Active nanocomposite films based on soy proteins-montmorillonite- clove essential oil for the preservation of refrigerated bluefin tuna (Thunnus thynnus) fillets

Ignacio Echeverría, M. Elvira López-Caballero, M. Carmen Gómez-Guillén, Adriana N. Mauri, M. Pilar Montero

PII: S0168-1605(17)30412-9
DOI: doi:10.1016/j.ijfoodmicro.2017.10.003
Reference: FOOD 7692

To appear in: *International Journal of Food Microbiology*

Received date: 2 February 2017
Revised date: 28 August 2017
Accepted date: 1 October 2017

Please cite this article as: Ignacio Echeverría, M. Elvira López-Caballero, M. Carmen Gómez-Guillén, Adriana N. Mauri, M. Pilar Montero , Active nanocomposite films based on soy proteins-montmorillonite- clove essential oil for the preservation of refrigerated bluefin tuna (Thunnus thynnus) fillets. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Food(2017), doi:10.1016/j.ijfoodmicro.2017.10.003

Active nanocomposite films based on soy proteins –montmorillonite- clove essential oil for the preservation of refrigerated bluefin tuna (*Thunnus thynnus*) fillets

Ignacio Echeverría[a], M. Elvira López-Caballero[b], M. Carmen Gómez-Guillén[b], Adriana N. Mauri[a,*], M. Pilar Montero[b]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, CCT La Plata- CONICET and Facultad de Ciencias Exactas, Universidad Nacional de La Plata). Calle 47 y 116 S/N°, B1900AJJ La Plata, Buenos Aires, Argentina.

[b] Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN, CSIC). Calle José Antonio Novais, 10, 28040 Madrid, España.

[*] Corresponding author: Tel. / Fax: +54 221 4890741.

E-mail address: anmauri@quimica.unlp.edu.ar (A.N. Mauri).

Abstract

This manuscript evaluates the potential application of active nanocomposite films based on soy protein isolate (SPI)-montmorillonite (MMT)-clove essential oil (CEO) to the preservation of muscle fillets of bluefin tuna (*Thunnus thynnus*) during refrigerated storage, and furthermore **analyzes** whether the clay diffuses from the package to food. SPI films with: CEO (SPI-CEO), MMT (SPI-MMT), or both CEO and MMT (SPI-MMT-CEO), were prepared and used to **cover** tuna fillets during 17 days of storage at 2 °C. Polyethylene films were also used as control. Protein films nanoreinforced with 10 g MMT / 100 g SPI and activated with CEO were able to decrease microbial growth (evaluated by **TVBN** and microorganism counts) and lipid autooxidation (evaluated according to the TBA index, FTIR and color parameters) of **tuna** fillets during the storage period studied. The presence of clay seemed to favor the release of the active principles of clove oil by prolonging its antimicrobial **(especially effective to inhibit *Pseudomonas* spp.)** and antioxidant activity over time without observing the **diffusion** of the clay's own metals (Si and Al) from the nanocomposite materials to the muscle of fish. These results are encouraging for the use of nanocomposite films in food packaging.

Keywords: *Active nanocomposite packaging, Fish preservation, Montmorillonite-soy proteins nanocomposites, Clove essential oil; Metal release*

1. Introduction

The use of nanocomposites in food packaging is expected to enhance the shelf life of foods considerably due to their improved physicochemical properties, such as mechanical and barrier properties, transparency, and thermal, chemical and dimensional stability (Smolander and Chaudhry, 2010). It also can be used as carriers of active agents and controlled release systems in the field of active food packaging. The diffusion rate of active compounds through the material can be modified by differences in the polymer network crooslinking and the increased tortuosity of the road ahead, both caused by nanofiller`s presence (Mascheroni et al., 2010).

During the last decades nanoclay-based nanocomposites have gained more importance in research studies and the packaging industry due to their ease availability, processing, and low costs compared to those with other nanofillers (Giannelis, 1996). **Among natural nanoclays, montmorillonite (MMT) is possibly one of the most commonly used as a reinforcing agent in many biopolymer films. Nanoclay-based nanocomposites** have been proved in different food packaging applications such as processed meats, cheese, confectionery, cereals, boil-in-bag foods, as well as in extrusion coating applications for fruit juices and dairy products, or co-extrusion processes for the manufacture of bottles for beer and carbonated beverages (Smolander and Chaudhry, 2010). But most applications have been proved with nanocomposites prepared from synthetic thermoset and thermoplastic polymers. Those based on bionanocomposites are mainly being studied and developed (Condes et al., 2016).

In a previous work (Echeverría et al., 2016) **nanoclay-based** cocomposite films **made of** soy protein and **MMT acquired** noticeable antioxidant and antimicrobial properties

through the addition of clove essential oil to the formulation. This essential oil also exerted a plasticizing effect -reverse to the strengthening produced by the montmorillonite-, and favored the **nanoclay** exfoliation in the protein matrix. Furthermore, **MMT** appeared to facilitate the release of some active compounds and occasionally modify the antimicrobial activity of films as well as to release some of its Si and Al ions in contact with water.

It is known, that the determination of a certain activity by an in vitro technique does not ensure that the studied material can gain the ability to protect any particular food during storage (Salgado et al., 2015). In these regard, although in vitro antimicrobial potential of films activated with clove essential oil had been reported, it was not always revealed when the films were applied in foods (Gómez-Estaca et al., 2010; Salgado et al., 2013) This may be attributed, for example, to the higher resistance of the native microbiota and microbial interactions compared to the strains from collections, or to the material interactions with the internally packaged product or the immediate external ambient (Salgado et al., 2013). These reasons lead to the necessity to prove possible applications of packaging materials in real systems.

Bluefin tuna (*Thunnus thynnus*) is an interesting food system to evaluate the developed active nanocomposite films as a food packaging material since it has a large commercial and nutritional value (Chini et al., 2008). Its flesh is highly demanded and appreciated by consumers -especially for raw consumption in products such as "sashimi" or "sushi"-, or semi cooked, but tends to deteriorate mainly by lipid autoxidation reactions and microbial growth.

Finally the successes of these advancements will be strictly dependent on exploration of worldwide regulatory issues for these materials. There are many aspects to be

studied in order to demonstrate product safety (Duncan, 2011), such as nanofiller migration through polymer films and its possible transfer to food when dealing with a food package.

The main objective of the present study was to evaluate the potential application of active nanocomposite films based on soy protein isolate-MMT- clove essential oil to the preservation of muscle fillets of bluefin tuna (*Thunnus thynnus*) during refrigerated storage, and furthermore analyze whether the **MMT minerals** diffuses from the package to food.

Based on previous results, three systems were selected to study the possible application as active packaging material to preserve bluefin tuna fillets during refrigerated storage: protein and nanocomposite (with 10 % MMT) films activated with clove essential oil, which present interesting antioxidant and antimicrobial properties, and the same nanocomposite film but without CEO, because this system showed a significantly high release of total phenols present in soy protein isolates, such as isoflavones; chlorogenic, caffeic and ferulic acid suggesting that the intercalated-exfoliated MMT may facilitate the release of these compounds (Echeverría et al., 2016). Polyethylene bags were used in the present work as control wrapping, because this is the typical polymer packaging used for this application.

2. Materials and Methods

2.1. Materials

Soy protein isolate (SPI; SUPRO 500-E) was kindly supplied by DuPont N & H (Brazil). The protein content of SPI, as measured by the Kjeldahl method, was 85 ± 2% (w/w, dry weight; N x 6.25). Clove essential oil (*Syzygium aromaticum* L.) (CEO; Eladiet SA.,

Spain) was used to activate the films. **Both eugenol and β-caryophyllene have been reported to be the main constituents of the clove essential oil, with percentages of 85% and 4.5%, respectively (Vasile et al., 2017), although these percentage might vary slightly depending on the origin of oil.** Sodium montmorillonite without organic modification (Cloisite® Na^+) was supplied by Southern Clay Products (USA). It has a cation-exchange capacity of 92.6 meq/100 g, a typical interlayer distance of 11.7 Å, a bulk density of 2.86 g ml^{-1}, and a typical particle-size distribution between 2 and 13 µm. Glycerol (p.a., Anedra, Argentina) was used as a film plasticizer. Transparent, low density polyethylene films (Unión Bolsera Madrileña, Madrid, Spain) were used as control wrapping. Bluefin tuna used was bought frozen at a local market (Tarragona, Spain).

