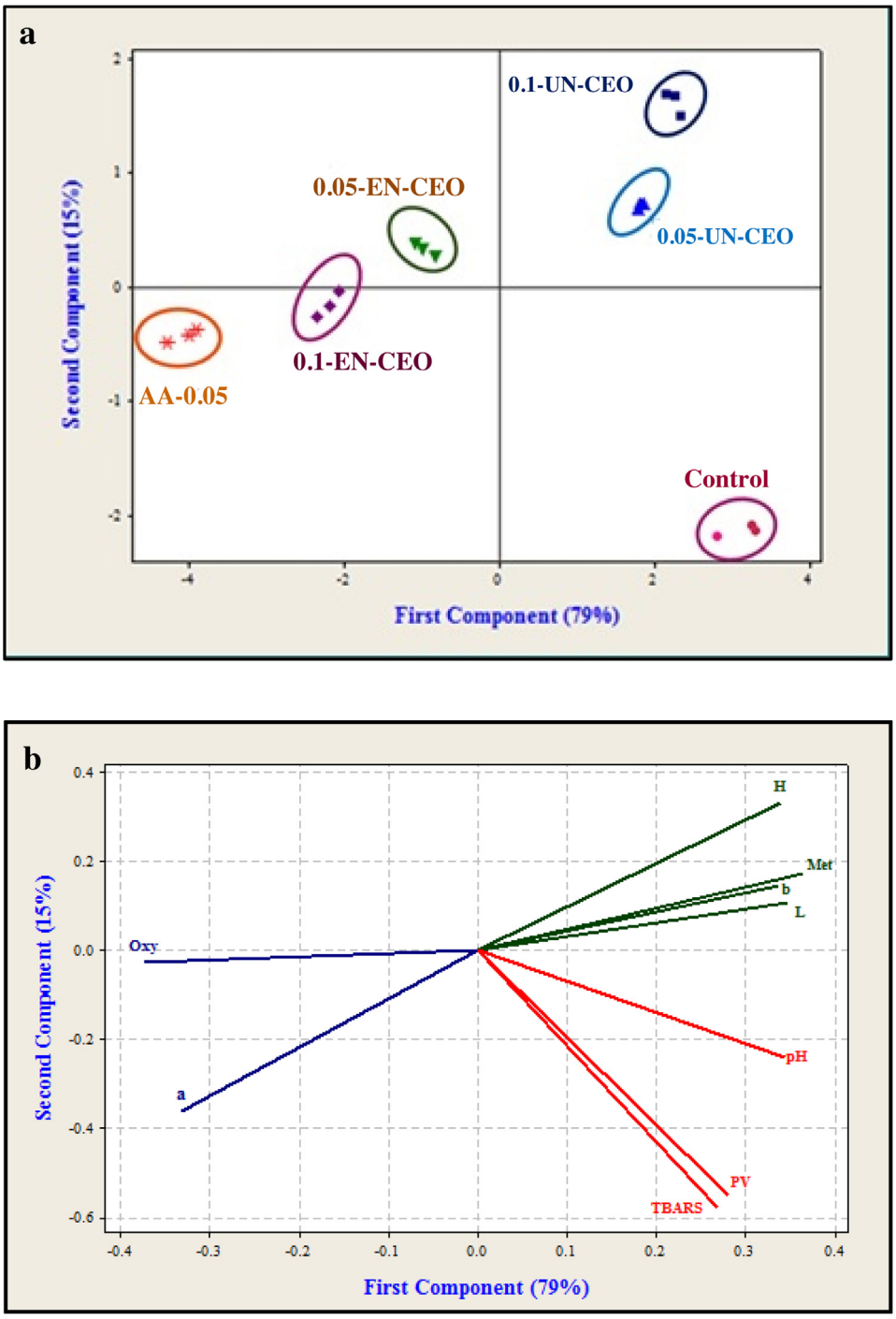

Fig. 5. Principle component analysis (PCA) score (a) and loading plots (b) derived from physicochemical characteristics of beef patties at the 8th day of refrigerated storage. For abbreviations see Fig. 1.

that unencapsulated CEO at high concentration may induce oxidation of ferrous-oxymyoglobin to ferric-metmyoglobin. The gradual rise in the MetMb content of different formulations during storage (Table 1) confirmed the observed decline in redness and an increase in hue angle values. Since the upper MetMb content for the consumer's preference was 30–40%, control, 0.05-UN-CEO, 0.1-UN-CEO formulations reached it on the 6th day, 0.05-EN-CEO on the 8th day and 0.1-UN-CEO and AA-0.05 did not reach below 30 until the end of the experiment. Thus, according to our results, ascorbic acid and encapsulated CEO extremely improved the red color stability of beef patties. Cinnamaldehyde, the major fraction of CEO, is an unsaturated aromatic aldehyde, which is routinely added as a food flavoring [6]. Increasing discoloration of beef patties exposed to high concentration of unencapsulated CEO may be explained by the results of Lynch and Faustman [34]. These researchers demonstrated that aldehyde compounds can reduce myoglobin stability by promoting OxyMb oxidation, enhancing the prooxidant function of MetMb and enzymatically the decreasing reducing ability of MetMb. So far, limited reports have been published on the influence of CEO or it's constitutes on the meat color.

