**Interest of black carob extract for the development of active biopolymer films for
cheese preservation**

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Abstract

The aim of this work was to develop bio-based packaging materials based on black carob extracts (BCE) and κ -carrageenan (κ -c) blends, and to study their impact for cheese preservation. Firstly, black carob extracts were obtained with and without alcohol pre-treatment (BCE-a and BCE-b), respectively, and its chemical composition and antioxidant properties was analysed. Protein and polyphenol contents were higher in the extract without alcohol pre-treatment (BCE-b). Different bio-based films were prepared by mixing BCEs with κ -c at different ratios, and the morphological, thermal, mechanical and water barrier properties of the films were evaluated. The antioxidant efficacy of BCE: κ -c films prepared with both BCE extracts was further compared when applied on the preservation of cheese slides. Results showed that skipping the ethanolic extraction pre-treatment gave rise to more ductile films due to the presence of sucrose and other small sugars or the higher protein content in the BCE-b extract, which could have a plasticizer effect on the biopolymer network. Mixtures with κ -c increased the rigidity of the films. A good correlation between barrier properties of films and the antioxidant efficacy on cheese samples was observed, suggesting that not only the antioxidant properties but also the physicochemical properties of the films had a direct impact on cheese quality and preservation. Films prepared with both BCEs at 50:50 BCE: κ -c ratio and those prepared with BCE-a at 25:75 BCE: κ -c ratio, presented good antioxidant capacity and a good compromise for physicochemical properties. However, from the economical point of view, 50:50 BCE-b: κ -c films are more suitable since the extraction protocol of BCE-b was simpler and the yield was three-fold higher.

Keywords: Active Films, Antioxidant, Barrier properties, Black carob extracts, κ -carrageenan

1. Introduction

Over the last decade, plastic pollution has become one of the most urgent environmental concerns, food packaging being demonstrably associated with high levels of waste, either terrestrial or marine litter, as well as low rates of reuse or recycling. Therefore, one of the strategies to counteract this problem is the development of biodegradable materials obtained from renewable resources, known as biopolymers. In fact, smart combinations of raw materials and processes, and new processing strategies are being lately promoted, making biopolymers competitive for food packaging applications (Galus, 2018).

Specifically, biopolymers derived from biological resources, like proteins and polysaccharides (Shojaee-Aliabadi et al., 2014), offer the greatest opportunities since their biodegradability and environmental compatibility are ensured. The combination of both proteins and polysaccharides may advantageously use the distinct functional characteristics of each film-forming compound and may improve the physicochemical and functional properties of the overall matrices by means of electrostatic interactions (Ahmed, Aschi, & Nicolai, 2018; Lopes, Michelon, Forster, Cunha, & Picone, 2019; Abdelgawad & Hudson, 2019). Furthermore, their use has been tailored to provide them with an active role in food preservation, e.g. imparting antioxidant and antimicrobial properties of interest for food safety purposes. This can be achieved by trapping active and bioactive substances within the biopolymer matrices in order to improve their functional properties (Xie et al., 2019; Yegappan, Vignesh, Sivashanmugam, & Jayakumar, 2018). For this aim, the most commonly used active components are polyphenols due to their strong antioxidant properties, which help to protect food ingredients from oxidation and microbial deterioration.

76 The use of polyphenols-enriched extracts from herbal and medicinal plants as bioactive
77 ingredients in edible films and coatings has been the focus in recent works (Quan,
78 Benjakula, Sae-leaw, Balange, & Maqsood, 2019; González et al., 2019; Velderrain-
79 Rodríguez, Acevedo-Fani, González-Aguilara, & Martín-Belloso, 2019). Furthermore,
80 food by-products are sources of different valuable compounds, such as proteins,
81 polysaccharides and polyphenols which are useful materials for developing edible and
82 biodegradable films (Grassino, Zhu, Barba, Brnčić, & Rimac Brnčić, 2018).

83 In particular, black carob (*Prosopis nigra*) constitutes a food source for human and
84 animals from the Monte desert in Argentina. *Prosopis nigra* mesocarp flour is produced
85 by milling dry pods without seeds. This flour has many beneficial properties like
86 antioxidant and anti-inflammatory activities and is able to inhibit enzymes related to the
87 metabolic syndrome (Pérez et al., 2014; Pérez, Zampini, Alberto, & Isla, 2018; Pérez et
88 al., 2020).