2.2. Chemical composition of Bluefin tuna muscle

The protein content was determined by a LECO FP-2000 nitrogen/protein analyser (Leco Corp., St. Joseph, MI, USA), according to the Dumas method (A.O.A.C., 2000). A nitrogen-to-protein conversion factor of 6.25 was used. Ash and moisture contents were evaluated following AOAC (2000). Fat content was determined according to Bligh and Dyer (1959). **Briefly, the muscle (100g) was homogenized with an amount of chloroform (100ml) and methanol (200ml) to form two phases, one miscible with the lipid phase and another containing all the non-lipidic rest. The purified lipid extract was isolated, and once the chloroform was evaporated, the fat content was weighed and expressed in %.** All determinations were performed at least in triplicate.

2.3. Film preparation

Films were prepared by casting taking into account the results of previous studies (Echeverria et al., 2016). Five g of SPI were dispersed in 80 ml of distilled water at room temperature by magnetic stirring and the pH of the dispersion adjusted to 10.5 with 2N NaOH. Different amounts of MMT powder (0, and 0.5 g corresponding to 0 and 10 g/100 g SPI) plus 1.25 g of glycerol were likewise dispersed in 20 mL of distilled water followed by sonication (Sonics & Materials Inc., Sonics Vibra-cell model VCX 130, USA) for 1 minute.at 80 % amplitude. Both SPI dispersion and MMT-glycerol suspension were mixed under magnetic stirring for 1 hour at room temperature, 0.5 ml of clove essential oil (CEO) was added, and the resulting dispersion was mixed 30 more minutes. Finally, 10 ml of film-forming dispersions were cast onto polystyrene Petri dishes (144 cm^2) and dried in an oven (Binder FD240, Germany) at 60 °C for 3 h. Films were preconditioned at 20 °C and 58 % relative humidity (RH) for 48 h before being characterized. Based on the presence/absence of montmorillonite (MMT) and clove essential oil (CEO), several soy protein isolate (SPI) films were formulated, namely, SPI-MMT, SPI-CEO and SPI-MMT-CEO. **The measured thickness of the different films was, respectively, 74.4 ± 6.3, 77.4 ± 5.9 and 100.1 ± 5.3 µm. Transparent, low density polyethylene bags of 58±3 µm thickness were used as control wrapping.**

2.4. Fish processing

Fresh peeled Bluefin tuna (*Thunnus thynnus*) fillets, acquired at a specialized local market in Madrid, were cut into portions of approximate size of 10 cm x 10 cm x 1 cm. They were wrapped (coated above and below with individual films) and stored on acrylic plates at 2° C (± 1° C) for two weeks. According to film nomenclature

described above, several fish batchs were studied (SPI-MMT, SPI-CEO, SPI-MMT-CEO and polyethylene bags used for the control batch).

2.5. Chemical analyses of fish

2.5.1. Total volatile basic nitrogen (TVBN). The measurement of TVBN is based on the distillation in alkaline medium of volatile nitrogen-containing compounds associated to the deterioration of fish products, and subsequent quantitation by titration as described by Antonacopoulos and Vyncke (1989). **Un-wrapped** Fillets samples (10 g) were homogenized with 90 mL of perchloric acid (6% v/v) in an Osterizer (at 5000 rpm for 1 min) to precipitate proteins. The mixture obtained was filtered through a Whatman n°1 paper, washed with 5 mL of perchloric acid, and adjusted to 100 mL. The filtrate was distilled in a Tecator AB device (model 1002, Kjeltec Systems, Sweden). The distillate was collected on boric acid (0.3% w/v) and was titrated with 0.05 mol/L HCl (Salgado et al., 2013). Results were expressed as mg nitrogen (TVBN) per 100 g of sample. Determinations were performed in quadruplicate.

2.5.2. pH determination. **Un-wrapped** fillet samples (5 g) were homogenized with 50 mL of distilled water using an Osterizer (at 5000 rpm for 1 min). The pH of the mixture was measured with a pHmeter (MeterLab model pHM 93, Denmark). Determinations were performed by sextuplicate.

***2.5.3. Thiobarbituric Acid Reactive Substances (TBARS) index*.** The determination of TBARS is based on the great reactivity of thiobarbituric acid with carbonyl groups of

aldehydes and ketones, which increase as a consequence of lipidic oxidation (Vyncke, 1970). **Un-wrapped** fillets samples (15 g) were homogenized with 30 mL of 7.5% w/v trichloroacetic acid (TCA; Panreac Química S.A.U., Barcelona, Spain) in an Osterizer device (at 5000 rpm for 1 min). The mixture was left to stand for 30 min and was subsequently filtered through Whatman N°1 paper. The filtrate (or appropriate dilutions in TCA) was subjected to the colorimetric reaction with thiobarbituric acid (TBA; Sigma-Aldrich Chemical Co., St Louis, USA). The reaction was performed at 90°C for 40 min, and the absorbance at 538 nm was immediately read in a spectrophotometer (Shimadzu model CPS-240, Japan), as described by Salgado et al., (2013). A calibration curve was prepared using 1,1,3,5-tetraethoxypropane (TEP; Sigma-Aldrich) as the standard. Results were expressed as mg of malonaldehyde (MAD) per kg of sample. Determinations were performed in quadruplicate.

2.6. Microbiological analyses of fish

Microbiological assays were performed as described by Salgado et al. (2013). Briefly, **fillets were un-wrapped** and a total of 10 g of minced bluefin tuna muscle were collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 mL of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min processing in a Stomacher blender (model Colworth 400, Seward, London, UK), appropriate dilutions were prepared for the following bacteriological determinations: (i) total bacterial counts (TBC) on spread plates of Iron Agar **(Microkit Ibérica, S:L. Villanueva de la Cañada, Madrid, España)** 1% NaCl incubated at 15 °C for 3 days; (ii) total mesophile counts on pour plates of Plate Count Agar, PCA **(Oxoid)**, incubated at 30 °C for 72 h; (iii) H_2S

producing organisms, as black colonies, on pour plates of Iron Agar **(Microkit Ibérica)** incubated at 15 °C for 3 days; (iv) luminescent bacteria on spread plates of Iron Agar **(Microkit Ibérica)** 1% NaCl incubated at 15 °C for 5 days **(counts were carried out in dark conditions)**; (v) Pseudomonas on spread plates of Pseudomonas Agar Base (Oxoid) with added CFC (Cetrimide, Fucidine, Cephalosporine) supplement for Pseudomonas spp. (Oxoid) incubated at 25 °C for 48 h; (vi) Enterobacteriaceae on double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h, and (vii) lactic acid bacteria **(LAB)** on double-layered plates of MRS Agar (Oxoid) incubated at 30 °C for 72 h. All microbiological counts are expressed as the log of the colony-forming units per gram of sample (log cfu/g). All analyses were performed at least in duplicate.

2.7. FTIR-Attenuated Total Reflectance (ATR) spectroscopy

Infrared spectra between 4000 and 650 cm^{-1} were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (PerkineElmer Inc, Waltham, MA, USA) equipped with an ATR prism crystal accessory. Measurements were performed at room temperature using pieces of protein films (approximately 3x3 cm^2), which were placed on the surface of the ATR crystal, and pressed with a flat tip plunger until spectra with suitable and stable peaks were obtained. For each spectrum 16 scans of interferograms were averaged and the spectral resolution was 4 cm^{-1}. Background was subtracted using the Spectrum software version 6.3.2 (PerkineElmer Inc., USA). All experiments were performed at least in duplicate.