3.5. Principal component analysis

In the present study, we subjected the data set derived from physicochemical evaluation of beef patties to PCA in order to identify the similarities and differences among 6 types of beef patties formulations (18 samples). The variables taken into consideration were pH, PV, TBARS, OxyMb (%), MetMb (%), a^*, b^*, L^* and H*. PCA resulted in 9 principal components (PCs) that PC1 and PC2 were accounted for 79 and 15% of the total variance, respectively. In other words, 94% of the total variance in the 9 considered variables can be compressed into two new variables (PCs). We demonstrated that the first highest-ranking component manifests clear differentiation among the patties treated with UN- and EN-CEO (Fig. 5a). In fact, similar positions of various formulations in the PCA plot demonstrated their similar physicochemical profiles. We recognized the main components accountable for separating the different formulations by analyzing the corresponding loading plots (Fig. 5b). The b^*, L^*, H^* and MetMb (%) were located on the upper right side of the loading plot, indicating that the discoloration and brownish color were indicators of UN-CEO treated beef patties even more than the control sample. The control patties were separated on the PC1 because of their high pH, PV, and TBARS values, while the dispersion of AA-0.05 and 0.1-EN-CEO, located in the lower left side of loading plot and along the PC2, was a function of high a^* and OxyMb content. 0.05-EN-CEO patties were located the nearby center of the loading plot and did not associate with any of the physicochemical characteristics.

3.6. Sensorial changes of beef patties

All sensory attributes of samples were significantly influenced by the storage time, formulation and their interaction ($p < 0.05$). The control, 0.05-UN-CEO and 0.1-UN-CEO beef patties assumed unacceptable color after day 4. Instead, the red color of AA-0.05, 0.05-EN-CEO, and 0.1-EN-CEO remained almost acceptable up to the end of the storage. The acceptance of the red color of these samples was in accordance with a^*, H, OxyMb and MetMb data, which in turn retarded surface color fading. The mean off-odor scores reduced from 4.4 (0th day) to 2.7 (4th day) in control and from 4.5 (0th day) to 2.9 (8th days) in AA-0.05 treatment, but the scores of the samples treated with UN-CEO and EN-CEO at two concentration levels were higher than 3 until the 8 day (Table 2). The development of unfavorable odor in meat was mainly related to lipid oxidation products such as MDA (higher than 0.5 mg MDA/kg) and microbial spoilage. At both unencapsulated CEO concentrations, the assessors could smell only the strong cinnamon odor. Therefore, we evaluate acceptance of cinnamon odor by panelists in the present work. Beef patties treated with 0.1% of unencapsulated CEO received scores below 3 at the beginning, indicating an unacceptable CEO odor. The scores of CEO odor of beef patties containing encapsulated CEO decreased with the storage time; however, these treatments were not judged as unacceptable by panelists on the 8th of the storage due to slow release of CEO molecules from CSNPs. So, encapsulation provides an effective tool to achieve the satisfactory sensory characteristics while preserving antioxidant and antimicrobial activities. The overall acceptability of the control, 0.05-UN-CEO and 0.1-UN-CEO after day 4, was significantly declined below the rejection limit ($p < 0.05$) (Table 2). Higher overall acceptability scores of 3.8 and 4.4 after 8 days for 0.05-EN-CEO and 0.1-EN-CEO correlated well with lower discoloration, TBARS values and microbial counts and above all higher OxyMb content. These results reveal that the strong odor of CEO was reasonably prevented through nanoencapsulation especially at higher concentrations. Our findings on the improvement of the organoleptic attributes of meat products by encapsulation are in accordance with those already reported for chilled pork [8].

Table 2
Changes in sensory attributes of beef patties with different formulations during 8 days of refrigerated storage.