89 The aim of this work was to develop edible films containing carob seed extracts for
90 active food packaging applications. Films were developed using different mixtures of
91 carob seed extracts with κ -carrageenan (κ -c), a linear sulphated polysaccharide with
92 high molecular weight derived from red seaweeds that has demonstrated excellent film-
93 forming and gas barrier properties, which make it an ideal matrix for active packaging
94 materials for food applications (Wang, Yuan, Cui, & Liu, 2018; Falcó, Randazzo,
95 Sánchez, López-Rubio, & Fabra, 2019). Specifically, κ -c has about 25% to 30% of
96 sulphate content and forms clear films with excellent mechanical and structural
97 properties with a tensile strength higher than their counterparts prepared with ι - or γ -
98 carrageenan films (Falcó et al., 2019, Farhan & Hani, 2017, Shojaei-Aliabadi et al.,
99 2014). Initially, the chemical composition and antioxidant properties of the black carob
100 extract (BCE) obtained by two different procedures was analysed. Then, films were

prepared by mixing BCE with κ -c and the morphological and physicochemical properties were assessed. Finally, the effectiveness of the films prepared with BCE extracts in reducing oxidation of cheese during storage was evaluated.

2. Materials and Methods

2.1. Reagents

κ -carrageenan was kindly provided by Ceamsa (Pontevedra, España). Commercial semi-hard cheese (Gouda cheese; protein 24.1%, fat 31%, salt content 1.8%) was purchased from a local supermarket in Valencia, Spain. The diammonium salt of 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic) acid (ABTS), potassium persulphate ($K_2O_8S_2$), β -carotene (purity $\geq 97.0\%$) and potassium iodide (KI) were acquired from Sigma-Aldrich. Ethanol 96% and sodium hydroxide (NaOH) were obtained from Scharlab. Glycerol was purchased from Panreac.

2.2. Preparation and characterization of black carob extracts

Black carobs were collected in February 2019 at Santa María, Catamarca, Argentina. Carob pods were dried at 50°C , ground with a low speed blade mill (Metvisa, Argentina) and sieved to separate the seeds with attached endocarp. This last fraction was which were further ground with a high-speed blade mill (Numak, Argentina) and sieved to obtain the seed flour and a fibrous residue corresponding to the fruit endocarp. Firstly, two different extracts were obtained from black carob seed flour by applying a sequential extraction process using 70% ethanol (v/v) first, in an ultrasonic bath for 90 min at 25°C by three times. After that, the residue was subjected to an alkaline extraction process. Briefly, black carob seed flour residue was gently stirred in a cool bath with 0.5% (w/v) aqueous sodium hydroxide (NaOH) at pH 10 and using a solid-solvent ratio of 1:4 (w/v) for 20 min. Then, the suspension was centrifuged at $\sim 9692g$

for 35 min at 4 °C (Sorvall RC-50) and the solid residue was re-extracted two-fold with the same solution and centrifuged. All supernatants were pooled and adjusted to their isoelectric point (pH 5.0) for protein precipitation (Cattaneo et al., 2014). The pellet obtained after centrifugation was freeze-dried and stored at -20 °C (BCE-a). For comparative purposes, a second black carob extract was also prepared by following the same procedure already described but skipping the alcoholic pre-treatment (BCE-b).

2.2.1. Protein content analysis

The protein content in the BCE-a and BCE-b extracts was calculated based on the nitrogen content estimated with Kjeldahl method (BÜCHI K350, Switzerland), multiplied by a factor of 6.25 (AOAC, 1998). All determinations were performed in triplicate.

2.2.2. Carbohydrate composition

The monosaccharide composition was determined by acid hydrolysis followed by chromatographic analysis (Morais de Carvalho et al., 2017). The samples were hydrolysed with 2 mol L⁻¹ trifluoroacetic acid (TFA) at 121 °C for 3h. The samples were dried under a stream of air and dissolved in H₂O. The hydrolysed monosaccharides were analysed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an ICS-3000 system (Dionex) equipped with a CarboPac PA1 column (4×250 mm, Dionex). All experiments were performed in triplicate.

2.2.3. Ash content

The ash content was determined by calcination (AACC 08-01, 2000). Samples were placed in a crucible and heated in a muffle oven at 550 °C for 12 h. Then, the weight of the crucible was recorded and ash content was calculated by weight difference.

2.2.4. Phenolic content

Total phenolic content was estimated by the Folin-Ciocalteu colorimetric assay, (Singleton, Orthofer, & Lamuela-Raventos, 1999). Briefly, Folin-Ciocalteu reagent was mixed with different concentrations of the samples, 0.4 mL of sodium carbonate (16 %) were added and the samples were incubated during 30 min at room temperature before absorbance values were read at 765 nm. A calibration curve was built using gallic acid as a standard, and the total phenolic content was expressed as mg gallic acid equivalents in 100 g of dry weight of extract (mg GAE/100 g DW). All determinations were carried out in triplicate.