2.8. Color

Fish color was determined using a Minolta Chroma meter (CR 300, Minolta Chroma Co., Osaka, Japan). A CIE Lab color scale was used to measure the degree of lightness (L), redness (+a) or greenness (-a), and yellowness (+b) or blueness (-b) of the protein products. The instrument was standardized using a set of three Minolta calibration plates. Samples were homogeneously dispersed on the plate surface with color coordinates of $L_{standard}$= 97.3, $a_{standard}$= 0.14 and $b_{standard}$=1.71. Total color difference (ΔE) was calculated from:

$$\Delta E = \sqrt{(L_{sample} - L_{standard})^2 + (a_{sample} - a_{standard})^2 + (b_{sample} - b_{standard})^2} \quad \textbf{(1)}$$

Values were expressed as the means of nine measurements on different areas of each sample.

2.9. Determination of metals

2-4 g of fillets were used for testing the metal release from the nanocomposites films. Samples were digested in triplicate in a Microwave Digestion LabStation (Milestone Inc., model Ethos 1, USA). Digestion was performed in closed vessels made of high density Teflon TFM, using nitric acid and hydrogen peroxide as reagents (Panreac). The reaction product was a 100 ml volumetric flask up to volume with water and deionized (Type I).

The concentration of metals was measured using an atomic absorption spectrophotometer (Perkin-Elmer, Zeeman PC 5100 model, USA). Aluminum, magnesium and silicon were determined by air-acetylene flame using a hollow cathode lamp trielemental (Ca, Mg, Zn). Sodium was determined by air-acetylene flame without using lamp (Emission Spectroscopy). Each metal was determined

separately with a calibration curve of different concentrations, prepared from commercial specific pattern of each metal (Panreac). All experiments were performed at least in duplicate.

2.10. Statistical analysis

Results were expressed as mean ± standard deviation, and the data were compared by analysis of variance (ANOVA). Means were tested with the Tukey's HSD (honestly significant difference) test for paired comparisons, at a significance level $p<0.05$, through the use of the Origin Pro 8.5 SR0 v8.0724 software (OriginLab Corporation, USA).

3. Results and discussion

3.1. Effect of film composition on fish microbial growth during refrigerated storage

Figure 1 showed the TVBN values obtained for the samples under study. The initial value for bluefin tuna fillets was 13.3 mg TVBN/100 g, indicating that the fish was of good quality **(EUR-Lex - 32005R2074, 2005).**

TVBN level of the control sample increased progressively during refrigerated storage, reaching values of 30 mg TVBN / 100 g muscle at the end of the first week and 40.3 mg TVB-N / 100 g at the end of the studied period (15 days). Samples covered with soy protein films with clove essential oil (SPI+CEO), with montmorillonite (SPI+MMT) and with both CEO and MMT (SPI+MMT+CEO) showed similar TVBN values, that were not modified until the eighth day of storage, when they started to increase to 30 - 33 mg TVB-N / 100 g, remaining below the control sample at the end of the storage. It is

worth noting that except the control sample, the others did not exceed the critical limit of 35 mg TVB-N / 100 g (EUR-Lex - 32005R2074, 2005) until at least 15 days preservation and also that from a microbiological point of view the shelf life of samples covered with soy protein films seemed to exceed in one week that of the control.

It is well known that changes in pH during refrigerated storage vary by species of fish and other factors such as the microorganism contamination degree and storage conditions (Ben-Gigirey et al., 1999). Figure 2 showed the pH of fillets covered with the studied films during their refrigerated storage. The initial pH for these samples was 5.88, slightly higher than the one reported by other authors for refrigerated samples of tuna (*Thunnus obesus*) (Ruiz-Capillas and Moral, 2005). The pH increase was significant only at 8 and 12 days for all protein films and the control one respectively. Almost in all the storage period the pH values of samples packaged in soy protein films remained higher than those in polyethylene, but differences became less important at the end of the assay, when the pH of all samples was among 6.14 and 6.29 ($p \leq 0.05$). Selmi and Sadok (2008) observed similar pH values (6.27) when working with tuna (*Thunnus thynnus*) packed in polyethylene bags with or without the addition of thyme, which remained constant during 18 days of storage at 0 °C. If the increase in pH is associated with the growth of microorganisms and/or protein decomposition, these results contrasted with TVBN determinations. The higher pH of soy protein films could be attributed at least in part to the alkaline pH of filmogenic dispersions used to promote the polypeptide chains solubilization. But it should be noted that the pH values reached at the end of storage did not exceed 7.5, which is the maximum stipulated for fresh fish sale (Article 272, Código Alimentario Argentino).

The deterioration of fish stored aerobically typically consists of non-fermenting Gram-negative psychrophile bacillis. Therefore, under aerobic conditions of low temperature, the flora is mainly composed by *Pseudomonas spp.* and *Shewanella putrefaciens* (López-Caballero et al., 2002; Chouliara et al., 2004). The mesophilic and enterobacteria count rate is also considered an index of fish quality (del Valle Marquez Figueroa et al., 2008; Topic Popovic et al., 2010). Table 1 shows microbiological analysis of samples covered with the different studied films during their refrigerated storage. Initially the total bacterial count at 15 °C in the tuna was 3.6 log CFU/g, a value almost equal to that obtained by mesophilic bacteria (3.65 log CFU/g). These values are similar to those reported for fresh tuna by del Valle Marquez Figueroa et al. (2008), who found values of 3.3 log CFU/g for total mesophilic. The colonies number of H_2S producers (presumably *S. putrefaciens*) and luminescent colonies (presumably *P. phosphoreum*) (Lopez-Caballero et al., 2005) were below the detection limits of the technique (<2 log CFU/g). *Pseudomonas spp.* counts were close to those of total bacteria (3.49 log CFU/g), whereas both lactic bacteria and enterobacterias counts were near to 1.5 log CFU/g values.

Counts remained nearly constant during the early days of **storage** in the control batch. After 8 days, a gradual increase was observed specially in the counts of total bacteria, mesophilic and *Pseudomonas* spp., which held up to above **8** log CFU/g at the end of the **storage.**

Lactic acid bacteria were below the detection limit during most of the studied period probably due to their mesophilic character and the storage temperature used (2 ± 1 ° C). Enterobacteria also remained close to the detection limit practically until 15 days, at which reached 2.4 log CFU/g (Table 1).

In general total bacteria and mesophilic flora counts (incubated at 15°C) were very similar during storage (Table 1). *Pseudomonas spp*. counts also achieve similar values to those obtained for total flora in common culture media. This fact, combined with low counts for H_2S-producers and luminescent colonies, would point *Pseudomonas spp*. as responsible for the spoilage of refrigerated tuna, as they were the majority flora observed.

Fillets covered by the SPI + MMT films developed similarly as the control for almost the entire period studied, showing that nanocomposite films did not contribute to the stability of tuna during storage under refrigeration (Table 1). But in tuna covered with films activated with CEO (SPI + CEO), the microorganisms counts remained 1 or 2 log lower than the control, being these differences more notorious between 8 and 15 days of **storage**. The antimicrobial action of clove oil can be attributed to its hydrophobic nature which allows essential oils and their compounds to alter the structure of lipids present in the cell membrane and mitochondria of bacteria, making them more permeable (Outtara et al., 1997). Gómez-Estaca et al. (2010) also applied gelatin/ chitosan films activated with clove essential oil and observed an increase in the shelf-life of cod and salmon during chilled storage by reducing the microbial counts or even totally inhibiting them, respectively..

