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Sunflower protein films incorporated with clove essential oil have potential application for the preservation of fish patties

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ABSTRACT

The incorporation of sunflower protein films with clove essential oil and the application of the resulting materials on the preservation of sardine patties were studied. The addition of clove essential oil to formulations based on sunflower protein concentrates allowed to prepare biodegradable and edible films with increased antioxidant properties and important *in vitro* antimicrobial properties. The presence of clove essential oil modified protein interactions reducing water solubility and glass transition temperature of resulting films, but it did not modify markedly their moisture content, thickness, color, opacity, water vapor permeability and mechanical properties. When applied to the preservation of refrigerated sardine patties these films allowed to retard fish lipidic auto-oxidation and to slightly delay the growth of total mesophiles, proving once again the importance of checking the films functionality in real food systems.

1. Introduction

Several proteins have been studied for their ability to form edible and/or biodegradable films (Cuq, Gontard, & Guilbert, 1998; Gennadios, 2002). Agro-industrial proteins are particularly attractive in this regard due to their lower cost as compared with other protein sources, making them interesting raw materials for the preparation of materials useful for food packaging (Embuscado & Huber, 2009). Among them, sunflower (*Helianthus annuus*) proteins can be extracted from the residual pellet of oil industry through the use of low cost simple procedures. The resulting protein products (concentrates and isolates) retain a residual content of phenolic compounds (mainly chlorogenic and caffeic acids) that are naturally present in sunflower seeds and cannot be completely eliminated due to their interaction with proteins (González-Pérez & Vereijken, 2007; Salgado, Molina Ortiz, Petruccelli, & Mauri, 2011). Previously, we studied the formation of films by casting using sunflower protein concentrates and isolates with different levels of such phenolic compounds. Our findings revealed that the presence of such compounds only modified films' color and conferred them antioxidant but not antimicrobial properties (Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2012; Salgado, Molina Ortiz, Petruccelli, & Mauri, 2010).

One great advantage of materials based on biopolymers is that they can be used as vehicles for additives, like antioxidant and/or antimicrobial agents, vitamins, flavors and pigments, thus acting as compound-releasing packaging that help to improve the quality and increase the shelf-life of the foods (Han, 2005). Synthetic antioxidants (BHA, BHT and n-propyl gallate) and antimicrobials (propionic, ascorbic, benzoic and sorbic acids and their salts) were investigated at the beginning (Cagri, Ustunol, & Ryser, 2004; Han, 2005; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2008; Valencia-Chamorro, Palou, del Río, & Pérez-Gago, 2011). Nowadays, the current trend in active food packaging materials is to incorporate natural additives such as vegetable extracts (Gómez-Estaca, Giménez, Gómez-Guillén, & Montero, 2009), phenolic compounds (Gemili, Yemenicioglu, & Altinkaya, 2010; Jongjareonrak et al., 2008) and protein hydrolyzates (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009; Salgado, Fernández, Drago, & Mauri, 2011). Essential oils categorized as GRAS (generally recognized as safe) by U.S. Food and Drug Administration can also be considered as potential alternatives to synthetic additives. In this sense, several essential oils – bergamot (*Citrus bergamia*), cinnamon (*Cinnamomum verum*), citronella (*Pelargonium citrosum*),

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coriander (*Coriandrum sativum*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), lavender (*Lavandula stoechas*), lemongrass (*Cymbopogon citratus*), oregano (*Origanum vulgare*), pimento (*Pimento officinalis*), rosemary (*Rosemarinus officinalis*), sage (*Salvia officinalis*), tarragon (*Artemisia dracunculus*) and thyme (*Thymus vulgaris*) – have been incorporated into protein-based films – soybean protein isolate, whey protein isolate, sodium caseinate, gelatin and hake protein isolate – in order to confer them antioxidant and antimicrobial properties (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Atarés, Bonilla, & Chiralt, 2010; Atarés, De Jesús, Talens, & Chiralt, 2010; Campos, Gerschenson, & Flores, 2011; Emiroğlu, Yemiş, Coşkun, & Candoğan, 2010; Giménez, Gómez-Guillén, López-Caballero, Gómez-Estaca, & Montero, 2012; Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011; Oussalah, Caillet, Salmieari, Saucier, & Lacroix, 2004; Pires et al., 2013; Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011; Seydim & Sarikus, 2006; Tongnuanchan, Benjakul, & Prodpran, 2012; Zinoviadou, Koutsoumanis, & Biliaderis, 2009). The advantage of applying such essential oils through the use of films, instead of applying them directly on foods, is that it allows to attain the desired goal with lower oil concentrations, thus limiting unwanted flavors and odors to the food (Gutierrez, Barry-Ryan, & Bourke, 2009; Sánchez-González et al., 2011).

In particular, clove (*Syzygium aromaticum*) essential oil – which contains mainly eugenol – is an effective inhibitor of the growth of *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus* in various agar mediums (Cressy, Jerrett, Osborne, & Bremer, 2003; Mytle, Anderson, Doyle, & Smith, 2006). Previous work has proven the *in vitro* antimicrobial activity of edible films incorporated with clove essential oil against health-related microorganisms (pathogens or probiotics) or food spoilers (Burt, 2004; Fernández-Pan, Royo, & Maté, 2012; Giménez et al., 2012; Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010). However, it must be highlighted that positive results obtained using films with such a high clove essential oil concentration do not always represent the behavior in real systems (Gómez-Estaca et al., 2010).

Microorganisms are the main responsible of fish spoilage. Psychrotrophic Gram-negative, rod-shaped bacteria belonging, among others, to the genera *Pseudomonas*, *Moraxella*, *Vibrionaceae*, *Shewanella*, etc., dominate the microbiota of temperate water fish (Gram & Huss, 1996). In addition, sardine (*Sardina pilchardus*) lipids are very prone to suffer autoxidation reactions (Serdaroğlu & Felekoğlu, 2005); therefore, this species constitutes an interesting system for assessing the efficiency of protein films with both antioxidant and antimicrobial properties.

The aims of the present study were: i) to endow antioxidant sunflower protein films with antimicrobial properties by including clove essential oil, ii) to evaluate the effect of the presence of this oil on the mechanical, barrier, thermal and micro-structural properties of the films, and iii) to determine the efficacy of the developed materials for ensuring the stability of sardine patties during refrigerated storage.

2. Materials and methods

2.1. Raw materials

The sunflower protein concentrate (**SPC**) was obtained from the residual pellet of an oil industry (Santa Clara, Molinos Río de La Plata, Argentina). Aqueous dispersions of the sunflower oilcake (67 g/L) were stirred for 1 h and their pH was adjusted to 9 with 3 mol/L NaOH. Solid–liquid separation was performed in a basket type centrifuge with filtering material (0.8–1.0 mm pore size) (ITA-UNL, Argentine) at 2100 × *g* and 20 °C; and the filtrate was collected. The residue was subjected to a second extraction of proteins as described above. The filtrates of both extractions were pooled, the pH was adjusted to 9 with 3 mol/L NaOH, and the mixture was subjected to isoelectric precipitation by adjusting the pH to 4.5 with 3 mol/L HCl. The mixture was stirred for 30 min and separation of the precipitate was carried out in a Westfalia centrifuge (Westfalia SAADH205 model, Germany) at 20 °C. The resulting precipitate was washed and centrifuged once more under the same conditions described above. This washed precipitate was resuspended in water (approximately 0.5 L/kg precipitate). The suspension was passed through a Manton-Gaulin two-stages homogenizer (Gaulin Corp., USA) with 2×10^5 and 5×10^5 Pa in the first and second stage respectively, the pH was adjusted to 9 with 3 mol/L NaOH, and the solution was spray-dried using a Niro Atomiser spray drier (Niro Atomiser Production Minor, Denmark) with an inlet temperature of 170–190 °C and an outlet temperature of 80–90 °C (Salgado, López-Caballero, et al., 2012). Chemical composition of **SPC** was determined. Moisture and ash values were determined by gravimetric measure (AOAC 935.29 and AOAC 923.03, 1995). Protein content was determined by the Kjeldahl method (AOAC 920.53, 1995) using 5.55 as nitrogen-to-protein conversion factor. Phenolic compounds were measured by UV spectrophotometry at 324 nm using chlorogenic acid (Chemika Fluka, Germany) as the standard (Salgado, López-Caballero, et al., 2012). Extraction of lipids was performed in a Soxhlet apparatus using *n*-hexane (Cicarelli, Argentina) as solvent and its quantification was carried out by gravimetric measure (AOAC 922.06, 1995). Total soluble sugars were quantified by the spectrophotometric anthrone method at 620 nm in 64% v/v sulfuric acid, using glucose (Sigma Aldrich Chemical Co., St. Louis, USA) as the standard (Salgado, López-Caballero, et al., 2012). The percent content of fibers was calculated by difference. All determinations were performed at least in duplicate.