2.2.5. Antioxidant activity

The ABTS^{•+} (2,2-azinobis (3-4ethylbenzothiazoline)-6-sulfonic acid) radical cation scavenging activity of the black carob extracts was determined according to Re et al. (1999). Briefly, a 7 mM ABTS solution was prepared in sodium phosphate buffer at pH 7.4 and mixed with 2.45 mM of potassium persulphate. This solution was left overnight in the dark to yield the ABTS^{•+} radical cation. Prior to use, the ABTS^{•+} was diluted in sodium phosphate buffer, pH 7.4, with an initial absorbance of $\sim 0.700 \pm 0.02$ (1:50 ratio) at 734 nm, at room temperature. Free radical scavenging activity was assessed by mixing 0.2 mL of diluted ABTS^{•+} with 20 μ L of the extracts and monitoring the change in absorbance after 6 min (until a steady state was achieved). A calibration curve was developed by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity of the extracts was expressed as μ g Trolox equivalents (TE)/g DW. All determinations were carried out in triplicate.

2.2.6. β -carotene-linoleic acid bleaching assay

The antioxidant capacity of the extracts was determined by the formation of conjugated diene hydroperoxides arising from linoleic acid oxidation, which results in the discoloration of β -carotene. To this end, a modification of the method described by

Velioglu, Mazza, Gao, & Oomah, (1998) was employed. In brief, 1 mg of β -carotene was dissolved in 5 mL chloroform. 1 mL of this solution was placed on a rotary evaporator and the chloroform was evaporated. Then, 25 μ L of linoleic acid and 200 mg Tween 40 were added and mixed with stirring. After that, 50 mL of aerated ultra-pure water, obtained by bubbling air through the water for 15 min, was transferred to the flask and stirred vigorously. Approximately 0.24 mg of the extracts or 0.2 mL of water as a control, were transferred to test tubes and then 5 mL of the β -carotene emulsion were added. The samples were incubated in a water bath at 50 °C for 2h. The absorbance of each sample was measured at 470 nm using a spectrophotometer. Measurements were carried out in triplicate. The concentration of extract (μ g DW/mL) that inhibits 50% of discoloration of β -carotene (IC_{50}) was determined.

2.3. Preparation and characterization of films

Black carob extracts prepared with and without alcoholic pre-treatment, BCE-a and BCE-b, respectively, were selected for the development of films. Specifically, eight different films were obtained by the solvent-casting method using different BCE: k-c ratios (100:0, 75:25, 50:50, 25:75) and a pure κ -carrageenan film was also prepared for comparative purposes.

The film-forming aqueous dispersions were prepared by dissolving 1% (w/w) κ -c in distilled water at 90 °C for 30 min. In parallel, freeze-dried BCE was dispersed in alkalized distilled water (pH 9) with 2% (w/v) NaOH under stirring for 1 h at room temperature until the extract was completely solubilized. Different aliquots of these solutions were added to the κ -c aqueous solution to give the required final BCE: k-c ratio in the mixtures. Then, the amount of glycerol required to obtain a BCE/ κ -c:plasticizer ratio of 1:0.3 was incorporated. Afterwards, film-forming dispersions, containing 1g of total solids, were spread evenly over a Teflon casting plate (5 cm

diameter) resting on a level surface. Films were formed by drying for approximately 24h at 40 °C and 45 RH%. These conditions were established after previous experiments to ensure that homogeneous and flawless films were obtained. Dry films could be peeled intact from the casting surface. Before morphological and physicochemical characterization, samples were equilibrated for one week at 53% RH (relative humidity) and 25 °C by using an oversaturated solution of $\text{Mg}(\text{NO}_3)_2$. Film thickness was measured with a Palmer digital micrometre to the nearest 0.0025 mm at five random positions, before mechanical, optical and water vapour barrier properties.

2.3.1. Scanning electron microscopy (SEM)

Microstructural analysis of the films was carried out using a Scanning Electron Microscope (Hitachi S-4800). Films in duplicate were frozen in liquid nitrogen and randomly broken to explore the cross-section of the samples. Films were mounted on M4 Aluminium Specimen Mounts and fixed on the support using double-side adhesive tape. Samples were gold–palladium coated and observed using an accelerating voltage of 10 kV.