Finally in tuna fillets covered by nanocomposite films with 10 g MMT/100 g SPI activated with CEO, counts of **most of the microorganisms were reduced especially until 12 d.** Meanwhile, the counts of lactic bacteria and enterobacteria remained almost below the detection limit throughout the storage

Especially for total counts at 15 °C, mesophilic and pseudomonas genus, films without clove exceed 7 log CFU/g at 12-15 days, while those containing the essential oil in

composition **did not at 15 days**(Table 1). Moreover, as mentioned before, clove essential oil activity was more evident for nanocomposite films (SPI + MMT + CEO) than for protein films (SPI + CEO), differing at least in 1 log cycle. These results suggest that active compounds of CEO could be releasing more slowly in nanocomposite films than in protein films so that a higher content of phenolic compounds would be active at the end of storage when MMT is present. Regarding this fact, it was reported that the addition of clove essential oil to other protein films (gelatin-egg white) nanoreinforced by sepiolite favored the diffusion of the essential oil increasing the antimicrobial activity of films (Giménez et al., 2012).

Biochemical index as TVBN is directly related to the growth of microorganisms and the formation of basic compounds that result from their metabolism and that lead to a raise in pH. In the present work, control batch registered the major microbial counts and basic compounds production. Gómez-Estaca et al (2010) reported a good correlation between biochemical idex and microbial growth indicating the viability of chitosan-clove films for cod preservation.

3.2. Effect of film composition on fish lipid autoxidation during refrigerated storage

The fish muscle contains long chain polyunsaturated fatty acids that have a variety of health benefits but are especially susceptible to oxidation reactions (Swanson et al., 2012; Ruxton et al., 2007; Preston Mason and Sherratt, 2016). Food lipids oxidationleads to a reduction in the product shelf-life due to changes in the taste and/or odor, texture deterioration, loss of muscle functionality, and nutritional quality reduction (Pereira de Abreu et al., 2010). Oxygen can also have a very negative effect on the muscle color, due to the oxidation of myoglobin to form metamyoglobin, which

imparts a brownish color (Bekhit and Faustman, 2005). The main oxidation mechanism of lipids is autoxidation. Subsequent reactions of oxidation, decomposition and polymerization lead to the formation of a complex mixture of intermediate and final oxidation products that include a wide variety of volatile and nonvolatile compounds, of different molecular weight and polarity, with different oxygen function, such as hydroperoxy, hydroxy, aldehyde, epoxy and ketones. (Chaijan et al., 2006; Pourashouri et al., 2009; Quitral et al., 2009).

Figure 3 shows the thiobarbituric acid index (TBA) of tuna muscle during the refrigerated storage. The initial level of malondialdehyde in the control sample was 0.18 mg MAL/kg muscle, remaining constant up to 4 days of storage. This stability to lipid oxidation may be attributed to the storage temperature and the oxygen barrier property of polyethylene bags used to cover the control batch. After 4 days the content of MAD began to increase exponentially up to a value of 1.8 mg MAD/kg muscle at the end of the study, due to the formation of secondary products of lipid oxidation such as aldehydes and other volatiles (Kolakowska, 2002). In a previous study with tuna stored at 0 °C in vacuum-sealed polyethylene bags (Selmi and Sadok, 2008), TBA levels remained at 0.34 mg MAD/kg of oil till the ninth day, and authors attributed this oxidation delay to the combined effect of temperature and vacuum packaging.

TBA values for samples covered with SPI-CEO, SPI-MMT and SPI-MMT-CEO films maintained its initial value during the entire storage period, except for the film with 10 g MMT/100 g SPI without addition of CEO, with which the TBA value began to increase during the last three days of storage, reaching 0.72 mg MAL/kg muscle. These results suggested that protein and nanocomposite films could protect tuna fillets from lipid autoxidation, being more effective when CEO is incorporated in the formulation.

Figure 4 shows ATR-FTIR spectrums between 3600 and 3050 cm^{-1} (A) and between 1780 and 1600 cm^{-1} (B) of fat extracted from fresh tuna covered with polyethylene (control) and nanocomposite films activated with CEO (SPI-MMT-CEO) at different storage days. Figure 4A shows the typical evolution of hydroperoxides during storage. Those generated in tuna fillets covered with polyethylene (batch control) increased during the first 12 days of storage (a higher absortion of the peak is observed) but then they were consumed to form more stable compounds such as aldehydes and ketones (Giménez et al., 2011). A similar trend happened with fillets covered with SPI-MMT-CEO films, although in this case the disappearance of these compounds begins to be evident from 12^{th} **storage** day.

Figure 4B shows the evolution of aldehydes and ketones that were accumulated during storage, through the increased intensity of peaks around 1728 cm^{-1} and 1712 cm^{-1} respectively, as well as with a decrease in the intensity and frequency of triglycerides peak, located around 1743 cm^{-1}. In these spectra, differences between the batch control (C) and the one covered with SPI-MMT-CEO film became significant from the 12th day of **storage**. Ketones accumulation and triglycerides degradation were clearly manifested at the 15th day of storage in both, the control sample (C4) and the sample covered with the active nanocomposite film (SPI-MMT-CEO)4, although in the latter case it did so with less intensity. This clearly indicated that the active nanocomposite film somehow protected lipid oxidation in the most advanced stages of **refrigeration storage.**

3.3. Effect of film composition on fish color during refrigerated storage

Figure 5 shows the variation of color parameters of fish covered by the different materials under study, during refrigerated storage. Lightness values (L) for all **batches** remained similar along **storage**. Only at the end of storage a variation of less than 10% was observed between the control batch and those added with CEO, with and without **MMT** (which showed the highest L values). The reddish coloration denoted by the a parameter decreased for all fillets coated with soybean films in the early days of **storage** and then remained constant, while in the control sample the decrease was more progressive, matching the rest of the **batches** from the 12^{th} day of storage. Meanwhile, a significant increase in b parameter (enhancing the yellow coloration) was observed in the samples covered with films containing clove essential oil during storage. These observations suggested that active compounds from the essential oil, such as phenols, could be transferred from the films to the muscle, protecting the fillets from oxidation and microbial growth, but also slightly modifying the appearance of fish. Finally *ΔE* variation summarized the changes in all parameters, seeming to be more influenced by lightness L.

3.4 Migration of MMT minerals from nanocomposite films to fish

As was previously mentioned, the nanofiller migration through polymer films to food is an important aspect to be studied in order to demonstrate product safety (Duncan, 2011). So MMT minerals (Na, Mg, Al and Si) were determined in tuna muscle. These results are shown in Table 2. Na and Mg contents in raw tuna muscle were not modified after 17 days storage in the control batch. However, in the other **batches** covered with SPI films with or without MMT or CEO a significant increase in Na content was observed while that of Mg remained constant. The increase in Na could

be attributed to the alkaline pH of film forming dispersions and not to MMT because films without the nanoclay showed a higher content of Na than nanocomposites.

Regardless of the absolute values found in the controls, the concentrations of Si and Al in tuna muscle did not increase when using films containing MMT in the formulation as packaging materials (activated or not with clove essential oil). In a previous study the mineral released from these nanocomposites films -determined by placing the materials in contact with water- was studied (Echeverria et al., 2016). Protein films containing MMT released a greater amount of Al and Si ions to water ($p<0.05$) than those without the **nanoclay,** but only in the case of silicon this release was significantly dependent on MMT concentration. It was also observed that the presence of CEO slightly hindered the release of Si in films with 10 g MMT/100 g SPI, which supports that **MMT** interacted with proteins and the essential oil, which favored its dispersion in the film matrix with a high degree of exfoliation-intercalation.

Results found in this study are very encouraging for the use of these materials as food packaging.

4. Conclusions

The application of active nanocomposite films based on soy proteins with 10 g MMT / 100 g SPI and clove essential oil managed to increase the quality of bluefin tuna (*Thunnus thynnus*) fillets during chilled storage. They promote the decrease of TVB index, the final count of microorganisms and lipid autoxidation. Results also evidenced that **MMT`s** minerals themselves did not diffuse to fish muscle and that **nanoclay´s** presence may favor the release of the active principles of clove oil by prolonging its antimicrobial and antioxidant activity over time.

These results are very promising for the use of active nanocomposite films as food packaging.

