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Abstract: Poly(vinyl alcohol-co-ethylene) (EVOH) films containing two different active ingredients (AIs), gallic acid (GA) and umbelliferone (UMB) at 5 wt. % and 15 wt. %, were successfully produced by solvent casting and extrusion. The effects of process techniques, AIs presence, typology and content on optical, morphological, thermal and mechanical properties of EVOH based films have been investigated. In addition, moisture content, migration and radical scavenging activity, antimicrobial and antifungal studies were performed, with the aim of evaluating the effect of AIs on structural and functional properties of realized food packaging systems. Results from colorimetric and transparency investigation underlined that the presence of AIs in EVOH copolymer induced important alterations, whereas migration to food simulant data restricted the use of produced films in contact with fatty foods. On the other hand, the use of AIs clearly induced positive antioxidant response, inhibitions of bacterial and antifungal growth, confirming the possibility of practically using these materials in the food packaging sector.

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Terni, 28/11/2017

Editorial Board

Polymer Degradation and Stability

Dear Editor,

Please find enclosed the manuscript entitle “**Effect of active ingredients on thermal, mechanical, antioxidant and antimicrobial properties of poly (vinyl alcohol-co-ethylene) films**” by F. Luzi, D. Puglia, F. Dominici, E. Fortunati, G. Giovanale, G.M. Balestra, L. Torre, that that I would like to submit for possible publication in Polymer Degradation and Stability.

In the present work, Poly(vinyl alcohol-co-ethylene) (EVOH) films containing two different active ingredients (AIs), gallic acid (GA) and umbelliferone (UMB) at 5 wt. % and 15 wt. %, were successfully produced by solvent casting and extrusion. The effects of process techniques, AIs presence, typology and content on optical, morphological, thermal and mechanical properties of EVOH based films have been investigated. In addition, moisture content, migration and radical scavenging activity, antimicrobial and antifungal studies were performed, with the aim of evaluating the effect of AIs on structural and functional properties of realized food packaging systems. Results from colorimetric and transparency investigation underlined that the presence of AIs in EVOH copolymer induced important alterations, whereas migration to food simulant data restricted the use of produced films in contact with fatty foods. On the other hand, the use of AIs clearly induced positive antioxidant response, inhibitions of bacterial and antifungal growth, confirming the possibility of practically using these materials in the food packaging sector.

I declare that all the authors mutually agree that it should be submitted to PDST, that this is the original work of the authors and that the manuscript was not previously submitted to PDST.

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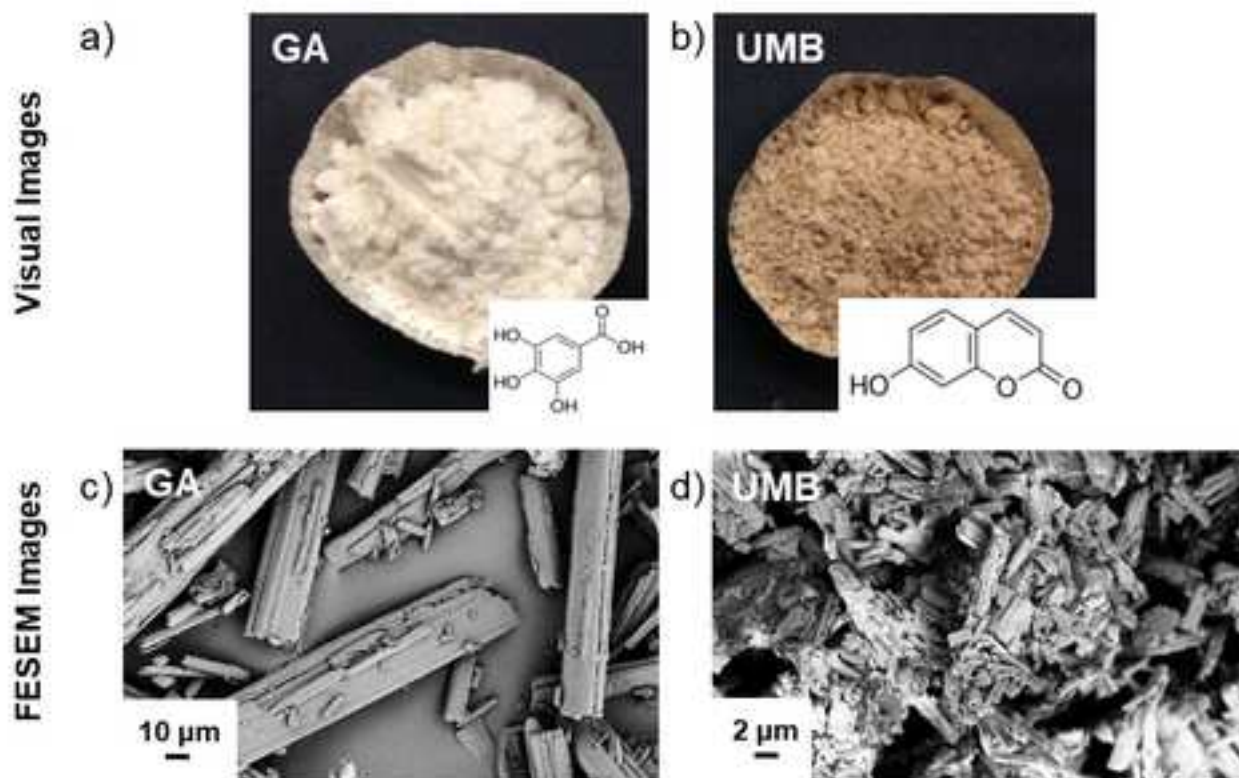
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Figure 1
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Panel A: AI Powders



