



Preparation and characterization of a chitosan film with grape seed extract-carvacrol microcapsules and its effect on the shelf-life of refrigerated Salmon (*Salmo salar*)

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ABSTRACT

Chitosan films with grape seed extract and carvacrol microcapsules (CMF) were prepared and their physico-chemical properties and effect on physico-chemical and microbiological parameters in refrigerated salmon were tested. CMF showed higher values of thickness (0.41 ± 0.04 mm), moisture content (13 ± 1 g water/100 g film), a (11 ± 3), b (12 ± 3), opacity ($20 \pm 1\%$), water vapor permeability (WVP) (4.4 ± 0.4) $\times 10^{-10}$ gPa⁻¹s⁻¹m⁻¹, oxygen permeability (O₂P) (1.3 ± 0.3) $\times 10^{-12}$ gPa⁻¹s⁻¹m⁻¹ and carbon dioxide permeability (CO₂P) (1.3 ± 0.3) $\times 10^{-12}$ gPa⁻¹s⁻¹m⁻¹ as compared to those of the chitosan control film (CCF). CMF showed lower values of L (66 ± 5) and water solubility ($17 \pm 1\%$).

The salmon packed into CMF presented on the 7th day of storage a lower value of TVB-N (37 ± 4 mg N/100 g fish) as compared to the CCF (42 ± 3 mg N/100 g fish) and control samples (CS; 66 ± 7 mg N/100 g fish). The CMF showed lower values of pH and lightness after 7 days of storage as compared to CS and CCF. The CMF showed also lower values of mesophilic and psychrophilic bacteria and *Pseudomonas* spp, reaching the maximum limit allowed for the first two only on the 7th day of storage.

CMF increases the shelf-life of refrigerated salmon to 4–7 days of storage due to the antimicrobial effect of the natural agents.

1. Introduction

It is important to find suitable materials in order to improve the efficiency and stability of packaging films produced from natural sources. Packaging films may be produced using different types of compounds, such as polysaccharides, proteins and lipids, alone or combined in blends or layers (Flores-López, Cerqueira, Jasso de Rodríguez, & Vicente, 2015). Chitosan (1,4-linked 2-amino-2-deoxy- β -D-glucan) is one of the most widely used natural compounds due to its characteristics such as high antimicrobial activity, biocompatibility, biodegradability and non-toxic profile. This polysaccharide was studied for application in different areas, such as food, pharmaceutical, medicine, agriculture, and environmental industries (Jiang, Deng, James, Nair, & Laurencin, 2014; Pinheiro et al., 2010, pp. 18–28; Ruiz-Navajas,

Viuda-Martos, Sendra, Perez-Alvarez, & Fernández-López, 2013).

Chitosan forms a transparent film with good mechanical properties, being an effective barrier to gases (CO₂ and O₂), showing high permeability to water vapor and flexibility and resistance to breakage in different types of packages, therefore, it was used for improving the quality and extending the shelf-life of fresh and processed foods (Aizpurua-Olaizola et al., 2016; Corrales, Fernández, & Han, 2014; Elsabee & Abdou, 2013; Fabra, López-Rubio, & Lagaron, 2014; Li, Li, Hu, & Li, 2013; Rubilar, Cruz, Silva et al., 2013; Sansone et al., 2014; Sathivel, Liu, Huang, & Prinyawiwatkul, 2007; Soares, Oliveira, & Vicente, 2015).

Chitosan, due to its intrinsic properties, was also used to prepare microcapsules (Estevinho, Rocha, Santos, & Alves, 2013; Hui et al., 2013; Song, Yu, Gao, Liu, & Ma, 2013). Microencapsulation can be

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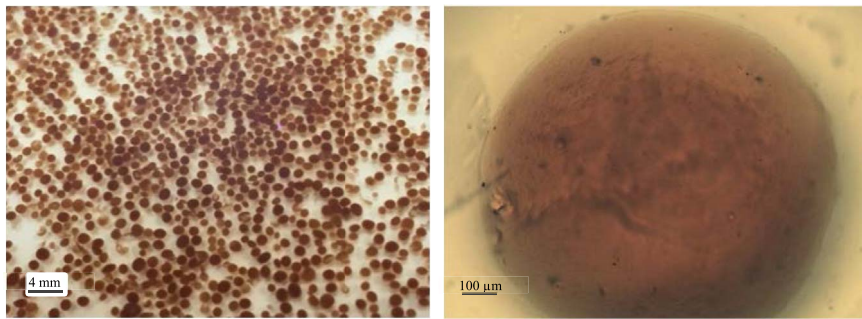


Fig. 1. Chitosan microcapsules with grape seed extract and carvacrol.

applied successfully to encapsulate natural compounds, such as essential oils and plant extracts, containing, for example, polyphenols with antimicrobial properties for use in food packaging (Nazzaro, Orlando, Fratianni, & Coppola, 2012).

Microencapsulation with natural extracts can benefit from new materials or packaging methodologies, capable to extend the shelf-life of highly perishable fresh foods like vegetables, fruit, fish and meat, increasing their quality and safety (Aizpurua-Olaizola et al., 2016; Nazzaro et al., 2012).

This technology is used for stabilization of particles and protection and/or isolation of their active core material from the surroundings, allowing materials to be handled more easily for the application (Bansode, Banarjee, Gaikwad, Jadhav, & Thorat, 2010). However, the degree of protection provided by microencapsulation is determined by the choice of the microencapsulation technique (Estevinho et al., 2013; Fabra et al., 2014; Fang & Bhandari, 2012; Ma & Sahai, 2013; Nesterenko, Alric, Silvestre, & Durrieu, 2013; Rosa et al., 2013; Sansone et al., 2014).

Carvacrol is the major component of oregano (*Origanum vulgare*) essential oil (50–86 ml/100 ml of oil) is a non-toxic phenolic monoterpene with proven antimicrobial activity against bacteria, fungi and yeasts and with insecticidal and anti-parasitic activity. It has high natural potential for preservation and safety of perishable foods (Costa et al., 2013; Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015a; Ramos, Beltrán, Peltzer, Valente, & Garrigós, 2014; Rubilar, Cruz, Silva et al., 2013).

Grape seed extract is another interesting compound, rich in polyphenols such as catechin, epicatechin, gallic acid and proanthocyanidins. These polyphenolic compounds have various biological effects, including antimicrobial and antioxidant properties (Corrales et al., 2014; Li, Miao, Wu, Chen, & Zhang, 2014; Li, Ai et al., 2013; Reddy et al., 2013; Rubilar, Cruz, Khmelinskii, & Vieira, 2013; Rubilar, Cruz, Silva et al., 2013; Terra et al., 2007). Moreover, Rubilar, Cruz, Khmelinskii et al., (2013) reported synergistic antimicrobial and antioxidant effects by combining carvacrol, grape seed extract and chitosan against different spoilage microorganisms.

This study aimed to develop chitosan films with microcapsules of grape seed extract and carvacrol, their physico-chemical characterization, and study of their effect on the physico-chemical and microbiological parameters of refrigerated salmon.