5. Acknowledgments

The authors would like to thank the Argentine National Research Council (CONICET), Argentine National Agency of Scientific and Technological Support (ANPCyT, PICT-2010-1837, PICT-2013-2124), La Plata National University (UNLP, Project 11/X618 and 11/X750), CYTED (309AC0382), and projects AGL2011-27607, AGL2014-52825, 201370E036 for their financial support

6. References

A.O.A.C., 2000. Official methods of analysis (17th ed.). Maryland, USA: Association of Official Analytical Chemistry.

Antonacopoulos, N., Vyncke, W., 1989. Determination of volatile bases in fish: a third collaborative study by the West European Fish Technologists Association (WEFTA). Zeitschrift für Lebensmitteluntersuchung und -Forschung A, 189, 309-316.

Bekhit, A.E.D., Faustman, C., 2005. Metmyoglobin reducing activity. *Meat Science,* 71, 407-439.

Ben-Gigirey, B., Vieites-Baptista-De-Sousa, J.M., Villa, T.G., Barros-Velazquez, J., 1999. Histamine and cadaverine production by bacteria isolated from fresh and frozen albacore (Thunnus alalunga). Journal of Food Protection. 62(8), 933-939.

Bligh, E. G., Dyer, W. J.,1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37(8), 911–917.

Chaijan, M., Benjakul, S., Visessanguan, W., Faustman C., 2006. Changes of lipids in sardine (Sardinella gibbosa) muscle during iced storage. Food Chemistry. 99 (1), 83-91.

Chouliara, I., Savvaidis, I.N., Panagiotakis, N., Kontominas, M.G., 2004. Preservation of salted, vacuum-packaged, refrigerated sea bream (*Sparus aurata*) fillets by irradiation: microbiological, chemical and sensory attributes. Zootecnia Tropical, 21, 351–359.

Código Alimentario Argentino (C.A.A.). Chapter VI, article 272, 2014. http://www.anmat.gov.ar/alimentos/codigoa/Capitulo_VI.pdf

Condés, M.C., Echeverría, I., Añón, M.C., Mauri, A.N., 2016. Nanocompouds as formulation aids. Section III, Chapter 13. In MP Montero, MC Gómez-Guillén, ME López-Caballero, & GV Barbosa-Cánovas (Eds), *Edible Films and Coatings: Fundamental and Applications*. (pp 241-261). CRC Press, Francis & Taylor Group.

Del Valle Márquez Figueroa, Y., Cabello, A.M., Villalobos, L.B., Zambuchini, B., Fiorini, D., Verdenelli, M.C., Orpianesi, C., Ballini, R., 2008. Inhibition of microbiological activity during sole (*Solea solea* L.) chilled storage by applying ellagic and ascorbic acids. Food Science and Technology, 41, 1733–1738.

Duncan, T.V., 2011. Applications of nanotechnology in food packaging and food safety: Barrier materials, antimicrobials and sensors". Journal of Colloid and Interface Science, 363: 1-24.

Echeverría I., López-Caballero M.E., Gómez-Guillén M.C., Mauri A.N., Montero M. P., 2016. Structure, functionality and active release of nanoclay- soy proteins films affected by clove essential oil". Food and bioprocess technology, 9, 11, 1937–1950

EUR-Lex - 32005R2074, 2005. Commission Regulation (EC) No 2074/2005 of 5 December 2005. http://data.europa.eu/eli/reg/2005/2074/oj.

Giannelis, E.P., 1996. Polymer layered silicate nanocomposites. Adv.Mater, 8, 29–35.

Giménez, B., Gómez-Guillén, M. C., López-Caballero, M. E., Gómez-Estaca, J., Montero, P., 2012. Role of sepiolite in the release of active compounds from gelatin-egg white films. Food Hydrocolloids, 27(2), 475-486.

Giménez, B., Gómez-Guillén, M.C., Pérez-Mateos, M., Montero, P., Márquez-Ruiz, G., 2011. Evaluation of lipid oxidation in horse mackerel patties covered with borage-containing film during frozen storage. Food Chemistry, 124, 1393-1403.

Gómez-Estaca, J., López de Lacey, A., López-Caballero, M. E., Gómez-Guillén, M. C., Montero, P., 2010. Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. Food Microbiology, 27(7), 889-896.

ICMSF, 1986. Microorganisms in Foods 2. Sampling for microbiological analysis: Principles and specific applications. 1986. 2nd Ed. International Commission on Microbiological Specifications for Foods.

Kamal, D., Preeti, B., Tapasya, S., Subrata, S., Parthaprasad, C., 2002. p53 dependent apoptosis in glioma cell lines in response to hydrogen peroxide induced oxidative stress. The International Journal of Biochemistry & Cell Biology, 34 (2), 148-157.

Kolakowska, A., 2002. Lipid oxidation in food systems. In Z. Sikorski & A. Kolakowska (Eds.), Chemical and functional properties of food lipids (pp. 133-165). London, UK: CRC Press.

López-Caballero, M. E., Álvarez, M. D., Sánchez, J. A., Moral, A., 2002. Photobacterium phosphoreum isolated as a luminescent colony from spoiled fish, cultured in model system under controlled atmospheres. European Food Research and Technology, 215, 390-395.

López-Caballero, M. E., Gómez-Guillén, M. C., Pérez-Mateos, M., Montero, P., 2005. A chitosan-gelatin blend as a coating for fish patties. Food Hydrocolloids, 19(2), 303-311.

Mascheroni, E., Chalier, P., Gontard, N., Gastaldi, E., 2010. Designing of a wheat gluten/montmorillonite based system as carvacrol carrier: Rheological and structural properties. Food Hydrocolloids, 24(4), 406-413.

Pereira de Abreu, D.A., Paseiro Losada, P., Maroto, J., Cruz, J.M., 2010. Evaluation of the effectiveness of a new active packaging film containing natural antioxidants (from barley husks) that retard lipid damage in frozen Atlantic salmon (Salmo salar L.). Food Research International. 43 (5), 1277-1282.

Pourashouri, P., Shabanpour, B., Auburg, P., Daghigh Rohi, J., Shabani, A., 2009. An investigation of rancidity inhibition during frozen storage of Wels catfish (*Silurus glanis*) fillets by previous ascorbic and citric acid treatment. International Journal of Food Science and Technology, 44 (8), 1503-1509.

Preston Mason, R., Sherratt, S.C.R., 2016. Omega-3 fatty acid fish oil dietary supplements contain saturated fats and oxidized lipids that may interfere with their intended biological benefits. Biochemical and Biophysical Research Communications

http://dx.doi.org/10.1016/j.bbrc.2016.12.127

Quitral, V., Donoso, M.L., Ortiz, J., Herrera, M.V., Araya, H., Aubourg S.P., 2009. Chemical changes during the chilled storage of Chilean jack mackerel (*Trachurus murphyi*): Effect of a plant-extract icing system. LWT - Food Science and Technology. 42 (8), 1450-1454.

Ruiz-Capillas, C., Moral, A., 2005. Sensory and biochemical aspects of quality of whole bigeye tuna (Thunnus obesus) during bulk storage in controlled atmospheres. Food Chemistry, 89, 347–354.

Ruxton, C., Reed, S., Simpson, M., Millington K., 2007. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J Hum Nutr Diet.*, 20(3), 275-285.

Salgado, P.R., López-Caballero, M.E., Gómez-Guillén, M.C., Mauri, A.N., Montero, M. P., 2013. Sunflower protein films incorporated with clove essential oil have potential application for the preservation of fish patties. Food Hydrocolloids, 33(1), 74-84.