The chemical composition of the **SPC** (in dry basis) was: 70.4% (±0.8) proteins, 17.2% (±1.7) fibers, 4.9% (±0.6) carbohydrates, 4.0% (±0.1) ashes, 2.5% (±0.1) phenolic compounds, and 1.0% (±0.1) lipids.

Films were incorporated with a commercial clove essential oil (**CEO**) (Eladiet S.A., Barcelona, Spain).

In order to perform assays on a real system, sardine patties were produced using sardines (*S. pilchardus*) purchased in a local market (Madrid, Spain).

2.2. Films preparation

Films were prepared by casting, after dispersing **SPC** (5% w/v) and glycerol (1.5% w/v, Anedra, Argentina) in distilled water. Dispersions were agitated in a magnetic stirrer for 30 min at room temperature and their pH was adjusted to 11 with 2 mol/L NaOH. **CEO** (0.75 mL/g **SPC**) was added to the formulations in order to obtain antimicrobial films. Filmogenic dispersions (with or without **CEO**) were mixed in a magnetic stirrer for 30 min, and then homogenized using an Ultra-Turrax device (Ultra-Turrax T25, IKA-Werke, Germany) at 20,000 rpm for 2 min to improve the dispersion of **CEO**. Aliquots (12.5 mL) of each filmogenic dispersion were poured on polystyrene Petri dishes (64 cm^2) and then dehydrated at 60 °C for 5 h in an oven with air flow circulation (Yamato, DKN600, USA). Films (**SPC** and **SPC** + **CEO**) were conditioned during 48 h at 20 °C and 58% relative humidity (RH) in desiccators with saturated solutions of NaBr before being peeled from the casting surface.

Films were prepared and characterized in CIDCA (La Plata, Argentina). Within the first 7 days after preparation, some of the produced films were sent in closed casings to ICTAN (Madrid,

Spain), and upon arrival they were conditioned under identical conditions before being used for the measurement of antioxidant and antimicrobial properties and FTIR, as well as for the fish storage trial. No visible phase separation (lipid exudation) in **SPC** + **CEO** films was evidenced at any time of the study.

2.3. Characterization of films

Film Thickness was measured using a digital coating thickness gauge (Check Line DCN-900, USA). Measurements were done at five positions along the rectangular strips for the tensile test, and at the center and at eight positions around the perimeter for the water vapor permeability (WVP) determinations. The mechanical properties and WVP were calculated using the average thickness for each film replicate. **SPC** and **SPC** + **CEO** films presented an average thickness of 70 ± 10 μm and 80 ± 10 μm, respectively.

Moisture Content (MC) was determined after drying in an oven at 105 °C for 24 h, according to ASTM method D644-99 (ASTM, 2004). Small specimens of films collected after conditioning were cut and placed on Petri dishes that were weighed before and after oven drying. MC values were determined in triplicate for each film, and calculated as the percentage of weight loss relative to the original weight. Presumptive evaporation of volatile aroma compounds from **CEO** was not considered.

Opacity: Each film specimen was cut into a rectangular piece and placed directly inside a spectrophotometer test cell, and measurements were performed using air as the reference. A spectrum of each film was obtained in an UV–Vis spectrophotometer (Beckman DU650, Germany). The area under the absorption curve from 400 to 800 nm was recorded, and the opacity of the film (UA/mm) was calculated by dividing the absorbance at 500 nm by the film thickness (mm) (Cao, Fu, & He, 2007). All determinations were performed in quadruplicate.

Color: Films color was determined with a Konica Minolta Chroma Meter CR-400 (Konica Minolta Chroma Co., Osaka, Japan) set to C illuminant/2° observer. A CIELab color scale was used to measure the degree of lightness (L^*), redness ($+a^*$) or greenness ($-a^*$), and yellowness ($+b^*$) or blueness ($-b^*$) of the films (CIE, 1976). The instrument was calibrated using a white standard plate with color coordinates of $L^*_{standard} = 97.55$, $a^*_{standard} = -0.03$ and $b^*_{standard} = 1.73$. Films color was measured on the surface of this standard plate and total color difference (ΔE^*) was calculated from Equation (1).

$$\Delta E^* = \sqrt{\left(L^*_{film} - L^*_{standard}\right)^2 + \left(a^*_{film} - a^*_{standard}\right)^2 + \left(b^*_{film} - b^*_{standard}\right)^2} \quad (1)$$

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

Water vapor permeability (WVP) tests were conducted using ASTM method E96-00 (ASTM, 2004) with some modifications. Each film sample was sealed over a circular opening of 0.00185 m^2 in a permeation cell that was stored at 20 °C in a desiccator. To maintain a 75% relative humidity (RH) gradient across the film, anhydrous silica (0% RH_c) was placed inside the cell and a saturated NaCl solution (75% RH_d) was used in the desiccator. The RH inside the cell was always lower than outside, and water vapor transport was determined from the weight gain of the permeation cell. When steady-state conditions were reached (about 1 h later), eight weight measurements were made over 5 h. Changes in the weight of the cell were recorded and plotted as a function of time. The slope of each curve ($\Delta m/\Delta t$, g H_2O/s) was obtained by linear regression (Microsoft® Office Excel 2007). WVP (g H_2O/Pa s m) was calculated as:

$$WVP = \frac{(\Delta m/\Delta t)}{P_v^{H_2O} \cdot (RH_d - RH_c) \cdot A} \cdot d \quad (2)$$

Where $\Delta m/\Delta t$ = slope obtained from the regression analysis of weight gain data as a function of time (g H_2O/s), $P_v^{H_2O}$ = vapor pressure of water at saturation at test temperature (2339.27 Pa at 20 °C), $RH_d - RH_c$ = relative humidity gradient across the film – expressed as a fraction – (=0.75), A = permeation area (m^2) and d = film thickness (m). Each WVP value represents the mean value of at least three samples taken from different films.

Water solubility (WS) of the films was determined following the method described by Gontard, Duchez, Cuq, and Guilbert (1994) with slight modifications. Film portions were weighed (diameter = 2 cm; $P_o \sim 0.10–0.15$ g) and placed in an Erlenmeyer flask (250 mL) with 50 mL of distilled water (containing 0.02% w/v sodium azide) and then sealed and shaken at 100 rpm for 24 h at 20 °C (Ferca, TT400 model, Argentina). The solution was then filtered through Whatman n°1 filter paper (previously dried and weighed) to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h (P_f). WS was calculated as follows:

$$WS = \frac{\left(P_0 \cdot (100 - MC) - P_f\right)}{P_0 \cdot (100 - MC)} \cdot 100 \quad (3)$$

Where P_0 = initial film weight (g), P_f = final dry film weight (g), MC = moisture content (%). All tests were carried out in triplicate.

Mechanical properties: The tensile strength, elongation at break and Young's modulus, of the films were determined following the procedures outlined in the ASTM methods D882-02 (ASTM, 2004), taking an average of six measurements for each film and using at least two films per formulation. The measurements were made at 20 °C and 65%RH in a controlled room. The films were cut into 6 mm wide and 80 mm long strips, and mounted between the grips of a texture analyzer (TA.XT2i, Stable Micro Systems, Surrey, England). The initial grip separation was set at 50 mm and the crosshead speed at 0.5 mm/s. The tensile strength (σ = force/initial cross-sectional area) and elongation at break (ε) were determined directly from the stress–strain curves using Texture Expert V.1.15 software (Stable Micro Systems, Surrey, England), and the Young's modulus (E) was calculated as the slope of the initial linear portion of this curve.