2. Materials and methods

2.1. Preparation of chitosan films with microcapsules

2.1.1. Preparation of chitosan film forming solution

Chitosan films 1.25 g/100 ml were prepared according to Rubilar, Cruz, Silva et al. (2013) with some modifications. Chitosan film forming solution was prepared by dissolving 20 g of chitosan (Sigma-Aldrich) in 1000 ml of glacial acetic acid (concentration of 1 ml glacial acetic acid/100 ml deionized water) (Panreac Química SA) and homogenized with an Ultra-Turrax homogenizer (T25, Ika-Labortechnik, Staufen,

Germany) at 9500 rpm for 20 min. The solution was kept at room temperature for 24 h at 20 °C. Then, the chitosan solution was filtered through non-woven cheesecloth in order to remove any undissolved particles. After filtration the solution was stirred on a hot plate (40 °C) and glycerol (0.8 g) was added to chitosan (for the final volume of 100 ml) and mixed for 30 min. The chitosan solution was degassed in an ultrasonic bath (Bandelin Sonorex Super RX102H, Berlin, Germany) for 30 min. Subsequently, 20 ml of the chitosan solution was placed to dry in Petri dishes (dia 8.5 cm, Normax) at a temperature of 20 °C during 48 h.

2.1.2. Preparation of microcapsules

Based on the methods reported by Wu and Yao (2013) and Rubilar, Cruz, Silva et al., (2013), 3.48 ml of Tween-80 (VWR) was added to the chitosan solution prepared in the previous section and mixed for 60 min at 40 °C. Then, 34.2 g of grape seed extract (Groupe Grap SUD) and 3.2 ml of carvacrol (Sigma-Aldrich) were added and mixed using an Ultra-Turrax homogenizer at 9500 rpm for 30 min. Finally, the solution was degassed in an ultrasonic bath (Bandelin Sonorex Super RX102H, Berlin, Germany) for 30 min. Then, the chitosan solution with grape seed extract and carvacrol was added drop wise using a micro syringe (BD Micro-Fine, 0.3 mm internal diameter needle) to a sodium tripolyphosphate solution (0.8 g/100 ml deionized water) (Sigma-Aldrich). The microcapsules were produced by ionic gelation during 30 min and, after the crosslinking time, were removed from the sodium tripolyphosphate solution and washed in distilled water (Fig. 1).

After 24 h of drying, 6.3 ml of chitosan microcapsules were placed in the chitosan films (approx. 1400 microcapsules, 4.5 cm²). After drying, each film was cut and stored in desiccators at 25 °C and 57% relative humidity (obtained using a NaBr solution) until testing (Fig. 2).

2.2. Determination of physico-chemical properties of the developed films

2.2.1. Film thickness

The film thickness was determined with a digital micrometer (No. 293-5, Mitutoyo, Japan). Ten thickness measurements were taken randomly on each of the samples tested. The mean values were next used to calculate water vapor permeability (WVP), oxygen permeability (O₂P) and carbon dioxide permeability (CO₂P).

2.2.2. Water solubility

This parameter was determined according to the method reported by Cuq, Gontard, Cuq, and Guilbert (1996). Solubility is defined as the content of dry matter solubilized after 24 h immersion in water. The initial dry matter content of each film was determined by drying to constant weight in an oven at 105 °C. Film disks (2 cm diameter) were cut, weighed (M_i), and immersed in 50 ml of water. After 24 h of immersion at 20 °C with agitation (60 rpm), the samples were taken out and dried to constant weight (M_f) in an oven at 105 °C, to determine the weight of dry matter that was not solubilized in water. The solubility of each film was then determined as follows:

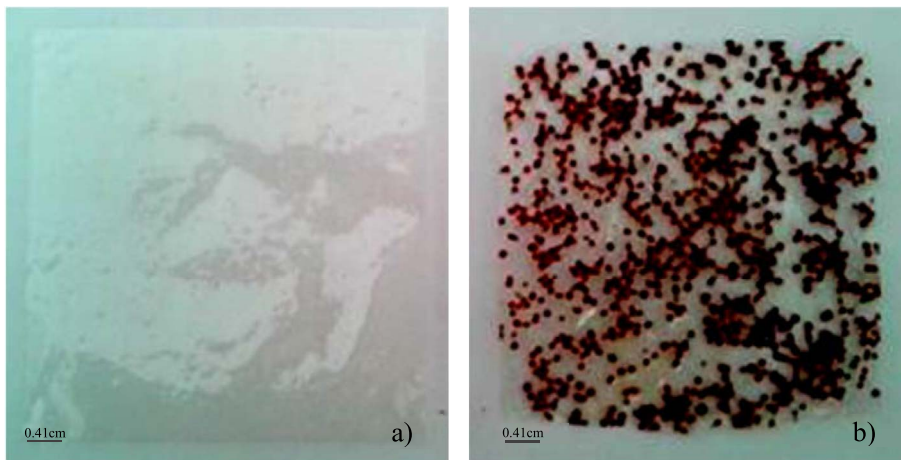


Fig. 2. a) Chitosan control film (CCF) and b) chitosan film with grape seed extract and carvacrol microcapsules (CMF).

$$\text{Solubility in water(\%)} = \frac{(M_i - M_f)}{M_i} \times 100 \quad (1)$$

Here, M_i is the initial mass and M_f is the final mass of the sample. Three replicates were obtained for each sample.

2.2.3. Moisture content

About 50 mg of film were dried at 105 °C during 24 h (until the equilibrium weight was attained). The weight loss of the sample was determined, and moisture content was calculated using the following equation:

$$\text{Moisture content} = \frac{(M_i - M_f)}{M_i} \times 100 \quad (2)$$

Here M_i and M_f are the masses of the initial and dried samples, respectively. Three replicates were obtained for each sample.

2.2.4. Color

The color of the films was determined with a Minolta colorimeter (CR 400; Minolta, Osaka, Japan). A white color plate was used as a standard for calibration and as the background for color measurements of the films. L , a , b values of each film were evaluated by reflectance measurements (Rao, Kanatt, Chawla, & Sharmam, 2010). The measurements were repeated five times for each film. The opacity of the films was determined as the relationship between the opacity of each sample on the black standard (Y_b) and the opacity of each sample on the white standard (Y_w) (Cerqueira et al., 2009a).

2.2.5. Fourier-transform infrared (FTIR) spectroscopy

The infrared spectra of the films were determined with a Fourier-transform infrared spectrometer (FTIR; Perkin Elmer 16 PC spectrometer, Boston, USA), based on the method reported by Rubilar, Cruz, Silva et al. (2013), using the attenuated total reflectance mode. Each spectrum results from 16 scans at 4 cm^{-1} resolution in the spectral range from 650 to 4000 cm^{-1} . All readings were performed at room temperature (20 °C). FTIR spectroscopy was used as a tool to investigate the interactions between chitosan and grape seed polyphenols and carvacrol by measuring the absorbance in the 650–4000 cm^{-1} wavenumber range at 4 cm^{-1} resolution. In the case of overlapping peaks, deconvolution was performed to calculate the contribution of the individual peaks using Peakfit software version 4.12 (SYSTAT Software Inc., Richmond, CA, USA). Deconvolution was used to estimate the peak area related to the specific vibration. The film spectra were deconvoluted with a smoothing filter of 15%. Each spectrum was baseline-corrected and the absorbance normalized between 0 and 1.