Salgado, P.R, Ortiz, C.M., Musso, Y.S., Di Giorgio, L., Mauri, A.N., 2015. Edible films and coatings containing bioactives. Current Opinion in Food Science, 5, 86–92

Selmi, S., Sadok, S., 2008. The effect of natural antioxidant (*Thymus vulgaris* Linnaeus) on flesh quality of tuna (*Thunnus thynnus* (Linnaeus)) during chilled storage. Pan-American Journalof Aquatic Sciences, 3 (1): 36-45

Smolander, M., Chaudhry, Q.., 2010. Nanotechnologies in food packaging. In Q. Chaudhry, L. Castle & R. Watkins (Eds.), Nanotechnologies in Food (pp. 86-101). Cambridge: RSC Publishing.

Swanson, D., Block, R., Mousa, S.A., 2012. Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. Advances in Nutrition, 3: 1–7.

Topic Popovic, N., Benussi Skukan, A., Dzidara, P., Coz-Rakovac, R., Strunjak-Perovic, I., Kozacinski, L., Jadan, M., Brlek-Gorski, D., 2010. Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. Veterinarni Medicina 55, (5), 233–241

Vasile, C., Sivertsvik, M., Mitelu, A.C., Brebu, M.A., Stoleru, E., Rosnes, J.T., Tănase, E.E., Khan, W., Pamfil, D., Cornea, C.P., Irimia, A., Popa, M.E., 2017. Comparative analysis of the composition and active property evaluation of certain essential oils to assess their potential applications in active food packaging. Materials (Basel) 10, 45:1-24.

Vyncke, W., 1970. Direct determination of the thiobarbituric acid value in trichloracetic acid extracts of fish as a measure of oxidative rancidity. Fette, Seifen, Anstrichmittel, 72, 1084-1087.

Figure legends

Figure 1. Total volatile basic nitrogen (mg TVBN/100 g product) of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

Figure 2. pH of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

Figure 3. Thiobarbituric acid reactive substances index (mg MAD/kg product) of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

Figure 4. FTIR spectra for hydroperoxides (**A**) and aldehydes and ketones (**B**) of Bluefin tuna fillets covered with polyethylene (C) and with soy protein isolated films with montmorillonite and clove essential oil (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). The numbers (1, 2, 3, 4) next to the acronyms (C and SPI-MMT-CEO) refer to days 4, 8, 12 and 15 of the tuna refrigerated period, respectively.

Figure 5. Values of luminosity (*L*), chromaticity (*a* y *b*) and color difference (*ΔE*) of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolated films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), and clove

essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

Table captions

Table 1. Microbiological counts (log CFU/g) for tuna steaks covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C).

Table 2. Sodium (Na), magnesium (Mg), aluminum (Al) and silicon (Si) contents for tuna steaks covered with polyethylene (Control) and with soy protein isolated films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C).

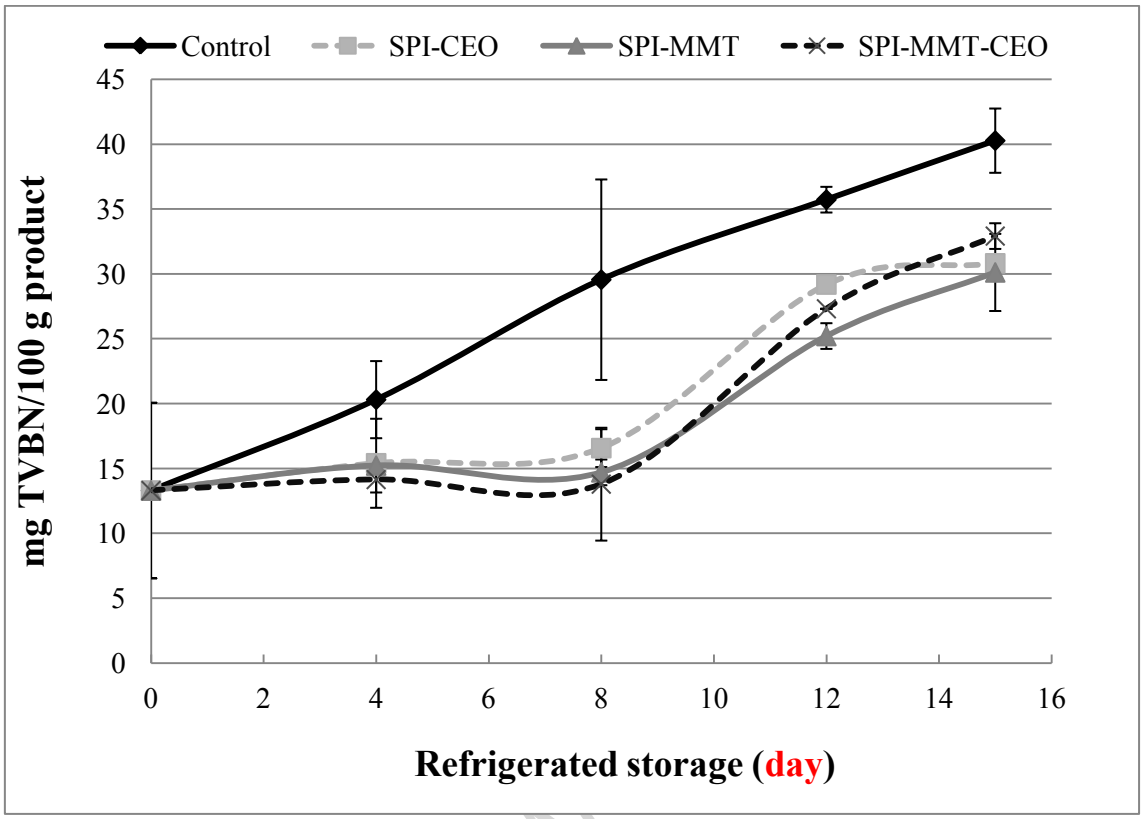

Figure 1. Total volatile basic nitrogen (mg TVBN/100 g product) of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

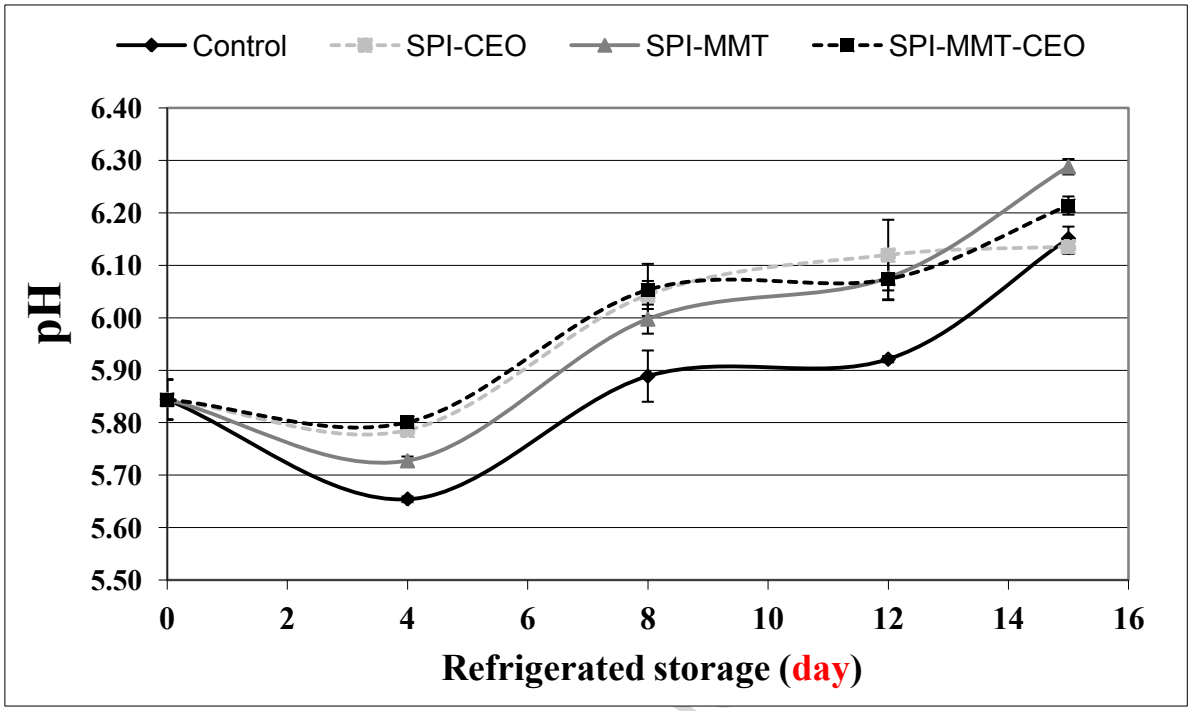

Figure 2. pH of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), and clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