2.3.2. Opacity of the films

Spectral transmittance of the samples was recorded, in triplicate, using an 8453 Agilent UV–Vis spectrophotometer. A suitable size of rectangle film samples was directly inserted in a quartz cuvette and scanned in the UV-vis range 200–700 nm with an empty cuvette as a reference, according to Rodsamran & Sothornvit (2019).

The film opacity was calculated by dividing the absorbance at 600 nm by the thickness of the film strip.

2.3.3. Thermogravimetric analysis (TGA)

Thermogravimetric curves (TG) were recorded with a Setaram TG/DTA92 (SETARAM Instrumentation, France). The samples were heated from 30 to 800 °C with a heating

rate of $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ under nitrogen atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

2.3.4. Mechanical properties

Tensile tests were carried out on a Mecmesin MultiTest 1-i (1 kN) machine (Virginia, USA) with the Emperor™ software, according to ASTM standard method D882-09 18 (ASTM, 2009). Tensile strength (TS), elongation percentage at break (EAB) and elastic modulus (E) were determined from the stress-strain curves. Equilibrated specimens (1 cm wide and 8 cm long) were mounted in the film extension grips and stretched at 50 mm min^{-1} until breaking. Eight replicates of each formulation were tested.

2.3.5. Water vapour permeability (WVP)

Direct permeability to water was determined from the slope of the weight loss versus time curves at $24\text{ }^{\circ}\text{C}$. Films were placed between the aluminium top (open O-ring) and bottom (deposit for the silica) parts of a specifically designed permeability cell with screws. A Viton rubber O-ring was placed between the film and bottom part of the cell to enhance seal ability. Cups with aluminium films were used as control samples to estimate solvent loss through the sealing. These permeability cells containing deionized water (100% RH) were then placed in an equilibrated relative humidity cabinet at 53% RH and $25\text{ }^{\circ}\text{C}$. The weight loss through a film area of 10 cm^2 was monitored and plotted as a function of time. Water vapour permeation rate was calculated from the steady-state permeation slopes obtained from the regression analysis of weight loss data vs. time, and weight loss was calculated as the total cell loss minus the loss through the sealing. Permeability was obtained by multiplying the permeance by the average film thickness. The tests were done, at least, in triplicate.

2.3.6. Water uptake

The water uptake was estimated from sorption experiments at 25 °C and 100% RH by means of weight gain using a Precisa Gravimetrics AG SERIES 320XB analytical balance (Dietikon, Switzerland). Square specimens with a total surface area of 6.25 cm² were cut from the films and their initial weight was registered. The assays were carried out at least in triplicate.

2.4. Challenge tests

Slices of Gouda cheese (5 cm x 5 cm) were coated on both sides with films that were prepared the day before and placed in the dark under refrigeration conditions (4 °C) for 20 days. The lipid oxidation of cheese samples was determined, in triplicate, by means of the peroxide index. In brief, the lipid content was extracted with 50 mL chloroform-methanol (2:1 v/v) and homogenised using a rotor–stator homogeniser. Next, 10 mL of CaCl₂ solution were added to the mixture and shaken vigorously for 15 seconds. Then, it was centrifuged at 4500 x g for 30 min and evaporated under vacuum. The extracted lipid content was then subjected to peroxide value (PV) analysis. In this analysis, cheese fat (1.5 g) was dissolved in a mixture of chloroform and acetic acid (2:3 v/v) and mixed with an oversaturated KI solution (0.5 mL). Next, the emulsion was kept in the dark for 5 min, followed by the addition of 30 mL of distilled water. The PV was then determined by titration with a solution of sodium thiosulphate (0.001 N), using starch as an indicator. The peroxide value was expressed as peroxide milliequivalents per kilogram of lipid.

2.5. Statistical analysis

Data of each test were statistically analyzed. The statistical analysis was carried out by means of IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Comparison of the means was done employing the Tukey's Honestly Significant Difference (HSD) at the 95% confidence level.

275

276 **3. Results and Discussion**

277 *3.1. Characterization of the black carob seed flour extracts*

278 Black carob was valorised in this work to evaluate the possibility of generating active
279 films for food packaging applications. The composition of the extracts subjected to the
280 extraction processes described in Section 2.2 are compiled in Table 1, while their
281 detailed carbohydrate composition is displayed in Table 2. Alkaline extracts were
282 mainly composed of proteins and carbohydrates but also contained significant amounts
283 of ashes and phenolic compounds (Table 1). The alkaline extraction yield was around
284 6% when no pre-treatment was applied, and it decreased to 2% within the alcoholic pre-
285 treatment was applied, due ethanol soluble low molecular weight compounds being
286 washed off. Protein was the main component in the extracts (48.8% and 53.1% to BCE-
287 a and BCE-b, respectively), this being higher than reported values for the *P. alba* and *P.*
288 *nigra* mesocarp flour (until 11 and 12%) and lower than *P. nigra* seed germ flour
289 (65.5%) (Rodriguez et al., 2019; Pérez et al., 2020; Mamone et al., 2019) and cotyledon
290 flour extract from *Prosopis alba* seed (Cattaneo et al., 2014).