2.2.6. Water vapor permeability (WVP)

This parameter was determined gravimetrically based on the

method ASTM E96-92 (1990), and following Cerqueira, Lima, Teixeira, Moreira, and Vicente (2009b). The film was sealed on the top of a permeation cell containing distilled water (100% RH; 2337 Pa vapor pressure at 20 °C), placed in a desiccator at 20 °C and 0% RH (0 Pa water vapor pressure) containing silica. The cells were weighed each 2 h for 10 h. Steady-state and uniform water pressure conditions were achieved by maintaining the air circulation constant outside the test cell by using a miniature fan inside the desiccator (McHugh, Avena-Bustillos, & Krochta, 1993). The WVP was estimated using regression analysis from Eq. (3) adapted from Sobral, Menegalli, Hubinger, and Roques (2001):

$$\text{WVP} = \frac{w \times x}{t \Delta P} \quad (3)$$

Here, A is the permeation area (0.005524 m^2), ΔP is the difference of partial vapor pressure of the atmosphere (2337 Pa at 20 °C), x is the average thickness of the films and the term w/t was calculated by linear regression using the data points for the weight gain vs. time, within the constant rate interval. The measurements were repeated ten times for each film.

2.2.7. Oxygen and carbon dioxide permeabilities (O_2P and CO_2P)

O_2P and CO_2P were determined based on the method ASTM D3985-02 (2002) and described by Cerqueira et al. (2009b). O_2 and CO_2 concentrations were determined by gas chromatography (Chrompack 9001, Middleburg, Netherlands) with a Porapak Q column 0.149–0.177 mm, 2 m \times 0.3175 cm \times 2 mm stainless steel to separate the CO_2 and with a molecular sieve 5A column 0.149–0.177 mm, 1 m \times 0.3175 cm \times 2 mm to separate the O_2 , followed by a thermal conductivity detector (TCD) at 110 °C. Helium at 23 ml min^{-1} was used as carrier gas. A mixture containing the following proportion of CO_2 , O_2 and N_2 (0.1:0.2:0.7) was used as the standard for calibration. Three replicates were obtained for each sample, in each case (O_2P and CO_2P). Three measurements were taken for each replication.

2.2.8. Optical microscopy

Chitosan microcapsules with carvacrol and grape seed extract were observed using a microscope (Motic BA 200, Motic, Xiamen, China) with a camera (Moticam 3.0 MP) and the Motic Images Plus 2.0 software.

2.3. Refrigerated salmon shelf-life study

2.3.1. Sample preparation

Atlantic salmon (*Salmo salar*) skinless and boneless fillets were purchased at the local market in Faro, Portugal. The samples were cut approximately into 4 \times 4 \times 1 cm^3 chunks. The samples were divided into three groups: Group 1 – control samples (CS), Group 2 – control

Table 1

Physico-chemical properties of the chitosan film with microcapsules and chitosan control film. All parameters were analyzed in triplicate.

	Film with microcapsules (CMF)	Control Film (CCF)
Thickness (mm)	0.41 ± 0.04	0.04 ± 0.01
Water solubility (%)	17 ± 1	34 ± 4
Moisture content (g water/100 g film)	13 ± 1	8 ± 2
Color		
<i>L</i>	66 ± 5	95 ± 3
<i>a</i>	11 ± 3	-0.5 ± 0.1
<i>b</i>	12 ± 3	4.8 ± 0.2
Opacity (%)	20 ± 1	11 ± 1
WVP (gPa ⁻¹ s ⁻¹ m ⁻¹)	(4.4 ± 0.4) × 10 ⁻¹⁰	(4.4 ± 0.4) × 10 ⁻¹¹
O ₂ P (gPa ⁻¹ s ⁻¹ m ⁻¹)	(1.3 ± 0.3) × 10 ⁻¹²	(10 ± 1) × 10 ⁻¹⁵
CO ₂ P (gPa ⁻¹ s ⁻¹ m ⁻¹)	(1.3 ± 0.3) × 10 ⁻¹²	(5 ± 2) × 10 ⁻¹⁵

chitosan film (CCF) and Group 3 – chitosan film with microcapsules of grape seed extract and carvacrol (CMF). The films were placed on the upper surface of the salmon chunks.

2.3.2. Storage

Each sample group was placed in polystyrene trays (22.5 × 13.8 × 3.8 cm) and wrapped with polyethylene film. The samples were stored during 14 days in a refrigerated chamber (Aralab, Fitoclima ClimaPlus 400, São Domingos de Rana, Portugal) at 5 °C and 65% relative humidity.

2.3.3. Physico-chemical parameters

2.3.3.1. pH. The pH determination was performed using a pH meter (Crison, micro pH 2001, Barcelona, Spain) equipped with a pH electrode (Crison, 52 31). Six measurements were performed for each sample.

2.3.3.2. Total volatile basic nitrogen (TVB-N). The TVB-N values for all salmon samples were determined by the Conway method, using NP 2930:2009 standard (IPQ, 2009). The results were expressed in mg of nitrogen per 100 g of sample.

2.3.3.3. Moisture content. The determination of moisture was performed with an infrared balance (Mettler LP16M and PM100, Zurich, Switzerland). Four grams of salmon were dried at 160 °C and measured for each sample.

2.3.3.4. Color. The color of the salmon samples was measured using a colorimeter (Dr. Lange, Spectro-color, Düsseldorf, Germany) in the Hunter *Lab* scale, D65 illuminant and 10° observer. Three measurements were run per salmon sample.

2.3.3.5. Texture. The texture of the samples was measured using a texturometer (Brookfield, LFRA Texture Analyzer 1500, Harlow, UK) with a stainless steel probe 4 mm in diameter (TA34), test speed of 0.5 mm/s, target distance of 5 mm and the TextureProLite v1.1 software. Texture profile analysis (TPA) was performed on all of the samples and the parameter values for hardness, cohesiveness, adhesiveness and springiness were obtained. Three measurements were run per salmon sample.

2.3.4. Microbiological analyses

Samples were analyzed for mesophilic and psychrophilic bacteria using plate count agar (PCA, Liofilchem) and *Pseudomonas* spp using cetrimide agar (Scharlau). Each sample (10 g) was diluted 1:10 in sterile distilled water and the mixture was homogenized for 2 min in a stomacher (model 400, Seward, UK). After serial dilutions in PBS solution (Merck), aliquots of 1 ml were inoculated in PCA and cetrimide

agar, and the plates were incubated at 37 ± 2 °C for 48 h, 7 ± 2 °C for 7–10 days and 44 ± 2 °C for 48 h to determine mesophilic bacteria, psychrophilic bacteria and *Pseudomonas* spp., respectively. All analyses were run in duplicate.

2.4. Statistical analysis

The experimental data were processed with SPSS software - Statistical Product and Service Solutions version 22. An analysis of variance (ANOVA) was performed to detect significant differences among the samples. The significance level was set at 0.05. The least significant difference (LSD) was used as a post-hoc test to detect which pair/pairs of samples presented significant differences.