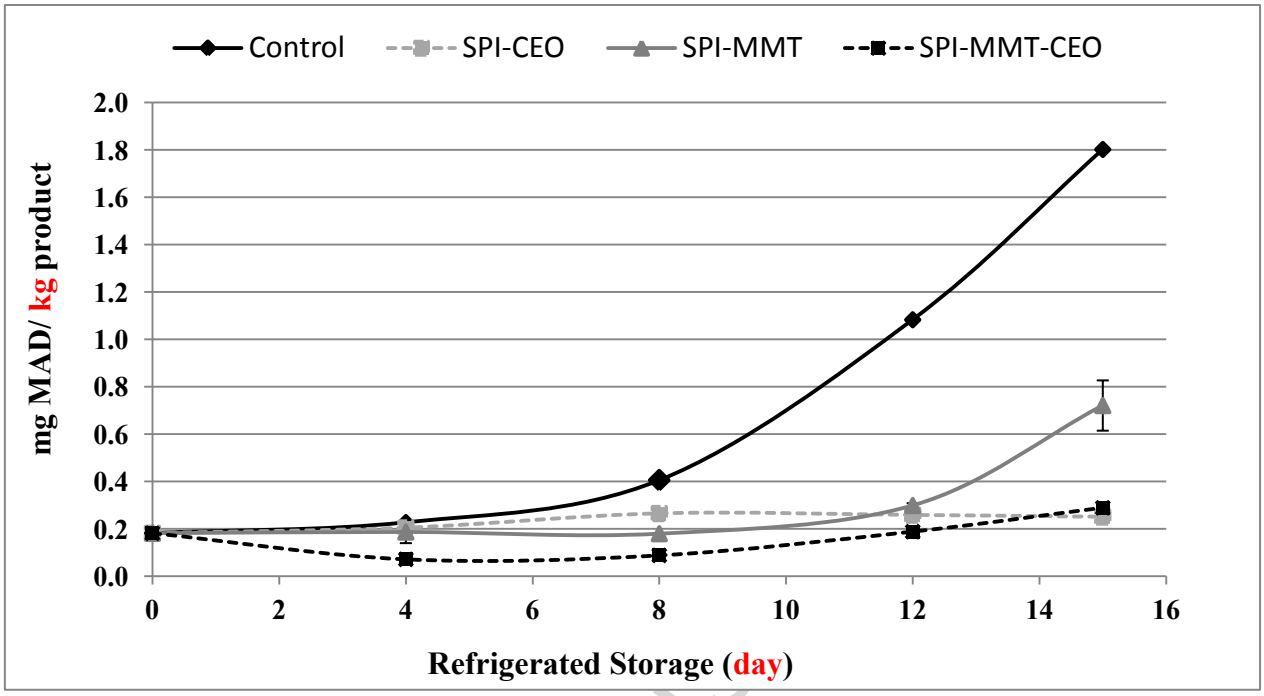

Figure 3. Thiobarbituric acid reactive substances index (mg MAD/kg product) of Bluefin tuna fillets

covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

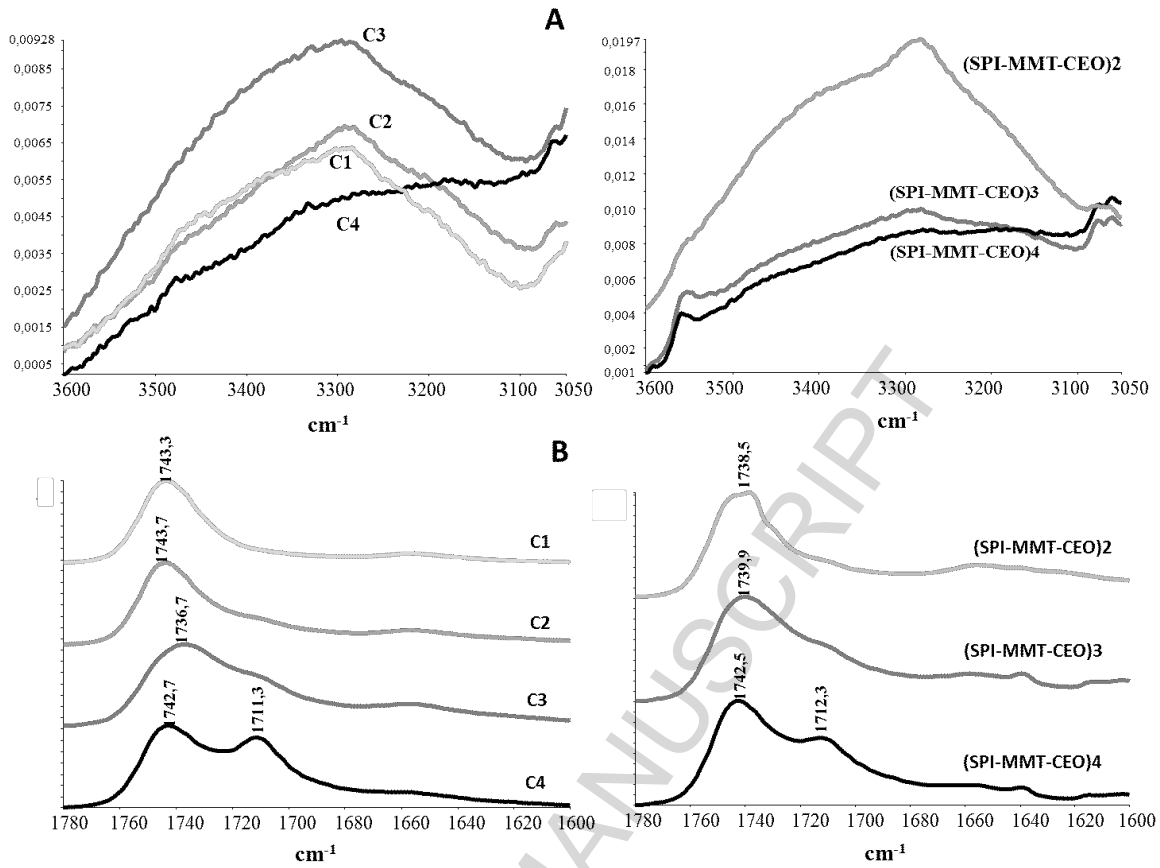

Figure 4. FTIR spectra for hydroperoxides (**A**) and aldehydes and ketones (**B**) of Bluefin tuna fillets covered with polyethylene (C) and with soy protein isolated films with montmorillonite and clove essential oil (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). The numbers (1, 2, 3, 4) next to the acronyms (C and SPI-MMT-CEO) refer to days 4, 8, 12 and 15 of the tuna refrigerated period, respectively.

