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**Research Article**

# Comparison of free and nano-encapsulated Safran (*Crocus sativus* L.) petal extract effects on some quality indexes of rainbow trout (*Oncorhynchus mykiss*) fillets

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**Abstract**

Fish as a main source of omega-3 and protein is widely consumed across the world. However, fish muscle is susceptible to putrefaction during storage. The aims of this study were to compare the effect of free and nano capsulated saffron petal extract on (bio) chemical, microbial and sensory properties of rainbow-trout fillet. Free saffron-petal extract (FSPE) and its nano capsulate (NSPE) were prepared by enzymatic-hydrolysis and coating with the core (FSPE), wall (50:50 maltodextrin/whey-protein concentrates MD/WPC) ratio of 1:4 respectively. The fillets were soaked into three solutions of FSPE and NSPE at 10% w/v as well as ionized water (control), dried with ambient-air, packed in aerobic condition and polyethylene bags and kept in refrigerator (15-day at 4±1ºC). Scanning-electron microscopy and Fourier-transform infra-red spectroscopy of NSPE confirmed the successful coating of particles and interactions between the FSPE and MD/WPC. The total-viable and psychrophilic bacteria-count of samples treated with saffron-petal extract was significantly lower than those of control samples. The difference between NSPE and FSPE samples was not significant ($p$>0.05). The lowest thiobarbituric-acid reactive substances (TBARS) and total-volatile basic nitrogen (TVB-N) value obtained in NSPE samples was 1.44 mg MDA/kg and 16.44 mgN2/100g after 9 days at 4ºC storage ($p$<0.05). The best sensory score was obtained for NSPE samples. The shelf-life of control fish was 5-8 days, while this time for NSPE and FSPE samples were 9-13 and 8-13 respectively. Overall, encapsulation of free saffron petal extract with maltodextrin/whey protein concentrate is d microbialrecommended for its higher protection against chemical an degradation.

**Keywords:** Rainbow trout fillet, Enzymatic saffron-petal extract, Maltodextrin, Whey protein concentrate, Nano-capsulation, TBA and TVB-N

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## Introduction

Fish can be considered as one of the best quality proteins for human body. In addition, it is a rich source of polyunsaturated fatty acids (PUFAs) and omega-3, vitamins and various minerals (e.g. phosphorus, selenium, zinc and iron). Along with increased customer awareness, the desire to consume such a healthy and high-quality food has increased (Khoshnoudi-Nia *et al.*, 2018). Rainbow trout (*Oncorhynchus mykiss*) belonging to the order *Salmonidae*, is widely cultured due to its high compatibility with fish farming conditions and annual production. High contents of protein (essential amino acids), omega-3 and omega-6 in rainbow trout make it a nutritional meal among people (Kolanowski 2010; Javadian *et al.*, 2017). However, the nutritional components (mainly proteins and PUFAs) in this fish and other sea foods are highly susceptible to degradation and putrefaction during storage (Khoshnoudi-Nia *et al.*, 2018). Generally shelf life of sea foods is highly affected by lipolytic oxidation, enzymatic reactions and microbial activities. Therefore, using antioxidant and antimicrobial preservatives, can be considered to decrease undesirable changes (Jouki *et al.*, 2014). Although synthetic antioxidants and preservatives were used to protect food products for many years, but they make serious side-effects on human health. This is the main reason that incorporating natural antioxidants instead of synthetic compounds have been considered widely to retard unfavorable chemical reactions and/or microbial growth (Hosseini *et al.*, 2009; Hashemi *et al.*, 2013).

Saffron *(Crocus sativus*), a perennial plant belonging to *Iridaceae* family, is a well-known plant with a wide range of applications in food and drug industries. The most important and valuable part of the plant is dark-red stigma which is known as the world's most costly spices (Moghaddasi, 2010). Since each saffron flower contains 2mg of stigma (based on dry weight), approximately 150000 flowers should be harvested to achieve 1kg of dried stigma. Saffron petal is the main by-product of saffron flower, and despite the fact that it is unusable for farmers, it contains considerable amounts of phenolic compounds including flavonoids (kaempferol, 12.6%), anthocyanins (pelargonidin 3-glycoside, pelargonidin 3, 5-glycosides, petunidin, 3, 5 cyanidin-diglycosides, and delphinidin 3-glycosides), carotenoids (crocin and crocetin) and glycosides (Khoshbakht Fahim *et al.*, 2012). Many researchers confirmed that the above-mentioned compounds have antioxidant, antimicrobial and other biological properties and there is a good potential to use them as natural preservatives (Hosseini and Shariatmadar 1994; Hosseini *et al.*, 2018; Wali *et al.*, 2020). However, due to susceptibility of phenolic compounds, they may be subject to degradation and loosing biological



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activity when exposed to oxygen, high temperature and even processing conditions, so tackling these problems and extent bioactivity should be considered (Donsì *et al.*, 2011).

Nano-capsulation is a new processing procedure, which covers susceptible substances with protective biopolymers and shield their bioactivities, when they are mixed with food during processing, handling and storage (Jafari, 2017). Various hydrocolloids have been studied to encapsulate anthocyanins. Carbohydrates such as Arabic gum (de Araujo Santiago *et al.*, 2016), maize starch (García-Tejeda *et al.*, 2015), maltodextrin (Jafari *et al.*, 2016; Suravanichnirachorn *et al.*, 2018; da Rosa *et al.*, 2019) as well as proteins including gelatin (Mahdavi *et al.*, 2016), whey protein concentrate (Moser *et al.*, 2017; Wang *et al.*, 2021), and soy protein isolate (Šaponjac *et al.*, 2017) have been extensively investigated for protection of anthocyanins.