291 The carbohydrate analysis revealed significant differences between both extracts. BCE-
292 b extracts were richer in carbohydrates, the difference being a high contribution of
293 glucose and fructose, compared to BCE-a. These sugar units were mainly ascribed to
294 the sucrose, fructose and free glucose content, which were soluble in ethanol and were
295 thus washed off during the pre-treatment in BCE-a. Galacturonic acid, galactose and
296 arabinose were present in both extracts, being enriched in BCE-a, and probably arise
297 from galacturonan, arabinan, or arabinogalactan pectin components. Both extracts
298 contain polyphenol compounds (~0.035 and 0.048 % in BCE-a and BCE-b,
299 respectively). Thus, the alcoholic pre-treatment affected the amount of polyphenols and

sugars in the BCE, being higher when this pre-treatment was not applied (BCE-b) and having a direct impact on the antioxidant capacity (Table 1). On the other hand, polyphenols are able to interact with proteins via either non-covalent (hydrophobic, ionic, and hydrogen bonding) or covalent bonds (You, Luo, & Wu, 2014) and the resulting protein-polyphenol conjugates have been reported to have more emulsifying and antioxidant activities (Quan et al., 2019). Since the BCE-b would clearly be more interesting not only from the functional properties but also from an economical perspective, it was selected for the production of active films and compared to their counterpart prepared with the alcoholic pre-treatment (BCB-a).

Table 1. Characterization of BCE extracts.

Samples	Yield (%)	Phenolic compounds (mgGAE/100gDW)*	Protein (%)	Total sugar (%)	Ash (%)	TEAC (µg TE/g DW)**	β-carotene (IC ₅₀ = µg DW/mL)** *
BCE-a	2	34.8 (5.9) ^a	48.8 (0.1) ^a	20.6 (8.0) ^a	8.5 (2.0) ^a	369 (13) ^a	300 (38) ^a
BCE-b	6	47.6 (3.3) ^b	53.1 (0.4) ^b	37.9 (6.5) ^b	6.4 (1.2) ^a	543 (38) ^b	300 (32) ^a

Mean values (standard deviation). Values with different letters in the same column are significantly different ($p \leq 0.05$). *mg of gallic acid equivalents per 100 grams of dry weight (DW). **µg Trolox Equivalent per gram of DW. *** Concentration that inhibits 50% of discoloration of β-carotene. DW: dry weight

Table 2. Carbohydrate composition of the BCE extracts.

Samples	Sugar unit concentration (g/ 100g extract)								
	Fuc	Rha	Ara	Gal	Glc	Man	Fruc	GalA	GlcA
BCE-a	<0.5	<0.5	4.8 (1.6)	5.2 (1.7)	1.5 (0.5)	2.1 (0.6)	0.9 (0.4)	4.7 (2.1)	<0.5
BCE-b	<0.5	<0.5	3.8 (0.6)	1.5 (0.4)	16.1 (3.8)	<0.5	9.6 (2.1)	5.6 (1.0)	<0.5

Mean values (standard deviation). Values with different letters in the same column are significantly different ($p \leq 0.05$). Fuc: fucose; Rha: rhamnose; Ara: arabinose; Gal: galactose; Glc: glucose; Man: mannose; Fruc: fructose; GalA: galacturonic acid; GlcA: glucuronic acid.

3.2. Characterization of films

3.2.1. Optical properties

The black carob extracts were used to generate films from aqueous suspensions by mixing them with κ -c at different ratios. Figure 1 (A) shows the visual appearance of the produced films with BCE-a and BCE-b. As observed, all the films were translucent and black carob extracts provided a brownish tonality, being accentuated as the content of black carob extracts increased in the biopolymer matrices. Interestingly, the films from BCE-b presented an opaquer appearance which may be due to the presence of a higher amount of polyphenols and other impurities. As observed in Figure 1 (B), all the BCE-containing films were more opaque than pure κ -c films, this being more patent in the case of BCE-b, which could be attributed to a greater content of polyphenols in this BCE fraction.