Panel B: AI Solutions

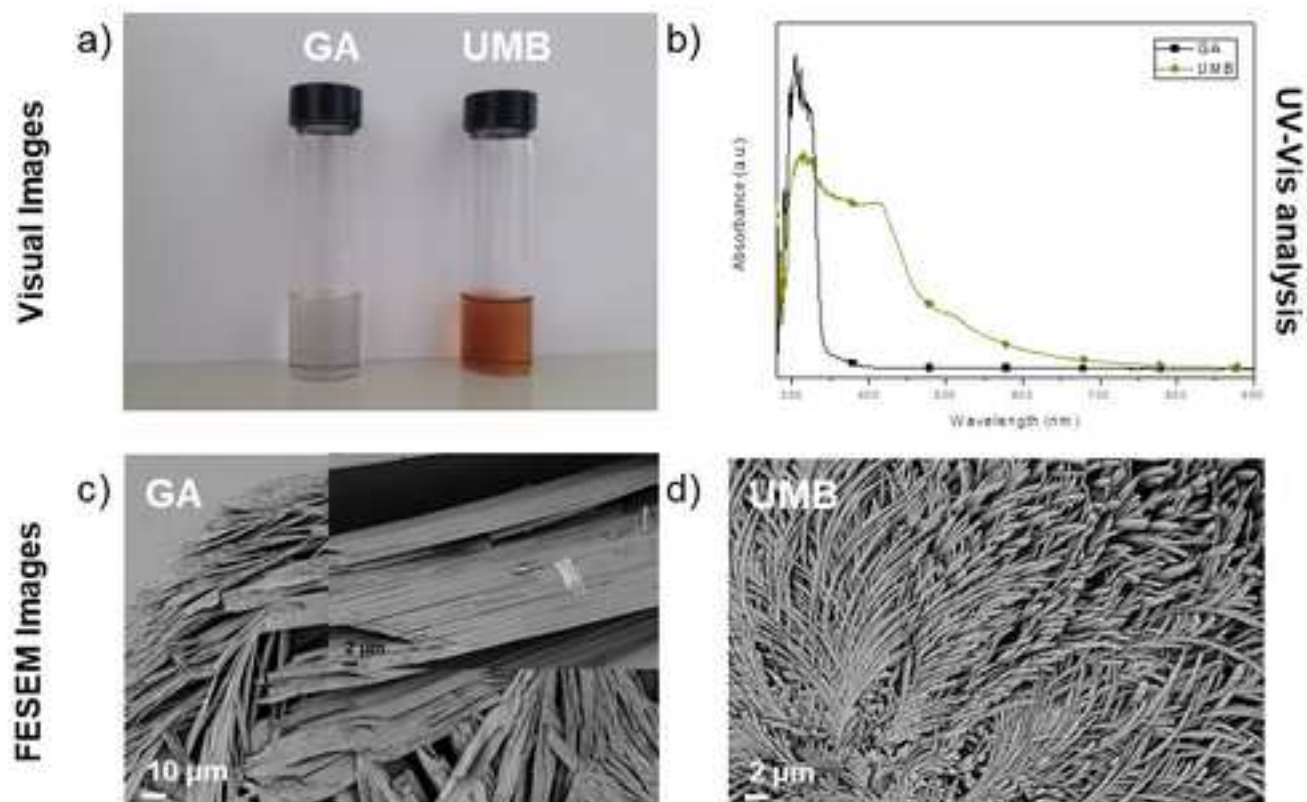


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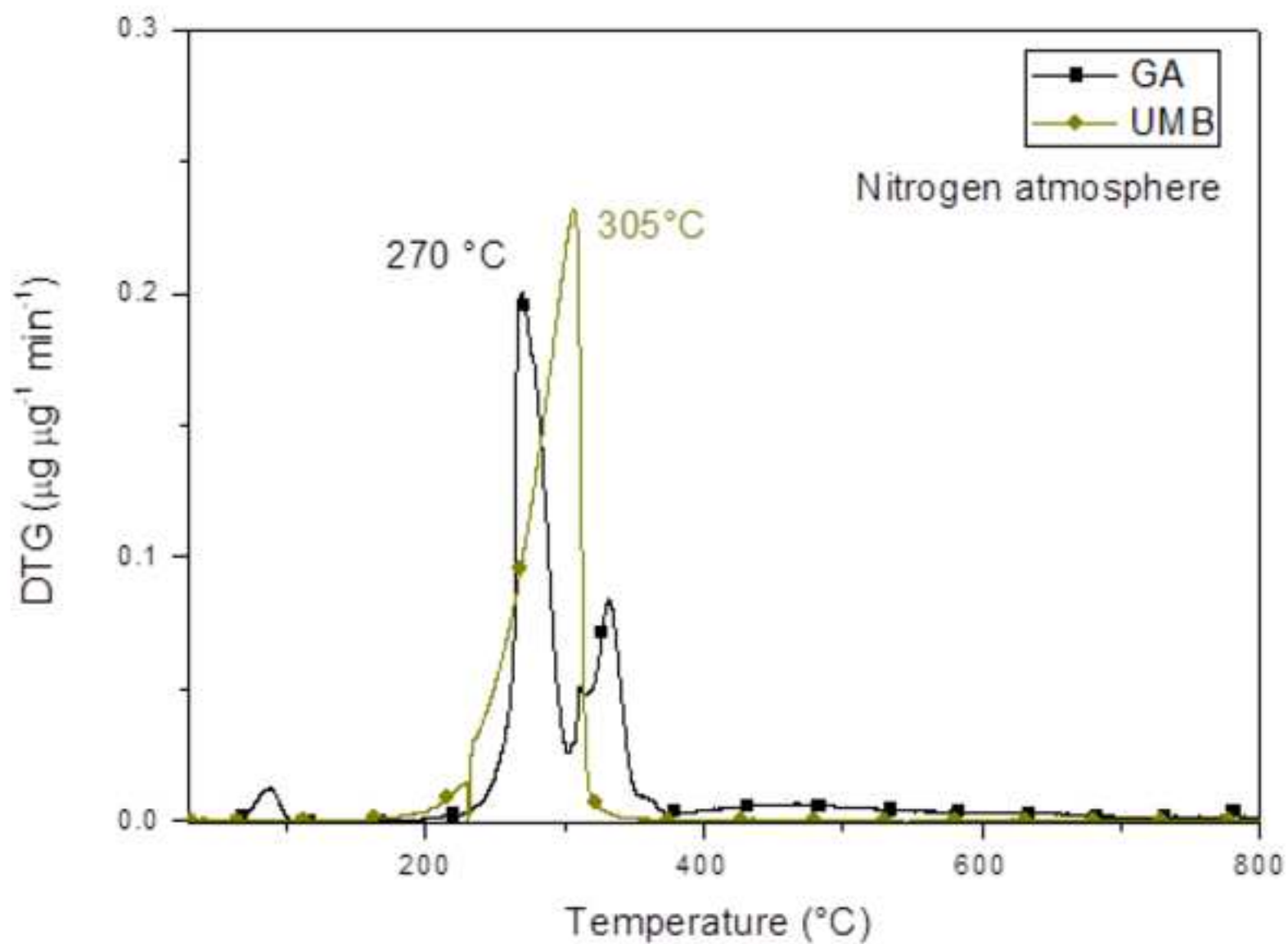


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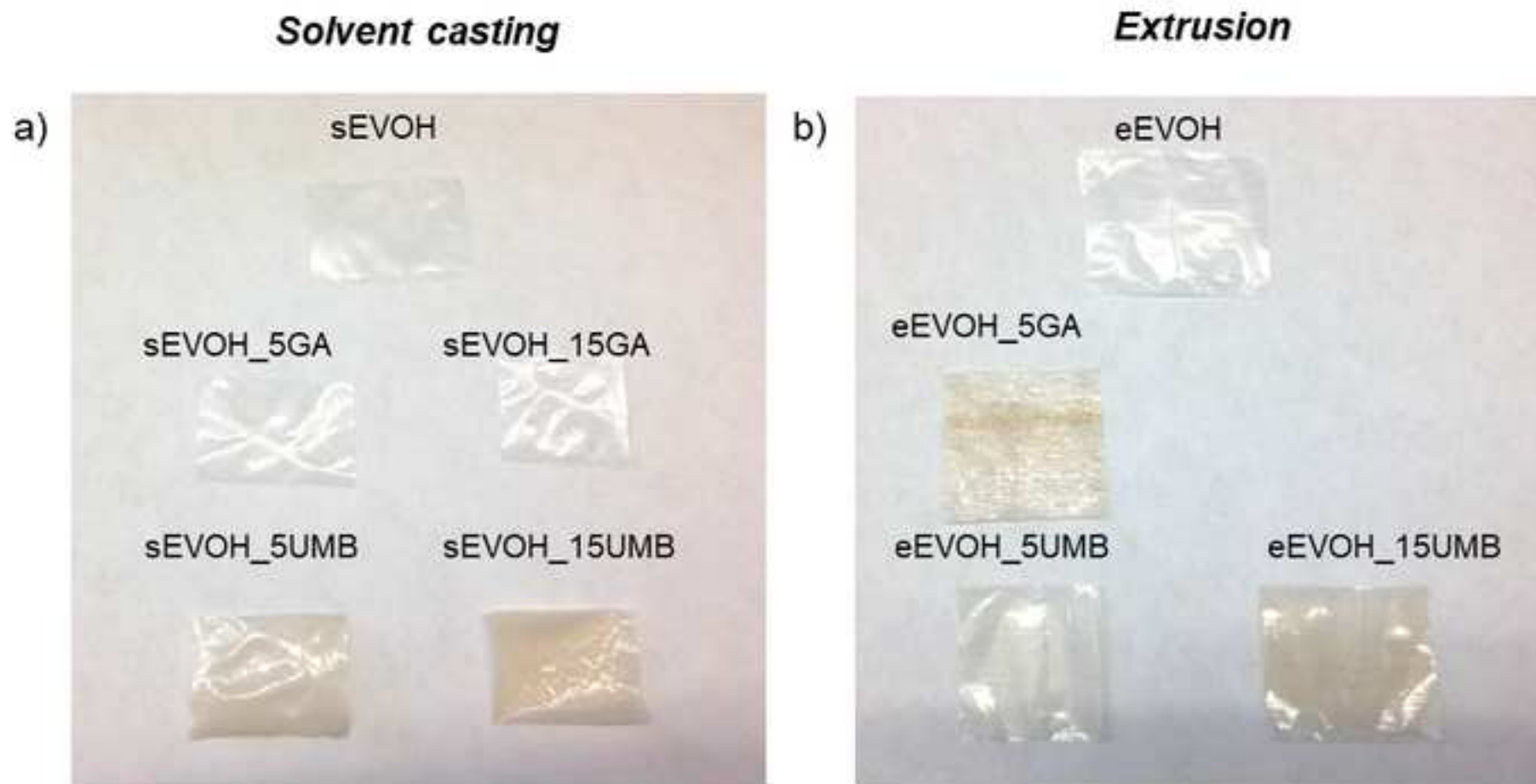