Maltodextrin (MD) as a biopolymer has effective drying property and because of its low price there is economic feasibility in purchasing it (Sharif *et al.*, 2020). Therefore, its combination with other carrier agents might be a financial advantage (Nayak and Rastogi, 2010). On the other hand, hygroscopic nature of MD and its moisture absorbance as a surface coating material of particles made it sticky and consequently increase aggregation of particles when this biopolymer is used alone (Both *et al.*, 2018; Sharif *et al.*, 2020). In this regard, several studies have shown that the combination of polysaccharide (such as maltodextrin) and protein will result a better coating of susceptible materials compared with individual use of MD or WPC (Kanakdande *et al.*, 2007; Moser *et al.*, 2017; Norkaew *et al.*, 2019; Pieczykolan and Kurek, 2019). Among various protein source, whey proteins exhibit very functional properties such as emulsifying and gelling features and typically are used to develop nanoparticles (Abaee *et al.*, 2017; Ramos *et al.*, 2019). For these reasons the combination of maltodextrin and whey protein concentrate was selected as wall material to encapsulate saffron extract.
Although some studies dedicated on saffron petal extract and its chemical components, not enough studies are focused on capsulation of saffron petal extract and its possible application for preserving sensible and perishable foods. Therefore, the aims of this study were to prepare saffron petal extract by enzymatic method, encapsulate the extract with MD/WPC layer as a coating agent and compare the effects of free saffron petal extract (FSPE) and nano-capsulated saffron petal extracts (NSPE) on shelf-life of rainbow-trout fish fillet.

## Materials and methods

### *Materials*

Maltodextrin (dextrose equivalent: 20˚) and whey protein concentrate were prepared from Allan & Robert (France).



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Saffron petals were purchased from a local shop in Mashhad city (Iran). After vacuum-oven drying of saffron petals at 50ºC for 48h, it was powdered and kept in tight polyethylene bags (with minimum air) at room temperature until use. All other chemicals used in this study were of analytical grade and purchased from Merck Chemical Company (Darmstadt, Germany).

*Enzymatic extraction of saffron petals*

Enzymatic extraction was done according to Lotfi *et al. (*2015) with slight modification. In brief, 1g of saffron petal powder was mixed with 10mL of acetate buffer (pH=3.5) and homogenized for 1min. Then the mixture of three enzymes (pectinase, cellulase, and hemi-cellulase) solution with commercial name of Pectine X Ultra SP-L (Novozyme Company, Denmark) was added to the prepared solution of saffron petal at 5% (v/v) concentration and held for 120min (reaction time) at 40°C. The resulting solution was stirred for 1h in a dark place and then centrifuged at 3000g force to separate its insoluble materials.

*Nano-capsulation of saffron petal extract*

The free saffron petal extract (FSPE) solution with 10% w/v concentration was capsulated with the coating layer of mixed maltodextrin (MD)/whey protein concentrate (WPC) with ratio of 1:4 based on Gortzi *et al. (*2006) method with some modifications. The mixed powder of coating material was dissolved in water/ethanol (W/E) solution at ratio of 1:3 (v/v) and its ethanol solvent was separated in a rotary evaporator (Gortzi *et al.*, 2006). The prepared extract of FSPE was then added to the mixture of MD/WPC at the core: wall ratio of 4:1 (v/v). After mixing the FSPE and coating layer for about 1h, the solvents were evaporated (by $N_2$ gas) and the resulting bioactive compounds (solid residue) were dissolved in 2mL of phosphate buffer (at pH 7.4). Then it was uniformed (with bench top Hielscher homogenizer UP2000) at 200 bar for 15min at 35$^{o}$C and kept at a dark and cool place for 2h (to prevent color change of anthocyanins in saffron petal extract). Finally, it was centrifuged (equipped with cooling system) at 6500rpm and the resulting nano-capsulated saffron extract (NSPE) product was freeze-dried for 24h at -45ºC under a 2-mbar vacuum conditions.

*Particle size distribution and morphology*

Morphology, surface appearance and mean diameter of NSPE particles with MD/WPC layer were evaluated by using Scanning Electron Microscope (SEM) equipment (XL 30 model, Philips, Netherland) with an accelerated voltage of 20kV. The NSPE particles were placed on aluminum stubs and sizzle coated with gold. The micrographs were prepared after complete drying of NSPE. The magnitude of the prepared pictures was 100k. It is necessary to mention that



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SEM system creates an image with 3 dimensions by detecting reflected or knocked-off electrons, therefore, this method was used to get surface information (such as roughness or contamination) of particles after encapsulation. However, in TEM (transmission electron microscopy), the electrons pass through a sample with 2 dimensions to generate an image. In most of the research work focused on encapsulation of antioxidants (such as anthocyanin), the morphology and particle size were examined solely on the basis of SEM images (de Araujo Santiago *et al.*, 2016; García-Tejeda *et al.*, 2016; Begum and Deka 2017; Moser *et al.*, 2017; Ratanapoompinyo *et al.*, 2017).

*FTIR spectroscopy analysis*

The FTIR (Fourier-transform infrared) spectroscopy analysis performed on three powders including NSPE, mixed coating materials of the MD/WPC and FSPE to recognize distinctive functional groups in resulting NSPE powder. Briefly 2mg of each powder was dispersed with 10mg potassium bromide powder and after complete mixing it was pressed in a specific disk at the pressure of about 5 bars for 3min. After placing the disc in a sample cup of FT-IR spectrophotometer (Shimadzu, IRAffinity-1S, Japan) and obtaining infrared spectrum, it was scanned from 500 to 4000 $cm^{-1}$ for more than 10 times to increase the signal to sound ratio. Each measurement was carried out with an average of 32 scans at 4 $cm^{-1}$ resolutions.

*Rainbow trout fillet preparation*

Fourteen rainbow trout (*Oncorhynchus mykiss*) fishes (each one with average weight of 550±20 g) were captured from a local fish farm in Sari (North of Iran) and immediately transferred to food analysis Lab of our department after placing in an ice box. After removing internal organs, the fish samples were beheaded, filleted and finally washed with drinking water. The resulting fillets (each one with 100±20 g) were randomly divided into enough samples to make 3 treatments, measuring their different quality indicators (chemical, microbiological) and sensory evaluation at 4 storage time intervals. The prepared samples were stored at 4$^{o}$C before treating with FSPE, NSPE solutions and ionize water (as control).

