Cebrián-Lloret, V.; Göksen, G.; Martínez-Abad, A.; López-Rubio, A.; Martínez-Sanz, M. (2022). Agar-based packaging films produced by melt mixing: Study of their retrogradation upon storage. Algal Research. 66:1-10. https://doi.org/10.1016/j.algal.2022.102802

AGAR-BASED PACKAGING FILMS PRODUCED BY MELT MIXING: STUDY OF THEIR RETROGRADATION UPON STORAGE

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Abstract

The ability of agar with different purification degrees to produce packaging films has been evaluated and the stability of the obtained materials after prolonged storage has been investigated. The less purified agar resulted in films with higher water vapour permeability and lower mechanical performance than pure commercial agar. However, the commercial agar film required the addition of a plasticiser to produce films that could be manipulated. It has also been observed that prolonged storage at 53% RH results in a change in the semi-crystalline structure of the agar and in water-polysaccharide interactions. As a result, pure agar films undergo a rigidizing effect resulting in unmanageable films after 7 days of storage. The presence of glycerol improved the stability of the films by limiting the structural changes up to 14 days of storage. In contrast, the films from the least purified agar extract, seemed to be less affected by moisture, showing a higher stability during storage. This points to the potential of the less purified extract to be used as an additive to reduce costs and improve the storage stability of pure agar films.

Keywords: seaweed; valorization; retrogradation; agar; biopolymers; packaging

1. Introduction

The excessive production of petroleum-derived plastics has become a major problem in recent years. This is mainly due to the fact that these materials are not biodegradable; consequently, they accumulate in natural ecosystems for hundreds of years causing severe environmental problems [1–3]. Although recycling strategies have been promoted worldwide over the last decade, the replacement of conventional plastics with biodegradable polymers obtained from renewable natural resources, i.e. biopolymers, is being studied and considered as a more sustainable alternative to address this problem in the long term. However, the production costs of biopolymers are too high to compete against conventional petroleum-based polymers on the market nowadays. On top of that, their properties are not yet comparable to those of benchmark synthetic polymers, especially in terms of mechanical and barrier properties. Moreover, the raw materials commonly used for the production of biopolymers come from land-based crops and, thus, they compete with their main use as food sources. This is why, as an alternative, marine resources (e.g. aquatic plants or seaweeds) are being explored as a source for obtaining biopolymers [4–8]. The cell walls of seaweeds are rich in polysaccharides, whose composition depends on the seaweed species, being cellulose, the most important structural component providing mechanical strength, while other polysaccharides are responsible for different functionalities. In particular, sulphated polysaccharides (i.e. carrageenan and agar) are highly relevant to the food industry, due to their extensive use as gelling agents, thickeners and stabilizers [9,10]. Amongst them, agar, which is typically found in the cell walls from some red seaweeds (Rhodophyceae) [11], has a great industrial relevance as gelling agent, not only for food applications, but also for microbiology. This polysaccharide contains two main components: agarose and agaropectin. Agarose constitutes the gelling fraction and consists of alternating units of

β-D-galactopyranosyl and 3,6-anhydrous-α-L-galactopyranosyl. On the other hand, agaropectin presents a structure similar to agarose, but contains 5-10% sulphate esters in addition to other residues such as methoxyl groups and pyruvic acid [12–14]. The agar extraction protocol is very well established at industrial scale; it involves the application of alkaline pre-treatments, followed by high temperature and pressure extraction and several filtration processes and freeze-thawing cycles to purify the product [6,15]. Since this is a very time and energy consuming process, efforts are being made to develop more energy-efficient extraction protocols. For instance, previous studies have reported on alternative methods for obtaining less purified agar fractions with good antioxidant properties, reducing the total extraction time and the amount of extraction steps [13,16,17]. Although the less purified agars produced by means of these simplified extraction protocols may not be suitable for applications where high purity is a requirement, they might be valuable for the development of bio-based packaging materials with a more sustainable character and reduced production costs. In fact, a recent work showed that the presence of other polysaccharides (mainly floridean starch) and proteins in less purified agar-based extracts had a positive effect on the mechanical and water barrier performance of the films produced by the casting methodology [13]. This work showed that agar-based films have promising properties for the development of sustainable bio-based films for food packaging applications. However, the solvent casting methodology used lacks industrial applicability. Inspired by the existing works reporting on the processing of other commercial biopolymers, in this work we have developed a simple methodology to produce agar-based films by means of melt mixing and hot pressing.

Amongst the most popular plant-derived polysaccharides currently used for bio-based packaging production, starch is undoubtedly one of the most promising materials due to

its abundance, cost-effectiveness and excellent film-forming capacity [18]. Although starch can be processed through different techniques, the basis for its processing lays in the gelatinization phenomenon: under adequate heat and moisture conditions, the semi-crystalline structure of starch is partially or completely destroyed (phenomenon known as cooperative melting), hence producing an amorphous material which can be easily processed. Interestingly, after processing, upon cooling and storage, the amylose and amylopectin chains in starch can re-associate to form a more ordered structure via hydrogen bonding [19,20]. This process, referred to as retrogradation, leads to the modification of several properties such as opacity, mechanical performance and vapour barrier capacity of the films; thus, in food packaging applications the shelf life and quality of the packaged product can be strongly affected due to changes in starch structure upon storage [21]. Based on the behaviour of starch, we developed a methodology in which agar-based extracts are subjected to heat and high moisture conditions during processing in an internal mixer. This allows to dissolve the agar molecular chains, which then are able to re-associate upon cooling, similarly to the gelation process. Since the formation of bundles of agar double helices has been shown to result in the formation of semi-crystalline structures [13], it is reasonable to hypothesize that the properties of agar-based materials may also be modified with storage time due to re-crystallization processes taking place. Thus, the aim of this study was to determine the processability of agars with different degrees of purity (one commercial grade with high purity and one less purified agar-based extract produced by a more energy-efficient extraction protocol) by means of the melt mixing technique and evaluate the performance properties of the obtained films. Furthermore, the evolution of these properties upon prolonged storage has been assessed to determine the effect of possible re-crystallization processes in the produced films.

2. Materials and methods

2.1 Materials

The seaweed *Gelidium sesquipedale* and the commercial grade agar PRONAGAR were kindly donated by Hispanagar (Burgos, Spain). The dried seaweed was ground to powder before further processing. Glycerol, used as plasticizer, was purchased from Panreac Quimica, S.A. (Castellar Del Vallés, Barcelona, Spain).

2.2 Production of the less purified agar-based extract

A less purified agar-based extract was produced from the raw seaweed by applying a hot water treatment, as previously described by Martinez-Sanz et al.[13]. Briefly, 50 g of dried seaweed powder were immersed in 500 mL of distilled water and heated at 90 °C for 2 h. Then, the agar-based solution was separated from the solid residue by filtration using a muslin cloth when the solution was still hot. The filtrate was allowed to form a gel upon cooling and it was subsequently frozen overnight at -21 °C. The material was then subjected to two freeze-thaw cycles (-21°C/25°C) to remove water-soluble impurities. Finally, the obtained gel was freeze-dried [13]. The obtained agar-based extract, coded as HW, has been previously characterized, showing a total carbohydrate content of ca. 39-42% (from which galactose represented 74%), ca. 11-14% proteins, ca. 35% ash and ca. 3% polyphenols [13, 22].