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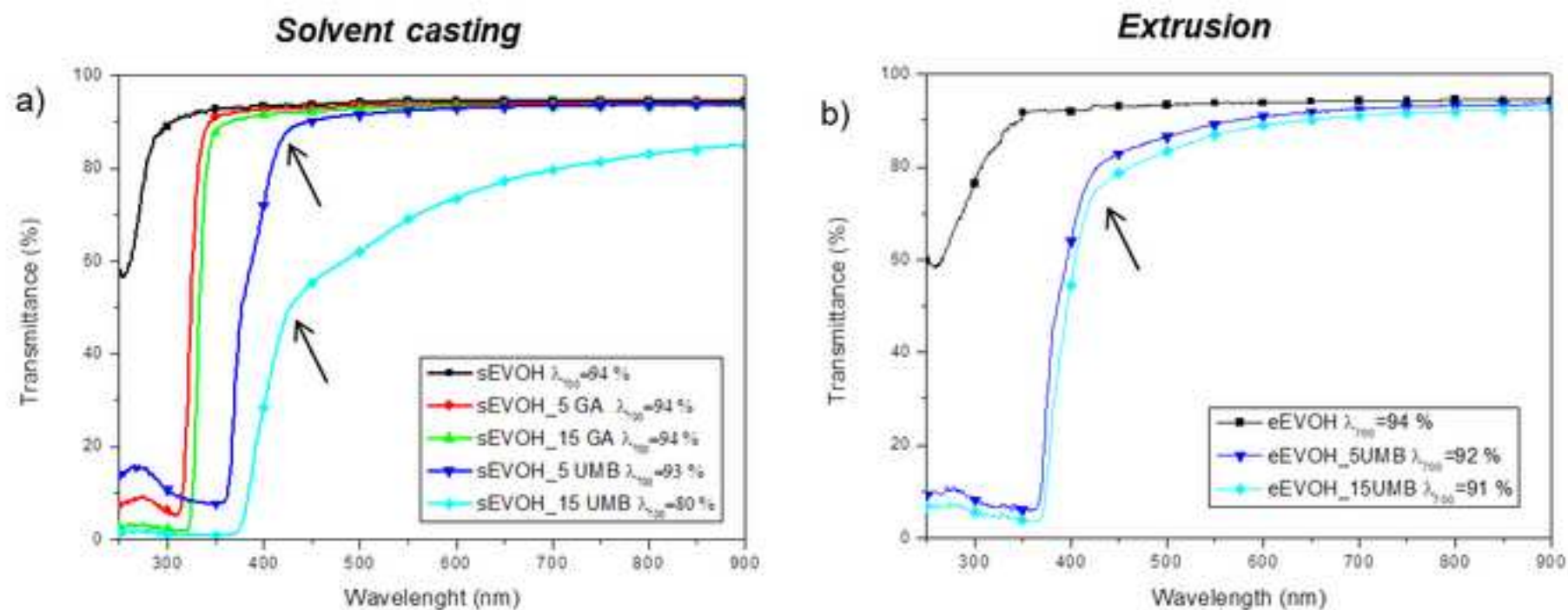


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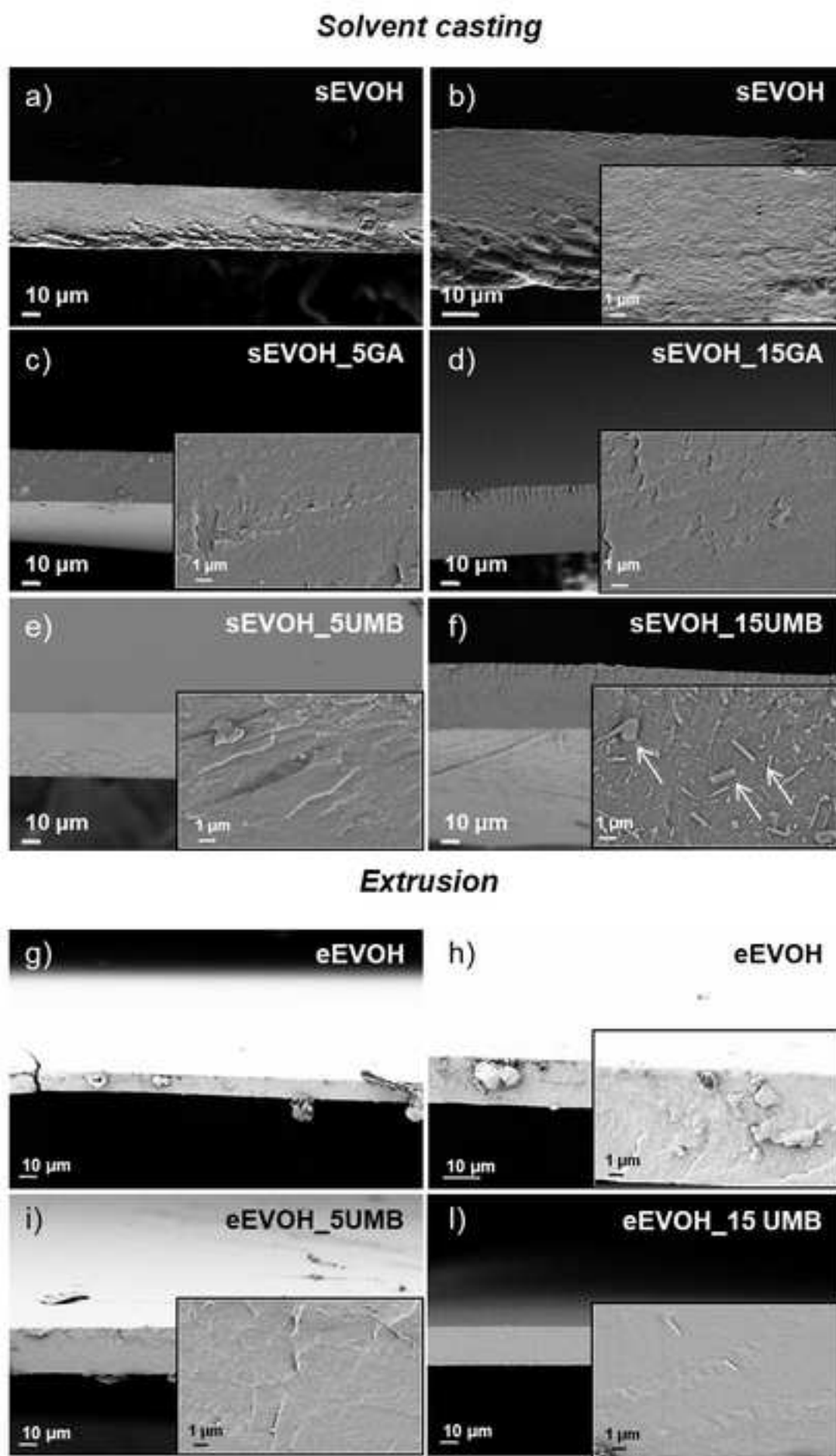