Differential solubility of proteins: Protein solubility of the films was determined according to the method described by Salgado et al. (2010). Briefly, pieces of films (~0.1 g) were weighed and placed into a tube containing 1 mL of water or buffer. Five different buffer systems, all at pH 7.5, were used: a) 0.1 M phosphate buffer containing 0.1 M NaCl (PB); b) PBD buffer: PB with 0.1% sodium dodecyl sulfate (SDS, Anedra, Argentine); c) PBU buffer: PB with 6 M urea (Riedel-deHaën, Germany); d) PBDU buffer: PB with 0.1% SDS and 6 M urea, and e) PBDUM buffer: PB with 0.1% SDS, 6 M urea

and 2.5% mercaptoethanol (ME, Sigma–Aldrich, Germany). The tubes were shaken for 24 h at 20 °C. Suspensions were then centrifuged at 9000 × *g* for 20 min and the protein content in the supernatant was determined using a Bradford assay (Bradford, 1976). Standard curves using bovine serum albumin (BSA, Sigma–Aldrich Chemical Co., St. Louis, USA) were constructed for each buffer. For each type of film, at least two samples from four independent film preparations were solubilized. The soluble protein content was expressed as a percentage of the total amount of protein in the film, which was measured by the Kjeldahl method (AOAC 920.53, 1995).

Differential Scanning Calorimetry (DSC): Protein denaturation temperature and enthalpy (Td y Δ*H*, respectively) and film glass transition temperature (Tg) were determined by differential scanning calorimetry, using a DSC TA 2010 calorimeter Q100 V9.8 Build 296 (TA Instrument, New Castle, Del., USA) controlled by a TA 5000 module with a quench cooling accessory. Temperature and heat flow calibration of the equipment were carried out according to ASTM Standards, using lauric and stearic acids and indium as standards. Hermetically sealed aluminum pans containing 5 mg of films were prepared, and the capsules were scanned as follows: 1 min at 25 °C (to equilibrate samples), cooling until −80 °C at 10 °C/min, isotherm determination for 1 min, and heating from −80 to 150 °C at 10 °C/min. The system was cooled to −50 °C and was again heated to 150 °C, in both cases at 10 °C/min. Denaturation enthalpies (Δ*H*) and peak denaturation temperatures (Td in °C) were obtained from the corresponding thermograms (Universal Analysis V4.2E, TA Instruments, New Castle, Del., USA). Enthalpy values (Δ*H*) were expressed as J/g protein, taking into account the dry weight (determined by perforating the pans and heating overnight at 105 °C) and the protein content of sample (Salgado et al., 2011). The glass transition temperature (Tg) was considered to be the inflexion point of the base line, caused by the discontinuity of the specific heat of the sample, and it was calculated using the Universal Analysis V4.2E software (TA Instruments, New Castle, Del., USA) (Sobral, Menegalli, Hubinger, & Roques, 2001). All the assays were performed at least in duplicate.

FTIR-Attenuated Total Reflectance (ATR) spectroscopy: Infrared spectra between 4000 and 650 cm^{-1} were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc, Waltham, MA, USA) equipped with an ATR prism crystal accessory. Measurements were performed at room temperature using pieces of protein films (approximately 3 × 3 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 (Perkin–Elmer Inc., USA). All experiments were performed at least in duplicate.

Scanning electron microscopy (SEM): SEM characterization of cryofractured cross-section of the film samples was performed. Films were cryofractured by immersion in liquid nitrogen. Pieces of films were mounted on aluminum stubs using a double-sided tape and were coated with a thin gold layer using a cool sputter system (SCD 005, BAL–TEC, Switzerland). SEM images were acquired with a scanning electron microscope (XL-20, Philips, Netherlands), using an acceleration voltage of 20 kV for all the samples.

2.4. Antioxidant properties

The supernatants obtained in the WS test were used for testing the film antioxidant capacity based on two different antioxidative mechanisms: the radical scavenging capacity and the reducing capacity. The radical scavenging capacity was measured using two different radicals: $ABTS^{\bullet+}$ radical (ABTS assay) and Superoxide anion radical (Photochemiluminescence assay). The reducing capacity was measured following the ferric ion reducing capacity (FRAP) assay.

ABTS assay: The stock solution of $ABTS^{\bullet+}$ radical (7 mM of 2,2-azinobis-(-ethylbenzothiazoline-6-sulfonic acid) in 2.45 mM potassium persulfate) was kept in the dark at room temperature for 12–16 h. An aliquot of stock solution was diluted with distilled water to prepare the working solution of $ABTS^{\bullet+}$ radical with an absorbance of 0.70 ± 0.02 at 734 nm (Shimadzu spectrophotometer CPS-240 model, Japan). Samples (20 μL) were mixed with 980 μL of ABTS reagent. The mixture was then left to stand at 30 °C for 10 min and absorbance values were read at 734 nm. Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC) per g of protein film based on a standard curve relating the concentration of Vitamin C (Sigma–Aldrich, USA) to the degree of absorbance reduction caused by such vitamin. All determinations were performed in triplicate.

FRAP assay: Samples (30 μL) were incubated (at 37 °C) with 90 μL of distilled water and 900 μL of FRAP reagent (containing 2,4,6-tripyridyl-s-triazine (TPTZ, Sigma–Aldrich) and $FeCl_3.H_2O$ in sodium acetate buffer pH 3.6). Absorbance values were read at 595 nm after 30 min (Shimadzu spectrophotometer CPS-240 model, Japan). Results were expressed as mmol $FeSO_4{\cdot}7H_2O$ equivalents per g of protein film based on a standard curve of $FeSO_4{\cdot}7H_2O$, which relates the concentration of $FeSO_4{\cdot}7H_2O$ (μmol) to the absorbance at 595 nm. All determinations were carried out in triplicate.

Photochemiluminescence (PCL) assays: The luminol-photochemiluminescence assay was carried out in the PHOTOCHEM® (Analytik Jena AG, Germany) system with the kits of antioxidant capacity of water-soluble substances (PCL-ACW) and antioxidant capacity of lipid-soluble substances (PCL-ACL), where the luminol plays a double role as photosensitizer and radical detecting agent. The hydrophilic and lipophilic antioxidants were measured using the ACW kit (1.5 mL of buffer solution pH 10.5, 1 mL of water, 25 μL of photosensitizer and 10 μL of the sample solution) and the ACL kit (2.3 mL of methanol, 200 μL of buffer solution, 25 μL of photosensitizer and 10 μL of the sample solution) respectively, following the instructions of the manufacturer. Calibration curves were performed with ascorbic acid and Trolox® as standards for PCL-ACW and PCL-ACL respectively. The results were expressed as μmol of standard per mg of protein film. Three replicates were made for each test sample.

2.5. Antimicrobial properties

The antimicrobial activity of sunflower protein films was determined by the agar disk diffusion method against 26 microbial strains as previously described (Gómez-Estaca et al., 2010). The strains were obtained from the Spanish Type Culture Collection (CECT), and included: *Lactobacillus acidophilus* CETC 903, *Salmonella cholerasuis* CECT 4300, *Listeria innocua* CECT 910, *Citrobacter freundii* CECT 401, *E. coli* CECT 515, *Shigella sonnei* CECT 4887, *Pseudomonas aeruginosa* CECT 110, *Yersinia enterocolitica* CECT 4315, *Brochothrix thermosphacta* CECT 847, *S. aureus* CECT 240, *Bacillus cereus* CECT 148, *L. monocytogenes* CECT 4032, *Clostridium perfringens* CECT 486, *Aeromonas hydrophila* CECT 839T, *Photobacterium phosphoreum* CECT 4192, *Shewanella putrefaciens* CECT 5346T, *Pseudomonas fluorescens* CECT 4898, *Vibrio parahaemolyticus* CECT 511T, *Bacillus coagulans* CECT 56, *Bifidobacterium animalis* subespecies *lactis* DSMZ 10140, *Bifidobacterium bifidum* DSMZ 20215, *Enterococcus faecium* DSM 20477, *Lactobacillus helveticus* DSM 20075, *Debaryomyces hansenii* CECT 11364, *Aspergillus niger* CECT 2088, *Penicillium expansum* DSMZ 62841. All the strains were grown in BHI broth (Oxoid, UK) (supplemented with 3% NaCl for

V. parahaemolyticus and 1% NaCl for *P. phosphoreum*). Organisms were incubated at 37 °C excepting *A. hydrophila*, *P. fluorescens*, *S. putrefaciens*, incubated at 30 °C; *B. thermosphacta* at 25 °C and *P. phosphoreum* at 15 °C. In addition, *L. acidophilus* was incubated under CO_2 flow and *C. perfringens* under anaerobic conditions (GasPack, Anaerogen; Oxoid).