3. Results and discussion

3.1. Film characterization

3.1.1. Color and thickness

The physico-chemical properties of each of the films are presented in Table 1. As regards color, the CMF presented a lower *L* value, indicating that these films were darker than CCF. Positive values were obtained for *a* and *b*, which indicates that these films were in the zone of red and yellow. The change in the luminosity of the films was due to the incorporation of the active agents carvacrol and GSE, as verified in the study by Rubilar, Cruz, Silva et al. (2013). CMF showed positive *a* values while CCF displayed negative *a* values. This indicates that color changed to a reddish tone due to the incorporation of GSE (also of reddish coloration). Both CCF and CMF showed positive *b* values, though higher for CMF as a result of its more yellowish appearance, which may also be due to the incorporation of GSE. The presence of microcapsules also changed the opacity of the film, with the CMF around the double of the opacity of the CCF.

CCF showed similar thickness values, with the results also similar to those of several other studies, such as the one reported by Imran, Klouj, Revol-Junelles, and Desobry (2014) on the controlled release of nisin, sodium caseinate, poly-lactic acid and chitosan for active packaging applications. The CMF was about 10 times thicker than the CCF, due to the incorporation of the microcapsules. The increase in chitosan film thickness was also verified in the study by Ojagh, Rezaei, Razavi, and Hosseini (2010), as cinnamon essential oil was incorporated in increasing amounts into the films, and in the study by Martins, Cerqueira, and Vicente (2012) on the influence of the addition of β-tocopherol on the physico-mechanical properties of chitosan films.

3.1.2. Moisture content and water solubility

In what concerns the moisture content and water solubility, the CMF showed lower water solubility but presented higher value of moisture content. These results are similar to those obtained in the study reported by Rubilar, Cruz, Silva et al. (2013). The increase of the moisture content of the CMF may be due to GSE, whose main components have hydrophilic groups in their structure (Yamakoshi, Kataoka, Koga, & Ariga, 1999). The higher solubility of CCF can be attributed to the ability of water molecules to bind to the plasticizer (glycerol) and functional groups of chitosan, as described in the study by Ojagh et al. (2010) on the development and evaluation of chitosan films incorporating cinnamon essential oil. The lower solubility of the CMF may be due to the presence of carvacrol and its hydrophobic nature, preventing interaction with the water molecules as described in Rubilar, Cruz, Silva et al. (2013). The solubility of the films in water can provide indications about the film behavior in aqueous environments, being a measure of its water resistance, and therefore related to the hydrophilicity of the materials. This is also an important factor that determines the biodegradability of the films when used as packaging materials. The resistance of the films to water, as determined by their solubility, is critical for their potential applications. High solubility in

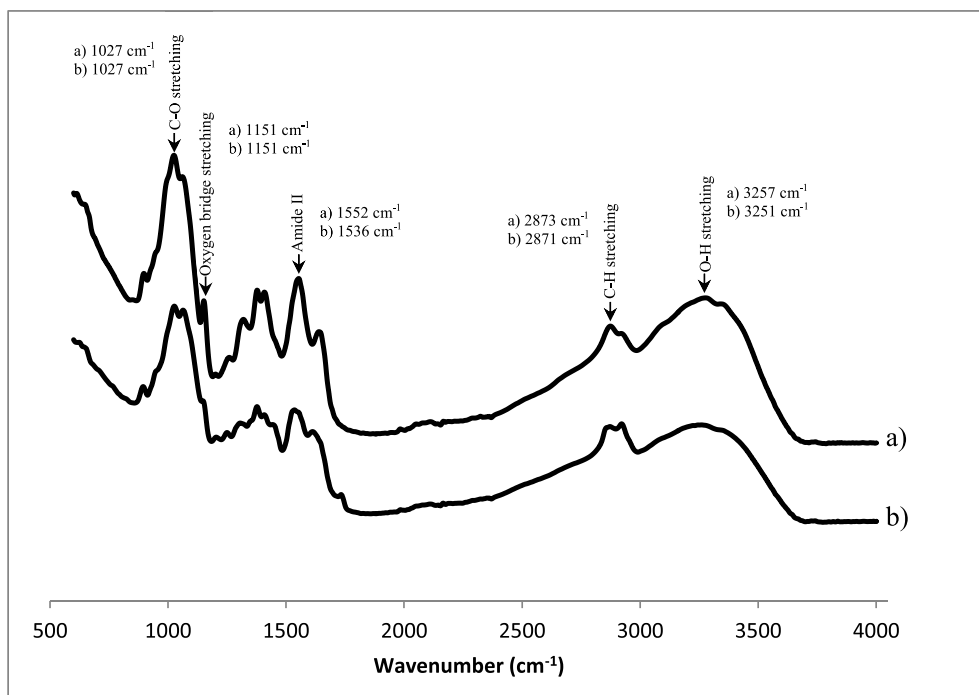


Fig. 3. Fourier transform infrared (FTIR) spectra of the studied films. a) chitosan control film (CCF); b) chitosan film with grape seed extract and carvacrol microcapsules (CMF).

water is sometimes desired when the film will be consumed simultaneously with the food. However, in other technological applications, low water solubility is required, as is the case with highly moist food packages (Rubilar, Cruz, Silva et al., 2013).

3.1.3. Water vapor (WVP), O_2 (O_2P) and CO_2 (CO_2P) permeabilities

CMF presented higher values of the tested permeabilities compared with the CCF (Table 1). The WVP required for an active film depends on the food to be packaged, and generally must be low to avoid or decrease the moisture transfer between the surrounding atmosphere and the food (Bourdon et al., 2011; Li et al., 2014). The transfer of water vapor generally occurs through the hydrophilic part of the film and depends on the ratio of the hydrophobic and hydrophilic constituents of the film (Bourdon et al., 2011; Jouki, Mortazavi, Yazdi, & Koocheki, 2014; Wu et al., 2014). There are several factors that can affect the WVP, such as film thickness and crystallinity (Jouki et al., 2014). Thus, the transfer of water vapor through a semicrystalline polymer occurs mainly in the amorphous phase, because the crystalline phase creates a much greater barrier to the passage of the water molecules. Therefore, as the percentage of crystallinity of a polymer increases, the gas permeability decreases (Bourdon et al., 2011; Rubilar, Cruz, Silva et al., 2013). Due to the release of GSE, which is hydrophilic, the water molecules are easier bonded by hydrogen bonds, as the additive has many hydroxyl groups, turning the polymer more permeable and allowing the absorption of water molecules by the film (Rubilar, Cruz, Silva et al., 2013). In addition, the presence of GSE may lead to less crystalline films, also leading to increased WVP (Rubilar, Cruz, Silva et al., 2013), as observed in this study where the WVP of the CMF were higher in relation to CCF. The study by Rubilar, Cruz, Silva et al. (2013) also demonstrated that lower concentrations of GSE and higher concentrations of carvacrol led to the reduction of WVP due to the hydrophobic nature of carvacrol, which affects the hydrophilic and hydrophobic balance of the film. The developed films presented similar WVP to fish gelatin-chitosan (1.5 g/100 ml) films incorporated with 1.2 g oregano oil/100 ml (2.11 ± 0.05) $\times 10^{-10}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$ (Hosseini et al., 2015a). In another study, chitosan (1 g/100 ml) nanocapsules formed by ionic gelation with TPP (0.4 g/100 ml) were embedded into a film with fish gelatin (4 g/100 ml) and the results similar to those of the present study were obtained. Namely, the control film showed a WVP of

(3.9 ± 0.2) $\times 10^{-10}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$ while the film with 8 g nanocapsules/100 g of fish gelatin presented a value of (2.5 ± 0.4) $\times 10^{-10}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$ (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015b).