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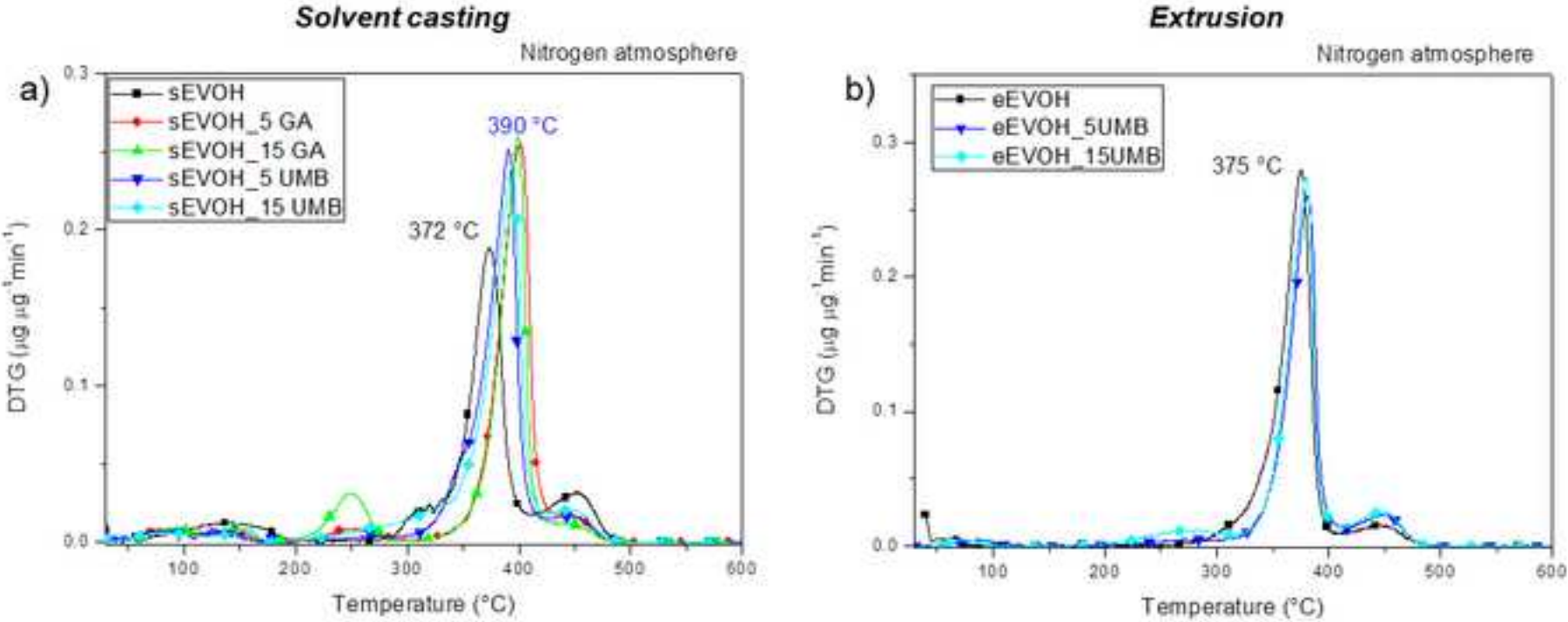


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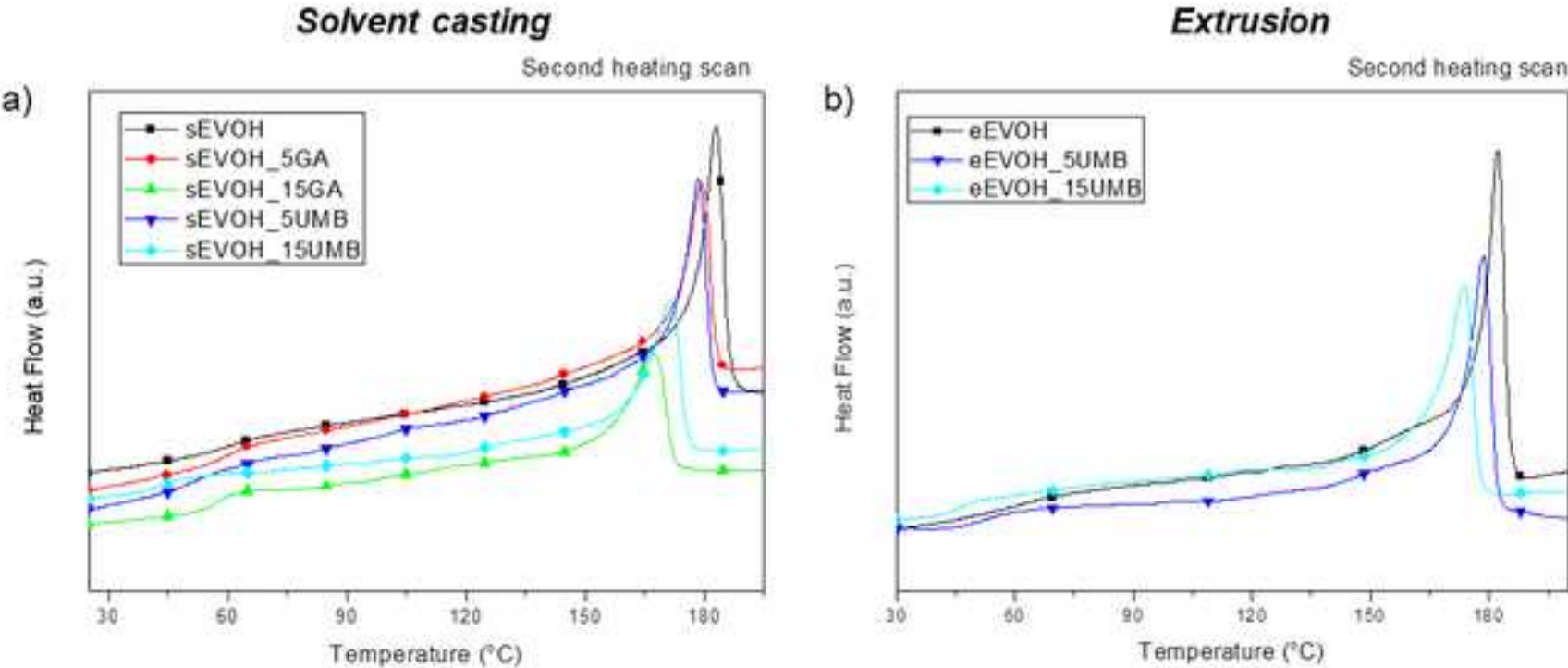