After incubation the inhibition area – considered as a measure of the antimicrobial activity – was measured with Adobe Acrobat® version 6 Professional software. Results were expressed as percentage of growth inhibition respect to the total plate surface. Each determination was performed in duplicate.

2.6. Use of sunflower protein films in fish preservation

Sardine patties were obtained for preservation studies, using sardines from the Cantabric Sea captured in May which were purchased in a local store (Madrid, Spain). The chemical composition of sardine was as follows: 21.7% (±0.8) proteins, 4.2% (±0.4) lipids, 1.4% (±0.1) ashes, and 74.5% (±0.6) humidity. For preparing the patties, 2 kg of sardine fillets were chopped, homogenized and mixed with salt (1% w/w NaCl, Panrec, Spain). The mixture was divided into portions of 50 g each, which were pressed to form discs or patties (high ≈1.5 cm; diameter ≈7 cm). The sunflower protein films used for these studies (**SPC** and **SPC** + **CEO**) were obtained by casting as described above (Section 2.2). The patties were placed on acrylic plates that had the films under study on their internal surfaces. In parallel, patties were placed on plates without sunflower protein films as a control (**Control**). The patties were stored at 2 °C (±1 °C) and were periodically sampled (0, 3, 5, 7, 10 and 13 days) to perform biochemical (pH, thiobarbituric acid reactive substances index –TBARS–, and total volatile basic nitrogen –TVBN–) and microbiological analyses.

pH: Patties 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.

Thiobarbituric Acid Reactive Substances (TBARS) index: The determination of TBARS was performed as described by Vyncke (1970). The method is based on the great reactivity of thiobarbituric acid with carbonyl groups of aldehydes and ketones, which increase as a consequence of lipidic oxidation. Samples of patties (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 Wathman 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). A calibration curve was prepared using 1,1,3,5-tetraethoxypropane (TEP; Sigma–Aldrich Chemical Co., St Louis, USA) as the standard. Results were expressed as mg of malonaldehyde (MAD) per kg of sample. Determinations were performed in quadruplicate.

Total volatile basic nitrogen (TVBN): The measurement of TVBN was based on the method described by Antonacopoulos and Vyncke (1989), which 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. Samples of patties (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 Wathman 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. Results were expressed as mg nitrogen (TVBN) per 100 g of sample. Determinations were performed in quadruplicate.

Microbiological assays: microbiological analyses were performed as follows: a total of 10 g of minced sardine 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 1% NaCl incubated at 15 °C for 3 days; (ii) total mesophile counts on pour plates of Plate Count Agar, PCA, incubated at 30 °C for 72 h; (iii) H_2S-producing organisms, as black colonies, on pour plates of Iron Agar incubated at 15 °C for 3 days; (iv) luminescent bacteria on spread plates of Iron Agar 1% NaCl incubated at 15 °C for 5 days; (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 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 in duplicate.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation. Results of films characterization were analyzed by one-way analysis of variance (one factor with two levels: **SPC** and **SPC** + **CEO**). Results of fish preservation assays were analyzed by two-way ANOVA (two factors: lot and storage time, in three and five levels, respectively: **Control**, **SPC** and **SPC** + **CEO** films; and 0, 3, 5, 7, 10 days). Means were tested with the Tukey's HSD (honestly significant difference) test for paired comparison, with a significance level $\alpha = 0.05$, using the SPSS® software (SPSS Statistical Software version 15.0, USA).

3. Results and discussion

3.1. Effect of the addition of clove essential oil on the physical properties of sunflower protein films

Sunflower protein films incorporated or not with clove essential oil (**SPC** + **CEO** and **SPC**, respectively) were found flexible and homogeneous. No lipid exudation occurred in **SPC** + **CEO** films despite the high **CEO** content added. Table 1 depicts the physico-chemical, mechanical and barrier properties of the resulting sunflower protein films. Both protein films (**SPC** and **SPC** + **CEO**) had no significant differences ($p > 0.05$) in their thickness and moisture content. Also, the addition of **CEO** to the formulation did not affect ($p > 0.05$) their water vapor permeability (WVP) being these values ($\approx 1.2{*}10^{-10}$ g H_2O/Pa m s) comparable to those reported for other protein films (Cuq et al., 1998; Embuscado & Huber, 2009; Gennadios, 2002; Gennadios, McHugh, Weller, & Krochta, 1994). In general, lipids really act as an effective moisture barrier in bilayer films formed by coating dried protein film with lipid dispersion (Weller, Gennadios, & Saraiva, 1998). Also, it has been reported that when lipids are dispersed into polymeric matrix its effect on WVP depends on several factors, such as the lipid type and concentration, hydrophobicity, particle size and emulsion stability (Debeaufort & Voilley, 1995; Kim, Marx, Weller, & Hanna, 2003; Pérez-Gago & Krochta, 2001; Weller et al., 1998). Our finding

Table 1
Physico-chemical, mechanical and barrier properties of sunflower protein concentrate films added or not with clove essential oil (**SPC** + **CEO** and **SPC**, respectively).

Properties	SPC	SPC + CEO
Thickness (μm)	70 ± 10[a]	80 ± 10[a]
Moisture Content (%)	28.6 ± 1.5[a]	28.9 ± 0.8[a]
Water Vapor Permeability ($*10^{10}$ g H_2O/Pa m s)	1.13 ± 0.10[a]	1.16 ± 0.09[a]
Water Solubility (%)	93.2 ± 1.7[b]	86.3 ± 1.4[a]
Tensile Strength (MPa)	2.3 ± 0.4[a]	2.5 ± 0.2[a]
Elongation at Break (%)	32.3 ± 3.9[b]	24.9 ± 1.7[a]
Young's Modulus (MPa)	0.22 ± 0.04[a]	0.25 ± 0.03[a]
L^* ($L^*_{standard} = 97.55$)	30.85 ± 1.68[b]	29.44 ± 1.20[a]
a^* ($a^*_{standard} = -0.03$)	−2.64 ± 0.99[a]	−1.66 ± 0.64[b]
b^* ($b^*_{standard} = 1.73$)	4.97 ± 1.04[b]	3.77 ± 1.02[a]
ΔE^*	66.87 ± 1.67[a]	68.18 ± 1.16[b]
Opacity (UA/mm)	12.9 ± 0.7[a]	13.1 ± 0.7[a]
Tg (°C)	−29.0 ± 0.3[b]	−33.0 ± 0.7[a]

Reported values for each sunflower protein film are means ± standard deviation ($n = 37$ for thickness; $n = 3$ for MC, WVP and WS; $n = 12$ for mechanical properties; $n = 18$ for color; $n = 4$ for opacity; $n = 2$ for Tg). Different letters (a, b) in the same row indicate significant differences ($p < 0.05$) according to Tukey's test.

suggest that distribution of **CEO** in sunflower protein film did not exert a barrier effect, evidencing that the hydrophilic protein matrix surrounding lipid droplets is the limiting factor for the film moisture barrier. Similar results were reported for whey protein films incorporated with oregano oil (Zinoviadou et al., 2009). On the other hand, some authors showed that the addition of different essential oils (cinnamon, ginger, citronella, coriander, tarragon and thyme) reduced the WVP of sodium caseinate or hake protein films (Atarés, Bonilla, et al., 2010; Pires et al., 2013), whereas a slight increase in WVP was also reported by adding clove or bergamot essential oils to gelatin films (Ahmad et al., 2012; Giménez et al., 2012).

Notwithstanding, sunflower protein films incorporated with **CEO** exhibited a reduction in water solubility (WS) as compared to control films (**SPC**) ($p < 0.05$). Ahmad et al. (2012) also reported that WS of gelatin films decreased when bergamot and lemongrass essential oils were incorporated to the formulation. Conversely, the addition of citronella, thyme, coriander and clove essential oils to hake protein films and gelatin-chitosan ones increased their WS due to the establishment of protein interaction with these essential oils, weakening the protein network (Gómez-Estaca et al., 2010; Pires et al., 2013). In the present study, reduction in WS was not proportional to the mass of oil added. High WS of **SPC** + **CEO** films suggest that in this assay an important part of the lipids are not retained in the insoluble fraction, either by having volatilized when determining the dry solids content (24 h at 105 °C) or not retained on the filter paper (11 μm pore size). It is noteworthy that the high water solubility of both sunflower protein films (**SPC** and **SPC** + **CEO**) − WS > 85% − could be advantageous for certain applications as to release of bioactive compounds.