(A)

Figure 1 (A). Contact transparency from BCE: κ-c films. Up: films prepared with BCE-a and down films prepared with BCE-b. From left to right: Pure BCE, pure κ-c, 25:75 BCE:κ-c ratio, 50:50 BCE:κ-c ratio, 75:25 BCE: κ-c ratio. **(B)** Opacity degree of the films. Black and white bars refer to films prepared with BCE-a and BCE-b, respectively.

3.2.2. Thermal stability

To determine the effect of both BCE on the thermal stability of the films, these were analysed by TGA. As deduced from the derivative thermogravimetric profiles shown in Figure 2, both BCE degraded at lower temperatures than the pure polysaccharide film. Pure κ-c film showed much sharper degradation peaks centred at 230 °C linked to the destruction of glycosidic bonds (Pascalau et al., 2012). The presence of BCE provoked a slight increase in the main degradation temperature ascribed to the κ-c, being higher in the case of BCE-b although showing a much broader degradation range. This might be explained by a higher abundance of proteins, bound polyphenols (resistant to alkaline treatment) and their heterogeneous interactions. Similarly, Alúdatt, Rababah, & Alli (2014) also reported an increase in the degradation temperature of flaxseed protein isolate in the presence of free and bound phenolic compounds. This effect was also

observed for soy protein after it was covalently modified with polyphenols (Rawel, Rohn, Kruse, & Kroll, 2002). Furthermore, the stability of κ -c has been previously reported to be significantly enhanced by mixing them with other proteins (Zhao et al., 2015; Li et al., 2019). Films prepared with more than 50% BCE-b presented broad peaks with an initial shoulder at $\sim 218^\circ\text{C}$, followed by the main degradation peaks, with maxima at $\sim 255^\circ\text{C}$, suggesting a saturation of this positive interaction with carrageenan and a decrease of the thermal stability of BCE-containing films.

Figure 2. Derivative thermogravimetric (DTG) curves of the films prepared with (A) BCE-a and (B) BCE-b at different BCE:κ-c ratios.

3.2.3. Morphology

The morphology of the films' cross-section was analysed by SEM and representative images are shown in Figure 3. A different internal arrangement was observed as a function of the film composition. While the films prepared from pure BCE showed a smooth and homogeneous microstructure, the samples containing κ -c showed structural discontinuities suggesting the lack of miscibility of **the** components. The incorporation of κ -c gave rise to a heterogeneous film matrix which behaved differently depending on the BCE- κ -c ratio, being rougher for those prepared with 50:50 or 25:75 BCE: κ -c ratios. Specifically, small crystals can be also observed which can be attributed to precipitation of small sugars and glycerol. Probably this effect did not occur in the wet films, such as pure BCE films, where water exerts a plasticizing role, thus favouring their solubilisation.

This different structural arrangement of BCE and κ -c, probably affecting the refracting index, also relates to their optical properties previously described.

Figure 3. SEM images from the cross-section of the tested films obtained from BCE-a (left) and BCE-b (right) extracts. From top to bottom: (A-B) Pure BCE, (C-D) 25:75 BCE-κ-c ratio, (E-F) 50:50 BCE- κ-c ratio, (G-H) 25:75 BCE- κ-c ratio, (I) pure k-c. Scale bars correspond to 30 μm.

The performance properties of the films were also tested to evaluate their potential as food packaging biopolymers. The mechanical properties were assessed by means of tensile testing and representative parameters, such as the elastic modulus (E), tensile

strength (TS) and elongation at break (EAB) obtained from the stress-strain curves, are gathered in Table 3. In general, the mechanical response of the films was largely affected by the extraction process from the black carob seeds. Pure BCE extract films presented poorer tensile properties than the well-known thermoplastic starch, alginate, soy protein, sodium caseinate or PLA films (Chotiprayon, Chaisawad & Yoksan, 2020, Fabra, Jiménez, Atarés, Talens, & Chiralt, 2009, Guitérrez-Jara, Bilbao-Sainz, McHugh, Chiou, Williams & Villalobos-Carbajal, 2020, Jin, Tang, Zhu, & Zhou, 2020; Liu et al., 2020, Monedero, Fabra, Talens & Chiralt, 2010) which could compromise their application as food packaging. Specifically, pure BCE extract films showed the lowest E and TS values, while pure κ -c films were more rigid and mechanically resistant. E and TS values of pure κ -c films were lower than those previously reported by Falcó et al. (2019) for carrageenan-based films, which can be explained by the presence of glycerol in the films developed in the present work since the plasticizer weakens the polysaccharide intermolecular forces and improves the molecular chain mobility. Comparing pure BCE-a and BCE-b, it should be highlighted that skipping the ethanolic extraction pre-treatment gave rise to more ductile and deformable films, most likely due to the presence of sucrose and other small sugars, or the higher protein content in the BCE-b which could have a plasticizer effect on the biopolymer network. However, the mechanical response of pure BCE films was not suitable to be applied as a packaging material due to their poor tensile properties, being the mixtures of BCE and κ -c more appropriate for this purpose. As expected, a notable increase of E and TS values and a trend to reduce EAB values was observed in κ -c-containing films which could be attributed to a greater compactness of the biopolymer chains (being higher for those prepared with BCE-a), supporting the beneficial effect of the carrageenan in the BCE extracts. Films prepared with BCE-b at 50:50 BCE: κ -c ratio presented very interesting

