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# Development of Antimicrobial Biocomposite Films to Preserve Quality of Bread

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## ABSTRACT

This study developed gelatin-based films with incorporation of microcrystalline cellulose as reinforcement material. Clove (*Syzygium aromaticum*), nutmeg (*Myristica fragrans*), and black pepper (*Piper nigrum*) oleoresins containing antimicrobial compounds of natural origin were incorporated into films. The mechanical, thermal, optical, and structural properties, as well as color, resistance to sealing and permeability to water vapor, light, and oil of the films were determined. Adding oleoresins to the gelatin matrix increased elongation of the material and significantly diminished its permeability to water vapor and oil. Evaluation of the potential use of films containing different oleoresins as bread packaging material was influenced by the film properties. The biocomposite film

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containing oleoresin from black pepper was the most effective packaging material for maintaining the bread's quality characteristics.

*Keywords: biocomposite films; gelatin; oleoresins; antimicrobial compounds; food quality*

## INTRODUCTION

Biocomposite materials are structures made up of biodegradable materials, where the main component in charge of providing cohesion and stability to the matrix is a polymer, which can be obtained from different sources. Proteins, polysaccharides, or a mixture of these, which can provide mechanical and/or barrier properties, are some of those commonly used to make up the polymer structure [1] [2]. Gelatin is one of the proteins with greatest industrial application due to its low melting point; in addition, it has a unique amino acid sequence, with high contents of proline, glycine, and hydroxyproline, which help in the formation of flexible films [3]. Gelatin is a good barrier against oxygen and carbon dioxide, but a poor barrier against water vapor [4]. To obtain materials with better flexibility, manageability, and extension ability, it is necessary to incorporate substances of low molecular weight, like plasticizers [5]. Also, compounds with antimicrobial and antioxidant activity can be incorporated, which help to improve the physical-chemical and microbiological properties of polymer materials and may improve the quality of the food they cover and/or increase its shelf life [6]. Different lipids (oleoresins and essential oils) have

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shown antimicrobial or antioxidant properties, which may be used for the development of biocomposite materials. Lipids are a good barrier against humidity, besides providing brightness and being a good barrier against $O_2$, $CO_2$, and water vapor. Thus, the formation of active biocomposite materials consists of incorporating compounds with antioxidant or antimicrobial properties to the polymer matrix, which permit, through the migration of the active compounds onto the food surface, the conservation and extension of its shelf life [7] [8]. Nevertheless, film properties are mainly influenced by the polymers used, active compounds, and elaboration method. The use of biocomposite materials as food packaging or wrapping will depend on the mechanical properties and the permeability to water vapor [9]. The aim of this work was to investigate the effect of adding natural antimicrobial compounds (clove, nutmeg, and black pepper oleoresins) incorporated into gelatin based biocomposite films, on their moisture content, water vapor permeability, oil permeability, tensile properties, structure, optics and thermal properties. Biocomposite films were evaluated as packaging material on bread slices stored at ambient conditions (around 25 °C and 75% relative humidity). The physical-chemical, microbiology, and sensory characteristics of bread were evaluated for nine days.

## 1. MATERIALS AND METHODS

### *1.1. Materials*

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To elaborate the films, food-grade gelatin was used with a bloom of 220 – 240 g (Gelco S.A., Barranquilla, Colombia), along with pharmaceutical-grade microcrystalline cellulose, Avicel® PC 105 (FMC Biopolymer, Brazil); 99% purity glycerol (Sigma Aldrich, USA); Tween 80 for synthesis (Merck, Germany); and clove, nutmeg, and black pepper oleoresins (TECNAS S.A., Colombia).

### *1.2. Minimum inhibitory concentration of the oleoresins*

The minimum inhibitory concentration (MIC) of the oleoresins against *S. aureus* and *E. coli* was determined by using the classic plate micro-dilution method by the National Committee of Laboratory Safety and Standards (NCLSS) [10]. Triplicates were performed of each concentration of natural antimicrobials (4, 2, 1, 0.5, and 0.25%). The lowest concentration of the natural antimicrobial that showed growth inhibition was considered the MIC.

#### *1.2.1. Physical-chemical analysis*

Gelatin particle size and MCC (Microcrystalline Cellulose) were determined by using a Zetasizer nano ZS90 (Malvern Instruments, Ltd., UK). Gelatin and MCC crystallinity were determined by using an X-ray diffractometer (Siemens, Karlsruhe, Deutschland) equipped with a Cu anode tube (which emits radiation of $\lambda = 0.154\ nm$) with a voltage of 40 kV and current of 30 mA and Kβ line filter, and a scintillation detector. The diffractograms were analyzed by using the Origin 8 program and crystallinity (% ICr) was determined by using equation 1.

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$$Ic_r = \frac{I_{200} - I_{non-cr}}{I_{200}} * 100 \quad (1)$$

Where:

$I_{200}$ = Maximum intensity value of the crystalline peak

$I_{non-cr}$ = Intensity value that separates both diffraction peaks

Identification of the principal functional groups of the MCC and the gelatin was made through Fourier transformed infrared spectroscopy (Perkin Elmer, Waltham, MA, USA). The spectra were obtained within a range from 4000 to 400 $cm^{-1}$ and analyzed by using the Origin 8 program. Thermal characterization of the MCC, the gelatin, and the oleoresins (nutmeg, black pepper, and clove) was carried out through differential scanning calorimetry (DSC 214 Polyma, Netzsch; Germany); scanning was from -20 to 140 °C at a rate of 5 °C / min.

The chemical composition of the oleoresins (nutmeg, clove, and black pepper) was determined by using a gas chromatograph AT 6890 Series Plus (Agilent Technologies, Palo Alto, California, USA), coupled to a selective mass detector (Agilent Technologies, MSD 5973) operating in full scan mode. The column used in the analysis was DB-5MS [5%-phenyl-poly-(methylsiloxane), 60 m X 0.25 mm X 0.25 µm] and the injection was conducted in split mode (30:1) with Viny = 2 µL.

### *1.3. Elaboration of films*

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Films were elaborated through the "casting" method following the methodology proposed by Andrade-Mahecha *et al*. (2012) [11] and by Mondragón *et al*. (2015) [12] with some modifications. The film-forming solution (FFS) was obtained from an aqueous suspension of microcrystalline cellulose (0.15 g / 100 g of FFS), which was magnetically agitated for 30 min at 35 °C. Simultaneously, two aqueous suspensions were also prepared; one containing gelatin (3 g / 100 g of FFS) with agitation at 60 °C for 30 min and another containing glycerol (0.45 g / 100 g of FFS) with agitation at 35 °C for 15 min. Thereafter, all the components were mixed and kept at 60 °C for 15 min under constant agitation. Next, the respective oleoresin containing the natural antimicrobial compounds (10 g (500 µL of oleoresin / 500 µL Tween 80 at 2%) / 100 g FFS) was added. The FFS was kept at 45 °C for 15 min, prior to being poured into nonstick molds to be subjected to convection drying (45 °C during 4 h) on a stove with forced-air circulation (FD-53, Binder, Germany).

### *1.4. Film characterization*

Film thickness was measured at five different points by using a digital micrometer (Mitutoyo, Corp., Kawasaki, Japan).