2.3 Preparation of agar-based films

Agar-based films were prepared by melt compounding, followed by compression molding, using formulations based on mixtures of agar and water, with and without the addition of a plasticizer. In the case of the pure commercial agar, the agar:water ratio used was 1:3 (w/w), while a higher ratio of 1:0.5 (w/w) was used in the case of the less purified

agar extract HW, since the agar content in that sample was lower. These ratios were selected on the basis of preliminary trials, to ensure a good balance between proper processability (i.e. enough water to aid the cooperative melting of agar) and good mechanical integrity of the obtained films (since too high water contents led to sticky materials, while too low water contents led to heterogeneous films). In the case of the pure commercial agar the obtained films showed a rigid behaviour and, therefore, we decided to explore the effect of adding a plasticizer on the final properties of the films. To do so, an additional formulation containing glycerol (30% with respect to the amount of agar in the mixture) was also prepared by adding the plasticizer to the commercial agar to form the final paste with water before the melt mixing step. The addition of plasticizer was not necessary in the case of the less purified agar films, which showed a much less rigid behaviour. It was hypothesized that the presence of other compounds in the extract could exert a plasticizing effect. The obtained pastes were then melt-mixed in a Brabender Plastograph (Germany) internal mixer at a temperature of 110 ℃ and 60 rpm for 2 min. Subsequently, 4 g of the obtained blends were spread evenly on Teflon films and placed in a compression mould (Carver 4122, USA) at a pressure of 16 tons and 110 ℃ for 4 min to form one film. The films were then stored in cabinets equilibrated at a relative humidity of 53% and 25℃ for the 30 days of the study. The samples were coded as follows: COMM (commercial agar), COMM+GLY (commercial agar with glycerol as plasticizer) and HW (less purified agar-based extract). Samples were taken for further analyses right after being processed (t=0) and after different storage periods (t=3, 7, 14 and 30 days).

2.4 Moisture content

The variability in the moisture content of the films over time was calculated from the difference between the weight after drying and the initial weight of the samples, before placing them in an oven at 60 °C for 24 hours.

2.5 Fourier transform infrared spectroscopy (FT-IR)

The films were analyzed by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolution in a wavelength range between 400-4000 cm^{-1} and averaging a minimum of 32 scans.

2.6 X-ray diffraction (XRD)

XRD measurements were carried out on a D5005 Bruker diffractometer. The instrument was equipped with a Cu tube and a secondary monochromator. The configuration of the equipment was θ–2θ, and the samples were examined over the angular range of 3°–60° with a step size of 0.02° and a count time of 200 s per step. Peak fitting was carried out using the Igor software package (Wavemetrics, Lake Oswego, Oregon), using the same protocol described in a previous work [13] The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

$$\chi^2 = \sum \left(\frac{y-y_i}{\sigma_i}\right)^2 \quad (1)$$

where y is a fitted value for a given point, y_i is the measured data value for the point and σ_i is an estimate of the standard deviation for y_i. The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize χ^2. The crystallinity index was determined from the obtained fitting results by applying the following equation:

$$X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (2)$$

where A_{Total} is the sum of the areas under all the diffraction peaks and $\Sigma A_{Crystal}$ is the sum of the areas corresponding to the crystalline peaks.

2.7 Scanning electron microscopy (SEM)

SEM was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-16 mm. Small pieces of the agar films were sputtered with a gold–palladium mixture under vacuum during 2 min before their morphology was examined.

2.8 Water vapor permeability (WVP)

Direct permeability to water was determined from the slope of the weight gain versus time curves at 24ºC. The films were sandwiched between the aluminum 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 sealability. These permeability cells containing silica were then placed in an equilibrated relative humidity cabinet at 75% RH and 25°C. The weight gain through a film area of 10 cm^2 was monitored and plotted as a function of time. Cells with aluminum films (with thickness of ca. 11 µm) were used as control samples to estimate weight gain through the sealing. The WVP was calculated according to the following equation:

$$WVP = \frac{\text{WVTR x L}}{\Delta P}$$

Where WVTR is the water vapor transmission rate (kg/s·m2) (calculated from the slope of the linear region of the weight gain vs. time, divided by the exposed film area), L is

the mean film thickness (m), and ΔP is the difference of vapor pressure between the two sides of the film (Pa). The tests were done at least in triplicate

2.9 Mechanical properties

Tensile tests were carried out at ambient conditions of typically 24ºC and 50%RH on a Mecmesin MultiTest 1-i (1 kN) machine (Virginia, USA) with the Emperor™ software. Pre-conditioned rectangular-shaped specimens with initial gauge length of 8 cm and 1 cm in width were cut directly from the films. A fixed crosshead rate of 25 mm/min was utilized in all cases. The elastic modulus (E), tensile strength (TS), and elongation at break (ε_B) were determined from the stress-strain curves, estimated from force–distance data obtained for the different films. At least, three specimens of each film were tensile tested as to obtain statistically meaningful results.

2.10 Statistical analysis

All data have been represented as the average ± standard deviation. Different letters show significant differences both in tables and graphs ($p \leq 0.05$). Analysis of variance (ANOVA) followed by a Tukey-test were used.

3. Results and discussion

In this work commercial agar and a less purified agar-based extract (described in section 2.2) were processed by melt mixing and compression moulding to prepare films for food packaging applications. As commented in section 2.3, adjusting the water content in the formulations was crucial to obtain homogeneous materials with a good mechanical integrity. Figure 1A shows the visual appearance of the films. As observed, the colour and transparency of the films were significantly affected by the agar purification. While

241 the films prepared from pure commercial agar were highly transparent, the HW films
242 presented a brownish coloration and were more opaque. This is most likely due to the
243 presence of other components such as proteins and polyphenols in the agar-based extract.
244 The films' surface morphology was analysed by SEM and representative images are
245 shown in Figures 1B-G. A noticeable morphological difference was observed between
246 the films right after being processed (t=0). Furthermore, the morphology of the films
247 evolved differently with storage time depending on the formulation. As it can be
248 observed, the COMM film showed a very smooth and homogeneous morphology, which
249 was maintained over storage time. In contrast, with the addition of glycerol the surface of
250 the COMM+GLY film became rougher and more heterogeneous, with small particles,
251 probably corresponding to glycerol, homogeneously distributed through the whole film
252 surface. Interestingly, these particles were no longer visible in the film after 30 days of
253 storage and the surface of the film was characterized by the appearance of large cracks,
254 which may be due to glycerol migration through the film and/or dehydration of the
255 material. In fact, previous studies have demonstrated that glycerol undergoes migration
256 in other polysaccharide-based materials, such as starch films [23]. The HW film presented
257 a very different microstructure, with significantly rougher surfaces and large particles
258 distributed along the surface, probably due to the presence of components other than agar
259 in these samples. Given the appearance of these particles, which resembled crystalline
260 clusters, and the high ash content previously reported for this type of agar [13], it is
261 suspected that they corresponded to minerals such as silica (SiO_2) and weddellite
262 ($CaC_2O_4 \cdot 2H_2O$), which have been previously reported to remain in the agar-based extract
263 [13]. The appearance of these particles changed after 30 days of storage, becoming more
264 diffuse and with a less defined structure, which may be attributed to the hydration of the
265 salts upon storage at a constant relative humidity of 53%.