Addition of **CEO** did not markedly modify the mechanical properties of films (Table 1). No significant differences ($p > 0.05$) were found in the tensile strength at break and the Young's modulus of both films, however, a slight but significant reduction in the elongation at break ($p < 0.05$) was observed after adding **CEO**. Different effects were observed on the mechanical properties when adding lipids to protein films. Gontard et al. (1994) reported that these effects depend on both the characteristics of the lipid and its capacity to interact with proteins. Thus, Giménez et al. (2012) showed that the addition of the same **CEO** (in the same proportion) to gelatin films produced a plasticizing effect (tensile strength increased and elongation at break decreased were **CEO** added), being largely attributed to interactions between the gelatin matrix and the **CEO**. Atarés, Bonilla, et al. (2010) and Atarés, De Jesús, et al. (2010) reported that cinnamon essential oil affected the mechanical behavior of soybean protein isolate films but not modified the mechanical properties of sodium caseinate-based films. It is evident that the effect of an essential oil depends on their capacity to interact with each protein.

Table 1 also shows CIELab color parameter and the opacity of sunflower protein films added or not with **CEO**. Films color was slightly modified ($p > 0.05$) by addition of **CEO** to the formulation (while L^* and b^* decreased, a^* was increased). This resulted in **SPC** + **CEO** films with greater total color difference (ΔE^*) than control films (**SPC**). However both films exhibited a greenish color (negative value of a^*) associated to the oxidation of phenolic compounds to o-quinones that takes place during the protein extraction in an alkaline medium in the production of the protein concentrate (Salgado et al., 2010). Film opacity usually increases in the presence of lipids (Gontard et al., 1994; Quezada-Gallo, Debeaufort, Callegarin, & Voilley, 2000). However, no significant differences ($p > 0.05$) were observed in the opacity of the resulting sunflower protein films. Similarly, Pires et al. (2013) reported that the addition of citronella, tarragon, thyme and coriander essential oils did not modify the opacity of the hake protein films.

3.2. Structural properties of sunflower protein films added or not with clove essential oil

The structural properties of films were assessed through SEM, DSC, ATR-FTIR and differential solubility assays. SEM of the cross-section area of both sunflower protein films (**SPC** and **SPC** + **CEO**) are shown in Fig. 1. **SPC** films showed continuous, homogeneous and compact microstructure (see Fig. 1a). Meanwhile, **SPC** + **CEO** films exhibited discontinuities in the form of micro-pores or cavities (see arrows in Fig. 1b). The existence of these micro-pores might be related to the evaporation of **CEO** during film formation (drying) (Sánchez-González et al., 2011). Similar results were reported for chitosan-based films incorporated with basil and thyme oils (Bonilla, Atarés, Vargas, & Chiralt, 2012), gelatin-egg white films incorporated with clove essential oil (Giménez et al., 2012), and gelatin films added with bergamot and lemongrass essential oils (Ahmad et al., 2012). These discontinuities in protein matrix may act as stress concentrators where an initial rupture could begin. Their presence would conduct to premature ruptures (at lower deformations). This behavior also was described by Atarés, De Jesús, et al. (2010) for SPI-based films incorporated with cinnamon essential oil. These authors reported that lipids dispersed in the protein matrix are unable to form a cohesive and continuous matrix (Atarés, De Jesús, et al., 2010).

Thermograms obtained by DSC of sunflower protein films showed that both protein matrices (**SPC** and **SPC** + **CEO**) are formed by proteins that still retain some of their native configuration (≈80%) since they exhibited the two denaturation endotherms at 103 °C and 126 °C approximately.

The ATR-FTIR spectra corresponding to the Amide I band (1600–1700 cm^{-1}) of **SPC** and **SPC** + **CEO** films and of the raw materials employed in their formulation (sunflower protein concentrate and **CEO**) are shown in Fig. 2a. While films obtained with **SPC** and **SPC** + **CEO** exhibited similar spectra, the analysis of their second derivatives (Fig. 2b) revealed that proteins in both films differed in their secondary structure. Proteins in **SPC** films, as well as those present in sunflower protein concentrate (derivative spectrum not shown), were mainly present in solvated helix conformation (1628 and 1633 cm^{-1}), with lower relative proportions of β sheet (1611 and 1620 cm^{-1}), random coil (1642 cm^{-1}), α-helix (1652 cm^{-1}), turns (1660 and 1667 cm^{-1}), and antiparallel β sheets (1675, 1682, 1692 and 1696 cm^{-1}) (Barth, 2007; Subirade, Kelly, Guéguen, &

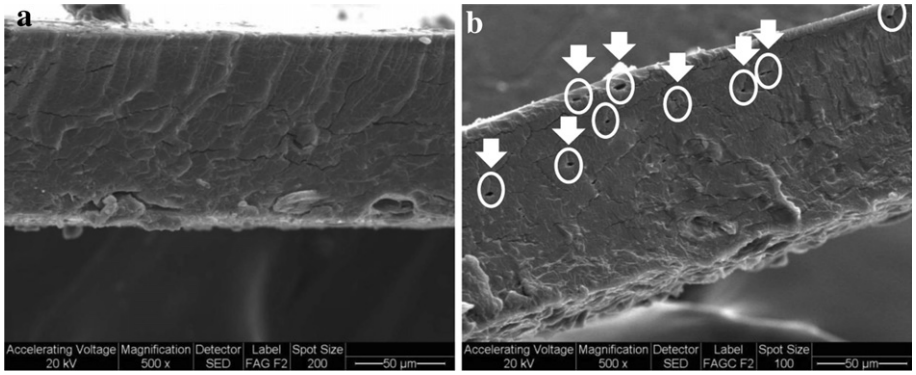

Fig. 1. SEM images (500×) of cross-sections of the sunflower protein concentrate films added or not with essential clove oil – **SPC** + **CEO** (panel **b**) and **SPC** (panel **a**), respectively.

Pézolet, 1998). On the other hand, spectrum of **CEO** presented an intense peak at 1638 cm^{-1} (overlapping with random coil) and other at 1604–1613 cm^{-1}, both corresponding to the alkene and aromatic –C=C– bonds, respectively (Giménez et al., 2012). In **SPC** + **CEO** films, proteins present a predominance of β sheets (1611, 1622 and 1624 cm^{-1}) and *random coil* (1637 cm^{-1}) at expense of solvated helix, with similar proportions of the other conformations that **SPC** films. This fact suggests an effect of **CEO** on the secondary structure of proteins.

The differential solubility of protein films in buffer systems able to disrupt different types of protein interactions was studied to determine the type and proportions of the interactions involved in film stabilization. The solubility profiles shown in Fig. 3 suggest that **SPC** films are mainly stabilized by hydrophobic interactions and hydrogen bonds, since their solubility increased in the presence of SDS (PBD) and urea (PBU), and also by disulfide bonds since solubility increased even further in the presence of mercaptoethanol (PBDUM). This film did not dissolve completely even in the presence of all these chemical agents, suggesting the potential formation of other types of interactions – most probably of covalent nature – that favor protein aggregation and reduce solubility. The presence of **CEO** modified the solubility profile of the film. The **SPC** + **CEO** protein matrix is mainly stabilized by hydrogen bonds and disulfide bonds. It is evident that the presence of SDS (PBD) did not increase film solubility as it did in the control film, suggesting a lower importance of hydrophobic interactions in the stability of the composite film. This may be due to the fact that in these films (**SPC** + **CEO**) the hydrophobic sites of proteins are more involved in interactions with **CEO** (of hydrophobic nature) than in hydrophobic interactions with other regions of the protein.

The glass transition temperature (Tg) of these materials also determined by DSC is shown in Table 1. The addition of **CEO** significantly ($p < 0.05$) decreased Tg of resulting films. This fact could be related to changes in the secondary protein structures and/or in the protein–protein interactions induced by the addition of **CEO** to film formulation. However, reduction in Tg, usually associated with a plasticizing effect, did not manifest in the mechanical or barrier properties discussed above.