properties, showing better tensile strength than the pure κ -c and other marine polysaccharides like agar (Moreno et al., 2020; Liu et al., 2019) but similar to thermoplastic starch (Fabra, Martínez-Sanz, Gómez-Mascaraque, Gavara & López-Rubio, 2018); and a good compromise for the water vapour permeability and antioxidant properties as it will be detailed below.

Table 3. Mechanical, water barrier properties and water sorption of the films obtained from the BCE extracts and κ -c.

	BCE: κ -c Ratio	E (MPa)	TS (MPa)	EAB (%)	WVP 10^{-14} (Kg-/Pa·s·m ²)	Water uptake (%)
BCE-a	0:100	2839 (304) ^a	28.2 (6.3) ^a	2.4 (0.9) ^a	19.3 (1.8) ^a	231 (2) ^a
	100:0	200 (28) ^b	4.0 (2.1) ^b	3.6 (1.4) ^a	11.7 (0.2) ^b	222 (6) ^{ab}
	75:25	1630 (179) ^c	16.3 (2.8) ^c	1.7 (0.1) ^a	11.2 (2.4) ^{bc}	188 (5) ^{cd}
	50:50	2162 (323) ^{ac}	11.6 (0.4) ^c	0.5 (0.1) ^b	8.0 (1.3) ^c	185 (14) ^{cde}
	25:75	4360 (270) ^d	22.5 (2.5) ^{ac}	0.6 (0.2) ^b	12.8 (0.8) ^b	151 (9) ^e
BCE-b	100:0	17.6 (1.1) ^e	0.9 (0.1) ^b	4.4 (1.2) ^a	22.6 (1.8) ^a	191 (20) ^{cd}
	75:25	1343 (164) ^c	19.7 (4.3) ^{ac}	4.2 (0.9) ^a	18.4 (2.0) ^a	192 (10) ^{bcd}
	50:50	1260 (212) ^c	27.4 (8.2) ^{ac}	4.5 (1.9) ^a	14.0 (1.4) ^b	171 (13) ^{ce}
	25:75	3836 (151) ^d	32.0 (6.3) ^a	1.5 (0.5) ^a	19.6 (0.2) ^a	210 (17) ^{abd}

Mean values (standard deviation). Values with different letters are significantly different ($p \leq 0.05$). E: Elastic Modulus; TS: Tensile Strength, EAB: Elongation at Break.

Table 3 summarizes the measured water vapour permeability (WVP) for the developed films. The WVP value of the pure κ -c films is in agreement with those from previous studies in which carrageenan cast films were obtained (Shojaee-Aliabadi et al., 2014; Rhim & Wang, 2014). It should be noted that the type of BCE affected the WVP, observing a statistically significant increase ($p < 0.05$) for pure BCE-b film, indicating that the removal of small sugars, some proteins or/and polyphenols by means of the ethanolic pre-treatment had a positive impact on the barrier performance of the films. In fact, the obtained values for BCE-b containing films did not show a clear tendency with the κ -c content since both BCE-b and the polysaccharide showed very similar values.

Interestingly, a significant decrease in the 50:50 BCE:κ-c ratio films, were found for both extracts which suggests a greater interaction between both components in the film matrices.

With regards to the water sorption capacity of the films, the results, also shown in Table 3, proved that the presence of BCE produced a decrease in the amount of water adsorbed by the films, probably ascribed to the presence of proteins in these extracts which are known to be less hydrophilic than polysaccharides (Zhang et al., 2019). In fact, the water sorption capacity of pure BCE-b (with higher amount of proteins, table 1), was lower than their counterpart prepared with BCE-a. These results point out the advantages of using heterogeneous extracts containing e.g. proteins and polyphenols rather than using pure carbohydrate fractions.