#### *1.4.1. Humidity content*

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Film humidity content was determined immediately after drying and after a conditioning period (58% RH during 48 h), according to the gravimetric method by ASTM D644-99 [13].

### *1.4.2. Water vapor permeability*

Water vapor permeability (WVP) was determined by following the ASTM E96-05 [14]. The films were placed on stainless steel cells subjected to external environments with different relative humidity (RH 2-33%, 33-66%, and 64-90%). Weight loss of the cells was monitored every hour for 9 h.

### *1.4.3. Mechanical properties*

Mechanical tests were carried out on a texture analyzer (TA-XT plus, Stable Micro Systems, England), at an operating rate of 1 mm/s and separation of 40 mm between clamps. The mechanical properties evaluated were: tensile strength (MPa), elongation (%), and young's modulus (MPa), according to ASTM D882-02 [15].

### *1.4.4. Oil Permeability*

Oil Permeability was determined by following the methodology proposed by Yan *et al*. (2012) [16], using the following equation:

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$$Po = \frac{\Delta W \times FT}{A \times T} \quad (2)$$

Where $\Delta W$ is the weight variation of the filter paper (g), FT is the film thickness (mm), A is the area of effective contact ($m^2$), and T is the storage period (days).

*1.4.5. Measurement of resistance to sealing*

Film samples (7.62 X 2.54 cm) were thermally sealed for two seconds, one on top of another with an area of 2.54 cm X 0.2 cm, using a manual thrust sealer (Hongzhan, KS-100, China). Resistance to sealing was determined through the ASTM F-88 [17], using a texture analyzer (TA-XT plus, Stable Micro Systems, England). Resistance to sealing was calculated with the maximum force on the film width [18].

*1.4.6. X-ray diffraction (XRD)*

Film samples (4 x 4 cm) were analyzed in an X-ray diffractometer (Bruker D8-Advance, Germany) equipped with a tube with Cu anode (radiation of $\lambda$ = 0.154 nm) with voltage of 30 Kv, current of 35 Ma, filter for the $K_\beta$ line, and a scintillation detector. The diffractograms were analyzed by using the Origin 8 program.

*1.4.7. Fourier Transform Infrared (FTIR) Spectroscopy*

Identification of the principal functional groups was conducted via spectroscopy equipment (IR Prestige 21 SHIMADZU, Japan). Spectra were obtained within a

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171 wave number range of 4000 to 400 $cm^{-1}$ and analyzed by using the Origin 8
172 program.

173

174 *1.4.8. UV-vis light transmittance values*

175 Film light barrier (50 x 30 mm samples) was evaluated in wavelengths from 200 to
176 800 nm in a spectrophotometer (Thermo Scientific Genesys-10S-UV-Vis)
177 according to Leceta *et al.* (2015) [19].

178

179 *1.4.9. Color*

180 Film color was determined by using a spectrocolorimeter (Hunter Associates
181 Laboratory, Inc., Reston, VA, USA), according to Arfat *et al*. (2017) [9]. The color
182 difference (ΔE*) was calculated by using the following equation:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5} \qquad (3)$$

183 Where $\Delta E^*, \Delta a^* \text{and } \Delta b^*$ corresponded to the differences between the color
184 parameters of films containing oleoresins and the values of the reference film
185 (without oleoresin) ($L^* = 92.094,\ a^* = -1.46, b^* = 4.73$).

186

187 *1.4.10. Thermogravimetric Analysis (TGA)*

188 Non-isometric degradation measurements were conducted on a Thermo
189 Microbalance (TG 209 F1 Iris, Germany). Tests were run from 25 °C to 600 °C at a
190 heating rate of 10 °C/min in a nitrogen atmosphere (10 ml/min) [19].

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### *1.5. Influence of biocomposite films on bread quality*

The formulation of the artisan bread was carried out according to Colombian standard NTC 1363 [20]. Two lots of bread manufactured on different days were evaluated. In both lots, bread samples were packaged in reference films (without oleoresin) and with oleoresins (clove, nutmeg, and black pepper). The bread samples (8 cm x 8 cm) were wrapped with two sheets of the biocomposite material (10 x 10 cm), which were thermally sealed on four sides. The physical-chemical stability (humidity, pH, aw, and weight loss) was evaluated on days 1, 3, 5, and 9 of the storage period at 25 °C and 75% RH. The microbiological and sensory quality of the bread was evaluated at days 1, 4, and 9.

#### *1.5.1. Physical-chemical evaluation of the bread*

Humidity was determined by using the Colombian standard NTC 282 [21]. Five-gram amounts of the sample were taken in a metallic capsule, which was introduced with the samples into a stove with forced-air circulation (FD-53, Binder, Germany) between 100 and 110 °C and their weight was evaluated until constant weight. The pH was determined through NTC 1363 [20]. Water activity was determined by using a portable PawKit water activity (aw) meter (Aqualab, USA). Weight loss was calculated as the difference between the initial weight and the final weight of the bread sample.

### *1.6. Microbiological evaluation of the bread*

A microbiological count was conducted of *Escherichia coli* [22], *Staphylococcus aureus* positive coagulase [23], *Bacillus cereus* [24], along with detection of *Salmonella spp* [25], mold and yeast [26], according to that indicated in the technical standard for bread.

### *1.7. Sensory evaluation of the bread*

A descriptive sensory analysis was conducted with 10 untrained panelists following the NTC 3925 [27]. Attributes of aroma, flavor, hardness, and general acceptability were evaluated in a scale from 1 to 5.

### *1.8. Statistical analysis*

Each treatment was done in triplicate and the results of the properties were evaluated with 5% significance level ($p \leq 0.05$). Analysis of variance (ANOVA) and a multiple comparison test (Tukey) permitted identifying significant differences among the treatments. The results were analyzed through the SPSS Statistics 22.0 software for Windows (SPSS Statistical software, Inc., Chicago, IL, USA).

## 2. RESULTS AND DISCUSSIONS

### *2.1. Physical-chemical and microbiological study of the components of the biocomposite material*

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Black pepper showed, antimicrobial activity against both bacterial strains, starting at 0.5 % (0.5 µL / 100 µL), while clove started at 1 % (1 µL / 100 µL) against *Staphylococcus aureus* and as of 2 % (2 µL / 100 µL) against *Escherichia coli*. Likewise, nutmeg inhibited the growth of *Staphylococcus aureus* as of 0.5 % (0.5 µL / 100 µL) and as of 1 % (1 µL / 100 µL) against *Escherichia coli*.

The gelatin used in this research has a Bloom of 220 – 240 g, which is related to the gel's mechanical elasticity, an important parameter in its capacity and gelling force, for provoking deformation at a certain concentration and temperature.

Figure 1 (a) shows the gelatin's particle size distribution, which had unimodal distribution with a broad range of sizes comprised between 3.8 – 1905 µm. A large peak was observed where there are most of the particles with approximate sizes of 549.5 µm with 10.58% volume, this particle diameter is close to the mean diameter (D [4.3] = 476.987 µm).

Figure 1 (b) displays the gelatin's X-ray diffractogram, showing a dominant peak amply widened at 20.08°, which is characteristic of amorphous materials, indicating that gelatin does not have a periodic structure, given that it comprised of proteins with different structures [28].