Sensory attribute	Treatments	Storage time (days)		
		0	4	8
Red color	Control	$3.7 = 1.1^{a}$	$2.9 = 0.7^{bc}$	$2.2 = 0.4^{b}$
	0.05-UN-CEO	$3.9 = 0.7^{a}$	$2.7 = 1.3^{c}$	$2.4 = 0.7^{b}$
	0.1-UN-CEO	$3.6 = 1.3^{a}$	$2.4 = 0.9^{c}$	$1.8 = 0.6^{b}$
	0.05-EN-CEO	$4.0 = 0.8^{a}$	$4.3 = 0.6^{ab}$	$3.6 = 0.8^{a}$
	0.1-EN-CEO	$4.1 = 1.3^{a}$	$4.0 = 0.9^{abc}$	$3.8 = 0.7^{a}$
	AA-0.05	$4.7 = 0.9^{a}$	$4.5 = 0.7^{a}$	$4.4 = 0.4^{a}$
Off- odor	Control	$4.2 = 0.6^{a}$	$2.7 = 0.4^{c}$	$2.2 = 0.6^{b}$
	0.05-UN-CEO	$4.7 = 0.4^{a}$	$4.0 = 0.8^{bc}$	$3.5 = 0.5^{ab}$
	0.1-UN-CEO	$4.7 = 0.4^{a}$	$4.5 = 0.7^{b}$	$3.7 = 0.9^{ab}$
	0.05-EN-CEO	$4.8 = 0.4^{a}$	$4.5 = 0.7^{b}$	$3.6 = 0.9^{ab}$
	0.1-EN-CEO	$4.8 = 0.8^{a}$	$4.9 = 0.3^{a}$	$4.2 = 0.7^{a}$
	AA-0.05	$4.5 = 0.4^{a}$	$2.9 = 0.9^{c}$	$2.4 = 0.7^{b}$
CEO odor	Control	nd	nd	nd
	0.05-UN-CEO	$3.7 = 0.8^{ab}$	$3.5 = 0.7^{ab}$	$3.2 = 0.9^{b}$
	0.1-UN-CEO	$2.6 = 0.5^{b}$	$2.4 = 0.9^{b}$	$2.7 = 1.0^{b}$
	0.05-EN-CEO	$4.7 = 0.4^{a}$	$4.4 = 0.6^{a}$	$4.0 = 0.6^{a}$
	0.1-EN-CEO	$4.6 = 0.8^{a}$	$4.1 = 0.8^{a}$	$3.7 = 0.6^{ab}$
	AA-0.05	nd	nd	nd
Overall acceptability	Control	$3.5 = 1.1^{a}$	$2.8 = 0.8^{b}$	$2.5 = 0.9^{bc}$
	0.05-UN-CEO	$4.2 = 0.9^{a}$	$2.8 = 1.0^{b}$	$2.2 = 0.7^{c}$
	0.1-UN-CEO	$3.9 = 0.9^{a}$	$2.6 = 1.1^{b}$	$1.7 = 0.6^{c}$
	0.05-EN-CEO	$4.3 = 0.8^{a}$	$4.3 = 0.9^{a}$	$3.8 = 0.7^{ab}$
	0.1-EN-CEO	$4.3 = 1.1^{a}$	$4.2 = 0.7^{a}$	$4.4 = 0.7^{a}$
	AA-0.05	$3.9 = 0.9^{a}$	$3.6 = 0.9^{ab}$	$2.8 = 0.8^{bc}$

For each sensory attribute at each day, values in the same column with the same lowercase letter (a, b, c,. . .) are not significantly different ($p < 0.05$). nd = Not determined. Control: sample without any cinnamon essential oil; 0.05-UN-CEO, 0.1-UN-CEO and 0.05-EN-CEO, 0.1-EN-CEO and AA-0.05samples containing 0.05 and 0.1% unencapsulated cinnamon essential oil and 0.05 and 0.1% encapsulated cinnamon essential oil and 0.05% ascorbic acid, respectively.

4. Conclusion

Our findings indicate that unencapsulated CEO can be useful to prevent lipid oxidation and microbial spoilage in beef patties. But, it had an adverse effect on the red color of samples and MetMb formation had a similar trend as the control sample or even faster in 0.1-UN-CEO formulation. Indeed, the magnitude of the antioxidant and antimicrobial activities decreased during the experiment in these treatments, which may be associated with evaporation of CEO. The use of CSNPs as a carrier of CEO looks promising, as lower lipid oxidation, microbial growth, and higher red color stability were obtained, especially after 4 days of storage. Although CEO has good preservative properties, its strong flavor and adverse effect on red color will finally limit its utilization in patties due to lower sensorial scores. It was also revealed that some of the negative sensorial attributes of the patties can be overcome by addition of encapsulated CEO due to sustained release during storage and masking strong CEO taste and odor. This study provides support for expanding the use of nanoencapsulation techniques to design the delivery system for utilization of natural preservative in functional food.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.04.002 .

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