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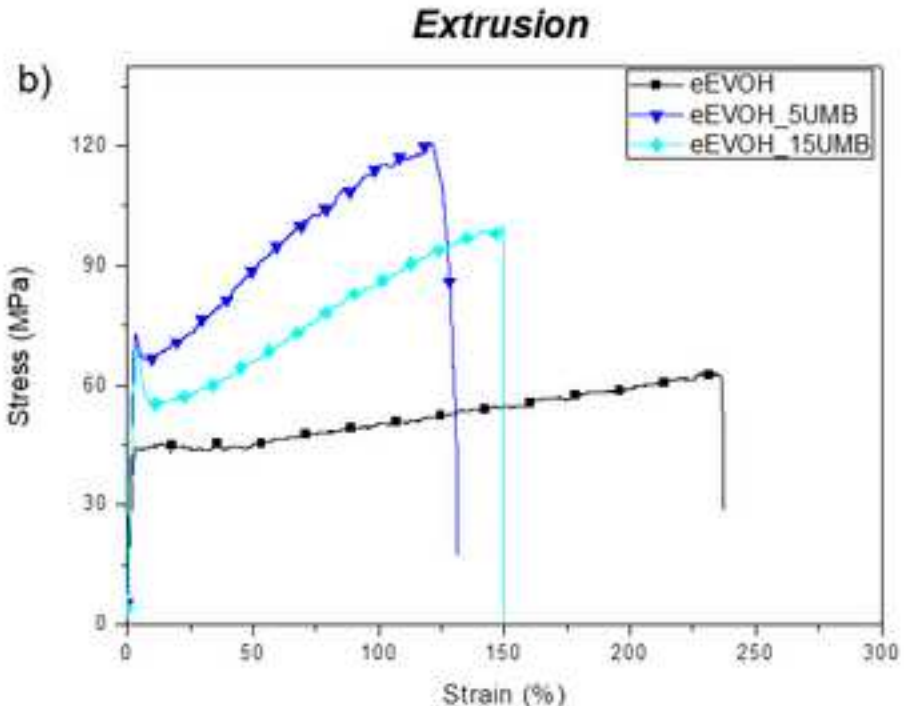
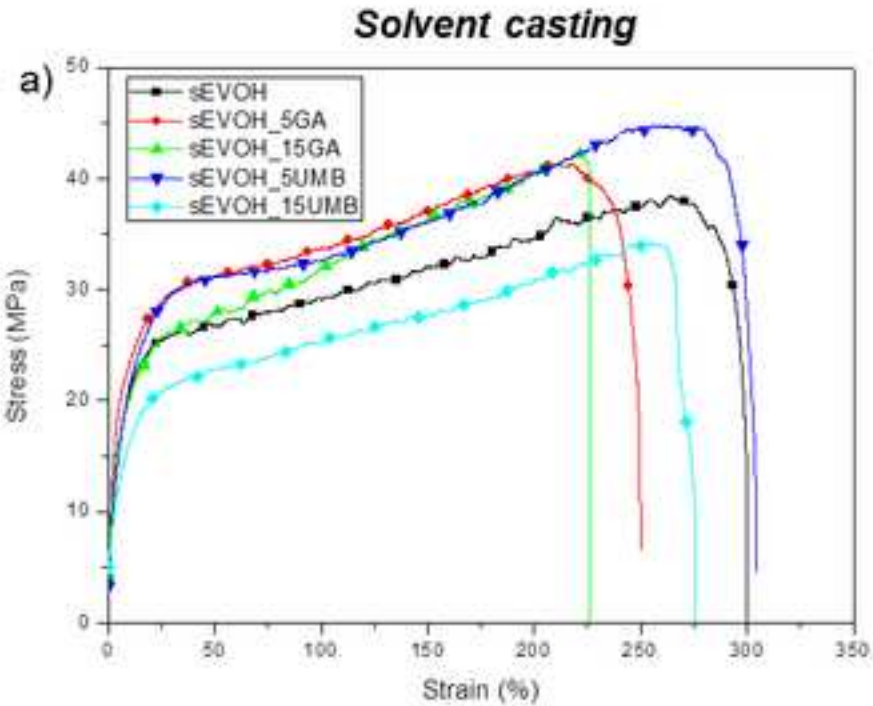