*Fish coating process*

The prepared fillets were divided into three groups and randomly soaked for 15min separately in 10% (w/v) ratio solutions of FSPE, NSPE and ionized water. The impregnated fillets were dried by a wind-blowing fan at ambient temperature (20$^{o}$C) for 15min, packed in polyethylene bags in aerobic condition and kept in refrigerator (4±1$^{o}$C). The following chemical and microbial parameters of each group of fillets were measured during 15-days cold storage (in 3 replicates) with 3days intervals.



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*Thiobarbituric acid reactive substances (TBARS)*

TBARS values of fish samples were measured based on colorimetric method (at 530nm; Spectrophotometer: Hitachi, U-3300; Tokyo, Japan) and expressed by mg of malonaldehyde (MDA) per kg of sample (Sun *et al.*, 2001). The TBARS value of 2mg MDA/kg fish filet is out of acceptable range and it means the spoilage initiation point for fresh fish has started (Lakshmanan *et al.*, 1999; Goulas and Kontominas, 2005).

*Total-volatile basic nitrogen (TVB-N)*

The TVN-B value of each fish sample, (which is one of the core indicators for evaluation of fish freshness) was measured using Kjeldahl method and presented as mg N/100g of fish sample (Goulas and Kontominas, 2005). Measuring TVB-N in meat products is essential, because it is the core indicator for evaluation of fish freshness and when this value exceeds 20mg N/100g represents the starting point of spoilage (Pons-Sanchez-Cascado *et al.*, 2005; Cheng and Sun 2015).

*Microbial analysis*

The Total Viable microorganism (TVM) of each fish sample was prepared and measured according to standard plate count agar (PCA; Merck, Germany) and based on ISO 8443: 2003 (ISO, 2003). The resulting plates were incubated at 35°C for 48 hours. Psychrotrophic Total Count (PTC) were also incubated on plate count agar (PCA) media at 7°C for 10 days (ISO, 2001). The microbial load was recorded in terms of log of colony forming units per gram ($log_{10}$CFU/g) of each sample. The maximum acceptable limit for microbial load (TVM or PTC) is considered 7 log CFU/g (ICMSF, 1986).

*Sensory evaluation*

Sensory evaluation of fish fillets cooked (at 120ºC for 15min) was performed by using 20 experienced panelists (10 men and 10 women) at the age-range of 25-35 years during 15-days storage with 3-day intervals. At each sampling time, three forms of cooked fillets were randomly given to judges to evaluate their sensorial attributes under fluorescent (natural day) light and room temperature (20-24$^{o}$C). Drinking water for cleansing the panelist's taste was provided. The panelists were asked to check the color, texture and taste (combined with flavor) and express their acceptance scores for each sample according to hedonic scale (Chytiri *et al.*, 2004; Ozogul *et al.*, 2017) with some modifications. Scores of 1 to 5 were assigned respectively for "very bad" to "very good" on each attribute of every treatment. Since the level effects of different attributes (color, texture and taste) on overall acceptance score (OAS) of cooked fish are not similar, the method was modified and 20 panelists made three sets of sensory evaluation tests on three (color, texture and taste) attributes of cooked fish fillets with no treatment



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(similar to the control samples). After this treatment they assigned importance or weight levels of 1, 2 and 3 respectively for their color, texture and taste attributes (Frøst *et al.*, 2005). After performing the sensory evaluation of treated and non-treated samples, the OAS of every panelist for each sample and specific storage time was obtained by the following equation:

OAS (overall acceptance score) = (color score*1+texture score*2 + taste score*3)/ (1+2+3)

An overall acceptance score≥ 3.0 (out of maximum 5.0) was considered as the borderline for freshness acceptability of the treated fillets as recommended by (Barassi *et al.*, 1987).

*Statistical analysis*

Data analysis was performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA). A complete randomized design was used to get the chemical, microbiological and sensory data from the fillets treated with FSPE, NSPE and Control samples during 15-day of storage. The sensory data were based on mean±SD of at least three replications. Moreover, the use of these parametric tests was justified to be sure that the obtained sensory data (n>30: 20 panelists×6 interval times×3 replications) approximately normally distributed by checking the central limit theorem as described by Yu and Behrens (1995). The Analysis of variance (ANOVA) and Tukey's multiple range test ($p$<0.05) was used to compare the obtained hedonic scores and determine the significant or insignificant differences between the treated fish fillets.

**Results**

*Particle size distribution and morphology*

The Morphology (shape, uniformity and characteristics of the surfaces) of saffron petal extract capsules was investigated by using SEM with different magnifications (Fig. 1). The images show irregular particles with flake-like structure and porous surface. The encapsulated saffron petal powder exhibited a relatively uniform and small particle size (40.4 to 97.46 nm).

*FTIR spectroscopy of NSPE*

FTIR analysis was carried out to confirm the cross-linking interactions between MD/WPC and FSPE. The infrared absorption spectra of MD/WPC layer, FSPE and NSPE confirmed the strong absorption bands of these compounds at around 3500cm$^{-1}$ and characteristic peaks at around 1600cm$^{-1}$ (Fig. 2). Comparing the spectra of NSPE with FSPE and MD/WPC layer, there was a shift in vibration peaks from 3375 to 3495cm$^{-1}$. The spectra of NSPE showed various sharp bands at 2921, 1640, 1447, and 1237. The spectra of NSPE differed from spectra of MD/WPC and FSPE. The spectra of NSPE retained characteristic peak of MD/WPC at 2350cm$^{-1}$ and the peak of MD/WPC layer at 1470cm$^{-1}$ has become more prominent. The characteristic peak of FSPE at 1160cm$^{-1}$



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was incorporated into MD/WPC. However, the peak spectra of FSPE disappeared at 1237cm$^{-1}$. In addition, the characteristic peak of FSPE at 1070cm$^{-1}$ shifted to 1080cm$^{-1}$ for NSPE. A region between 700-1200cm$^{-1}$, known as finger print region, was observed different in FSPE from spectra of NSPE.