3.3. Challenge tests

Challenge tests on coated cheese slices at refrigerated temperature (4 °C) were carried out under *in-vivo* storage, mimicking the realistic scenarios of commercially available cheese slices packed and stored under refrigeration conditions in order to ascertain the antioxidant effectiveness of BCE-a and BCE-b extracts. For comparative purposes, pure κ-c films and uncoated cheese slides were also analysed. The oxidative stability of fat cheese was measured in terms of the peroxide value (PV) after 10 and 20 days of storage.

Figure 4 summarizes the peroxide value (PV) of uncoated and coated sliced cheese samples as a function of storage time at 4 °C. The initial PV of cheese sample was 0.25 mEq O₂ / Kg, in agreement with other studies (Mortensen, Sørensen, & Stapelfeldt, 2002). As observed, different ranges of PV were reached and significant differences between uncoated sample and those coated with the different films were detected. An

increase in PV values was observed with storage of uncoated samples, reaching PV value of 4.20 mEq O₂ / Kg after 20 days of storage.

After 10 days, coated samples showed lower PV values than uncoated cheese slides, thus evidencing the antioxidant effect of the films. Similarly, some researchers have reported the efficacy of polyphenol antioxidants incorporated into biopolymer packaging materials for cheese preservation (Di Pierro et al., 2011; Bonilla & Sobral Paulo, 2019, Göksen et al., 2020). However, a very different behaviour was observed between both pure BCE films. Cheese slides coated with pure BCE-b extract showed the lowest PV value after 10 days' storage, which might be related to the greater polyphenol content and antioxidant capacity of this extract (see Table 1). However, for longer storage times, a sharp increase ($p<0.05$) in the PV value was observed for cheese samples coated with pure BCE-b films, being significantly higher ($p<0.05$) than those obtained either for pure BCE-a films or for films prepared with different ratios of BCE:κ-c. This evidences that not only the polyphenol content but also the WVP of the films played an important role in the oxidation phenomena of coated samples. In fact, BCE-b films were more permeable (see table 3) than their counterparts prepared with BCE-a.

Pure κ-c films also presented lower PV values than the uncoated samples through the first 10 days. This protective effect could be attributed to a greater WVP observed in these films, which was in the same range as films prepared with BCE-b content higher than 75%, or/and the antioxidant capacity of the sulphated galactans, as reported in red seaweeds from which carrageenans are obtained (Rocha de Souza et al., 2007).

Although the protective effect in delaying fat oxidation could be guaranteed with pure BCE-a films, their poor mechanical properties would limit their application as a food packaging. Thus, a good compromise between physicochemical and functional

properties is needed. The results presented in this work indicate that films prepared both BCE at 50:50 BCE (a or b):κ-c ratio and those prepared with BCE-a at 25:75 BCE:κ-c ratio, presented very promising properties, with good antioxidant properties and a good compromise for mechanical and water barrier properties, both being very relevant in food preservation. However, from an economical perspective, 50:50 BCE-b:κ-c films are more adequate since the extraction protocol of BCE-b was simpler and the yield was three-folds higher.

Figure 4. Peroxide Value (meq O₂/kg) of uncoated and coated cheese slides for 20 days storage at refrigerated conditions.

4. Conclusions

Novel antioxidant films based on black carob extracts have been successfully developed for cheese preservation. In general, coated sliced cheese samples showed lower PV values after storage at 4 °C than uncoated cheese slides, thus evidencing the antioxidant effect of the active compounds of the extracts. The overall results of the present work demonstrated a good correlation between barrier properties of stand-alone films and the

antioxidant efficiency on cheese samples, indicating that not only the antioxidant properties but also the physicochemical properties of the films played an important role on cheese preservation. In fact, although pure BCE-a films successfully prevented fat oxidation, their poor mechanical properties would limit their application as a food packaging. As a result, the best results in terms of mechanical and barrier properties were achieved using 50:50 BCE:κ-c films. However, from the economical point of view of the extraction process, films prepared with 50:50 BCE-b: κ-c are more suitable since the BCE extraction process was faster, simpler with reduced number of steps and the extraction yield of BCE-b was three-fold higher than BCE-a.

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696 **Highlights:**

697 Black carob extracts (BCE) and κ -carrageenan (k-c) active films were developed
698 Black carob extracts were obtained with and without alcohol pre-treatment
699 Protein and polyphenol contents were higher in the extract without alcohol pre-
700 treatment
701 Films prepared with 50:50 BCE: κ -c ratio showed very promising properties
702 The developed active films successfully improved cheese preservation
703