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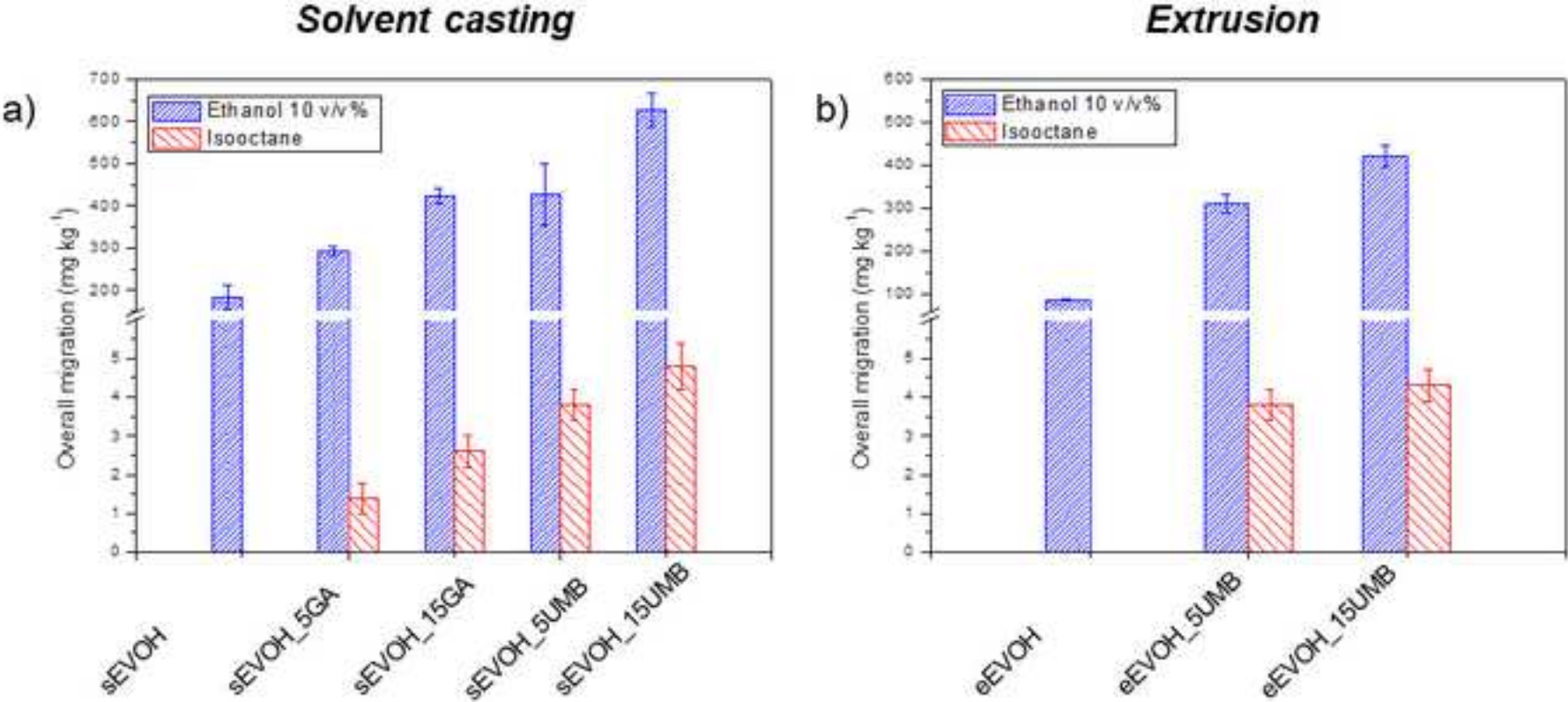
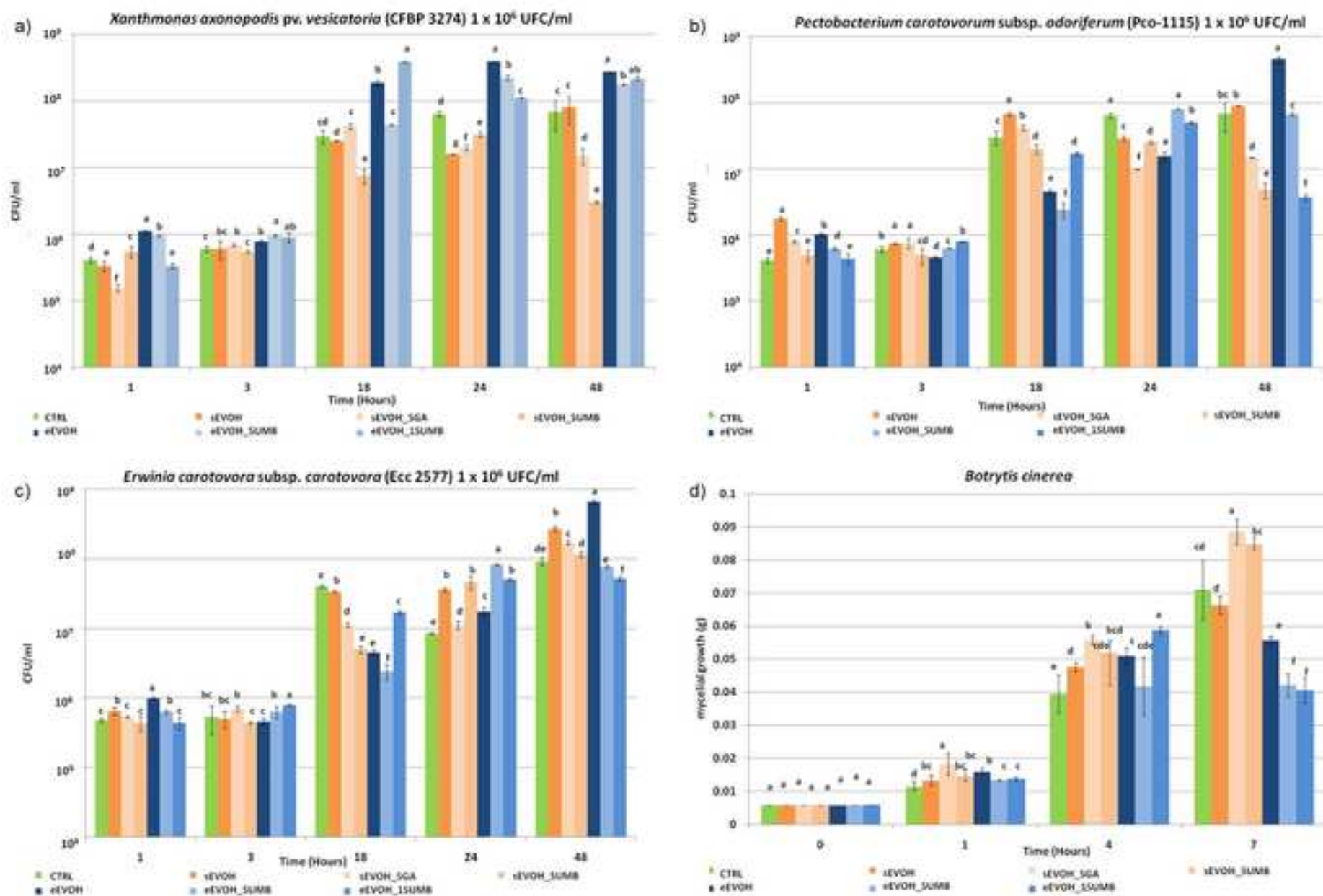
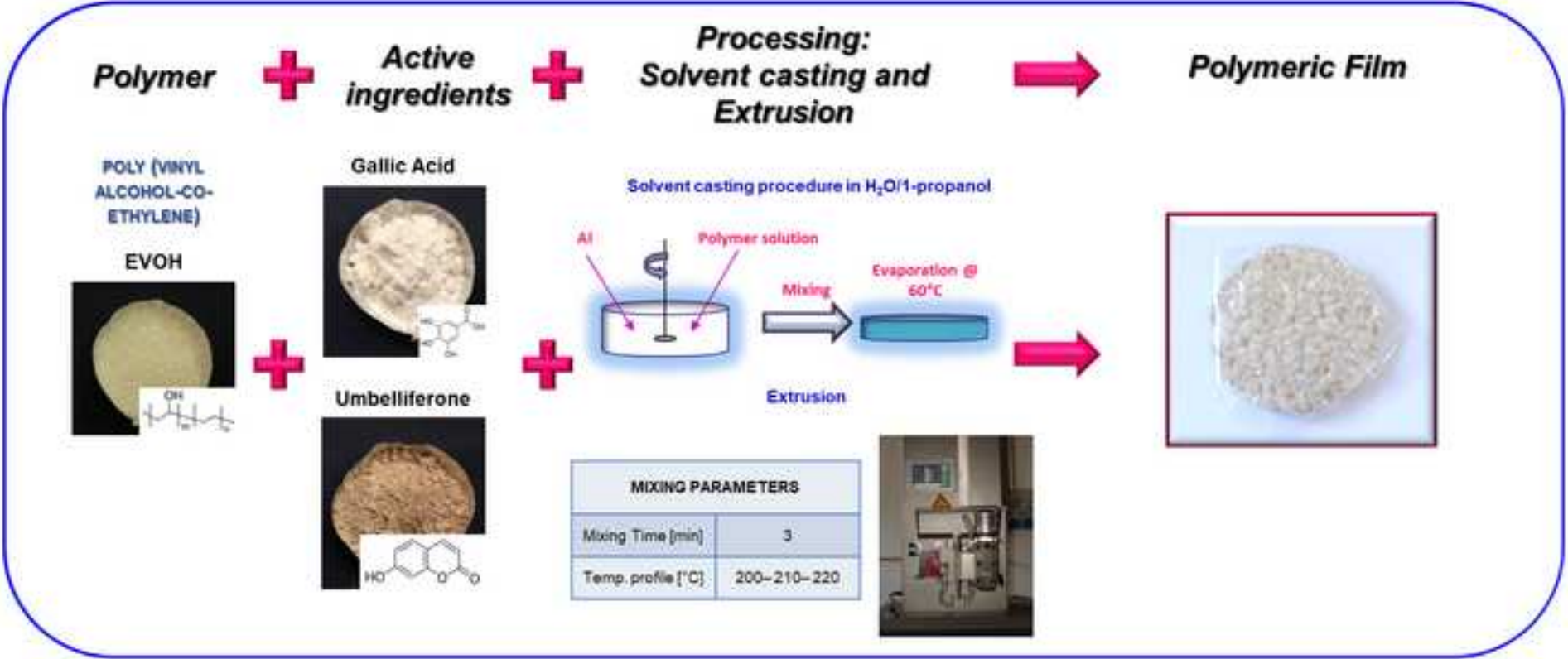


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Highlights

- EVOH based films were produced by solvent casting and extrusion.
- Gallic acid and umbelliferone were combined with EVOH to get active food packaging.
- Homogeneous distribution of active ingredients in EVOH was observed and transparent films were obtained.
- Gallic acid and umbelliferone in EVOH based formulations induced antioxidant, antimicrobial and antifungal effects.

Effect of active ingredients on thermal, mechanical, antioxidant and antimicrobial properties of poly (vinyl alcohol-co-ethylene) films

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Abstract

Poly(vinyl alcohol-co-ethylene) (EVOH) films containing two different active ingredients (AIs), gallic acid (GA) and umbelliferone (UMB) at 5 wt. % and 15 wt. %, were successfully produced by solvent casting and extrusion. The effects of process techniques, AIs presence, typology and content on optical, morphological, thermal and mechanical properties of EVOH based films have been investigated. In addition, moisture content, migration and radical scavenging activity, antimicrobial and antifungal studies were performed, with the aim of evaluating the effect of AIs on structural and functional properties of realized food packaging systems. Results from colorimetric and transparency investigation underlined that the presence of AIs in EVOH copolymer induced important alterations, whereas migration to food simulant data restricted the use of produced films in contact with fatty foods. On the other hand, the use of AIs clearly induced positive antioxidant response, inhibitions of bacterial and antifungal growth, confirming the possibility of practically using these materials in the food packaging sector.

Keywords: gallic acid, umbelliferone, poly (vinyl alcohol-co-ethylene), food packaging, active packaging, antioxidant and antimicrobial properties.

29 **1. Introduction**

30 In the last years, traditional food packaging systems are considered unsuitable to offer the shelf-life
31 and commercialization conditions required by the sector, precisely in the case of fresh or
32 minimally-processed foods [1]. In general, it is required to them to cover and contain foods and to
33 protect them from environmental attacks as moisture, light radiation, oxidation process and/or
34 contaminations [2]. In this context, the widespread consumption of ready or semi-finished foods, as
35 the salad already washed and ready to be eaten or the meat in the supermarket fridge shelf ready to
36 be eaten or cooked, has led the development and the production of new packaging solutions with
37 increased properties in terms of antibacterial and antioxidant responses.