These results evidence that water may be playing an important role in the structural variation of the films upon storage. Thus, the moisture content of the films was monitored during the 30 days of the study (cf. Figure S1) in order to better understand the changes in the properties of the films during storage. In the case of the COMM films, the moisture content increased during the first 14 days of storage (from 16% w/w to 178% w/w) and after that, the moisture content of the film was stabilised. As expected, the presence of a highly hydrophilic plasticizer such as glycerol led to a higher moisture content in the films at t= 0 (68% w/w). In that case, the moisture also increased during the first 14 days of storage (up to 676% w/w), and after that, the films dehydrated significantly (reaching a moisture content of ca. 246% w/w). In contrast, the HW films showed a less pronounced increase in the moisture content during the first 7 days of storage (from 110% w/w to 170% w/w) and after that, the moisture content slightly decreased and then remained quite stable until the end of the study (reaching a final value of ca. 100% w/w). This hydration-dehydration phenomenon may be related to a structural re-organization of the agar and changes in the type of interactions being established with water. These structural changes seemed to be less evident in the HW films, which is reasonable given the lower agar content in this material. It should also be noted that for the as-prepared films, the moisture content was the highest in the HW films, even though the amount of water added to process the formulations in the internal mixer was lower in that case (cf. section 2.3). This may be related to the more amorphous structure of agar (as suggested by the XRD results presented later) and explain why these materials did not require the addition of any plasticizer to obtain flexible and easy-to-handle films, since bulk water seemed to act as a plasticizer.

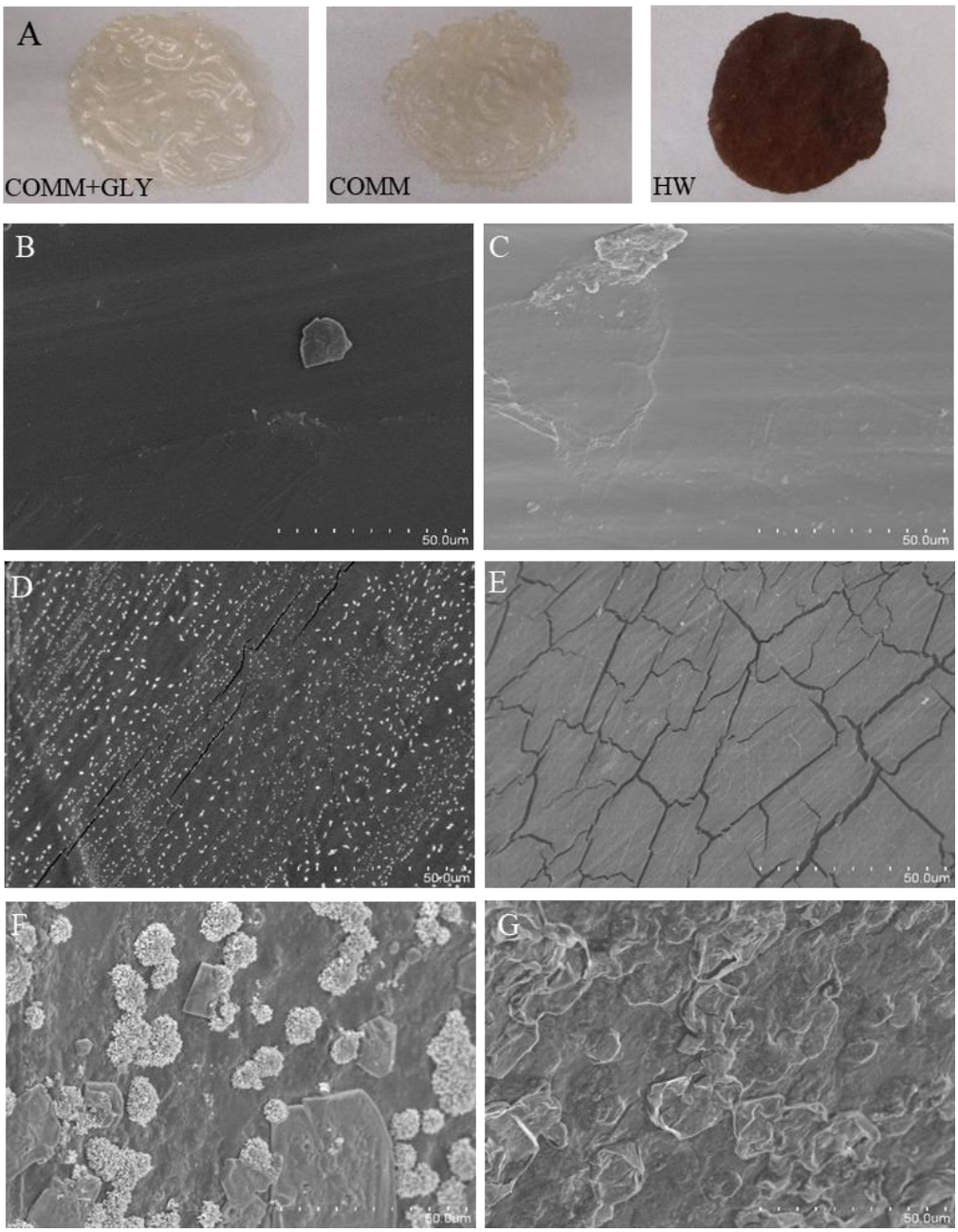


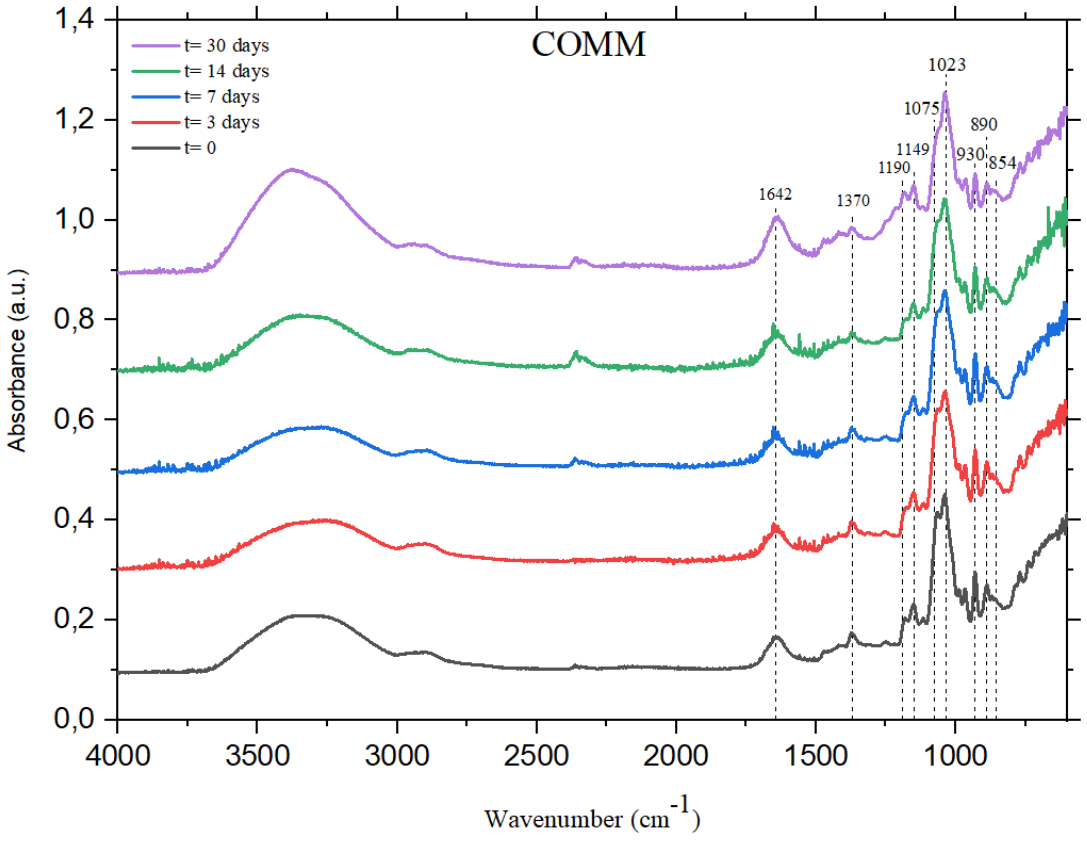
Figure 1. (A) Visual appearance of the agar-based films after being processed (t=0). (B-G) SEM images of the surface of agar films: (B) COMM t= 0; (C) COMM t= 30 days; (D) COMM+GLY t= 0; (E) COMM+GLY t= 30 days; (F) HW t=0 and (G) HW t= 30 days. Scale bars correspond to 50 μm.