CMF had $O_2P = (1.3 \pm 0.3) \times 10^{-12}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$ while the CCF showed a lower value $O_2P = (10 \pm 1) \times 10^{-15}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$. The O_2P of the food packaging materials is of great importance for food preservation, since oxygen is the factor that can cause oxidation and initiate various deterioration reactions. By nature, polysaccharide films provide a good barrier to oxygen, since their hydrogen network is well organized (Bourdon et al., 2011; Jouki et al., 2014; Rubilar, Cruz, Silva et al., 2013). O_2P depends on the films' crystallinity, the higher the crystallinity the lower the oxygen transmission (Rubilar, Cruz, Silva et al., 2013). According to Rubilar, Cruz, Silva et al. (2013) the films' O_2P is increased by the incorporation of high GSE concentrations. Nevertheless, $O_2P = (6 \pm 1) \times 10^{-13}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$ was obtained in chitosan films with the incorporation of natural extracts at the same concentrations as used in the microcapsules of the present study.

As regards CO_2P , the CMF permeability was higher $CO_2P = (1.3 \pm 0.3) \times 10^{-12}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$, than that of CCF, $CO_2P = (5 \pm 2) \times 10^{-15}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$. Generally, the gas permeability is dependent on the film crystallinity, with the increased crystallinity causing a reduced CO_2P (Bourdon et al., 2011). In the study by Rubilar, Cruz, Silva et al. (2013) chitosan films incorporating GSE and carvacrol were developed, having lower $CO_2P = (3.7 \pm 0.4) \times 10^{-13}$ gPa $^{-1}$ s $^{-1}$ m $^{-1}$ as compared to CMF.

3.1.4. Fourier transform infrared spectrum (FTIR)

Fourier transform infrared spectra of all of the films are shown in Fig. 3. FTIR was used to evaluate possible chemical interactions between chitosan and the bioactive compounds introduced as microcapsules. The peaks obtained in the FTIR showed the same behavior in CCF and CMF, however there was an artifact flattening of the CMF FTIR due to existence of the microcapsules. As the film is quite heterogeneous the peak amplitudes correspond to the mean value, so the peak amplitudes differ slightly between the two films studied (CCF and CMF), as described by several authors, depending on the amount of extracts added and the amplitudes of the spectrum (Hosseini et al., 2015a; Rubilar, Cruz, Silva et al., 2013). At 1027 cm $^{-1}$ a C-O stretching

for CCF and CMF was observed, this peak corresponds to the hydroxyl group (OH) of the saccharine structures (Arancibia, Alemán, López-Caballero, Gómez-Guillén, & Montero, 2015; Bonilla, Fortunati, Atarés, Chiralt, & Kenny, 2014; Silva-Weiss, Bifani, Ihl, Sobral, & Gómez-Guillén, 2013), especially glycerol used as plasticizer (Hosseini et al., 2015a). The oxygen bridge elongation mode at 1151 cm^{-1} was observed in both films (CCF and CMF). This mode is also typical for saccharin structures (Arancibia et al., 2015; Bonilla et al., 2014; Hosseini et al., 2015a), indicating that the length of the glycosidic chain was slightly modified with the addition of microcapsules into the film (Silva-Weiss et al., 2013). Some of the peaks were transferred to lower wavenumbers with the incorporation of the extracts, for example, the 1552 cm^{-1} and 1536 cm^{-1} peaks for the CCF and CMF, respectively, which correspond to the Amide II group (Bonilla et al., 2014; Hosseini et al., 2015a; Rubilar, Cruz, Silva et al., 2013). A peak at 1712 cm^{-1} was also observed for the CMF. This peak, according to Silva-Weiss et al. (2013), is related to the presence of quercetin in the microcapsules of the film, as well as the ester-bond between chitosan and the polyphenol extracts. A C-H group stretching at 2873 cm^{-1} and 2871 cm^{-1} was also observed suggesting that hydrogen bonds may occur between the chitosan carbon groups and the hydroxyl groups of the polyphenols present in the extracts (Silva-Weiss et al., 2013). Finally, there was a stretching of the hydroxyl group (O-H) at 3257 cm^{-1} and 3251 cm^{-1} for CCF and CMF, respectively, representing intermolecular bonds of chitosan molecules, mainly polymeric hydrogen bonds (Arancibia et al., 2015; Hosseini et al., 2015a; Silva-Weiss et al., 2013; Vodnar, 2012). The peak amplitude decreased in CMF due to presence of polyphenols, which form intramolecular and intermolecular hydrogen bonds (Li et al., 2014).

Li et al. (2014) studied a gelatin film (3 g/100 ml) with incorporated GSE, confirming the results presented in this work. The addition of the extract was verified by the presence of C-O and C-C bonds between 1000 cm^{-1} and 1300 cm^{-1} , respectively. Also, C=O and N-H stretching representing Amide II between 1500 cm^{-1} and 1675 cm^{-1} , for gelatin film and gelatin film with incorporated GSE, respectively. In another study (Hosseini et al., 2015b), chitosan film (1.5 g/100 ml) and another with oregano oil (1.2 g/100 ml) showed spectra similar to the ones obtained in this study. The films showed a peak at 3327 cm^{-1} , which corresponds to a stretching oscillation of the hydroxyl (OH) group, another one at 1546 cm^{-1} of the amide group II, and the peaks located between 1042 and 1045 cm^{-1} corresponding to the hydroxyl group.

3.2. Refrigerated salmon shelf-life study

3.2.1. Physico-chemical parameters

Table 2 presents the physico-chemical parameters of fresh salmon. Luminosity of the salmon samples with CS, CCF and CMF films presented the same behavior (Fig. 4). From day 0 to day 1, a significant increase ($p < 0.05$) from 42 to values between 45 and 50 was observed, meaning that the salmon samples became lighter. Throughout the remaining days, from day 1 to day 14, the luminosity values of the samples were approximately constant. However, the samples in CMF throughout the storage showed luminosity values closer to those of the fresh salmon, as compared to CS and CCF samples. Moreover, the CMF samples at the end of the storage time were not as light and milky as the CS and CCF samples (Fig. 5). This fact may be due to the presence of polyphenols, in particular, to the proanthocyanidins present in grape seed extract, which when transferred from the film to the sample, maintained the samples slightly darker, confirming that chitosan alone does not have a positive effect on the samples (Rubilar, Cruz, Khmelinskii et al., 2013). In a study where the red snapper samples (*Pagrus pagrus*) were coated with a solution of chitosan (2 g/100 ml) and another solution of chitosan (2 g/100 ml) with oregano oil (0.1 g/100 ml) and stored at $4\text{ }^{\circ}\text{C}$ for 20 days, despite slight variations in the two treatments, the addition of oregano oil did not significantly affect

Table 2

Physico-chemical and microbiological parameters of fresh salmon for shelf-life study (analyzed in triplicate and duplicate, respectively).