It is evident that the presence of **CEO** leads to modifications in the protein secondary structures and to the establishment of protein–protein and protein-**CEO** interactions. These changes in structural properties of films only produced a reduction in Tg and water solubility not affecting their mechanical and barrier properties.

3.3. Antioxidant properties of sunflower protein films added or not with clove essential oil

The antioxidant properties of sunflower protein films as assessed by different methods (ABTS, FRAP and PCL) are shown in Table 2. Both films exhibited antioxidant capacity, which in the case of **SPC** was due to the natural phenolic compounds of sunflower and in the case of **SPC** + **CEO** was due to phenolic compounds present in both sunflower and clove. The increase of antioxidant capacity upon adding **CEO** to the formulation was remarkable; suggesting that at least a fraction of the oil incorporated to the film would not be involved in strong interactions with the protein matrix, thus maintaining its antioxidant properties. By comparing the different antioxidative mechanisms tested, it could be stated that the **CEO** compounds improved both the radical scavenging and

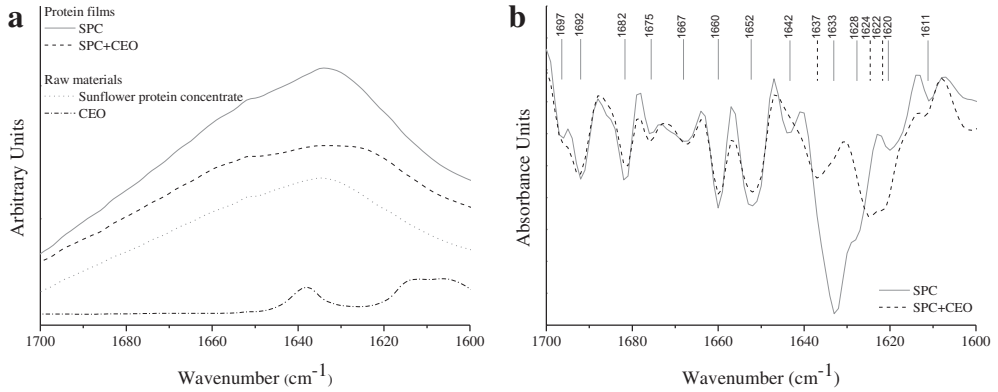

Fig. 2. a) Amide I band (1690–1600 cm^{-1}) from ATR-FTIR spectra of sunflower protein concentrate films added or not with essential clove oil – **SPC** + **CEO** (- - -) and **SPC** (—), respectively – and raw materials – sunflower protein concentrate (……) and **CEO** (-·-·-). **b**) Second derivative of Amide I band (1690–1600 cm^{-1}) from ATR-FTIR spectra of sunflower protein concentrate films added or not with essential clove oil – **SPC** + **CEO** (-·-·-) and **SPC** (—), respectively.

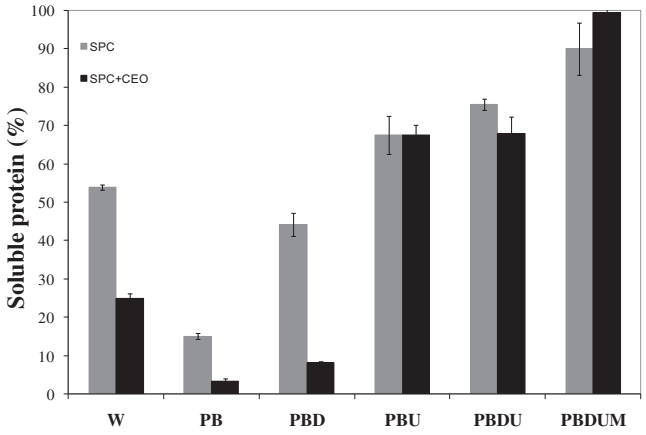

Fig. 3. Differential protein solubility of sunflower protein concentrate films added or not with clove essential oil (**SPC** + **CEO** and **SPC**, respectively) in media with different chemical activity: Water (W), 0.1 M sodium phosphate buffer (PB), PB containing 0.1% w/v SDS (PBD), PB containing 6 M urea (PBU), PB containing both 0.1% SDS and 6 M urea (PBDU), and PBDU with 2.5% v/v mercaptoethanol (PBDUM), all at pH 7.5. Reported values for each protein film are means ± standard deviation ($n = 4$).

reducing capacities of films. The radical scavenging ability was, however, considerably higher against the ABTS radical than against the superoxide anion radical, as deduced from the less difference found between **SPC** and **SPC** + **CEO** films in PCL assays. The phenolic compounds involved in the antioxidant effect, mainly chlorogenic acid, caffeic acid and eugenol, exhibited such effect in a wide range of conditions, including aqueous solutions at neutral pH (in the ABTS technique), acid pH (FRAP assay), and alkaline pH (PCL-ACW method), and also in media containing hexane (PCL-ACL assay).

3.4. Antimicrobial properties of sunflower protein films added or not with clove essential oil

The addition of **CEO** conferred important *in vitro* antimicrobial properties to sunflower protein films, which were originally devoid of them (Salgado, Drago, et al., 2012). The levels of antimicrobial activity of sunflower protein films incorporated with **CEO** are shown in Table 3. As revealed by these results, the films inhibited to a varying degree the growth of 26 strains of microorganisms selected because of their importance in health (such as probiotics or pathogens) or for being responsible for food spoilage, these findings being probably related to the presence of eugenol in the **CEO** added. The highest inhibition percentage corresponded to the yeast *D. hansenii* and to the molds *A. niger* and *P. expansum*. Regarding bacteria, the largest inhibition areas corresponded to *P. phosphoreum* and *B. thermosphacta*, Gram-negative and Gram-positive bacteria, respectively (Table 3). Regarding the sensitivity of microorganisms to essential oils, Outtara, Simard, Holley, Piette, & Bégin (1997) reported that cell wall lipopolysaccharides of Gram-negative bacteria may prevent active components from reaching the cytoplasmic membrane. It has been reported that the addition of clove essential oil to gelatin and gelatin-chitosan films results in the inhibition of spoilers and potentially pathogenic microorganisms (Gómez-Estaca et al., 2010). Our results show that **CEO** retains its antimicrobial activity after its addition to sunflower protein films.

Table 2
Antioxidant capacity of the sunflower protein concentrate films added or not with clove essential oil (**SPC** + **CEO** and **SPC**, respectively), evaluated by the ABTS, FRAP and PCL assays.

	ABTS (mg/g)	FRAP (mmol/g)	PCL-ACW (μmol/g)	PCL-ACL (μmol/g)
SPC	33.2 ± 1.1[a]	200.1 ± 14.2[a]	34.5 ± 2.1[a]	149.8 ± 1.0[a]
SPC + **CEO**	1194.1 ± 77.0[b]	5733.3 ± 92.5[b]	229.0 ± 6.6[b]	1767.0 ± 11.8[b]

Reported values for each sunflower protein film are means ± standard deviation ($n = 3$). Different letters (a, b) in the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

Table 3
Antimicrobial activity (%) of sunflower protein concentrate films added with clove essential oil (**SPC** + **CEO**) over different microorganisms.

Microorganisms	Antimicrobial activity (%)
Aeromonas hydrophila	32.66 ± 17.59[a, b, c, d]
Aspergillus niger	38.32 ± 11.24[c, d, e]
Bacillus cereus	25.61 ± 5.22[a, b, c, d]
Bacillus coagulans	37.21 ± 5.13[b, c, d, e]
Bifidobacterium animalis subespecie lactis	25.52 ± 9.85[a, b, c, d]
Bifidobacterium bifidum	27.42 ± 14.04[a, b, c, d]
Brochothrix thermophacta	40.20 ± 0.87[d, e]
Citrobacter freundii	24.14 ± 5.54[a, b, c]
Clostridium perfringens	21.15 ± 0.71[a]
Debaryomyces hansenii	60.74 ± 9.90[f]
Enterococcus faecium	22.07 ± 1.14[a, b]
Escherichia coli	25.68 ± 1.43[a, b, c, d]
Lactobacillus acidophilus	22.00 ± 1.81[a, b]
Lactobacillus helveticus	24.95 ± 5.64[a, b, c]
Listeria innocua	31.04 ± 0.51[a, b, c, d]
Listeria monocytogenes	23.34 ± 4.13[a, b, c]
Penicilium expansum	50.34 ± 2.48[e, f]
Photobacterium phosphoreum	38.20 ± 6.43[c, d, e]
Pseudomonas aeruginosa	27.09 ± 2.47[a, b, c, d]
Pseudomonas fluorescens	27.39 ± 8.13[a, b, c, d]
Salmonella cholerasuis	26.40 ± 3.51[a, b, c, d]
Shewanella putrefaciens	24.25 ± 4.99[a, b, c]
Shigella sonnei	22.04 ± 2.46[a, b]
Staphylococcus aureus	29.35 ± 3.52[a, b, c, d]
Vibrio parahaemolyticus	30.77 ± 9.80[a, b, c, d]
Yersinia enterocolítica	22.55 ± 4.79[a, b]

Reported values for each microorganism are means ± standard deviation ($n = 2$). Different letters (a–f) in the column indicate significant differences ($p < 0.05$) according to Tukey's test.