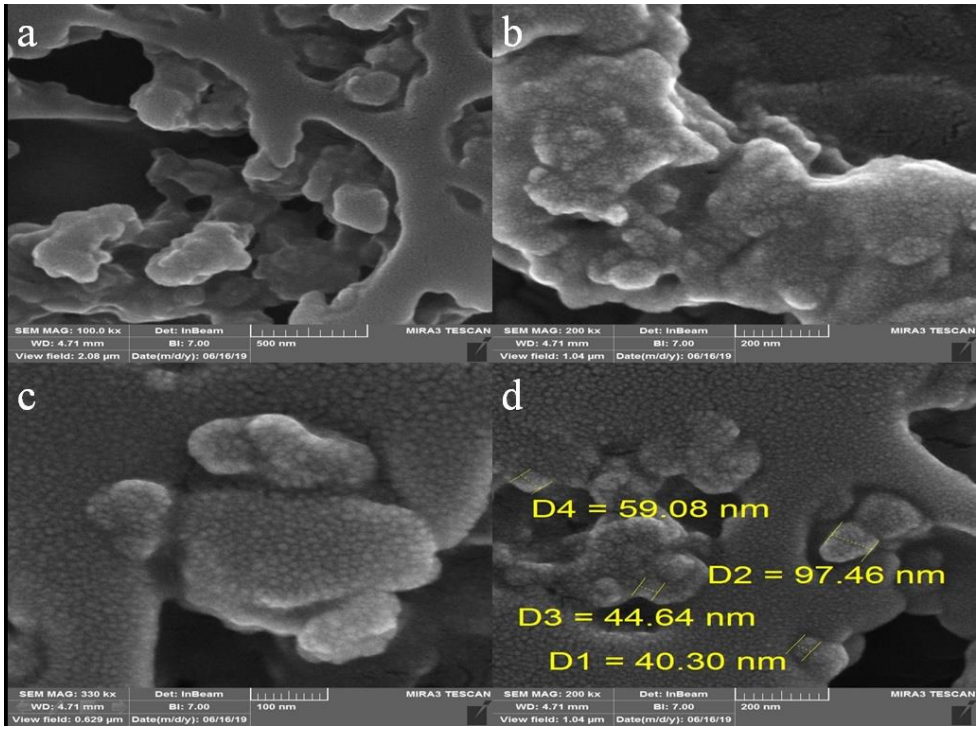


**Figure 1: Micrographs of nano-capsulated saffron petal extract (NSPE) prepared by scanning electronic microscope (SEM).**

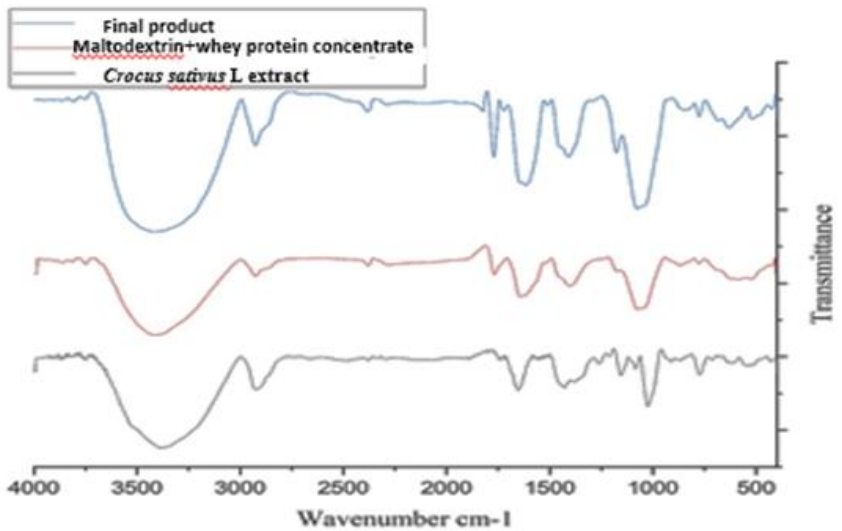


**Figure 2: FT-IR spectra of MD/WPC (maltodextrin+whey protein concentrate) layer, FSPE (free saffron petal or *Crocus sativus* L extract) and Nano-encapsulated saffron petal extract (NSPE or final product).**



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*TBARS (Thiobarbituric acid reactive substances) value*

The effect of FSPE and NSPE on TBARS value of treated fish fillets during storage time is illustrated in Figure 3a. The TBARS values in all treatments increased significantly ($p$<0.05) over time. However, the trend of increasing in TBARS values for fillets treated with FSPE was significantly lower than control samples ($p$<0.05). The comparison between fish fillets treated with FSPE and NSPE showed that the FSPE samples had insignificantly lower TBARS values than NSPE samples until 6$^{th}$ day of storage. However, after this time of storage, the situation became in reverse form and the TBARS values of NSPE samples became lower than those in FSPE samples. Considering the acceptable level of TBARS values in fish samples equal to 2mg MDA/kg, the fillets treated with FSPE were not acceptable after 9 days. While the TBARS value for NSPE sample were lower than 2mg MDA/kg for more than 12 days, the control samples reached to this level before 6 days of storage. Therefore using FSPE and NSPE in rainbow fish-fillets could extend shelf life by retarding the oxidative process more than 1.5 and 2 folds as compared to control samples, respectively.

*TVB-N (Total-volatile basic nitrogen) value*

The TVB-N of treated fillets increased from their initial value (9.54) to 44.07 (more than 5 times), 37.16 and 29.15mg N/100g respectively for control, FSPE and NSPE samples for 15 days storage. Although the TVB-N content of the FSPE and NSPE were insignificantly lower than the control samples up to 3-day of storage, these differences became highly significant ($p$<0.05) after 3-day storage (Fig. 3). The lowest TVB-N content during 6 initial day's storage was recorded for FSPE samples (18mg N/100g). Whereas no significant difference was observed between FSPE and NSPE samples at this day, the NSPE had significantly ($p$<0.05) lower TVB-N than FSPE samples on 12$^{th}$ and 15$^{th}$ day of storage. Based on the permissible threshold limit for generated TVB-N= 20mg N/100g of food product during storage, the Control, FSPE and NSPE samples had 7, 9 and 12 days of acceptable storage time (Fig. 3).