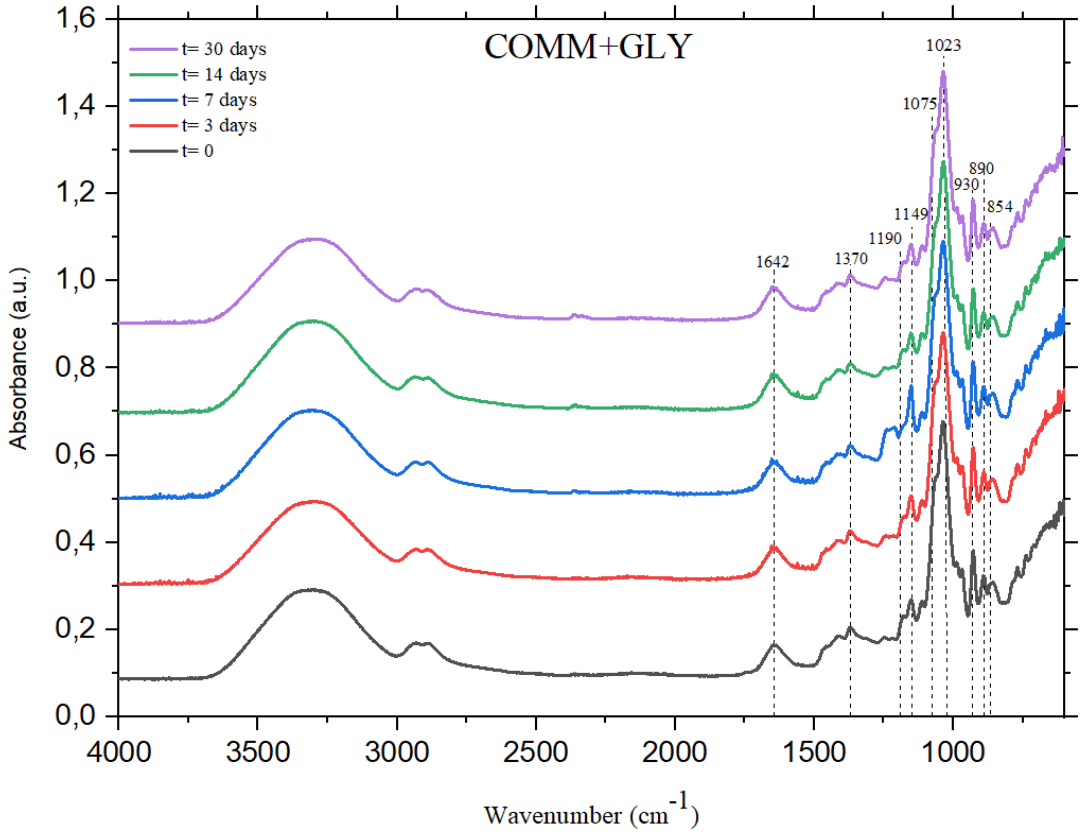
FT-IR characterization of the obtained films was carried out to identify changes in the molecular structure of the films over time. Figure 2 shows the spectra of the different

297 freshly prepared films (t=0) and after different storage periods. As observed, the three
298 films presented the most characteristic agar bands, located at 890 cm^{-1}, assigned to the C-
299 H bending at the anomeric carbon in β-galactopyranosyl residues, and at 930 cm^{-1},
300 associated with the vibration of the C-O-C bridge of the 3,6-anhydro-galactose
301 [12,13,24]. As expected, the relative intensity of these bands was stronger for the films
302 made from pure commercial agar, while they were much less intense in the HW film,
303 which is reasonable given the lower agar content in the less purified agar-based extract.
304 Other agar-characteristic bands, related to the sulfation degree, are those located at 1370
305 cm^{-1}, 1243 cm^{-1} and 1190 cm^{-1}, which are linked to the vibration mode of the sulphate
306 groups, the band at 1149 cm^{-1}, which is mainly associated to the vibration mode of the
307 ester-sulphate bond, and the small signal at 854 cm^{-1}, which is assigned to the sulphate at
308 C-4 of galactose [12,13,25]. Despite the lower agar content in the HW extract, the relative
309 intensity of most of these bands was quite high, which is due to the greater degree of
310 sulphation in this type of extracts, as previously reported [13,16]. The broad band at 1642
311 cm^{-1} confirmed the presence of significant amounts of bound water in all samples, which
312 is not surprising due to the hydrophilic character of agar. In the particular case of the HW
313 film, this band overlaps with the amide I band, due to the presence of considerable
314 amounts of proteins in this sample [13,26]. Interestingly, while the spectra from the
315 COMM+GLY and HW samples did not extensively change upon storage, some evident
316 changes were detected in the case of the COMM film. The most notable changes occurred
317 in the relative intensity of the bands located at 1190 cm^{-1}, 1075 cm^{-1} and 1023 cm^{-1}, which
318 are associated to C−C, C−O, C−H stretching and COH bending modes. Although it is
319 difficult to assign bands from this region to specific structural features, since they are
320 present in many different polysaccharides, it is interesting to note that the band at 1075
321 cm^{-1} has been previously related to vibrational modes within the amorphous phase of