Figure 1 (c) shows the gelatin's FTIR spectrum. The band at 3425 $cm^{-1}$ has Type A amides that correspond to stretching of N-H groups with hydrogen bonds; at 2928 $cm^{-1}$ there are the type B amides that corresponds to stretching of $CH_2$; the band at 1638 $cm^{-1}$ is attributed to type I amides; at 1543 $cm^{-1}$, we find the type II amides and at 1232 $cm^{-1}$ the type III amides [29] [30].

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Figure 1 (d) shows three thermal degradation stages. The first stage at 191 °C with 14.15% mass loss, corresponding to water elimination; the second stage at 434 °C has the highest mass loss (55.55%), which was associated to degradation of proteins of high molecular weight and a residual mass (ashes) of 22.97%.

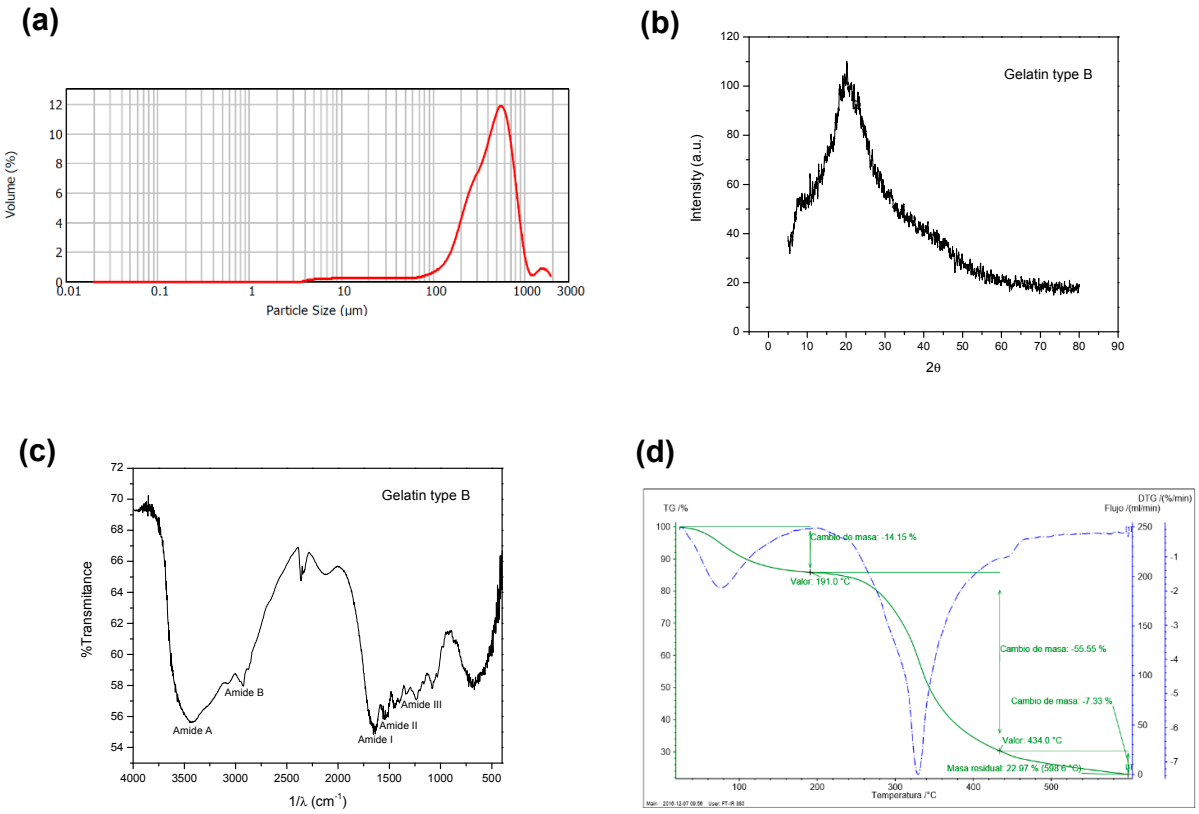


**Figure 1.** Structural and thermal characterization of type B gelatin. **(a)** Particle size distribution; **(b)** X-ray diffractogram; **(c)** FTIR spectrum; and **(d)** TGA thermogram.

Figure 2 (a) illustrates a unimodal distribution with size range between 0.41 and 138 µm. A large peak was observed where there is the majority of particles with

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266 approximate sizes of 26.3 μm with 9.06% volume; this particle diameter was close
267 to the mean diameter (D [4,3] = 25.9 μm).

268 Figure 2 (b) presents the microcrystalline cellulose diffractogram, with a peak in the
269 2θ angle at 22.59°, corresponding to the crystalline and a peak on the 2θ angle at
270 18.8°, corresponding to the amorphous material. The microcrystalline cellulose had
271 79.87% crystallinity, which coincides with the values reported in other studies [31]
272 [32] [33].

273 Figure 2 (c) shows the principal functional groups of the microcrystalline cellulose.
274 At 3399 $cm^{-1}$, stretching of the –OH groups was observed; the CH and $CH_2$ groups
275 are found at 2900 $cm^{-1}$; the band at 1427 $cm^{-1}$ is associated to the presence of
276 HCH groups and OCH vibrations; the band at 1374 $cm^{-1}$ is related to the vibration
277 of CH groups; the band at 1217 $cm^{-1}$ is attributed to C-C groups and at 1039 $cm^{-1}$
278 to stretching of C-O groups [34] [35].

279 Figure 2 (d) shows that the first stage of thermal degradation corresponds to water
280 elimination at 108 °C. Dehydration, decarboxylation, depolymerization, and
281 glycosyl decomposition take place in the second stage between 363 and 589 °C
282 [33] [36] [37].

283 The main active compounds identified in the nutmeg oleoresin were: Mirístic acid,
284 Miristicine, Elemicine, Sabinen and α-Pinen. For the clove oleoresin, Eugenol y
285 Acetate were identified (the most representative phenolic compounds). The black
286 pepper oleoresin presented Piperine as its main component, followed by trans-β-
287 Cariofilene y Limonen. All these compounds have antimicrobial and antioxidant
288 properties, as reported by other authors [38] [39] [40].