3.5. Performance of sunflower protein films in the preservation of sardine patties

Taking into account the *in vitro* antioxidant and antimicrobial properties of sunflower protein films, the next step was the evaluation of their efficacy in the preservation of sardine patties. Biochemical parameters such as pH of the product, TBARS index and TVBN were assessed (Fig. 4). In addition, numbers of total bacteria, total mesophiles, H_2S-producers, luminescent colonies, *Pseudomonas* spp., lactic bacteria, and *Enterobacteriaceae* were determined (Table 4).

The initial pH of sardine patties was 6.3 ± 0.1 (Fig. 4a). This value increased slightly (to 6.5 ± 0.1) during the cold storage of the product. Stamatis and Arkoudelos (2007) reported initial pH values of 6.2 for sardines, which raised to 6.8 at 13 days of storage at 3 ± 0.5 °C. In the present study no differences ($p > 0.05$) were found between the control sample (**Control**) and those coated with **SPC** and **SPC** + **CEO** films.

The results obtained in the TBARS assay are shown in Fig. 4b. This assay measures the amount of secondary products of lipid oxidation, particularly malonaldehyde (and other TBA-reacting components), thus providing information on the progress of oxidation in the product. The malonaldehyde level in the control sample (**Control**) was initially 11.2 mg MAD/kg product, but increased during storage to reach 16.8 mg MAD/kg product at 10 days. Kasmoglu, Denli, and Erhan (2003) reported initial values of

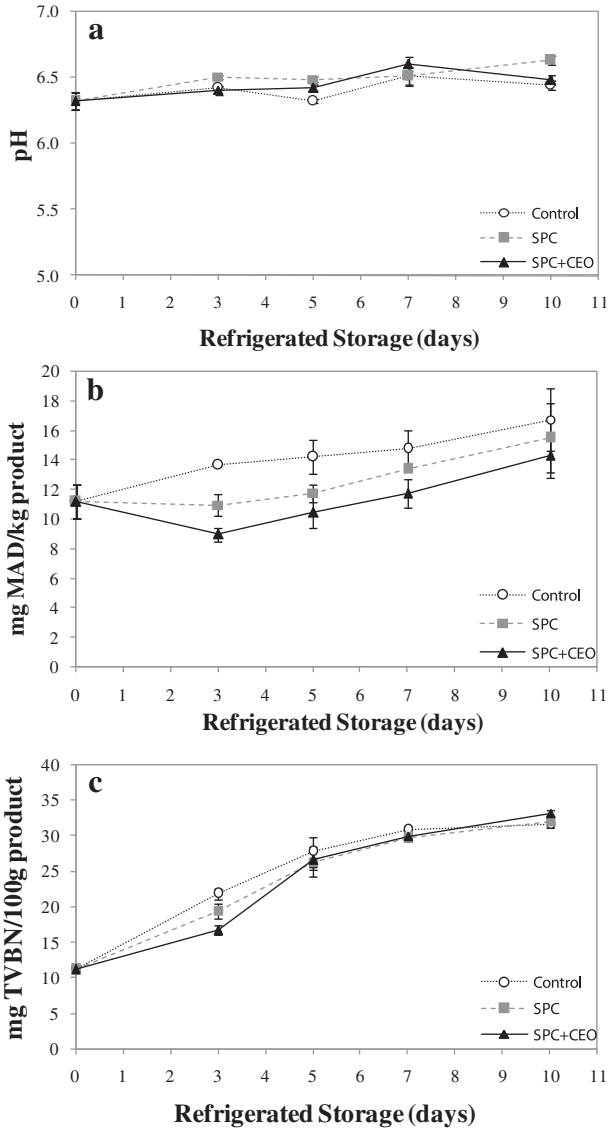

Fig. 4. **a**) pH, **b**) thiobarbituric acid reactive substances index (mg MAD/kg product), and **c**) total volatile basic nitrogen (mg TVBN/100 g product) of sardine patties uncovered (**Control**) and covered with sunflower protein concentrate films with or without clove essential oil (**SPC** + **CEO** and **SPC**, respectively), refrigerated at 2 °C (±1 °C). Reported values for each sample are means ± standard deviation ($n = 6$ for pH; $n = 4$ for TBARS and TVBN).

16.85 mg MAD/kg in chilled sardine, evolving to 7.43 mg MAD/kg at 10 day. Higher initial values of TBARS (17.2 mg/kg in sardine) have been reported by Chaijan, Benjakul, Visessanguan, and Faustman (2006), suggesting that lipid oxidation may occur during post-mortem handling to some extent. However, in another study with sardines stored at 3 °C lower MAD levels were reported, increasing from 0.83 to 2.35 mg MAD/kg at 3 days, and then declining up to 1.59 mg MAD/kg at 20 days of storage (Kenar, Özogul, & Kuley, 2010). This fluctuating evolution of secondary products of lipidic oxidation was not observed in the present study. Fluctuations in TBARS may arise from the interaction of TBA reactive products with other tissue constituents, or to malonaldehyde utilization by surviving microflora (Kasmoglu et al., 2003). The rate of malonaldehyde production was significantly ($p < 0.05$) lower in patties stored with clove-containing films (**SPC** + **CEO**) during the first 3 days of storage, indicating a noticeable delay in hydroperoxide (primary lipid oxidation products) degradation exerted by the **CEO** components, allowing TBARS remaining at the lowest values along the whole storage period. These results are related to the antioxidant properties of these films, being **SPC** + **CEO** the one with highest antioxidant power.

The TVBN measure is directly related with the growth of microorganisms and the formation of basic compounds that result from their metabolism and that lead to a raise in pH. TVBN values obtained for the samples under study are shown in Fig. 4c. The initial value for sardines was 11.2 mg TVBN/100 g, which agrees with values reported by Gökodlu, Özden, and Erkan (1998). This value falls in the middle of TVBN values reported for fresh sardine, which range from 5 mg TVBN/100 mg (Özogul, Polat, & Özogul, 2004) to 21.34 mg TVBN/100 mg (Kenar et al., 2010). The **CEO**-containing film reduced the TVBN content during the first days of storage ($p < 0.05$), but no significant differences between samples ($p > 0.05$) were found from the fifth day onwards. At the end of the follow-up the TVBN values of the different samples were slightly above 30 mg TVBN/100 g. Sardines containing up to 30 mg TVBN/100 g are usually regarded as “good” and those containing up to 35 mg/100 g as “marketable” (Kietzmann, Priebe, Rakov, & Reichstein, 1969).

At the beginning of the storage period the counts of total microorganisms in sardine patties was around 4 log cfu/g (see Table 4). Similar values have been reported for refrigerated whole eviscerated (Özogul et al., 2004) or filleted sardine (Kenar et al., 2010). The numbers of black colonies (presumably *S. putrefaciens*) and luminescent colonies (presumably *P. phosphoreum*) were below the detection limit of the technique. Counts of *Pseudomonas* spp. were similar to total bacterial counts, whereas those of lactic bacteria and *Enterobacteria* were close to 3 log cfu/g (see Table 4). At 3 days of storage an increment in microorganism counts was observed, especially in total viable organisms (15 °C). This increment is related to the increase of luminescent colonies, whose presence, due to requirements regarding incubation temperature and culture medium, is not reflected in the total mesophiles count (López-Caballero, Álvarez, Sánchez, & Moral, 2002; López-Caballero, Gómez-Guillén, Pérez-Mateos, & Montero, 2005). In general, counts progressed similarly for all the microorganisms, with no difference between samples. *Pseudomonas* species seemed to be involved in the deterioration of sardine patties since they represented the most prevalent microbiota at the end of the storage period assessed.