starch [27]. The fact that the relative intensity of this band (with respect to the one at 1023 cm^{-1}) changed along the storage time may be indicative of marked structural modifications taking place in the semi-crystalline fraction from agar. In addition, the shape and relative intensity of the broad band at 3030-3350 cm^{-1}, corresponding to hydrogen-bonded OH stretching, was significantly affected, with the relative intensity being lower at t=3-14 days and then increasing at t=30 days. It should also be noted that the shape of the bands within the region 3000-3700 cm^{-1} changed significantly from a broad band without any shoulders, which is characteristic of the presence of liquid bulk water [28], to a sharper band with different shoulders after 30 days of storage. Moreover, the relative intensity of the bound water, located at 1642 cm^{-1}, also increased after 30 days of storage. This may seem counterintuitive if compared to the evolution of the moisture content in the films. However, it should be considered that the gravimetrically determined moisture content is indicative of the overall amount of water, i.e. bulk and bound to the polysaccharide. Thus, while the overall water content in the films increased upon storage, the FT-IR results suggest that, due to structural re-organization of the agar molecular chains, the nature of interactions between the polysaccharide and water changed along the storage period. This phenomenon is expected to be linked to major changes in the semi-crystalline structure of agar (as demonstrated later by XRD). Despite the fact that the overall moisture content in the COMM+GLY films also varied upon storage, the relative intensity of the OH vibration and bound water bands (with respect to the band at 1023 cm^{-1}) remained quite constant during the whole experiment. This suggests that structural changes in the semi-crystalline structure of agar are limited due to the presence of glycerol. In the case of the HW films, changes occurred mostly in the relative intensity of the bands at 1190 cm^{-1}, 1075 cm^{-1} and 1023 cm^{-1}, suggesting that small structural

changes took place upon storage, while the relative intensity of the bands associated to bound water and OH vibration were slightly affected during storage.




COMM+GLY
t= 30 days
t= 14 days
t= 7 days
t= 3 days
t= 0
1642
1370
1190
1149
1075
1023
930
890
854
Absorbance (a.u.)
Wavenumber (cm-1)

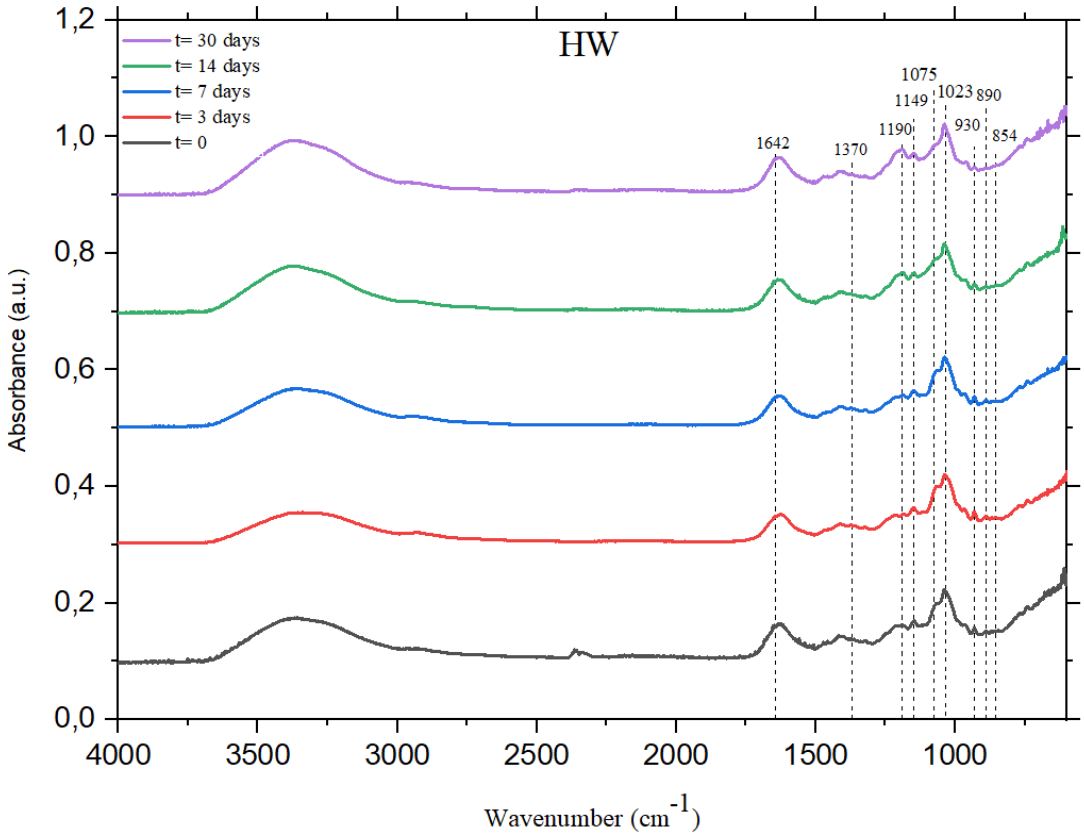
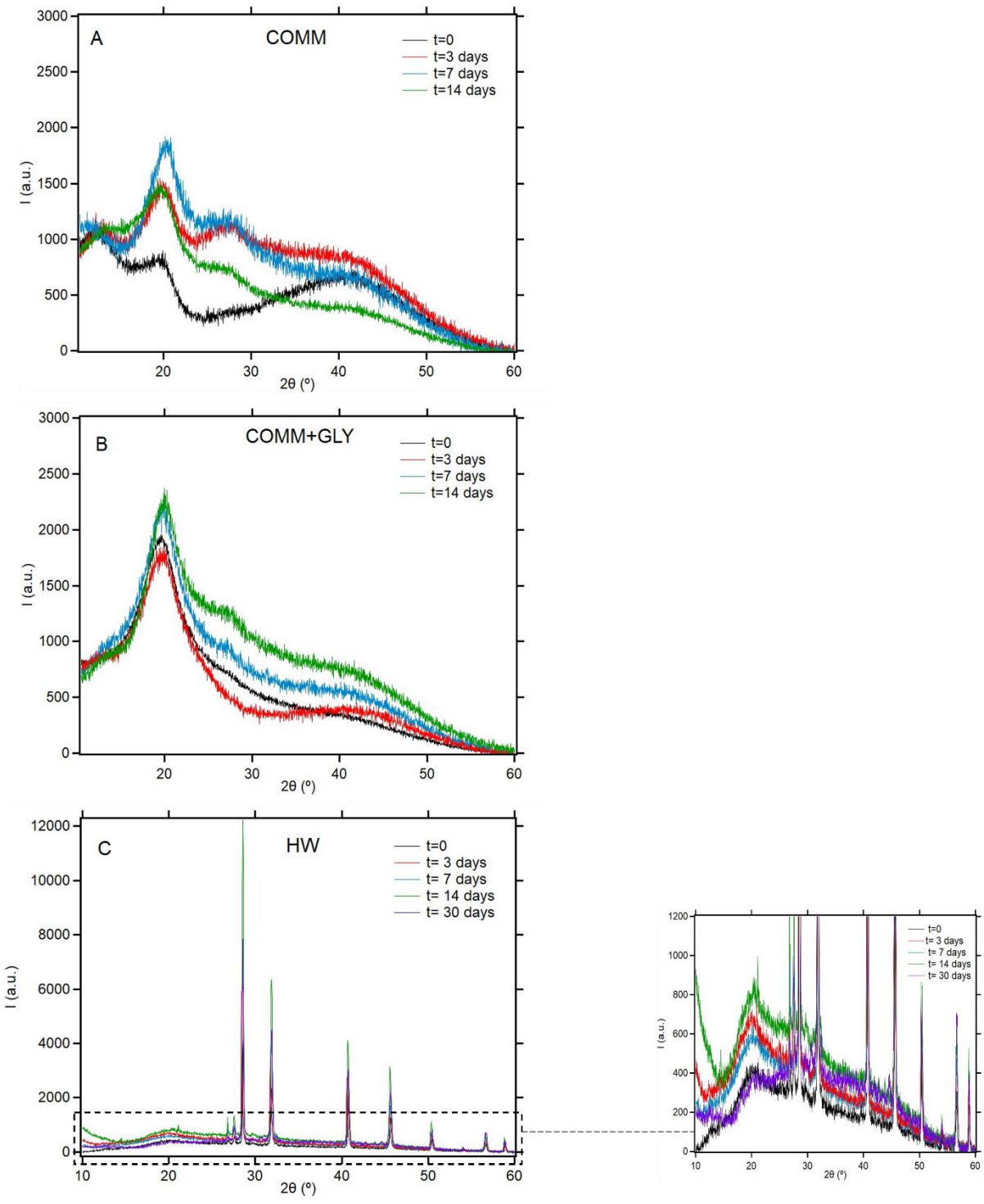