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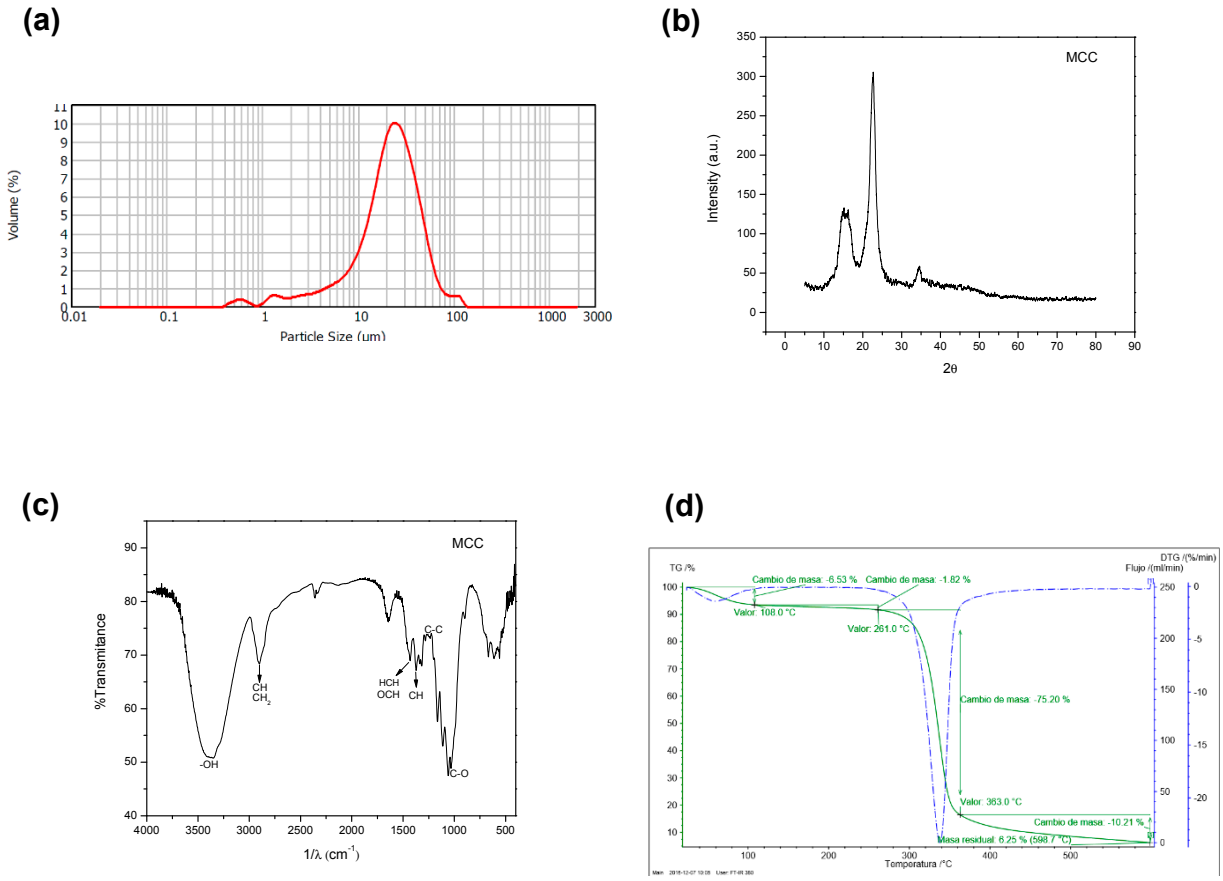


289 **Figure 2.** Structural and thermal characterization of MCC. **(a)** Particle size distribution; **(b)** X-ray
290 diffractogram; **(c)** FTIR spectrum; and **(d)** TGA thermogram.

291

292 *2.2. Characterization of films*

293 To obtain the films, 0.22 g of solution per each $cm^2$ of support plate permitted
294 obtaining a continuous cohesive matrix with thicknesses of 0.058 ± 0.0013 mm
295 containing clove oleoresin, 0.06 ± 0.0021 mm containing nutmeg oleoresin, and
296 0.062 ± 0.0018 mm containing black pepper oleoresin.

297

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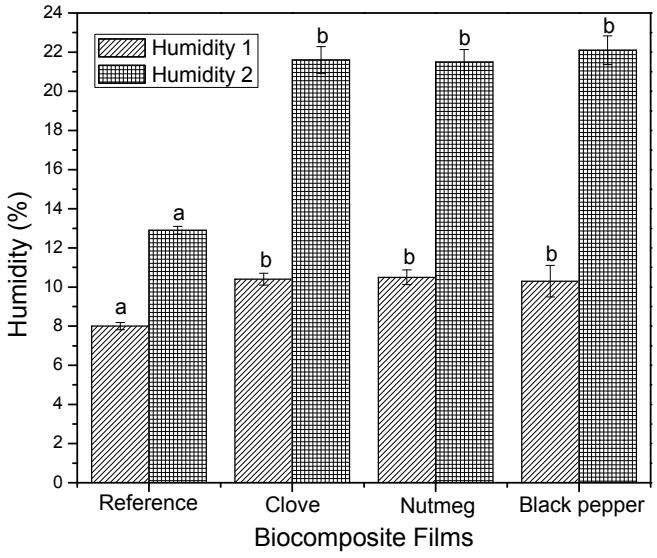
298 *2.2.1. Humidity*

299 Figure 3 presents the humidity of films after drying (Humidity 1) and after the
300 conditioning period (Humidity 2). The humidity content of the biocomposite films
301 containing oleoresins did not have significant differences after drying, obtaining
302 humidity values of 10.4 ± 0.1 g of water/100 g of biocomposite film. After
303 conditioning, the films increased their humidity content to 21.73 ± 0.32 g of
304 water/100 g of biocomposite film. Additionally, the reference films (biocomposites
305 without added oleoresin) had humidity contents of 8 ± 0.18 g of water/100 g of
306 biocomposed film after drying and 12.9 ± 0.19 g of water/100 g of biocomposite
307 film upon completing the conditioning period. The reference films had low humidity
308 after drying and after conditioning with respect to the biocomposite films with
309 oleoresins presenting significant differences ($p < 0.05$), which indicates that
310 oleoresins would be creating bonds with the OH groups of the plasticizer and its
311 polyphenolic groups, thus, maintaining the structure hydrated [41].

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313 **Figure 3.** Humidity content of biocomposite films with oleoresins and reference before and after
314 conditioning.

315

316 *2.2.2. Permeability to water vapor*

317 Upon adding nutmeg, clove, and black pepper oleoresins to the gelatin matrix,
318 microcrystalline cellulose and glycerol (reference film), the permeability gradient
319 diminished significantly from $4*10^{-7}$ g*m/m2*h*Pa to $4*10^{-9}$ g*m/m2*h*Pa (Figure
320 4). Likewise, upon varying the relative humidity, slight permeability changes were
321 observed among the same films. This permeability decrease with respect to
322 reference films is due to the hydrophobic nature of the oleoresins, which avoids
323 absorption and desorption of water molecules, increasing film hydrophobicity
324 because of the interactions between the oleoresin and the other film components,
325 diminishing the availability of the hydrophilic groups. The water molecules diffuse
326 into the continuous polymer phase, where the presence of oily molecules
327 introduces interruptions that increase the tortuosity for transference or mobility of
328 water molecules [42].

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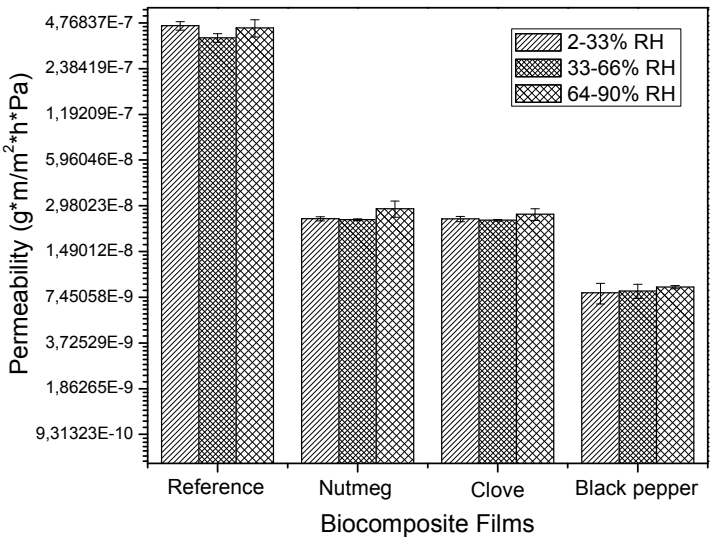


329

330 **Figure 4.** Permeability to water vapor of biocomposite films containing oleoresins and reference
331 (without oleoresin).