Physico-chemical	
pH	6.2 ± 0.1
TVB-N (mg N/100 g)	12 ± 3
Moisture content (g water/100 g fish)	60 ± 3
Color	
L	42 ± 2
a	15 ± 2
b	14 ± 2
Texture	
Hardness (g)	123 ± 26
Cohesiveness	0.19 ± 0.02
Adhesiveness (gs)	-61 ± 27
Springiness (mm)	1.9 ± 0.4
Microbiological	
Mesophilic bacteria (log(CFU/g))	3.9 ± 0.3
Psychrophilic bacteria (log(CFU/g))	4.9 ± 0.5
<i>Pseudomonas</i> spp. (log(CFU/g))	0.8 ± 0.5

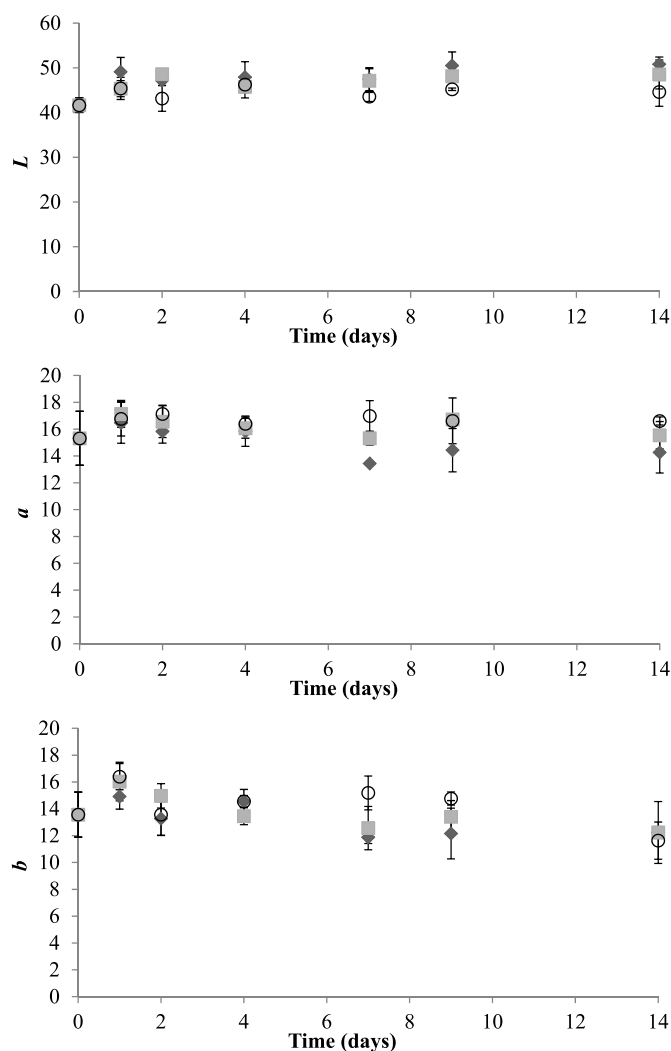


Fig. 4. Lab colour values for salmon samples during 14 days of storage at $5\text{ }^{\circ}\text{C}$. \blacklozenge control sample (CS), \blacksquare chitosan control film (CCF) and \circ chitosan film with grape seed extract and carvacrol microcapsules (CMF).

($p > 0.05$) the luminosity of the samples (Vatavali, Karakosta, Nathanailides, Georgantelis, & Kontominas, 2013). In a work reported by Nicorescu, Nguyen, Chevalier, and Orange (2014), the L values of salmon are in agreement with the results obtained in this work. At day 0

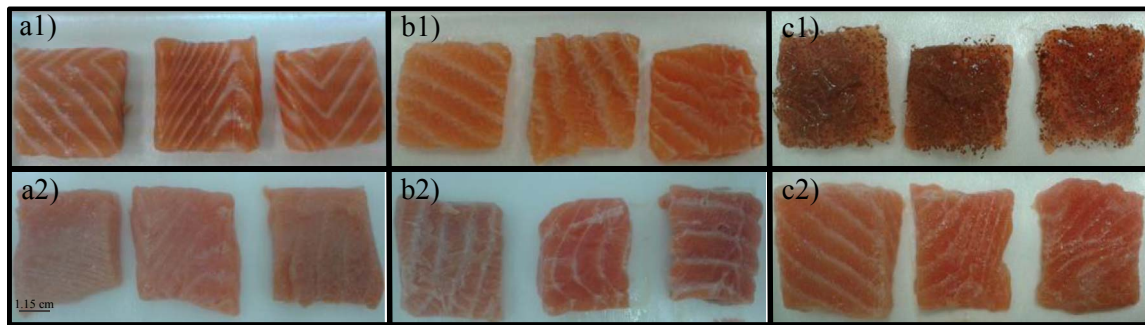


Fig. 5. Fresh samples: a1) control sample (CS), b1) chitosan control film (CCF) and c1) chitosan film with grape seed extract and carvacrol microcapsules (CMF); After 14 days in refrigerated storage: a2) CS, b2) CCF and c2) CMF.

the samples showed a value of approximately 46, with 47 ± 4 and of 47 ± 2 on day 7 stored at 4°C , for the control and samples irradiated with 30 J/cm^2 , respectively.

Regarding the a parameter, its values were approximately constant for all of the stored samples from day 0 to day 14 of storage. The reddish color of salmon was maintained throughout the storage; however, the a values for the salmon samples with CMF were slightly higher compared to CS and CCF samples, although not significantly (Fig. 4). These results are also corroborated with the results showed in Fig. 5, where the CMF presented a better preservation of the characteristic red color of salmon, which may be attributed to polyphenols, especially proanthocyanidins present in grape seed extract, as previously mentioned. According to Vatavali et al. (2013) a coating with chitosan (2 g/100 ml) and oregano oil (0.1 g/100 ml) also protected the red color of the red snapper (*Pagrus pagrus*) between the 6th and the 20th days of storage at 4°C .

The salmon samples presented almost constant values for the b parameter between the 11th and the 17th day (Fig. 4). All of the samples presented some variability throughout the storage period but no considerable changes were detected, besides the decrease of the salmon control sample at the 7th day of storage. These values are in accordance with the study reported by Vatavali et al. (2013) in which the values of b in red snapper (*Pagrus pagrus*) varied between 8 and 12, without significant differences between the control treatments, coating with chitosan (2 g/100 ml) and chitosan (2 g/100 ml) with oregano oil (0.1 g/100 ml).

In what concerns pH (Fig. 6a), a significant increase in the control samples ($p < 0.05$) was observed between the 4th and the 7th days of storage with values from 6 ± 1 to 6.6 ± 0.1 . The control samples showed a maximum value of 7.1 ± 0.1 at the 14th day of storage. These results confirmed that the control samples were degrading at a faster rate during the storage time. The higher pH values may be related to the fast deterioration of the product with the production of alkaline compounds due the autolysis of nitrogen compounds such as ammonia, trimethylamine and other biogenic amines, and further, due to deterioration by bacterial proliferation (Li, Li et al., 2013; Qiu, Chen, Liu, & Yang, 2014; Souza et al., 2010; Vatavali et al., 2013).