*Microbial analysis*

Total viable microorganism (TVM) and Psychrotrophic total count of rainbow-trout fillets with and without treatment during storage at 4°C after 15 days incubation are shown in Figure 4 (a and b). The initial number of bacteria in trout samples was 3.6 log CFU/g, indicating that the fish used in this study had good quality. The results showed that samples treated with FSPE can be maintained for longer time as compared with control ones. However, the difference between fillet samples treated with FSPE and NSPE was not significant ($p$>0.05). The maximum acceptable microbial limit (for both



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PTC and TVM values) in fresh and frozen fish is $10^7$ CFU/g. After 12 days of storage, the TVM of rainbow-trout fillet with FSPE was still below 7 log CFU/g, however this time was about 8 days in control samples. Based on PTC value the shelf-life of control, FSPE and NSPE samples of fish fillets were estimated 7, 14 and 14 days, respectively.

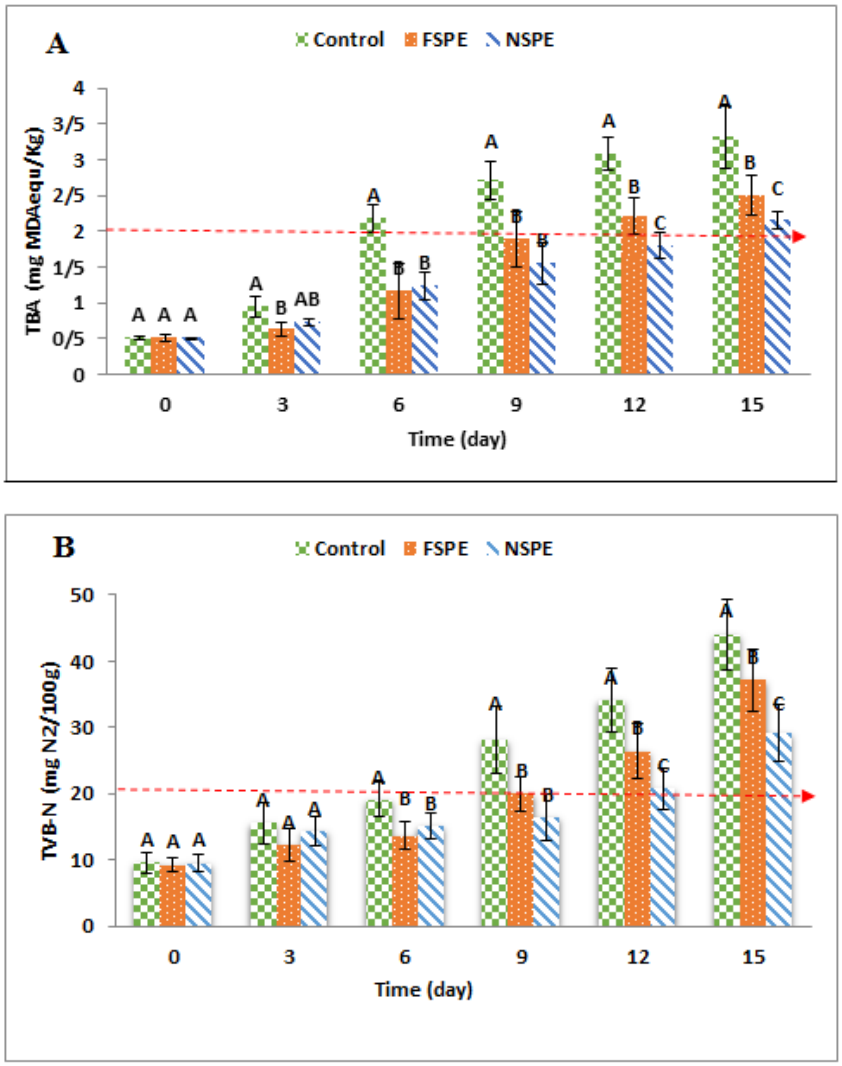


**Figure 3: The effects of free and nano-encapsulated saffron petal extracts (FSPE and NSPE) on amounts of A) thiobarbituric acid reactive substances (TBARS) and B) total-volatile basic nitrogen (TVB-N) of fish samples stored for 15 days at 4±1˚C. Different letters indicate significant difference among samples at each interval time of storage ($p$<0.05). Dashed line represents the threshold limit.**

*Sensory analysis*

Table 1 and Figure 5 show the sensory scores obtained for color, texture, taste (combined with flavor) and overall acceptance of treated and non-treated (control) samples of cooked-fish filets during storage.

As Table 1 shows clearly, there was no significant difference between colors, texture and taste of treated and control



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samples of fish samples up to 3th day of storage. However, significant ($p$<0.05) differences were observed for three sensory attributes between treated (fish fillets containing FSPE and NSPE) and control samples after 3 days of cold storage. In fact, the OAS (overall acceptance score) of FSPE and NSPE reached acceptable level (score ≥3), after 7 and 9 days of storage, respectively. However, OAS for control samples was not acceptable after 5-day of storage (Fig. 5). From the 6th day of storage, some samples were regarded not edible by panelists. Therefore the samples were estimated based on odor instead of taste.