332

333 *2.2.3. Mechanical properties*

334 Addition of oleoresins improved significantly ($p < 0.05$) tensile strength (MPa),
335 elongation (%), and Young's modulus (MPa) of biocomposite films compared to the
336 reference films (Figure 5). The results obtained showed that the films without
337 oleoresin were more rigid, possibly due to the restricted mobility of the molecules
338 present in the structure, which could limit their manipulation when used to wrap or
339 package foods. Furthermore, the addition of oleoresins and the higher final
340 moisture content permitted better molecular mobility of the polymer structure, thus,
341 providing greater elasticity to the films containing oleoresins. As noted in Figure 5,
342 when adding black pepper oleoresin, higher elongation values were obtained.
343 Some authors have indicated that the addition of oily substances permits better

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interaction between the polymer chains, which gives the material greater flexibility [43]. Incorporation of oleoresins to the polymer gelatin matrix broadens the potential use of these materials as food packaging.

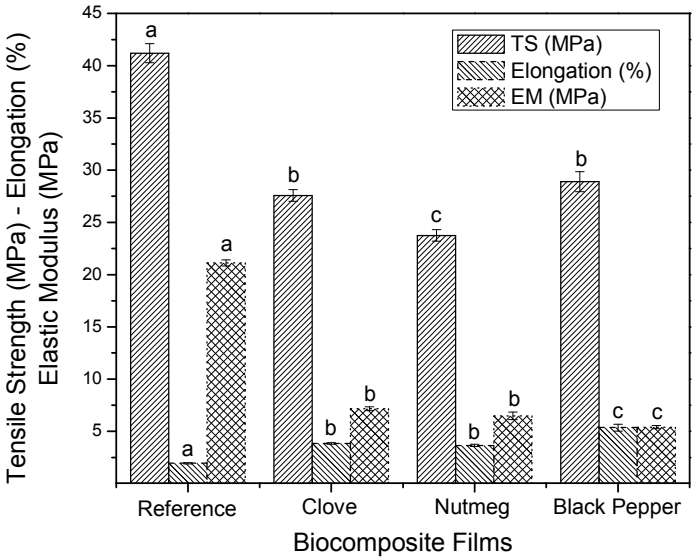


**Figure 5.** Mechanical properties of biocomposite films with oleoresins and reference.

### *2.2.4. Permeability to oil*

As shown in Figure 6, the reference films had a permeability to oil of 0.36 ± 0.0017 $g*mm*m^{-2}*d^{-1}$. Upon adding the oleoresins to the biocomposite material, said permeability diminished between 25% and 30%. Because of their hydrophobic nature, oleoresins manage to impede the passage of molecules of their same nature. Some oil molecules remain on the polymer matrix structure, adhering to the oleoresin molecules. Additionally, upon forming a network between the oleoresins and the other film components, the oil did not manage to effectively pass through

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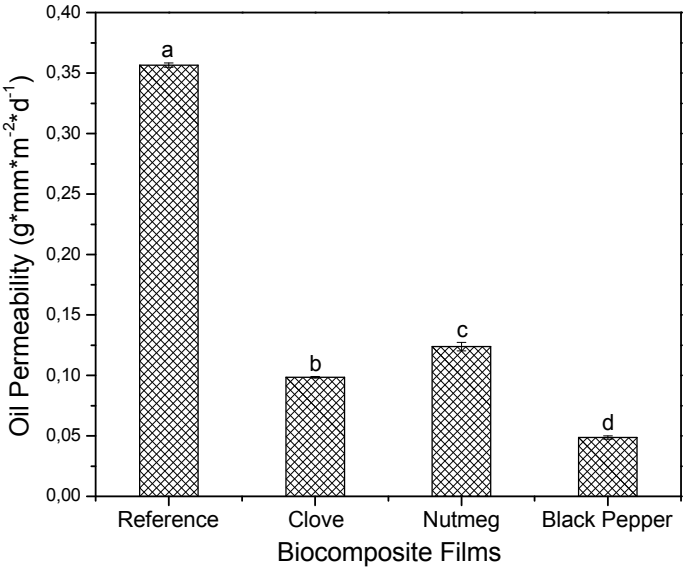
358 the matrix structure. Values of permeability to oil varied significantly in function of
359 the type of oleoresin. This result could be attributed to the physical properties of
360 the oleoresin, such as viscosity, which influenced the structure, resulting in a film
361 which was more compact and resistant.




362

363 **Figure 6.** Oil Permeability of biocomposite films with oleoresins and reference.

364

365 *2.2.5. UV-vis light transmittance values*

366 As noted in Figure 7, the capacity UV-vis light transmission of the gelatin films was
367 significantly reduced with the incorporation of oleoresins ($p < 0.05$). The reference
368 film had a higher transmittance value at 600 nm (75.39%), followed by the films
369 containing black pepper (73.04%), clove (62.81%), and nutmeg (51.34%)
370 oleoresins. This result was attributed to the dispersion of light due to the

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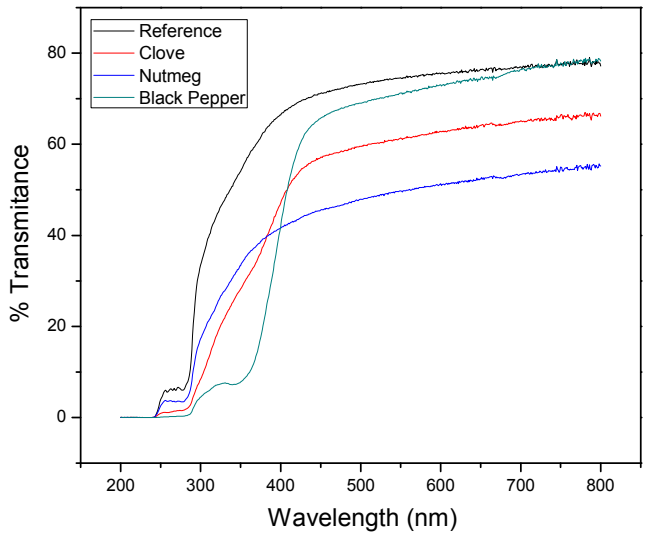
371 characteristic colors of each oleoresin. This property is important for food
372 protection, given that it can avoid photo-oxidation of organic compounds.




373

374 **Figure 7.** Light transmission of biocomposite films with clove, nutmeg, and black pepper oleoresins
375 and reference (without oleoresin).