In samples packed with CCF a pH increase from 6.3 ± 0.1 to 6.4 ± 0.1 from the 4th to the 7th day of storage was observed. Thus, these samples were degrading more slowly than the control samples. However, in the samples packed with CMF a more pronounced increase in pH was only observed between days 9 and 14 of storage, showing values from 6.3 ± 0.1 to 6.7 ± 0.1 . Thus, the CMF maintained the pH lower for longer time, delaying the degradation of the samples over at least 9 days, in what concerns the evaluated parameter. Similar results were reported for other fish species coated with chitosan. In a study with red snapper stored at 4°C the initial pH was 6.6. The pH of the samples coated with chitosan (2 g/100 ml) with or without oregano oil (0.1 g/100 ml) increased throughout storage (6.7 at 13 days storage), but was lower than that of the control samples (6.9 at 13th day of

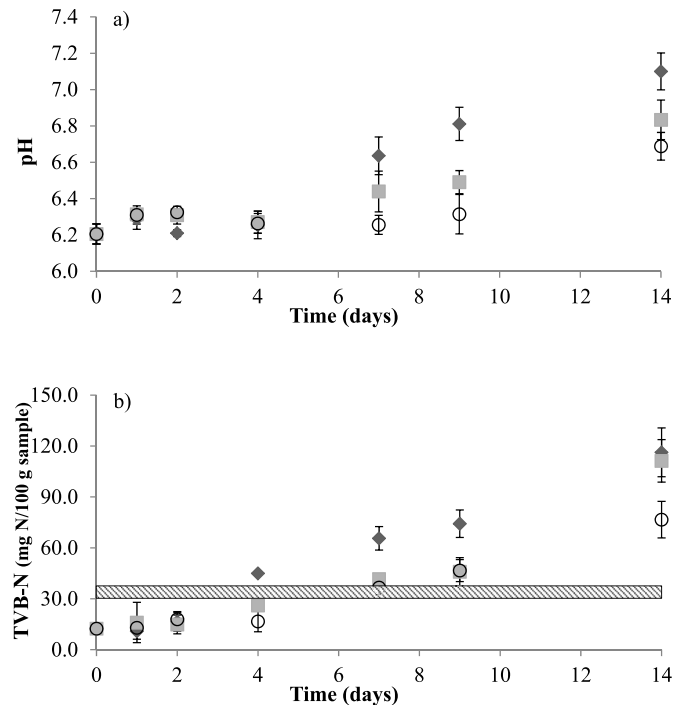


Fig. 6. a) pH and b) TVB-N values for salmon samples during 14 days of storage at 5°C . ♦ control sample (CS), □ chitosan control film (CCF) and ○ chitosan film with grape seed extract and carvacrol microcapsules (CMF). The horizontal area with pattern represents the rejection limit for fish flesh, which is 30–35 mg TVB-N/100 g, (CE) N.º 2074/2005.

storage, $p < 0.05$). The lower pH values are related to the antimicrobial action of the natural agents present in the CMF, which inhibit the bacteria that cause protein degradation and the production of basic compounds responsible for the pH increase (Vatavali et al., 2013).

In a study with redfish (*Sciaenops ocellatus*) the results were also in accordance with those presently reported. The pH of the control sample was higher than the pH of the samples coated with chitosan (1.5 g/100 ml) and grape seed extract (0.2 g/100 ml) ($p < 0.05$) during storage at 4°C (Li, Li et al., 2013).

The results for TVB-N are presented in Fig. 6b. Fresh salmon samples showed initial values of 12 ± 3 mg N/100 g of salmon. The TVB-N values increased throughout the storage time for all the studied samples showing on day 14 values of 116 ± 14 , 111 ± 13 and 77 ± 11 mg N/100 g of salmon for the CS, CCF and CMF, respectively. The CMF samples showed lower values than the other studied samples, maintaining the fish with low TVB-N values mainly between the 4th and the 7th days of storage. These differences may be attributed to the antimicrobial activity of chitosan and the extracts present in the microcapsules, contributing to the reduction of the bacterial population or the reduction of the capacity of the bacteria to perform oxidative

deamination (Souza et al., 2010). The values of TVB-N remained stable during the first two days of storage, however, an increase in the control samples exceeding 35 mg of N/100 g (established as the limit of the acceptability of salmon by Regulation (EC) No 2074/2005) was observed already on the 4th day.

CCF samples showed a significant increase from the 4th to the 7th days ($p < 0.05$) from 26 ± 1 to 42 ± 3 mg N/100 g of salmon, exceeding the maximum limit of 35 mg N/100 g of fish. The salmon samples packed with CMF also showed a significant increase from the 4th to the 7th days ($p < 0.05$). However, the value of TVB-N at day 7 (37 ± 4 mg N/100 g of salmon) was still within the maximum limit of 35 mg N/100 g of fish, indicating lower degradation of salmon stored at 5 °C with CMF. The antimicrobial activity of CMF may maintain or retard the growth of microorganisms that perform oxidative deamination of non-protein nitrogen compounds (Li, Li et al., 2013).

In a study with red fish fillets (*Sciaenops ocellatus*) the values of TVB-N also increased progressively ($p < 0.05$). The initial values were around 6 mg N/100 g and at the 20th day of storage at 4 °C, 51 and 38 mg N/100 g for the control and chitosan (1.5 g/100 ml) with grape seed extract (0.2 g/100 ml), respectively (Li, Li et al., 2013).

In another study carried out on carp samples (*Ctenopharyngodon idellus*) the values of TVB-N during 12 days of storage at 4 °C presented the same behavior as those in the present study. The control samples and those packed with Parafilm® showed an increase from 8 mg N/100 g to 55 mg N/100 g, while the samples packed in chitosan with oregano essential oil (4 ml oil/100 ml) were significantly lower ($p < 0.05$), showing 40 mg N/100 g on the last day of storage. Considering the limit value of 35 mg N/100 g, the shelf-life of samples packed in chitosan with oregano oil (4 ml oil/100 ml) was extended to 12 days (Wu et al., 2014).

Refrigerated sea bass (*Lateolabrax japonicus*) showed an increase from 11 mg N/100 g on day 0–100 mg N/100 g after 12 days of storage at 4 °C. The maximum limit of 35 mg N/100 g was exceeded on the 8th day of storage. However, chitosan coated samples (1.5 g/100 ml) presented at the end of storage (day 12) a value of 61 mg N/100 g and the ones coated with chitosan (1.5 g/100 ml) and citric acid (0.5 g/100 ml) presented still a lower value of TVB-N (48 mg N/100 g) (Qiu et al., 2014).

Regarding the texture of the samples, no significant differences ($p > 0.05$) were observed for the tested parameters throughout the storage. However, the CMF salmon samples were visibly more cohesive compared to the CS and CCF samples.

Although CS, CCF and CMF samples showed some variation in the moisture content throughout the storage due to drip loss, no significant differences were observed among samples, with the values remaining practically constant between 45 ± 3 g water/100 g of fish and 60 ± 3 g water/100 g of fish. Nevertheless, a pronounced decrease was observed from day 0 to day 1, indicating a greater loss of water in the samples.

3.2.2. Microbiological parameters

Table 2 also presents the microbiological parameters of fresh salmon. Fig. 7 shows the effect of the tested packages on the mesophilic bacteria, psychrophilic bacteria and *Pseudomonas* spp. growth in the refrigerated salmon. The CS and CCF samples, showed values of 7.2 ± 0.3 log(CFU/g) and 7.2 ± 0.6 log(CFU/g) for mesophilic bacteria, respectively, on the 4th day of storage at 5 °C, exceeding the maximum limit of 7 log(CFU/g). On the other hand, the CMF samples showed a pronounced increase only between the 4th and the 7th days of storage, from 4.8 ± 0.6 to 7.1 ± 0.3 log(CFU/g). Thus, the CMF delayed the deterioration of salmon samples due to the active compounds with antimicrobial and antioxidant properties that affect the cellular membranes and retard microbial growth (Rubilar, Cruz, Khmelinskii et al., 2013).