Sunflower protein films did not exert a definite inhibitory effect on the growth of the microorganisms evaluated (Table 4). It has been reported that the addition of **CEO** oil to gelatin and/or chitosan films reduces the growth of microorganisms in salmon and refrigerated cod (Gómez-Estaca et al., 2010). Our results, however, showed that the addition of **CEO** to sunflower protein films does not contribute to the microbiological stabilization of sardine patties during storage (Table 4). The polyphenol–protein interaction may reduce the activity of phenolic compounds (von Staszweski, Pilosof, & Jagus, 2011), although there was an evident antimicrobial activity of clove-containing films in the model system (agar disk diffusion), even on bacteria involved in fish spoilage (Table 3). On the other hand, the pH of the culture medium used in the model system (close to neutrality) is similar to that of sardine during storage. These findings make evident the existence of differences between results obtained with the model system and those from the real system (food). Factors that may explain such differences include the differential resistance of collection strains (more sensitive) and natural strains to antimicrobial agents, a potentially lower diffusion of the active agent in sardine muscle than in agar, an impaired penetration of clove compounds in the product (that contains not only the natural microbiota but also that acquired during

Table 4
Microbiological counts (log CFU/g) of total viable bacteria, total mesophiles, H_2S-producers microorganisms, luminescent colonies, *Pseudomonas* spp., *Enterobacteriaceae* and lactic bacteria for sardine patties uncovered (**Control**) and covered with sunflower protein concentrate films with or without clove essential oil (**SPC + CEO** and **SPC**, respectively), refrigerated at 2 °C (±1 °C).

Microorganisms	Lots	Refrigerated storage time (days)				
		0	3	5	7	13
Total viable bacteria	**Control**	$4.16 \pm 0.1^{a/x}$	$7.00 \pm 0.1^{b/y}$	$7.37 \pm 0.2^{a/y}$	$7.93 \pm 0.3^{a/z}$	$8.29 \pm 0.2^{a/z}$
	SPC	$4.16 \pm 0.1^{a/w}$	$6.82 \pm 0.1^{ab/x}$	$7.06 \pm 0.3^{a/x}$	$7.59 \pm 0.3^{a/y}$	$8.51 \pm 0.1^{a/z}$
	SPC + CEO	$4.16 \pm 0.1^{a/w}$	$6.61 \pm 0.2^{a/x}$	$6.77 \pm 0.4^{a/x}$	$7.61 \pm 0.4^{a/y}$	$8.95 \pm 0.1^{b/z}$
Total mesophiles	**Control**	$4.56 \pm 0.2^{a/x}$	$4.80 \pm 0.2^{a/x}$	$4.80 \pm 0.0^{b/x}$	$5.54 \pm 0.0^{b/y}$	$7.71 \pm 0.1^{a/z}$
	SPC	$4.56 \pm 0.0^{a/x}$	$4.69 \pm 0.3^{a/x}$	$4.92 \pm 0.1^{b/x}$	$5.28 \pm 0.0^{ab/y}$	$7.82 \pm 0.2^{a/z}$
	SPC + CEO	$4.56 \pm 0.2^{a/wx}$	$4.78 \pm 0.2^{a/xy}$	$4.27 \pm 0.4^{a/w}$	$5.16 \pm 0.3^{a/y}$	$8.10 \pm 0.1^{b/z}$
H_2S-producer microorganisms	**Control**	<2	$2.50 \pm 0.7^{a/x}$	$5.96 \pm 1.0^{a/y}$	$5.95 \pm 0.1^{a/y}$	$7.22 \pm 0.1^{b/z}$
	SPC	<2	$4.34 \pm 0.4^{b/x}$	$6.06 \pm 0.9^{a/y}$	$5.97 \pm 0.0^{a/y}$	$7.53 \pm 0.3^{b/z}$
	SPC + CEO	<2	$5.51 \pm 0.5^{c/v}$	$5.80 \pm 1.3^{a/v}$	$6.10 \pm 0.3^{a/v}$	$6.62 \pm 0.5^{a/v}$
Luminescent colonies	**Control**	<2	$6.11 \pm 0.2^{b/x}$	$6.50 \pm 1.1^{a/x}$	$6.90 \pm 1.4^{a/x}$	$7.25 \pm 0.1^{a/x}$
	SPC	<2	$6.07 \pm 0.1^{b/x}$	$6.77 \pm 0.1^{a/xy}$	$6.80 \pm 1.3^{a/xy}$	$7.23 \pm 0.1^{a/z}$
	SPC + CEO	<2	$5.42 \pm 0.6^{a/x}$	$6.34 \pm 0.5^{a/xy}$	$6.63 \pm 1.3^{a/xy}$	$7.30 \pm 0.4^{a/z}$
Pseudomonas spp.	**Control**	$4.29 \pm 0.0^{a/x}$	$4.71 \pm 0.0^{a/x}$	$4.40 \pm 0.6^{a/x}$	$6.27 \pm 0.3^{a/y}$	$7.73 \pm 0.3^{a/z}$
	SPC	$4.29 \pm 0.0^{a/x}$	$4.78 \pm 0.0^{a/x}$	$4.80 \pm 0.3^{a/x}$	$6.62 \pm 0.7^{a/y}$	$8.36 \pm 0.3^{ab/z}$
	SPC + CEO	$4.29 \pm 0.0^{a/w}$	$5.05 \pm 0.1^{b/x}$	$4.82 \pm 0.0^{a/wx}$	$6.48 \pm 0.4^{a/x}$	$8.79 \pm 0.6^{b/z}$
Lactic bacteria	**Control**	$3.40 \pm 0.1^{a/w}$	$3.54 \pm 0.1^{a/w}$	$3.62 \pm 0.0^{a/w}$	$4.40 \pm 0.1^{b/y}$	$5.48 \pm 0.1^{a/z}$
	SPC	$3.40 \pm 0.1^{a/y}$	$3.48 \pm 0.1^{a/y}$	$3.56 \pm 0.1^{a/y}$	$4.00 \pm 0.0^{a/y}$	$5.53 \pm 0.7^{a/z}$
	SPC + CEO	$3.40 \pm 0.1^{a/xy}$	$3.46 \pm 0.1^{a/xy}$	$2.93 \pm 0.8^{a/x}$	$4.15 \pm 0.0^{a/y}$	$6.16 \pm 0.4^{a/z}$
Enterobacteriaceae	**Control**	$3.05 \pm 0.0^{a/x}$	$2.90 \pm 0.2^{a/w}$	$1.00 \pm 0.0^{a/v}$	$3.42 \pm 0.0^{b/y}$	$4.35 \pm 0.0^{a/z}$
	SPC	$3.05 \pm 0.0^{a/x}$	$2.80 \pm 0.0^{a/w}$	$1.00 \pm 0.0^{a/v}$	$3.31 \pm 0.0^{a/y}$	$4.57 \pm 0.0^{b/z}$
	SPC + CEO	$3.05 \pm 0.0^{a/x}$	$3.06 \pm 0.1^{a/x}$	$2.33 \pm 0.0^{b/w}$	$3.28 \pm 0.0^{a/y}$	$5.22 \pm 0.2^{c/z}$

Reported values are means ± standard deviation ($n = 2$). 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 (v, w, x, y, z) 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.

processing) due to the thickness of patties, etc. Further studies are planned using a higher concentration of clove, compatible with a sensorially acceptable product. It may be also interesting to apply the films to other fish products (such as fillets) offering a larger contact surface with the films, and to study the microbial inhibition attained under these conditions.

4. Conclusions

The addition of clove essential oil to formulations based on sunflower protein concentrates allowed to obtain edible and biodegradable films with *in vitro* antioxidant and antimicrobial properties. While the presence of clove essential oil modified the interaction properties of proteins, reducing the water solubility and glass transition temperature of resulting protein films, it did not modify markedly its remaining physico-chemical properties. When applied to the preservation of refrigerated sardine patties these sunflower protein films allowed to retard their lipidic auto-oxidation and to slightly delay the growth of total mesophiles.

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