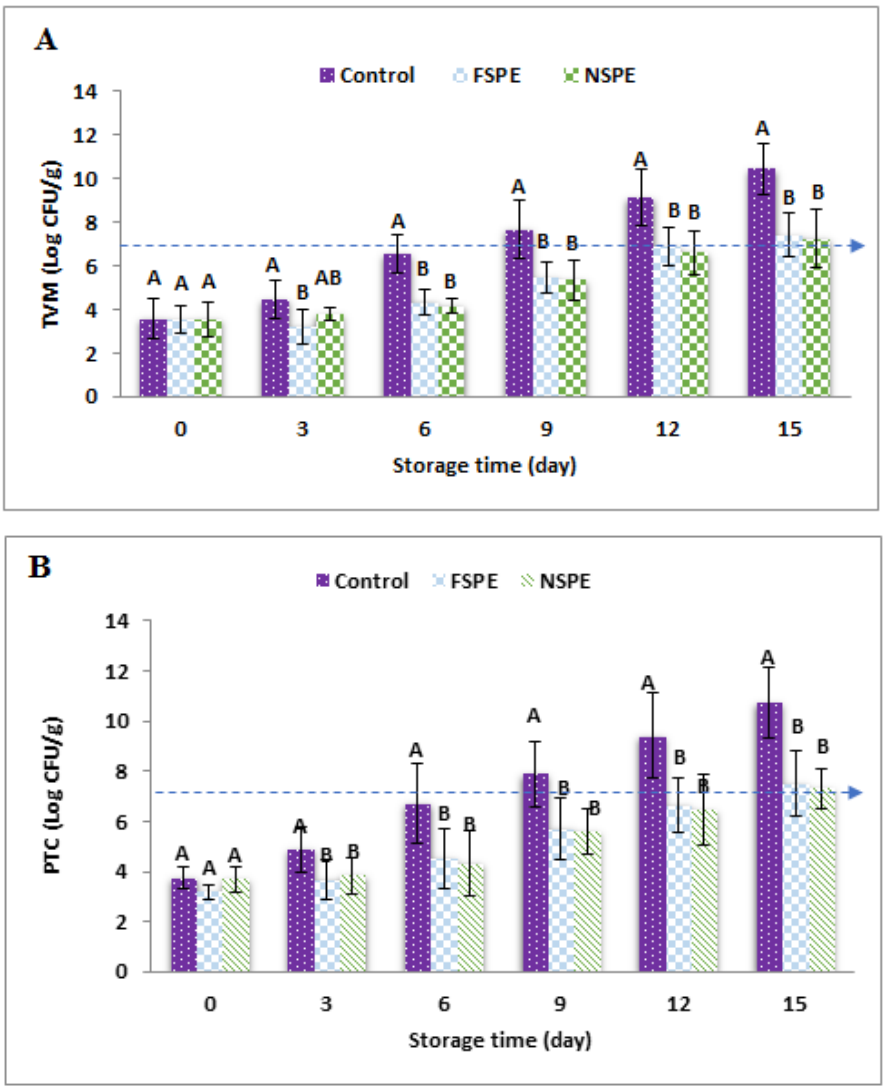


**Figure 4: The effects of free and nano-encapsulated saffron petal extracts (FSPE and NSPE) on A) total viable microorganisms (TVM) and B) psychrophilic bacteria count (PTC) of fish samples stored for 15-day at 4±1˚C. Different letters indicate significant difference among samples at each interval time of storage ($p$<0.05). Dashed line represents the threshold limit.**



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**Table 1: Sensory scores of control, free saffron petal extract (FSPE) and nano-capsulated saffron petal extract (NSPE) samples for color, texture and taste attributes of cooked rainbow-trout fillets within 15 days or cold storage with 3 days intervals.**

| Treatments | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 3 | 6 | 9 | 12 | 15 |
| | | | **Color** | | | |
| Control | $5\pm0.00^{aA}$ | $4.1\pm0.12^{bB}$ | $2.24\pm0.11^{cC}$ | $1.8\pm0.1^{cD}$ | $1\pm0^{bE}$ | $1\pm0^{bE}$ |
| FSPE | $5\pm0.00^{aA}$ | $4.22\pm0.14^{bB}$ | $3.76\pm0.13^{bC}$ | $2.92\pm0.18^{bD}$ | $2.08\pm0.23^{aE}$ | $1.1\pm0.1^{bF}$ |
| NSPE | $5\pm0.00^{aA}$ | $4.52\pm0.16^{aB}$ | $4.06\pm0.24^{aC}$ | $3.46\pm0.25^{aD}$ | $2.28\pm0.15^{aE}$ | $1.82\pm0.08^{aF}$ |
| | | | **Texture** | | | |
| Control | $5\pm0.00^{aA**}$ | $4.9\pm0.29^{bB}$ | $2.9\pm0.25^{bC}$ | $2.12\pm0.26^{bD}$ | $1.46\pm0.2^{bE}$ | $1.12\pm0.13I^{bE}$ |
| FSPE | $5\pm0.00^{aA}$ | $4.24\pm0.16^{aB}$ | $3.54\pm0.2^{aC}$ | $2.82\pm0.19^{aD}$ | $2.2\pm0.1^{aE}$ | $1.56\pm0.18^{aF}$ |
| NSPE | $5\pm0.00^{aA}$ | $4.3\pm0.18^{aB}$ | $3.54\pm0.2^{aC}$ | $2.86\pm0.23^{aD}$ | $2.18\pm0.16^{aE}$ | $1.64\pm0.11^{aF}$ |
| | | | **Taste and Flavor** | | | |
| Control | $5\pm0.00^{aA}$ | $4.24\pm0.17^{bB}$ | $2.34\pm0.24^{bC}$ | $1.2\pm0.16^{bD}$ | $1\pm0^{cE}$ | $1\pm0^{bE}$ |
| FSPE | $5\pm0.00^{aA}$ | $4.2\pm0.16^{bB}$ | $3.66\pm0.15^{aC}$ | $2.84\pm0.21^{aD}$ | $1.94\pm0.42^{bE}$ | $1\pm0^{bE}$ |
| NSPE | $5\pm0.00^{aA}$ | $4.54\pm0.12^{aB}$ | $3.88\pm0.22^{aC}$ | $3.08\pm0.29^{aD}$ | $2.3\pm0.16^{aE}$ | $1.56\pm0.17^{aF}$ |

* Each score resulted from the average of 60 numbers (20 panelists, every one tested 3 times).