HW
t= 30 days
t= 14 days
t= 7 days
t= 3 days
t= 0
1642
1370
1190
1149
1075
1023
930
890
854
Absorbance (a.u.)
Wavenumber (cm-1)

Figure 2. FT-IR spectra from the agar-based films after being processed (t=0) and after different storage times. All the spectra were normalized to the intensity of the band at 1023 cm^{-1}.

Previous characterization of the commercial agar and the less purified agar-based extract used in this work to produce the films showed that indeed these agars present a semi-crystalline structure [13]. Thus, to analyse the changes in the semi-crystalline structure of the agar-based films upon storage, they were characterized by XRD and the obtained patterns are shown in Figure 3. The XRD patterns from the three films at t=0 showed the same features previously reported for agars with semi-crystalline structure, with a well-defined peak at 19.0° and a shoulder at 13.9° [24,26] suggesting that these films presented a certain degree of order in their structure. Furthermore, two broad shoulders located at ca. 27° and 40° were clearly detected in all the samples but were less evident in the HW films. Such shoulders have been previously noted in the XRD patterns from other polysaccharides such as cellulose [29] and chitosan/glucomannan blends [30] and correspond to water molecules bound to the surface of the semi-crystalline polysaccharide. Thus, it seems that even though the amount of moisture in the HW films at t=0 was the highest, this was mainly bulk water, which was not strongly interacting with the polysaccharide. In contrast, in the case of the COMM and COMM+GLY films a certain fraction of water was strongly bound to the polysaccharide and adopting a partially ordered conformation. It should be noted that the agar crystalline peaks were more intense in the COMM+GLY than in the COMM sample at t=0, suggesting that the presence of glycerol induced the formation of a more crystalline structure after processing the material. This was indeed, reflected on the estimated crystallinity values (cf. Table 1), which were higher in the freshly prepared films containing glycerol. This increase in the

crystallinity due to the plasticizer addition has been previously observed in other polysaccharides such as chitosan [31] and thermoplastic starch [32]. On the contrary, due to its lower agar content, the agar characteristic peaks were much weaker in the HW film which, in turn, showed multiple intense and sharp peaks that were absent in the commercial agar films and also contributed to the overall crystallinity of the material. These peaks have been reported to appear in the XRD patterns from less purified agar-based extracts and were attributed to the presence of minerals such as silica and weddellite [6]. Interestingly, the semi-crystalline structure of the films evolved differently upon storage time. In the case of the COMM sample, it was clearly observed that the relative intensity of the crystalline peaks changed with storage time, suggesting a structural re-organization of the agar chains into different semi-crystalline conformations. Moreover, the contribution from the water shoulders seemed to be reduced after 14 days of storage. The agar crystallinity index reached a maximum of 27% at 7 days of storage and remained constant at 10% during the rest of the experiment. These results confirm that the structure of agar undergoes significant changes during the storage period, being water essential for these structural changes. In contrast, in the case of the in the COMM+GLY sample, the relative intensity of the agar-characteristic peaks was not strongly modified and the crystallinity index remained fairly constant throughout storage up to 14 days. Interestingly, contrarily to the COMM films, the contribution of the shoulders assigned to water slightly increased along storage. It should be noted that these two samples could not be measured at the end of the storage experiment (t=30d) since it was not possible to obtain completely flat film surfaces on the XRD sample holder due to an excessive rigidity of the films, hence preventing a correct measurement of the specimens. In the case of the HW film, the overall crystallinity increased after the first 3 days of storage and then remained fairly constant. The crystallinity corresponding to the agar fraction,

representing only 15-30% of the overall crystallinity, slightly increased with respect to the film at t=0, but the values were still very low, indicating the existence of a more amorphous agar. These results show that, in the absence of other components, the semi-crystalline structure of agar undergoes significant changes upon storage, which are most likely driven by variations in the moisture content and water re-organization within the film structure. These structural changes are minimized or delayed when glycerol is added as plasticizer into the film formulation or by the presence of other components in the HW film, hence providing materials with a better stability upon prolonged storage.



413

414 **Figure 3.** XRD patterns of the agar-based films after being processed (t=0) and after

415 different periods of storage. The patterns from the COMM films are shown in (A), while

416 (B) corresponds to the COMM+ GLY films and (C) to the HW films.

417

Table 1. Crystallinity index determined from the XRD patterns from the agar-based films after different storage times. In the case of the HW films, the crystallinity values estimated by considering only the agar characteristic peaks are additionally shown between brackets.

	Xc (%)				
	t=0	t=3 days	t=7 days	t=14 days	t=30 days
COMM	10	10	27	10	---
COMM+GLY	17	15	12	18	---
HW	12 [2]	21 [6]	23 [5]	20 [3]	18 [4]

The mechanical properties of the agar-based films are highly relevant to determine their suitability to be used as packaging materials. Therefore, they were evaluated by tensile tests and the most representative parameters obtained from the stress-strain curves are shown in Figure 4. It is evident that there was a large difference between the mechanical performance of the pure commercial agar films and those obtained from the less purified agar-based extract. For the freshly processed films, the COMM sample presented the highest elastic modulus (E≈1500 MPa) and tensile strength (σ≈34 MPa) values. The addition of glycerol had a clear plasticization effect, reducing both the elastic modulus (E≈640 MPa) and tensile strength (σ≈15 MPa), while increasing the elongation at break (from 10% for COMM to 32% for COMM+GLY). As expected, due to the lower agar concentration in the HW films, they presented poorer mechanical resistance, with low elastic modulus (E≈60 MPa), tensile strength (E≈3 MPa) and elongation at break (ε_B≈6%). Interestingly, the mechanical performance of the COMM films was drastically affected upon storage, with the three measured parameters significantly decreasing over time. This was also directly reflected in the appearance of the films since, after two weeks