38 Food products are very susceptible to rancidity and putrefaction, caused by oxidation of their lipids
39 and microbial growth [3]. In order to limit oxidation and deterioration in sensitive and delicate
40 foods, the addition of antioxidant ingredients and modified atmosphere packaging technology or the
41 design of a suitable vacuum system are the two most important and/or common options to be
42 considered [4]. In this scenario, the food packaging industry demands and requests progress and
43 development of innovative packaging and, thus, the food packaging sector is continuously
44 developing advanced systems, concentrating its research on the production of active and smart
45 packaging [5].

46 Smart and active packaging systems are based on the interactions with the food environment or the
47 food itself [6]. Smart packaging keeps under control the environment surrounding the food or the
48 condition of foods during storage and transport [5, 7], while active food packaging offers the
49 opportunity to include active ingredients into packaging systems, allowing the reduction of food
50 additives into the foods and improving at the same time the shelf-life of foodstuffs [8-10]. This new
51 concept of food packaging system is considered of crucial importance to improve and maintain
52 unchanged the safety, the quality and the organoleptic characteristics of food products during the
53 shelf-life [11].

54 The use of polymer-based packaging for food products shows many advantages respect to paper and
55 aluminium packaging and glass containers, since plastics are more flexible, in terms of reducing
56 weight and energy requirements for their production [12]. Within the wide list of polymeric systems
57 that could be considered as useful for the realization of food packaging systems, poly(vinyl alcohol-
58 co-ethylene) (EVOH) can be certainly considered as one of the most suitable, due to its specific
59 properties: in particular, poly(vinyl alcohol-co-ethylene) (EVOH) is a semicrystalline random
60 copolymer with excellent chemical resistance [13], high transparency and outstanding gas barrier
61 properties to hydrocarbons [14], especially when low content of ethylene (below 38 mol %
62 ethylene) is considered [15]. EVOH based systems have been increasingly used in the food-
63 packaging applications, being this sector characterized by stringent standards in terms of gas and
64 chemical resistance, aroma, water hydrocarbon permeation. The mechanical and barrier
65 characteristics of EVOH under dry conditions are attributed to the high inter- and intra-molecular
66 cohesive energy and semi-crystalline morphology [16].

67 However, the main problem of EVOH copolymers is their moisture content sensitivity, that
68 determines a significant reduction of their characteristics (thermal, mechanical and barrier
69 properties) at high relative humidity environments [17].

70 In order to limit this behaviour, EVOH in food packaging application is often used in multilayer
71 polymeric based systems, usually combined with hydrophobic polymers to modulate the final
72 properties of packaging devices [15] and limit water molecules absorption into copolymer chain.

73 The high affinity of EVOH to moisture determines a high water uptake in copolymer chains,
74 leading to a reduction of the gas barrier performance in high relative humidity environments. This
75 behaviour influences also the mechanical response [15].

76 Other than reduction of moisture absorption, another important issue is the need to prevent and/or
77 inhibit the microbial growth in food products, that is currently limited by considering the use of
78 natural extracts (coffee silver skin, green tea and catechin) and/ or essential oils (carvacrol and

hydroxytyrosol) as active ingredients (AIs) combined with polymeric thermoplastic matrices [11, 18, 19].

In this frame, we considered to extend the use of gallic acid and umbelliferone, two active ingredients (AIs) already largely applied in biomedical and pharmaceutical sectors [20, 21], to food packaging applications in a smart manner.

Gallic acid (GA) (3, 4, 5-trihydroxybenzoic acid) is a polyphenol extracted from plant entities, present in tea, berries, grapes and fruits [22]. It possesses a variety of biological properties, like anti-inflammatory, antioxidant, and anticancer effects [20, 21, 23]. While evidence of GA use as natural compound with potent antioxidant and antimicrobial activity in food systems is already available, no examples can be found for umbelliferone in this specific sector [24, 25].

Umbelliferone (7-hydroxycoumarin) is a natural phenolic compound widely spread in plants of the coumarin family, with recognized antioxidant properties [26, 27]. The *Umbelliferae* family is inclusive of economically important herbs such as alexanders, sanicle, asafoetida, angelica, celery, fennel, cumin, parsley and giant hogweed [27].

This research activity proposes the development, production and characterization of EVOH and EVOH_AIs based films for food packaging sector. In this work, two different active ingredients, gallic acid and umbelliferone (5 wt. % and 15 wt. %) were used as antimicrobial and antioxidant principles. Different formulations were prepared by both solvent casting and extrusion techniques with the idea to evaluate how the different process techniques and the introduction of natural active agents could influence the final properties of different EVOH based systems.

All produced systems were investigated in terms of thermal, morphological, optical and mechanical properties. Finally, functional properties of relevant importance for food packaging sector, such as overall migration, moisture content, antioxidant and antimicrobial activity were here intensely investigated.

103

104 2. Experimental Part

105 **2.1. Materials**

106 Poly(vinyl alcohol-co-ethylene) with 32 mol % ethylene content (EVOH 32) (density: 1.19 g/mL at
107 25 °C, melt index: 3.8 g/10 min (210 °C)), 1-Propanol reagent, ≥99.5%, gallic acid (GA)
108 ((HO)₃C₆H₂CO₂H, 3,4,5-Trihydroxybenzoic acid, with 98.5% of purity, average, average M_w =
109 170.12 kg mol⁻¹) and umbelliferone (UMB) (C₉H₆O₃, 7-Hydroxycoumarin with 98.5% of purity,
110 average M_w 162.14 kg mol⁻¹) were provided from Sigma-Aldrich® (Milan, Italy).

111

112 **2.2. Characterization of active ingredients (AIs)**

113 The morphological structures of active ingredients in powder state and after dissolution in 1-
114 propanol/water solution (10 wt/v %), were investigated by field emission scanning electron
115 microscope (FESEM, Supra 25-Zeiss). AIs powders were deposited on conductive adhesive, gold
116 sputtered and visualized, while few drops of the AI suspensions were deposited onto a substrate of
117 silicon, dried at room temperature and visualized after gold sputtering. A magnetic stirring at room
118 temperature for 1 hour and a sonication bath treatment (Ultrasonic bath-mod.AC-5, EMMEGI,
119 Italy) for 1 additional hour were applied in order to obtain a uniform dispersion of AIs in the
120 solvent. The absorbance study of AIs solutions (1-propanol/water) was investigated by UV-Vis
121 spectroscopy in the range 250-900 nm by using a Perkin Elmer Lambda 35 spectrometer.

122 Thermogravimetric measurements (TGA) of AI powders were performed under nitrogen flow by
123 using a Seiko Exstar 6300. Heating scans were performed from 30 to 800 °C at 10 °C min⁻¹ and
124 three repetitions of the test have been considered.

125

126 **2.3. Production of EVOH based systems**

127 ***2.3.1. Production of EVOH based films by solvent casting***

128 EVOH and EVOH_AIs based films combined with gallic acid and umbelliferone at 5 and 15 wt. %
129 (EVOH_5GA, EVOH_15GA, EVOH_5UMB and EVOH_15UMB) were prepared by solvent
130 casting method (sEVOH, where “s” indicates the solvent method) by using the procedure described

131 by Martinez-Abad and co-authors [28]]. EVOH was initially dissolved in 1-propanol/water (70:30
132 wt/wt%) in the ratio 8:92 (wt/wt%) under magnetic stirring at 100 °C for 2 h.
133 EVOH_AIs systems were obtained by mixing EVOH solution with a specific amount of the AIs
134 previously dispersed in 1-propanol/water. AIs dispersions were obtained by applying a two steps
135 procedure: firstly, GA and UMB were dispersed under magnetic stirring in 1-propanol/water
136 solution (10 wt/v%) for 1 h at room temperature (RT), after that a bath sonication treatment
137 (Ultrasonic bath-mod.AC-5, EMMEGI, Italy) was applied for 1 hour at RT. EVOH solution was
138 cooled down to approximately 60 °C and then mixed with the AIs solution by magnetic stirring for
139 1 h at RT. Finally, EVOH solutions were cast in a *Teflon*[®] Petri dish and dried in an oven at 60 °C
140 for 3 h. The films (of 140 mm diameter and 50-70µm thick) were stored and equilibrated for 2 days
141 in a desiccator by using silica salt, after the processing and before the characterizations [29]. **Table**
142 **1** summarizes the compositions of all produced formulations, in terms of AIs content, respect to
143 EVOH polymer (the “s” indicates the solvent casting method).