**Different uppercase letters in each row show significant differences among storage days ($p<0.05$). Similarly, different lowercase letters on each column display significant difference among the three treatments ($p<0.05$). FSPE: the samples coating with free saffron petal extract solution; NSPE: the samples coating with nano-encapsulated saffron petal extract solution.

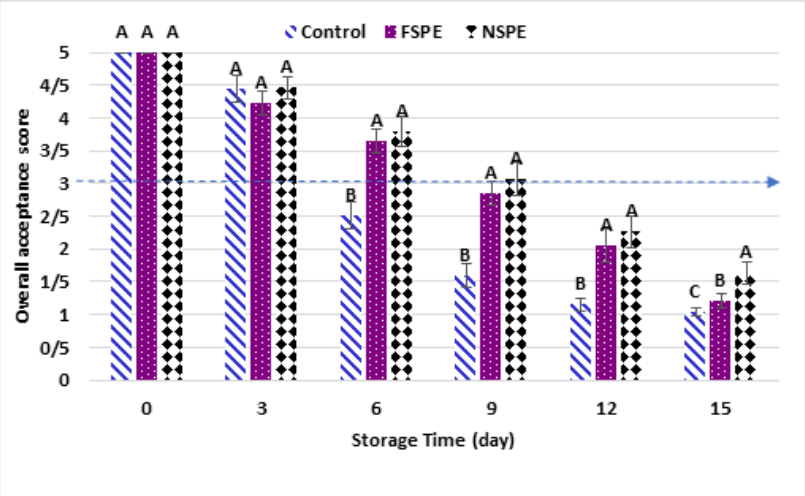


**Figure 5: The impregnation effects of rainbow-trout fish with free and nano-encapsulated saffron petal extracts (FSPE and NSPE) on overall acceptance scores (OAS) of cooked fillets stored for 15-day at 4±1˚C. Different letters indicate significant difference among samples in each interval time of storage ($p<0.05$). Dashed line represents the threshold limit.**

## Discussion

*Particle size distribution and morphology*

The main reason for using the combined form of MD and WPC was reducing hygroscopic nature, crack formation and irregularity in surface and shape of resulting nanoparticles. Because MD layer absorbs more moisture than WPC on the surface and makes sticky particles and consequently increased aggregation (Both *et al.*,



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2018; Sharif *et al.*, 2020). Encapsulation of saffron petal extract by MD/WPC was performed successfully because the maximum particle size of coated FSPE reached 97.46nm. Researchers obtained 100nm diameter for encapsulated particles when they coated saffron extract with a mixture of WPC and pectin (Esfanjani *et al.*, 2015). They explained that smaller particles release bioactive compounds in slower rate (longer time) than those with medium or large sizes, and therefore their antioxidants and antimicrobial efficiency would be significantly higher.

*FTIR spectroscopy of NSPE*

Absorption band (AB) of NSPE from 1000 to 3600 is related to different compounds. A region between 800 to 1200 (1153 $cm^{-1}$) in MD/WPC layer was assigned to C-O bond stretching related to anhydro-glucose ring vibration (Castro-Cabado *et al.*, 2016). The peak at 1069 and 1335$cm^{-1}$, refers to the aromatic ring C-H deformation and C-O angular deformations of phenols (Cai *et al.*, 2019), which is available in saffron petal extract. The AB in 1402 to 1764$cm^{-1}$ may belong to C=C and C=O bonds found in aromatic ring such as crocin (in saffron), benzopyran aromatic and pyran rings (Lee *et al.*, 2015). An absorption peak at 1629 can be assigned to amid I which is composed of stretching vibration of C=N and C-N groups available in WPC (Kutzli *et al.*, 2018). In addition OH bonds of hydroxyl and carboxyl groups (available in WPC and MD), asymmetric and symmetric stretching vibration of N-H bonds of amino groups (in WPC) appeared at AB range of 3600-3100$cm^{-1}$ (Castro-Cabado *et al.*, 2016; Kutzli *et al.*, 2018).

*TBARS value*

Since peroxide compounds are unstable products and convert to secondary products, the TBARS value is more reliable indicator for oxidation when a sample should be analyzed for shelf-life determination (German *et al.*, 1985). The increasing trend of TBARS value during storage time could be due to increase in free heme and/or other prooxidants in myofibrils degradation of fish muscle after death (Nanditha and Prabhasankar 2008; Khoshnoudi-Nia and Moosavi-Nasab, 2019a). Also, different aldehydes are produced as secondary oxidation products by breakdown of hydro-peroxides, which can be another reason for increasing TBARS in fish fillets (Tokur *et al.*, 2006).

Lower TBARS value of samples treated with FSPE is related to antioxidant properties of the extract and ability to absorb free radicals, to chelate metals and decompose the peroxides (Jouki *et al.*, 2014; Eskandari *et al.*, 2015; Moosavi-Nasab *et al.*, 2016; Mirzapour-Kouhdasht and Moosavi-Nasab, 2020). In fact, the bioactive compounds of FSPE (such as flavonoids, phenolic, and anthocyanin) effectively retarded oxidation of the lipid of fillets (Sadighara *et al.*, 2013,



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Ahmadian *et al.*, 2019; Moratalla-López *et al.*, 2019). TBARS value of FSPE samples was lower than that in NSPE samples on 6th day of storage. As extract is added directly to the samples, they show more antioxidant activity than when the extract is used as nanoparticles. One of the reasons for this phenomenon may be evaporation of the extract during preparation of nano/micro capsules (Alboofetileh *et al.*, 2014) .On the other hand, the controlled release of extract from nanoparticles, result in lower effectiveness in a short time as compared to using the essential oils without coating (Khoshnoudi-Nia and Sedaghat, 2019). The lower TBARS value for the NSPE-treated fish filets after 6 days storage could be related to protection effect of MD/WPC wall materials on the extract. Most probably, nano-capsulation of Saffron petal extract increased its stability and reduced the releasing rate of its bioactive compounds in comparison with FSPE, and therefore could prohibit oxidation reactions of fish fillets and extend their shelf life (Mahmoud *et al.*, 2016). Overall, while the TBARS values of NSPE and FSPE samples were< 2mg malondialdehyde/kg of fish filet where within the standard limit (Colle *et al.*, 2019) until 12 days of storage, the similar values in control samples exceeded this limit easily after 6th day of storage.