of storage, the material became extremely rigid and brittle, impeding a proper characterization of its mechanical properties. This trend in worsening the mechanical properties upon storage is in line with the results reported by Freile-Pelegrin et al. in their study on the biodegradability of agar films in a humid tropical climate, attributing this effect to a reduction in the molecular weight of agar [24]. Our results do not show any signs of agar hydrolysis upon storage, since the crystallinity of the films was not strongly modified. Instead, a re-organization of the semi-crystalline structure of agar was observed and the proportion of water tightly bound to the polysaccharide and forming part of its semi-crystalline structure was reduced after 14 days of storage. Thus, it seems that the loss of water within the agar semi-crystalline structure (even though the overall amount of moisture in the films increased) was the main driver for the rigidizing effect induced by storage. In contrast, the mechanical properties of the more ductile COMM+GLY films varied erratically for the first 14 days of storage, which may be a consequence of glycerol migration within the film structure and/or slight variations in the amount of water tightly bound to the polysaccharide (as suggested by XRD). This was followed by a rigidizing effect after 30 days of storage. At this point, the elongation at break decayed to approximately 5% and the elastic modulus increased up to 1100 MPa. Note that, at this storage time, the total amount of moisture in the films also experienced a sharp decrease (cf. Figure S1). Such phenomenon may be explained by an excessive migration of glycerol from the structure of the film, thus promoting dehydration and rigidization. On the other hand, the mechanical properties of the HW films remained quite stable throughout the storage time. In fact, the elongation at break was even slightly improved, reaching a similar value to that obtained for the COMM+GLY film ($\varepsilon \approx 7\%$) after 30 days of storage. This slight improvement may have been originated by structural changes taking place in the salts present in the agar-based extract, as evidenced by the SEM images

(cf. Figure 1). An important implication is that the mechanical properties of the agar films processed by melt mixing are superior to those from other reference biopolymers such as thermoplastic starch (E =29.8 MPa, σ=3.1MPa, ε_B=62.6%) [33,34] and comparable to the values reported for agar-based films processed by casting (E =29-1600 MPa, σ=6-38 MPa, ε_B=15-26 %) [35–37]. Although the pure agar presents much better mechanical performance than that from the less purified agar-based extract, the addition of plasticizer in the former is required to obtain films with a certain stability upon storage (up to ca. 14 days), while that is not needed in the case of the HW films. Thus, while the HW extract may not be competitive for the development of packaging films on its own, it may be an interesting choice as additive to reduce costs and improve the stability upon storage of pure agar films.

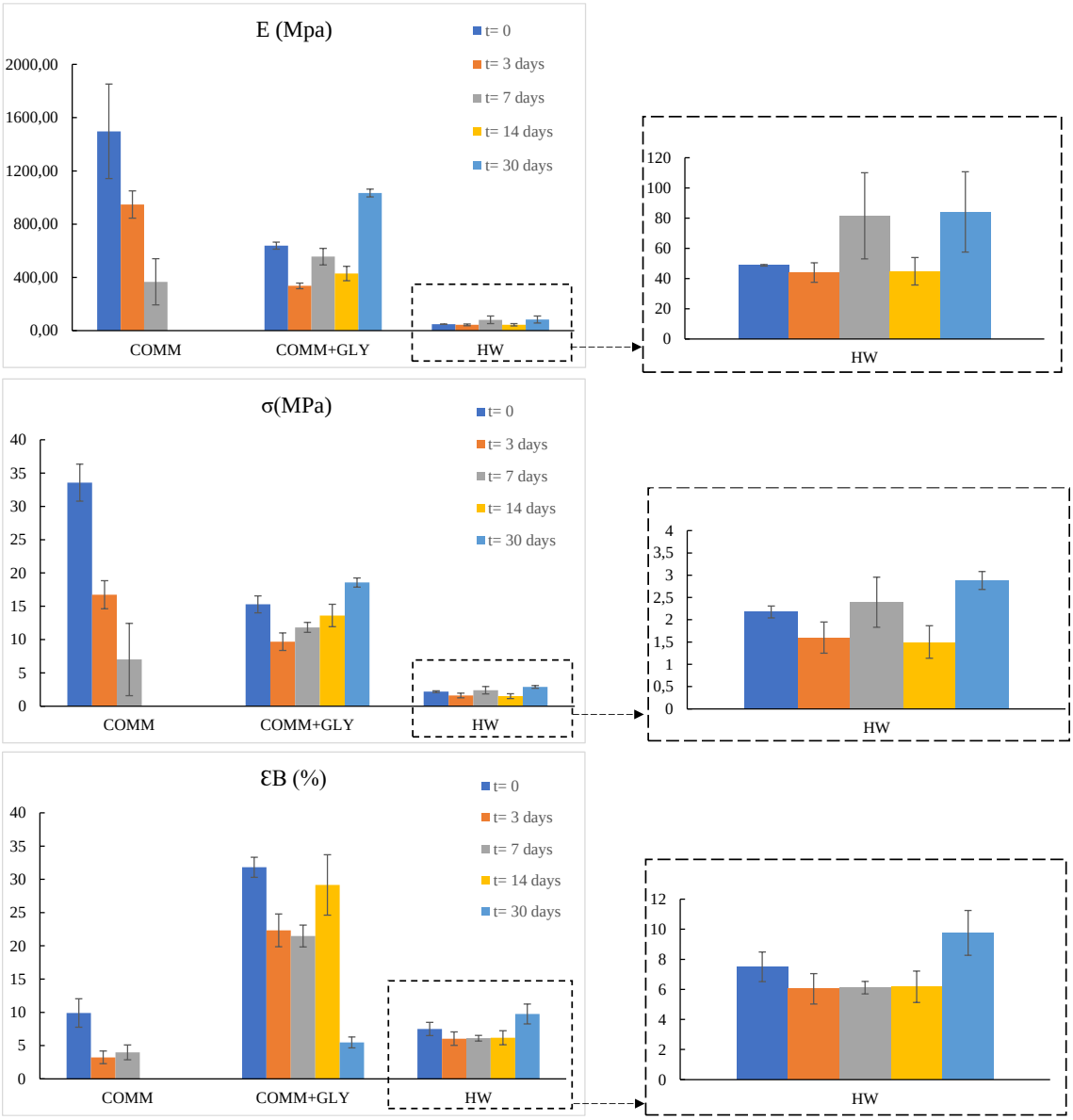


Figure 4. Mechanical properties of the agar-based films after different storage times. E: Elastic modulus, σ: tensile strength and $Ɛ_B$: elongation at break. Data correspond to the mean calculated values, n=3.