Similar results were found in the study by Wu et al. (2014) carried out in carp (*Ctenopharyngodon idellus*) stored at 5 °C and packed with

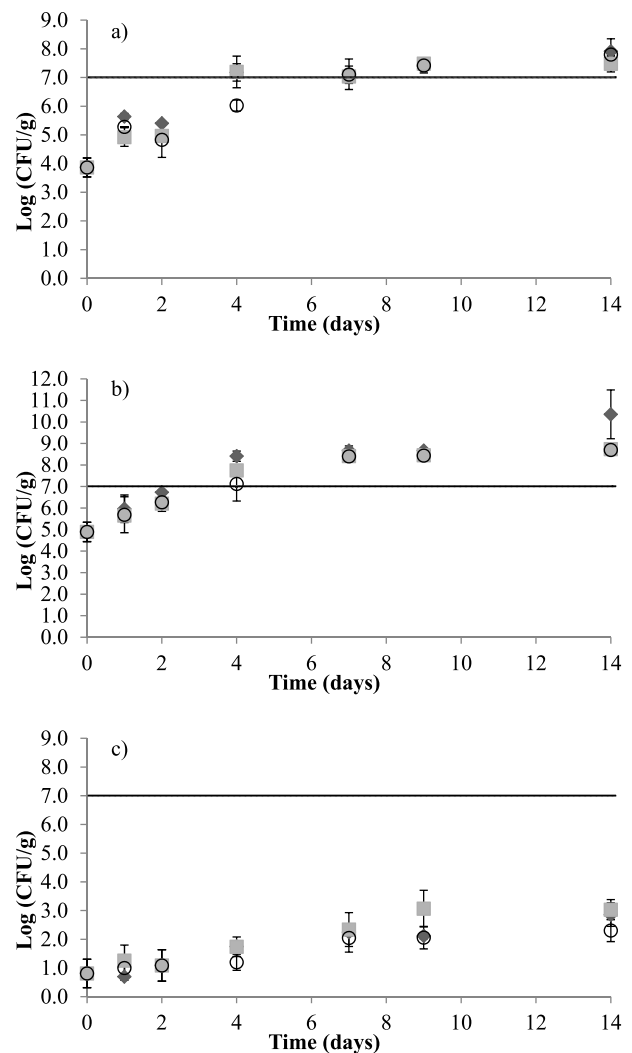


Fig. 7. a) Mesophilic, b) psychrophilic bacteria and c) *Pseudomonas* spp. values for salmon samples during 14 days of storage at 5 °C. ♦ control sample (CS), ■ chitosan control film (CCF) and ○ chitosan film with grape seed extract and carvacrol microcapsules (CMF). The horizontal lines represent the maximum limit (7.0 log(CFU/g)) allowed for consumption (Monitoring & Surveillance Series, 2010; Nowzari et al., 2013; Souza et al., 2010; Vatavali et al., 2013).

chitosan film incorporated with of oregano oil (4 ml oil/100 ml), which proved to have a good antimicrobial activity. The high microbial growth rate in the present study is due to the composition of the nutrient-rich fish, namely basic nitrogen, which makes it favorable for microbial growth (Nowzari, Shábanpour, & Ojagh, 2013; Souza et al., 2010).

In what concerns the psychrophilic bacteria, the CS exceeded 7 log (CFU/g) at day 4 of storage at 5 °C with 8.4 ± 0.2 log(CFU/g). CCF and CMF samples also reached the limit value at day 4 with 7.8 ± 0.3 log(CFU/g) and 7.1 ± 0.8 log(CFU/g), respectively. Nevertheless, the samples packed with CMF showed higher values of psychrophilic bacteria only at the 7th day of storage with 8.4 ± 0.1 log(CFU/g). These bacteria are the main group of microorganisms responsible for the deterioration of fresh fish stored at refrigerated temperatures (Nowzari et al., 2013). This shows that the release of natural agents into salmon samples has a beneficial effect, since it reduced the growth rate of the microorganisms studied.

The action of polyphenolic compounds and phenolic acids with antibacterial and antioxidant activity was significant. These compounds confer an inhibitory effect, primarily against Gram-positive microorganisms. Polyphenols can penetrate the bacterial semipermeable

membrane and react with cytoplasm or cellular proteins, destabilizing the microbial cells (Corrales et al., 2014).

According to Sallam, (2007), the results for salmon samples (*Onchorhynchus nerka*) are in agreement with the present work. An initial count of approximately 4 log psychrophilic bacteria (CFU/g) was recorded and at the 15th day of storage at 1 °C different samples treated with sodium acetate, sodium lactate and sodium citrate presented counts of 7.1, 8.2 and 8.5 log(CFU/g), respectively, whereas the control salmon samples showed a value of 10.4 log(CFU/g).

In relation to *Pseudomonas* spp. no significant differences were observed between day 0 and day 2 ($p > 0.05$) in all of the studied samples, the values ranged from the minimum of 0.7 ± 0.0 to the maximum of 1.1 ± 0.6 log(CFU/g). In the control samples an increase from day 4 to day 7 was observed from 1.8 ± 0.1 to 2.3 ± 0.1 log(CFU/g). In the CCF samples there were significant differences ($p < 0.05$) from day 2 to day 4 from 1.1 ± 0.6 to 1.7 ± 0.3 log(CFU/g).

In the samples packed with CMF there were no significant differences ($p > 0.05$) from day 0 to day 4. From the 4th day of storage a significant increase ($p < 0.05$) was observed in *Pseudomonas* spp. counts, with the salmon samples at the 7th day of storage in a higher degradation state. It was also found that the growth of the microorganisms in CMF was slower compared to CS and CCF samples. Nevertheless, the allowed maximum limit of 7.0 log(CFU/g) was not exceeded in any of the three cases under study.

In general, the microbial tests showed that salmon samples packed in CMF maintained acceptable values of mesophilic bacteria, psychrophilic bacteria and *Pseudomonas* spp. up to 4–7 days of storage.

4. Conclusion

The incorporation of microcapsules with grape seed extract and carvacrol affected the physicochemical properties of the developed films, increasing their thickness, moisture content, *a* and *b* color parameters, WVP, O₂P and CO₂P, when compared with the unmodified chitosan films. On the other hand, this study reports the beneficial effects of the CMF on salmon samples stored at 5 °C, especially maintaining the luminosity values closer to those of fresh salmon and by keeping the samples with lower TVB-N, pH values and bacterial counts for a longer period of time, with the samples remaining acceptable between the 4th and 7th days of storage.

The CMF revealed a great potential since it was placed on the upper surface of salmon only and even so showed a markedly positive effect. Moreover, CMF could also be suitable for packaging other type of food such as meat. Nevertheless, scale-up tests regarding this type of packaging are mandatory to assess its commercial and economic viability.

This type of packaging application could also be a sustainable alternative to petroleum-based plastic materials that are not biodegradable. The production of these packages will allow adding value to the by-products of several industries and also increasing the shelf-life of food products.

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