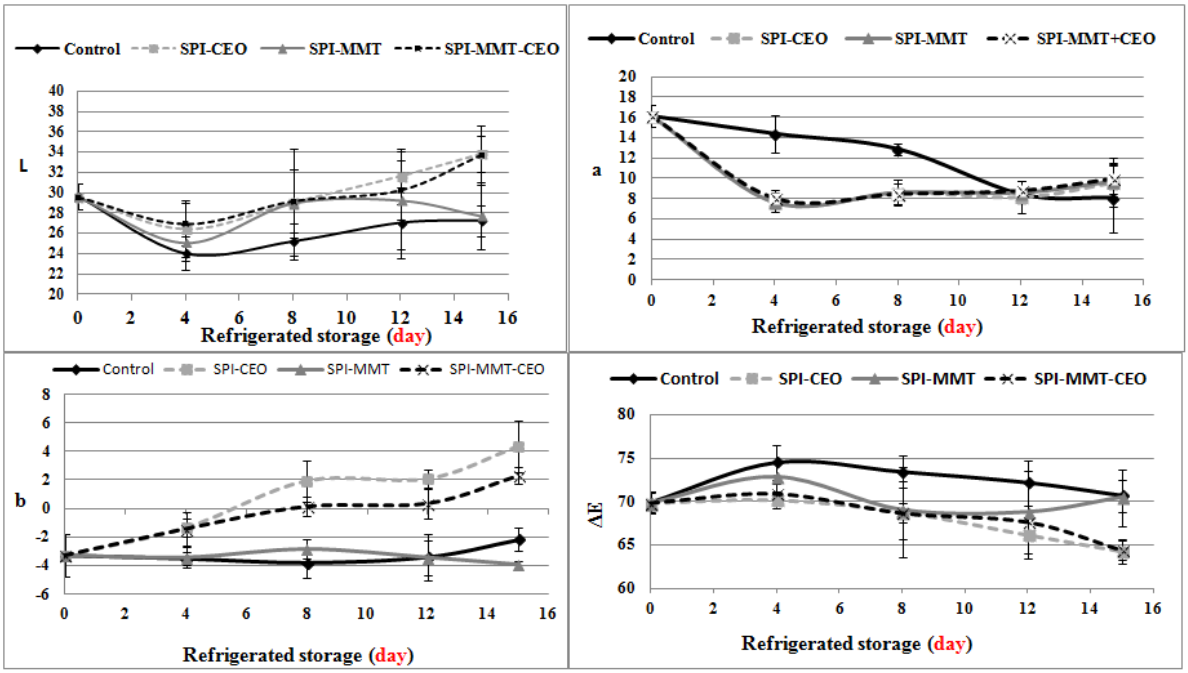

Figure 5. Values of luminosity (*L*), chromaticity (*a* y *b*) and color difference (*ΔE*) of Bluefin tuna fillets covered with polyethylene (Control) and with soy protein isolated films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), and clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation.

Table 1. Microbiological counts (log CFU/g) for tuna steaks covered with polyethylene (Control) and with soy protein isolate films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C).

Microorganisms	Batches	Refrigerated storage time (days)				
		0	4	8	12	15
Total viable bacteria	**Control**	3,6 ± 0,0	3,0 ± 0,3	4,3 ± 0,0	6,3 ± 0,0	8,6 ± 0,3
	SPI-CEO	3,6 ± 0,0	2,3 ± 0,4	2,9 ± 0,3	5,4 ± 0,1	6,9 ± 0,2
	SPI-MMT	3,6 ± 0,0	2,9 ± 0,1	4,9 ± 0,0	6,9 ± 0,0	8,3 ± 0,0
	SPI-MMT-CEO	3,6 ± 0,0	<2	2,7 ± 0,2	4,5 ± 0,0	6,5 ± 0,3
Total aerobic mesophiles	**Control**	3,6 ± 0,0	3,3 ± 0,0	4,3 ± 0,0	6,3 ± 0,1	8,4 ± 0,1
	SPI-CEO	3,6 ± 0,0	2,3 ± 0,0	3,2 ± 0,0	5,3 ± 0,0	6,9 ± 0,1
	SPI-MMT	3,6 ± 0,0	2,5 ± 0,4	4,7 ± 0,1	6,8 ± 0,0	8,3 ± 0,0
	SPI-MMT-CEO	3,6 ± 0,0	2,0 ± 0,2	2,9 ± 0,0	4,5 ± 0,0	6,4 ± 0,1
H_2S-producer microorganisms	**Control**	<2	<2	<2	<3,0	<5,0
	SPI-CEO	<2	<2	<2	<2	<4,0
	SPI-MMT	<2	<2	<2	<3,0	<5,0
	SPI-MMT-CEO	<2	<2	<2	<2	<4,0
Luminescent colonies	**Control**	<2	<2	<2	<3	<5,0
	SPI-CEO	<2	<2	<2	<2	<4,0
	SPI-MMT	<2	<2	<2	<3,0	<5,0
	SPI-MMT-CEO	<2	<2	<2	<2	<4,0
***Pseudomonas* spp.**	**Control**	3,5 ± 0,0	3,0 ± 0,1	4,4 ± 0,1	6,2 ± 0,0	8,7 ± 0,3
	SPI-CEO	3,5 ± 0,0	<2,0	3,1 ± 0,2	5,3 ± 0,0	6,9 ± 0,0
	SPI-MMT	3,5 ± 0,0	2,7 ± 0,0	4,8 ± 0,0	6,4 ± 0,0	7,8 ± 0,2
	SPI-MMT-CEO	3,5 ± 0,0	<2,0	2,9 ± 0,2	4,3 ± 0,0	6,4 ± 0,1
Lactic bacteria	**Control**	1,4 ± 0,1	1,9 ± 0,0	1,8 ± 0,0	<1	<1
	SPI-CEO	1,4 ± 0,1	<1,6	<1,6	<1	<1
	SPI-MMT	1,4 ± 0,1	<1,6	<1	<1	<1
	SPI-MMT-CEO	1,4 ± 0,1	<1,0	<1,6	<1	<1,6
Enterobacteriaceae	**Control**	1,9 ± 0,1	1,3 ± 0,4	<1,6	<1	2,4 ± 0,1
	SPI-CEO	1,9 ± 0,1	1,3 ± 0,4	<1	<1	<1
	SPI-MMT	1,9 ± 0,1	1,3 ± 0,5	<1,3	<1,3	2,6 ± 0,0
	SPI-MMT-CEO	1,9 ± 0,1	<1	<1	<1	<1

Table 2. Sodium (Na), magnesium (Mg), aluminum (Al) and silicon (Si) contents for tuna steaks covered with polyethylene (Control) and with soy protein isolated films with: clove essential oil (SPI-CEO), montmorillonite (SPI-MMT), clove essential oil and montmorillonite (SPI-MMT-CEO), refrigerated at 2 °C (±1 °C).

Batches	Minerals							
	Na (mg/100 g sample)		Mg (mg/100g sample)		Al (mg/100 g sample)		Si (mg/100 g sample)	
	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
Control	50,25 ± 5,73 ^{A}	51,80 ± 3,22 ^{aA}	36,10 ± 2,72 ^{A}	37,80 ± 0,81 ^{aA}	0,46±0,24^{A}	0,13±0,09 ^{aA}	0,34±0,05	0,23±0,16 ^{abA}
SPI-CEO	--	103,64 ± 6,65 ^{c}	--	37,65 ± 0,31 ^{a}	--	0,20±0,05 ^{b}	--	0,34±0,06 ^{b}
SPI-MMT	--	87,70 ± 3,03 ^{b}	--	39,83 ± 2,92 ^{a}	--	0,13±0,05 ^{a}	--	0,15±0,02 ^{a}
SPI-MMT-CEO	--	79,24 ± 5,33 ^{b}	--	37,03 ± 0,57 ^{a}	--	0,11±0,02 ^{a}	--	0,13±0,01 ^{a}

Reported values are means ± standard deviation. Different letters (a, b, c) in the same column indicate significant differences ($p < 0.05$) among the different lots for the same refrigerated storage time, according to Tukey's test. Different letters (A,B,C) in the same arrow indicate significant differences ($p < 0.05$) among the different refrigerated storage time for the same lot, according to Tukey's test.

Highlights

- Nanocomposite films with CEO (SPI-MMT-CEO) improved tuna quality at 2 ºC storage

- SPI-MMT-CEO films decrease Pseudomonads growth and lipid autooxidation

- Nanoclay minerals (Si and Al) did not diffuse from nanocomposite films to fish muscle

- MMT favor the release of CEO´s active principles extending the shelf-life of tuna