376

377 *2.2.6. Measurement of sealing resistance*

378 As shown in Figure 8, films containing oleoresins had significant changes with
379 respect to the reference films (without oleoresin) ($p < 0.05$). The reference films
380 had a resistance of 4.93 ± 0.05 MPa. On average, oleoresins increased film
381 resistance to sealing by 8% with respect to that of the reference film, achieving
382 greater fusion between the components when heat was applied through the sealer.

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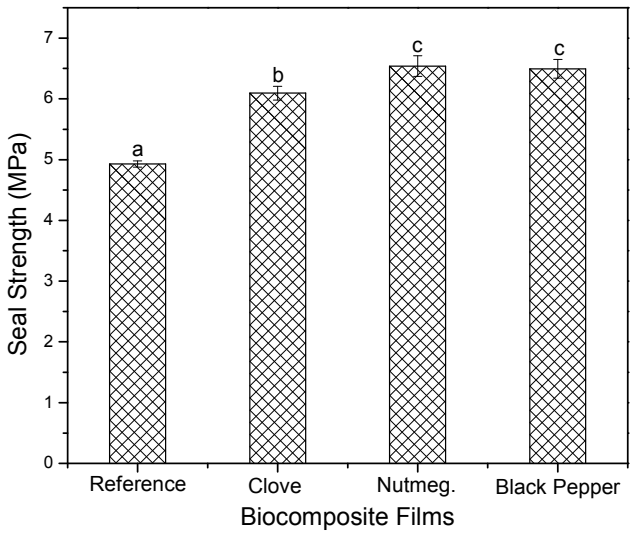


383

384 **Figure 8.** Resistance to sealing of biocomposite films with oleoresins and reference.

385

386 *2.2.7. X-ray diffraction (XRD)*

387 Film crystallinity is one of the determinant parameters for its application as
388 packaging material. Figure 9 (a) shows the diffractogram of the reference film,
389 which in the 2θ angle presented two peaks at 19.61° and 7.09°, with 54.18%
390 crystallinity. The diffractogram (b), which corresponds to the biocomposite film with
391 clove oleoresin presented two peaks in the 2θ angle at 21.33° and 7.76°, with
392 27.83% crystallinity. The diffractogram (c), corresponding to the biocomposite film
393 with nutmeg oleoresin had two peaks in the 2θ angle at 20.28° and 6.71°, with
394 34.68% crystallinity. The diffractogram (d), corresponding to the biocomposite film
395 with black pepper oleoresin had two peaks in the 2θ angle at 19.95° and 7.09°,
396 with crystallinity of 42.29%. When oleoresins were added to the polymer matrix,
397 crystallinity diminished with respect to the reference film. The value of the

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crystallinity obtained for the film containing black pepper oleoresin could indicate good compatibility and interaction with the other components.

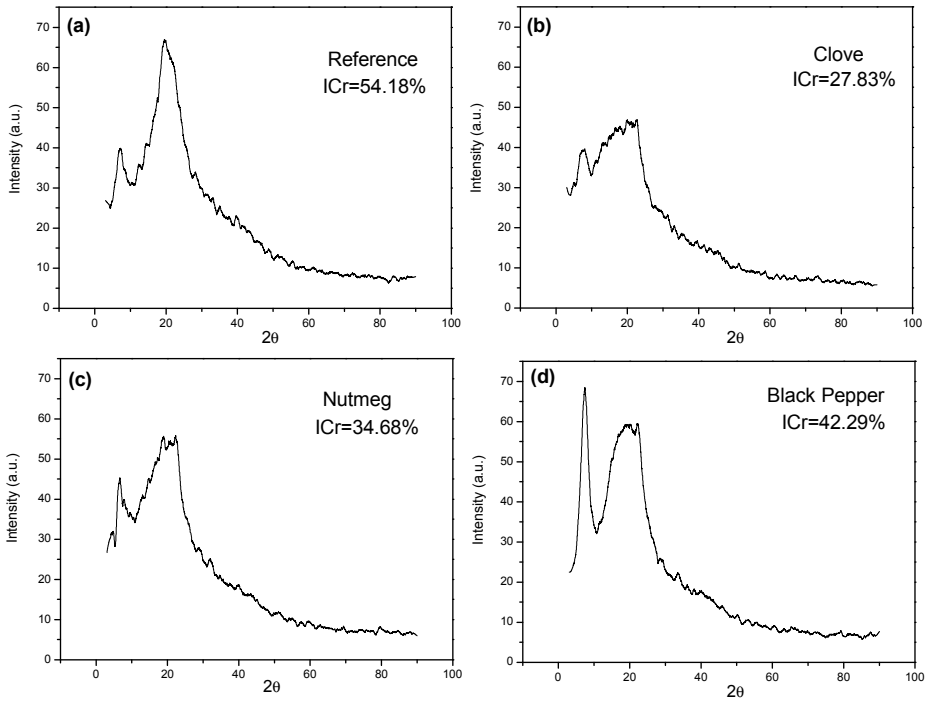


**Figure 9.** Diffractogram biocomposite films (a) Reference (without oleoresin); (b) with clove oleoresin; (c) with nutmeg oleoresin; (d) with black pepper oleoresin.

### *2.2.8. Fourier Transformed Infrared (FTIR) spectroscopy*

In the IR spectrum obtained for the reference films (Figure 10 (a)), it can be noted that the band at 3422 $cm^{-1}$ has OH and N-H groups characteristic of glycerol and gelatin (Type A amides A), respectively. At 2887 $cm^{-1}$, there are CH and $CH_2$ groups; the band at 1428 $cm^{-1}$ has the HCH groups and OCH vibrations; the band

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409 at 1347 $cm^{-1}$ is related to the vibration of CH groups; the band at 1219 $cm^{-1}$ is
410 attributed to C-C groups; and at 1045 $cm^{-1}$ to C-O group stretching. Most of the
411 functional groups coincide with that reported for microcrystalline cellulose and
412 gelatin. The (b), (c), and (d) spectra corresponding to films with clove, nutmeg, and
413 black pepper oleoresins, respectively, were quite similar amongst themselves, but
414 different from the reference film. Hydrogen bonds of O-H and N-H groups gave
415 way to a broad band at 3383 and 3199 $cm^{-1}$ because of the compounds in the
416 polymer matrix (gelatin, cellulose, and glycerol); C-H groups were found at 2946
417 $cm^{-1}$. The band at 1638 $cm^{-1}$ is attributed to C=O bonds; at 1597 and 1479 $cm^{-1}$
418 $NH_2$ primary amines and N-H secondary amines, respectively, and at 1065 $cm^{-1}$ C-
419 O groups. These results indicate that adding oily molecules propitiated a structural
420 change of the polymer matrix material.