*TVB-N value*

The amount of TVB-N in fish muscles are good indicator of the freshness levels during storage, because they generate off flavor and bad taste (Khoshnoudi-Nia and Moosavi-Nasab, 2020). Any effort to control bacterial load and enzymatic activity will decrease TVB-N content (Esfahani *et al.*, 2019; Moosavi-Nasab *et al*., 2021). The inhibitory effects of saffron petal bio-actives decreased TVB-N production in treated samples. A study on effects of aqueous and alcoholic saffron petal extract on quality and shelf-life of pacific white shrimp during iced-storage showed that the treated samples had significantly lower TVB-N due to its inhibitory effect against proteolytic bacteria and enzymes (Abbasvali *et al.*, 2016). Overall, after 9 days storage only NSPE samples had acceptable quality indicator compared to other treatments which could be related to protective effect and controlled release of bioactive compounds.

*Microbial analysis*

The effect of immersing fish fillets in various plant extract to reduce microbial growth depends on extract concentration, immersion time, fish species, microbial load and storage conditions (Savvaidis *et al.*, 2002). Although, measuring total viable microorganism (TVM) is a good quality indicator to show microbial population in fish fillets, major causes of microbial spoilage of fish fillets stored at 1 to 4$^{o}$C



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are related to aerobic and gram-negative psychrotrophic bacteria (PTC value). Because psychrotrophic bacteria affect the quality and shelf-life of fish by secreting various extracellular enzymes such as lipase and protease (Gram and Huss, 1996; Andevari and Rezaei, 2011). Since maximum acceptable limit for microbial load (TVM or PTC) is considered 7 log CFU/g, it seems that PTC value is more accurate and stricter index to evaluate microbial quality of fish samples as compared to TVM value (ICMSF, 1986).

Our results showed that FSPE had a positive significant effect on reducing microbial load of fillet samples mainly due to its anthocyanin compounds. In fact, different anthocyanins (such as delphinidin, quercetin and myricetin) of saffron petal extract have substantial antibacterial activity (Ghaheh *et al.*, 2014). Saffron petal extract contains phenolic compounds that inhibit different bacterial growth especially food borne pathogens such as *Bacillus cereus, Staphylococcus aureus, Salmonella enterica, Escherichia coli and Shigella dysenteries* (Vaquero *et al.*, 2007; Asgarpanah *et al.*, 2013; Jafari-Sales and Pashazadeh, 2020).

The differences between microbial quality of fish fillets treated (with ESPE and NSPE) or non-treated (control) were not significant during the first few days of 4$^{o}$C storage. However, the inhibition effects of FSPE fish-fillets on TVB-N and TBARS values were more than NSPE on 6th day of storage. Possible reasons for this could be as follows: (i) when FSPE was directly applied on the fillet samples, its microbial count was suddenly faced with more concertation of antibacterial agent as compared to samples treated with NSPE (Alboofetileh *et al.*, 2014). This lower initial load may have affected the microbial load of the product until the end of the storage period. (ii) In addition, the hydrocolloid of MD and WPC may be degraded (into simpler compounds during storage) by microorganisms and thus neutralize the positive effect of releasing bioactive compounds. However, several authors reported that nano-capsulation of essential oils or herbal extracts protect bioactive compounds against the effects of various environmental stresses and provide a long-lasting release of antibacterial compounds which in turn significantly inhibit and retard bacterial growth (Saloko *et al.*, 2014; Alves *et al.*, 2018; Bagheri Darvish *et al.*, 2020).

*Sensory analysis*

Shelf life of fish and marine products, like other foods, is highly related to consumer sensory acceptability. Sensory properties of fish can be used as one of the most efficient parameters to evaluate storage-ability and shelf-life of fish (Khoshnoudi-Nia and Moosavi-Nasab 2019b). There is a correlation between chemical and microbial properties with sensory attributes (Jouki *et al.*, 2014; Khoshnoudi-Nia and Moosavi-Nasab, 2019b). While the overall acceptance score of control



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sample diminished sharply after 3 days, the fish fillets impregnated with FSPE and NSPE maintained their acceptability (score >3 out of maximum 5) up to 9 days of storage. Reduction in the sensory scores of fish fillets during storage was most probably related to lipolytic oxidation as well as proteolytic degradation. In general, the best sensory score was obtained for sample treated with NSPE.

This study demonstrated that the combination of maltodextrin and whey protein concentrate is a suitable wall material to protect the bioactive compounds of saffron petal extract. The complete encapsulation of saffron petal extract was confirmed by the satisfactory results obtained in SEM test and FTIR spectroscopy analysis. Comparison of shelf life of treated rainbow-trout fillets and control samples based on chemical parameters (mainly TBARS and TVB-N), microbiological indicators (chiefly PTC and TVM) and sensory properties (overall acceptance) showed that the shelf-life of control fish was 5 (based on TBARS value and overall acceptance) to 8 days (TVM and PTC). However, the shelf life of NSPE and FSPE samples were estimated to be 9 (overall acceptance) to 13 (TBARS, PTC and TVM) and 8 (TBARS and sensory score) to 13 days (PTC) respectively at 4°C. The difference between protective effect of free and encapsulated extract was usually not significant. Although encapsulation of saffron petal extract by MD/WPC was successful, treating fresh fish with free extract is recommended due to simplicity and lower cost process. However, for longer storage at lower temperatures, the application of nano-capsules can be introduced as the best treatment to improve self-life of fish fillets by controlling release and protecting of bioactive compounds in long term.

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