The water vapor permeability (WVP) of the films was also characterized and the obtained results are shown in Figure 5. As observed, the freshly made COMM film exhibited the lowest water permeability ($8.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa), which decreased slightly during the two following weeks. As previously mentioned, this film could not be measured for the entire duration of the experiment due to its physical deterioration. It should also be noted

that the presence of glycerol in the COMM+GLY film had a slightly negative impact on the barrier capacity. This was mainly noticeable during the first two weeks of storage, in which the permeability values remained roughly stable (~$9.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa). The detrimental effect of hydrophilic plasticizers on the WVP of other polysaccharide-based films has been reported before and may be attributed to a distortion in the network of the hydrogen bound hydroxyl groups from the polysaccharide [38]. Finally, after 30 days of storage the permeability decreased to a minimum of $6.1 \cdot 10^{-14}$ Kg·m/s·m^2·Pa. This is in line with the rigidizing effect observed in the mechanical properties and would be consistent with the migration of a significant amount of the highly hydrophilic glycerol plasticizer from the film structure. On the other hand, the presence of other components in the HW extract resulted in a higher water permeability in the freshly made film ($1.3 \cdot 10^{-13}$ Kg·m/s·m^2·Pa). Surprisingly, the barrier capacity improved over time, reaching a permeability value comparable to that of the commercial agar films after 30 days of storage ($7.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa). Previous studies have reported that the improvement in the permeability of the less purified extract can be explained by the formation of partially intertwined three-dimensional networks at the molecular scale between the agar and the proteins contained in these materials [13,39]. Another possible explanation may be related to the more homogeneous integration of the salt particles in the film structure, as evidenced by the SEM characterization. It is also worth noting that the WVP values of the three films obtained in this study were lower than those previously reported by Rhim et al. for casting-processed agar films ($2.2 \cdot 10^{-14}$ Kg·m/s·m^2·Pa) [40] and even for reference biopolymers such as thermoplastic starch ($2.5 \cdot 10^{-14}$ Kg·m/s·m^2·Pa) [41], thus highlighting the potential of these materials to be used in packaging applications requiring high barrier properties. Once again, the results suggest that the use of the HW agar-based extract as additive in more purified agar films might be interesting to reduce costs, while

improving the stability of the films upon storage in terms of crystallinity and mechanical performance and having a positive effect on the water barrier capacity.

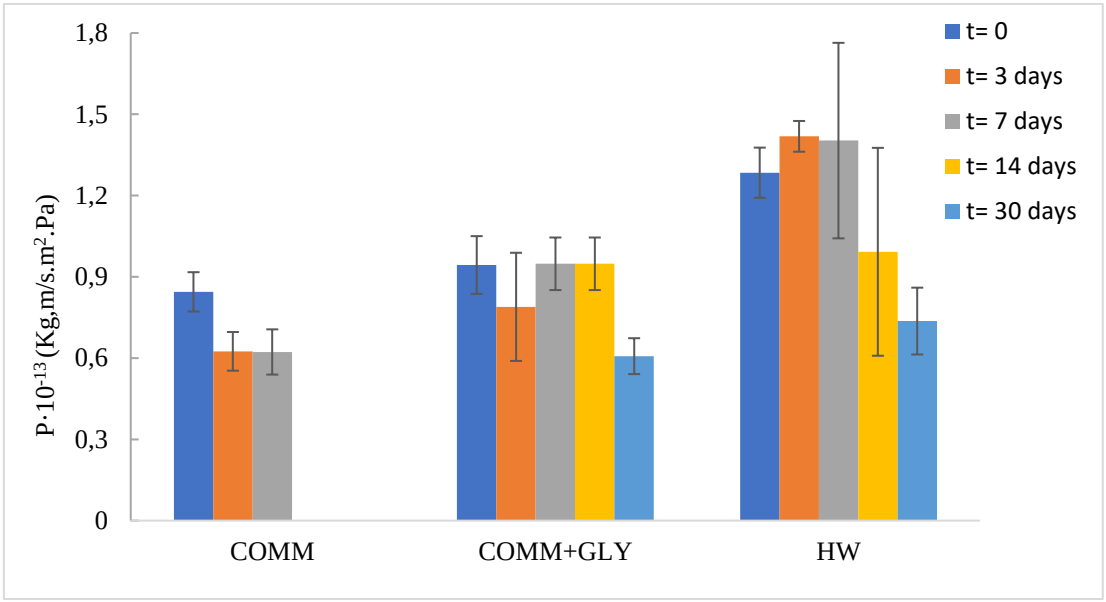


Figure 5. Water Vapor Permeability (WVP) of the agar-based films after different storage times. Data correspond to the mean calculated values, n=3.

4. Conclusions

The capacity of agars with different degrees of purity to form packaging films by means of the melt mixing technique has been evaluated and the stability of the obtained materials upon prolonged storage has been investigated. The degree of purification of the agar had a major impact in the properties of the films. In particular, the less purified agar-based extract yielded more opaque films with a brownish coloration, lower mechanical performance and higher water vapour permeability than the commercial pure agar. On the other hand, the extremely rigid behaviour of the commercial agar film required the addition of a plasticizer to produce films which could be manipulated without causing breakage. Indeed, the addition of glycerol produced more ductile films but reduced their mechanical resistance. Interestingly, this study has demonstrated that when the films are

stored at 53%RH after preparation, the semi-crystalline structure of agar undergoes significant changes and the proportion of tightly bound water is modified. As a result, the pure agar films undergo a rigidizing effect, which is also reflected in a reduction of the water permeability, making the films unmanageable after 7 days of storage. The presence of glycerol prevented these changes in the semi-crystalline structure of agar, improving the stability of the films up to 14 days of storage. However, the films were rigidized after 30 days of storage, most likely due to glycerol migration. Notably, the water-polysaccharide interactions seemed to be more limited in the films from the less purified agar-based extract, hence showing a greater stability upon storage. These results show the potential of the less purified agar-based extract, produced by a more energy efficient extraction protocol, to be used as additive to reduce costs and improve storage stability of pure agar films.

Acknowledgements

This work was financially supported by Hispanagar.

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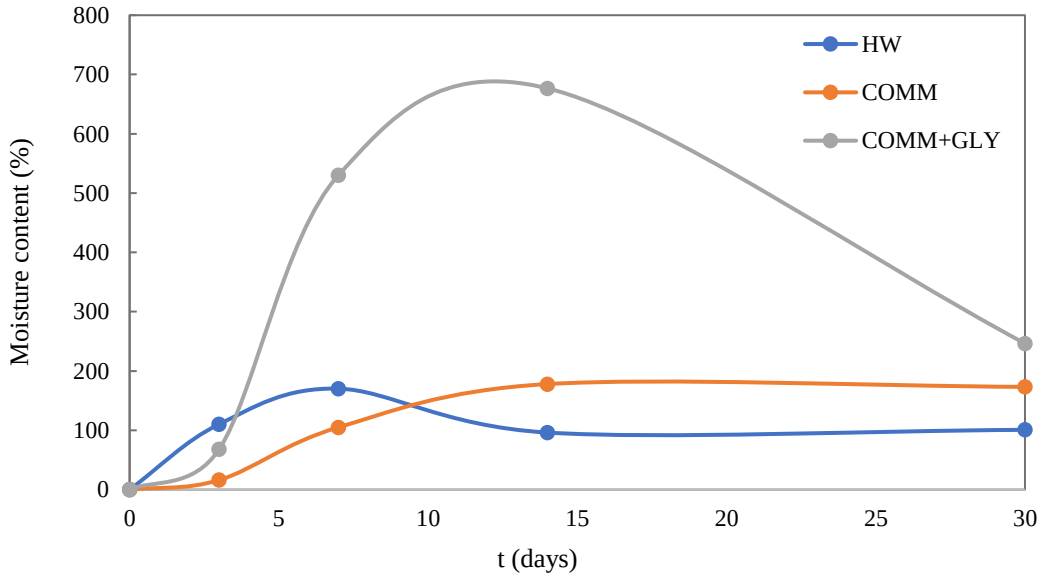
696 **Supplementary Material**



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698 **Fig S1.** Moisture content determined in the agar-based films at different storage times.