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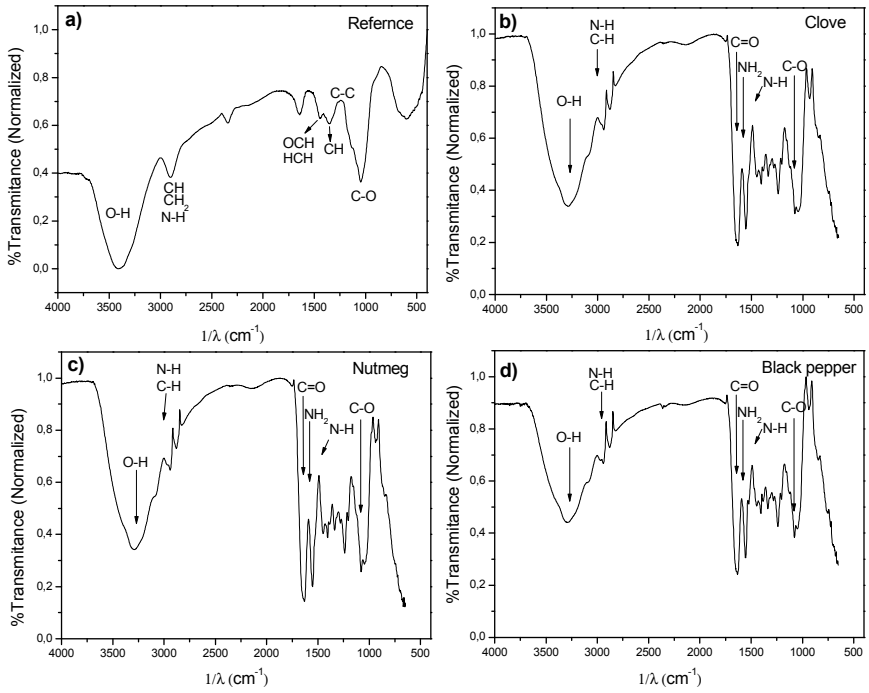


421

422 **Figure 10.** FTIR spectra of biocomposite films. (a) Reference (without oleoresin); (b) with clove
423 oleoresin; (c) with nutmeg oleoresin and (d) with black pepper oleoresin.

424

425 *2.2.9. Color*

426 The color parameters of the biocomposite films are shown in Table 1. Visually, the
427 films had a slightly yellow aspect and their transparency was reduced upon
428 incorporating the oleoresins. The films containing clove, nutmeg, and black pepper
429 oleoresins had ΔE of 4.66, 1.05, and 2.65, respectively, with respect to the
430 reference film.

431

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433 **Table 1.** CIELab color parameters obtained for biocomposite films.

| Biocomposite films | Color parameters | | | |
|---|---|---|---|---|
| | $L^*$ | $a^*$ | $b^*$ | $\Delta E^*$ |
| Reference | 92,09 ± 0.094 [a] | -1,46 ± 0.029 [a] | 4,73 ± 0.33 [a] | --- |
| Clove | 89,59 ± 0.22 [b] | -1,16 ± 0.039 [a] | 8,65 ± 0.47 [b] | 4,66 ± 0.51 [a] |
| Nutmeg | 91,30 ± 0.099 [a] | -1,42 ± 0.014 [a] | 5,44 ± 0.12 [a] | 1,05 ± 0.15 [b] |
| Black pepper | 90,45 ± 0.77 [b] | -1,91 ± 0.10 [a] | 6,73 ± 0.79 [c] | 2,65 ± 0.99 [c] |

434 Reported values correspond to the mean ± standard deviation. Different letters in the same column indicate
435 significant differences ($P < 0.05$). ΔE * calculated on the basis of the L *, a *, b * values of the reference film.

436

437 *2.2.10. Thermogravimetric analysis (TGA)*

438 All the thermograms (not shown) detected four changes of temperature and mass;
439 the first stage revealed a weight loss from 12.73% to 14.37% at temperatures
440 ranging from 150 to 161 °C for all films: This change is related to the loss of free
441 water present in the films and volatile compounds of the oleoresins. On the second
442 stage, the reference biocomposite films and clove films, at temperatures from 273
443 and 276 °C lost 15.52% and 16.75% mass, respectively; while the films with
444 nutmeg and black pepper at temperatures from 284 and 274 °C lost 20.42% and
445 20.61% mass, respectively. Within these temperature ranges, degradation
446 occurred of low molecular weight proteins of the polymer (gelatin) and other
447 compounds present in the plasticizer (glycerol). During the third stage, the
448 temperature changes for the biocomposite films ranged between 386 and 402 °C
449 with weight loss between 42.44% and 46.45%, where decomposition took place of
450 high molecular weight proteins present in the gelatin, as well as the degradation of
451 the microcrystalline cellulose due to the rupture of the glycosidic bonds. On the
452 fourth stage, the temperature changes occurred between 598.5 and 598.7 °C for all

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453 the films with weight loss between 15.73% and 16.81%, where degradation
454 possibly occurred of the nonvolatile phase of the oleoresins and other compounds
455 of high molecular weight present in the films.
456

457 *2.3. Physical-chemical evaluation of the bread*
458

459 *2.3.1. Moisture*
460 Table 2 shows that, during the first three days of storage, the bread samples
461 packaged in films containing oleoresins maintained desirable moisture contents
462 according to the norm, while the bread samples without film and those packaged in
463 reference films (without oleoresin) had a significant decrease ($p < 0.05$) in moisture
464 content. After the nine-day storage period, it was noted that the bread samples
465 packaged in films containing black pepper oleoresin achieved a lower loss of
466 moisture compared to the rest, which is desirable to preserve the product's texture
467 characteristics.
468

469 *2.3.2. pH*

470 The pH values obtained for all the samples evaluated during the storage period
471 remained within the quality range established by the norm [20]. At nine days of
472 storage, the bread samples packaged in reference films had higher pH values
473 compared to those packaged in films containing oleoresins (Table 2). This result
474 was associated to the presence of acid compounds in the oleoresins. Additionally,
475 bread samples stored without film had the lowest pH values, which could be

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476 caused by enzymatic and microbiological reactions catalyzed by the product's
477 exposure to the environment.

478

479 *2.3.3. Water activity ($a_w$)*

480 On the third day of storage, the bread samples packaged in films containing
481 oleoresins maintained a higher water activity as compared to the samples without
482 film and those packaged in reference films. The latter had the lowest values of $a_w$
483 due to the direct exposure of the product to the environment (Table 2). Among the
484 bread samples packaged in films containing oleoresins, those containing black
485 pepper had the highest values of $a_w$. This behavior coincides with the results on
486 humidity content of the bread and permeability to water vapor of said films.

487

488 *2.3.4. Weight loss (%wl)*

489 After three days of storage, the bread samples stored in films containing oleoresins
490 had the lowest weight loss values (20% – 28%). Upon completing the storage
491 period, the bread samples packaged with films containing clove and nutmeg
492 oleoresins had statistically similar weight loss values, while the samples packaged
493 in films containing black pepper oleoresin had the lowest weight loss values (13%).
494 These results confirm the greatest water vapor barrier exerted by the films when
495 incorporating black pepper oleoresin in their formulation.

496

497 **Table 2.** Physical-chemical characteristics of bread packaged in biocomposite films and stored during nine days at 25 °C and 75% RH.

| Bread packed in biocomposite films | Humidity | | | | pH | | | | aw | | | | %pp | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Día 1 | Día 3 | Día 5 | Día 9 | Día 1 | Día 3 | Día 5 | Día 9 | Día 1 | Día 3 | Día 5 | Día 9 | Día 3 | Día 5 | Día 9 |
| Reference | 26.34 ± 0.79 [a] | 17.94 ± 0.55 [a] | 9.61 ± 0.29 [a] | 7.11 ± 0.2 [a] | 5.73 ± 0.03 [a] | 5.71 ± 0.02 [a] | 5.67 ± 0.02 [a] | 5.64 ± 0.02 [a] | 0.84 ± 0.006 [a] | 0.75 ± 0.006 [a] | 0.58 ± 0.01 [a] | 0.44 ± 0.01 [a] | 45 ± 1.4 [a] | 54 ± 1.5 [a] | 34 ± 1.5 [a] |
| Clove | 25.69 ± 0.95 [b] | 21.09 ± 0.21 [b] | 11.61 ± 0.9 [b] | 8.86 ± 0.39 [b] | 5.73 ± 0.03 [a] | 5.65 ± 0.02 [a] | 5.63 ± 0.02 [a] | 5.59 ± 0.01 [a] | 0.84 ± 0.006 [a] | 0.79 ± 0.005 [a] | 0.63 ± 0 [a] | 0.54 ± 0.006 [b] | 27 ± 1.3 [b] | 29 ± 1.5 [b] | 17 ± 0.9 [b] |
| Nutmeg | 26.00 ± 0.55 [a] | 21.05 ± 0.53 [b] | 12.47 ± 0.9 [c] | 8.55 ± 0.59 [b] | 5.73 ± 0.03 [a] | 5.63 ± 0.02 [a] | 5.61 ± 0.02 [a] | 5.57 ± 0.02 [a] | 0.84 ± 0.006 [a] | 0.79 ± 0.01 [a] | 0.67 ± 0.047 [a] | 0.51 ± 0.01 [a] | 28 ± 1.5 [b] | 32 ± 1.5 [b] | 23 ± 1.7 [b] |
| Black pepper | 25.33 ± 0.49 [b] | 22.21 ± 0.85 [c] | 11.37 ± 0.67 [b] | 9.77 ± 0.45 [c] | 5.73 ± 0.03 [a] | 5.64 ± 0.02 [a] | 5.62 ± 0.02 [a] | 5.57 ± 0.02 [a] | 0.84 ± 0.006 [a] | 0.8 ± 0.01 [a] | 0.67 ± 0.01 [a] | 0.59 ± 0.006 [b] | 20 ± 0.8 [c] | 17 ± 0.9 [c] | 13 ± 1.3 [c] |
| Without film | 25.59 ± 0.3 [b] | 8.45 ± 0.69 [d] | 5.87 ± 0.25 [d] | 4.71 ± 0.81 [d] | 5.73 ± 0.03 [a] | 5.59 ± 0.02 [a] | 5.56 ± 0.01 [a] | 5.55 ± 0.04 [a] | 0.84 ± 0.006 [a] | 0.52 ± 0.01 [b] | 0.52 ± 0.006 [b] | 0.4 ± 0.01 [c] | 69 ± 1.8 [d] | 41 ± 1.5 [d] | 24 ± 0.7 [c] |

503 Reported values correspond to the mean ± standard deviation. Different letters in the same column indicate significant differences (P < 0.05).

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508 ### 2.4. *Microbiological evaluation of the bread*

509 Table 3 presents the microbiological quality of bread samples packaged in
510 biocomposite films on days 1, 4, and 9 of storage. Upon completing the storage
511 period evaluated, higher growth of mold and yeast occurred on bread samples
512 without film (2440 CFU/g) and in those packaged in reference films (460 CFU/g).
513 Similar behavior occurred in the *S. aureus* count (440 and 50 CFU/g, respectively).
514 In addition, the microbiological analyses evidenced no growth of *S. aureus* and
515 lower growth of mold and yeast occurred in the bread samples packaged in films
516 containing oleoresins. During the storage period, it was noted that using
517 biocomposite films containing black pepper oleoresin was the most effective
518 packaging material in terms of the product's microbiological quality.

519

520 **Table 3.** Microbiological analysis of the bread packaged in biocomposite films and stored during
521 nine days at 25 °C and 75% RH.

| **Bread packed in biocomposite films** | **count *S. aureus* (CFU/g)*** | | | **Count *molds and yeasts* (CFU/g)*** | | |
|---|---|---|---|---|---|---|
| | **Day 1** | **Day 4** | **Day 9** | **Day 1** | **Day 4** | **Day 9** |
| Reference | < 10 | < 10 | 50 | < 10 | 100 | 460 |
| Clove | < 10 | < 10 | < 10 | < 10 | 10 | 50 |
| Nutmeg | < 10 | < 10 | < 10 | < 10 | 10 | 40 |
| Black pepper | < 10 | < 10 | < 10 | < 10 | < 10 | 20 |
| Without film | < 10 | < 10 | 440 | < 10 | 80 | 2440 |

522

523 ### 2.5. *Sensory analysis of the bread*

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Figure 11 (a) displays the evolution of the aroma attribute of bread in Lot 2 during storage time. Note that on day 1, the maximum score was obtained (*I like it a lot*) for all the bread samples. On day 4, perceptions were different where the highest score was obtained by the sample packaged in the film with clove and reference (*I like it)* and the lowest scores were obtained by the samples packaged in films with black pepper and without film *(I neither like or dislike it).* Figure 11 (b) presents the flavor evolution of the bread packaged in biocomposite films through nine days of storage. It was observed that on day 1 the samples had the same score *(I like it a lot)*; on day 4, the samples packaged in films with clove and black pepper were better perceived *(I like it)* than those packaged in films with nutmeg, reference, and without film *(I neither like or dislike it).* Figure 11 (c) shows the hardness evolution of the bread packaged in biocomposite films during the storage period evaluated. On day 1, the panelists assigned the same score to all the bread samples (soft). On day 4, the samples packaged in films with black pepper were scored as soft; the samples packaged in films with nutmeg and clove were perceived as firm and the bread samples packaged in reference films and without film were perceived as very hard. The general acceptability of the bread packaged in biocomposite films is shown in Figure 11 (d). The sample with the highest acceptability during the storage period was that packaged in films containing black pepper, followed by those packaged in films containing clove or nutmeg. The samples with the best acceptability were those packaged in reference films and without film.

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545 **Figure 11.** Evolution of sensory attributes during nine days of storage (25 °C and 75% RH) of bread
546 packaged in biocomposite films. (a) Aroma; (b) flavor; (c) hardness; (d) general acceptability.

547

548 CONCLUSIONS

549 This study evidenced that clove, nutmeg, and black pepper oleoresins presented
550 inhibition against *S. aureus* and *E. coli*. Black pepper oleoresin was the compound
551 that inhibited both strains evaluated at a lower concentration (0.5%). Incorporation
552 of the oleoresins studied upon the polymer matrix managed to decrease the oil and
553 water vapor permeability values by 20 to 30%. Furthermore, the elasticity and
554 resistance to sealing, of biocomposite films based on gelatin and microcrystalline

555 cellulose was increased by 5%. Upon exploring the potential use of films containing
556 the different oleoresins, it was evident that the incorporation of black pepper
557 oleoresin was the most effective packaging material for maintaining the physical-
558 chemical, microbiological, and sensory quality of bread without adding
559 preservatives during nine days of storage.

560

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569

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