144

145 **2.3.2. Production of EVOH based films by extrusion**

146 Due to the hygroscopic nature of EVOH, GA and UMB, the different materials were pre-dried to
147 avoid any moisture trace in the copolymer and AIs structure and any undesirable hydrolysis reaction
148 during processing. EVOH copolymer pellets were dried in a vacuum oven at 95 °C for 6 h, while
149 the AIs were dried at 50 °C for 12 h.

150 **Table 1** summarizes the compositions of all different formulations, in terms of AI content respect to
151 EVOH copolymer (the “e” indicates the extrusion method).

152 EVOH and EVOH_AIs based films were processed by using a twin-screw microextruder (DSM
153 Explorer 5&15 CC Micro Compounder). Processing parameters (screw speed, mixing time and
154 temperature profile) were modulated to optimize the extrusion procedure. Screw speed at 50 rpm
155 was used for eEVOH based formulations. The temperature profile was set up at 200–210–220 °C in
156 the three different extrusion areas. The total processing time was established at 3 minutes. Neat

157 EVOH was mixed for 3 minutes, while UMB was added to the copolymer matrix after 2 minutes of
 158 mixing, so EVOH_UMB binary based formulations were mixed in total for 1 minute.
 159 eEVOH based formulations combined with GA were not processed and consequently not
 160 characterized, due to the presence of gases emission during the extrusion of the EVOH sample
 161 containing the lower amount of GA (eEVOH_5GA), that induced the formation of holes on the film
 162 surface (visible in the following Figure 3 b, that will be discussed later). On the basis of this result,
 163 we decided do not process the eEVOH_15GA sample.
 164 eEVOH based films with thicknesses between 40 and 60µm were obtained by extrusion.
 165

166 **2.4. Characterization of EVOH_AI based films**

167 **2.4.1. Morphology, transparency and colour analysis**

168 The transparency of different EVOH films produced by using solvent casting and extrusion was
 169 investigated by UV-Vis spectroscopy in the range 250-900 nm by using a Perkin Elmer Lambda 35.
 170 The microstructure of EVOH and EVOH_AI fractured surfaces was investigated by FESEM (Supra
 171 25-Zeiss) after gold sputtering and by using an accelerating voltage of 5 kV.
 172 Colour parameters of sEVOH and eEVOH films from CIELAB colour space were analysed by
 173 using a spectrophotometer (CM-2300d Konica Minolta, Japan). The results were investigated by
 174 using the SCI 10/D65 method, whereas CIELAB colour, as proposed by the Commission
 175 Internationale de l'Éclairage (CIE 1995), were used. The different produced formulations were
 176 positioned on a white standard substrate and L^* , a^* , and b^* parameters were determined. Samples
 177 were examined in triplicate, and three measurements were obtained at random locations on each of
 178 the produced films. The total colour difference ΔE^* between EVOH based systems was obtained as
 179 indicated in Eq. 1.

$$180 \quad \Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (Eq.1)$$

181 Gloss value was also determined by using the SCI 10/D65.

182

2.4.2. Thermal characterizations

Thermal characterization of EVOH and EVOH_AI based films produced by using solvent and extrusion techniques was performed by thermogravimetric measurements (TGA) and by differential scanning calorimetry (DSC). Thermogravimetric measurements were performed by using a Seiko Exstar 6300. Heating scans from 30 to 600 °C at 10 °C min⁻¹ under nitrogen flow were performed for each sample.

Differential scanning calorimetric measurements were performed on a TA Instruments DSC Q200 (TA Instruments Inc., USA) under nitrogen flow in the range from -25 to 210 °C at 10 °C min⁻¹, carrying out two heating and one cooling scans. The glass transition temperature (T_g) was investigated during cooling and second heating scan; crystallization temperature and enthalpy (T_c and ΔH_c) were determined from the cooling scan, whereas the melting temperature and enthalpy (T_m and ΔH_m) were determined from the second heating scan.

The crystallinity degree was evaluated according to the Eq. 2:

$$\chi = \frac{1}{(1 - m_f)} \left[\frac{\Delta H}{\Delta H_0} \right] * 100 \quad (\text{Eq. 2})$$

where ΔH is the enthalpy for melting or crystallization; ΔH_0 is melting enthalpy for a 100% crystalline EVOH sample and $(1 - m_f)$ is the weight fraction of EVOH in the sample.

The melting enthalpy at 100 % of EVOH was calculated according to Eq. 3 [30]:

$$\Delta H_0 = \alpha \Delta H_0^{\text{PVA}} + \beta \Delta H_0^{\text{PE}} \quad (\text{Eq. 3})$$

Where ΔH_0^{PVA} is enthalpy of melting for a 100% crystalline of poly(vinyl alcohol) (PVA) taken as 161.1 J g⁻¹ [31], while ΔH_0^{PE} is enthalpy of melting for a 100% crystalline of polyethylene (PE) taken as 290.0 J g⁻¹ [30] α and β are the weight fraction of vinyl alcohol ($\alpha=0.68$) and ethylene ($\beta=0.32$) in EVOH. ΔH_0 melting enthalpy for a 100% crystalline EVOH is taken as 202.4 J g⁻¹.

2.4.3. Mechanical characterization

207 The mechanical properties of EVOH and EVOH_AIs based films were evaluated by tensile tests, by
208 using rectangular probes (50 mm x 10 mm) on the basis of UNI ISO 527 standard with a load cell of
209 500 N, an initial gauge length of 25 mm and a crosshead speed of 5 mm min⁻¹. Young's modulus
210 (E_{Young}), the tensile strength (σ_B) and elongation at break (ϵ_B) were calculated from the stress-strain
211 curves. The analysis was carried out at room temperature and five samples were tested.

212

213 **2.4.4. Antiradical activity**

214 The radical scavenging activity of cast and extruded EVOH films was determined by using a
215 spectroscopic method according to the procedure proposed in literature by Byun et al. [32]]. The
216 different EVOH based samples (0.1 g) were cut into small pieces and immersed in 2 mL of
217 methanol for 24 h at RT. An aliquot of methanol extract (1 mL) was mixed with 1 mL of DPPH in
218 methanol (50 mg L⁻¹). The mixture was allowed to stand at RT in the dark for 60 min. The
219 absorbance was measured at 517 nm using a UV spectrometer (Lambda 35). The DPPH mixture
220 solution of methanol extracted from neat EVOH films produced by solvent casting and extrusion
221 was used as control. DPPH radical scavenging activity (RSA) was measured according to the Eq.4:

$$222 \quad RSA(\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} * 100 \quad (Eq. 4)$$

223 where A_{sample} is the absorbance of sample and $A_{control}$ is the absorbance of the control.

224

225 **2.4.5. Overall migration and moisture content**

226 The overall migration analysis of EVOH based films producing by using both techniques was
227 determined in triplicate in simulant A (10 % (v/v) ethanol water solution) and simulant D
228 (isooctane) according to Commission Regulation (EU) No 10/2011. The samples of 10 cm² were
229 immersed in 10 mL of simulant [33]. Samples were maintained at 40 °C for 10 days according to
230 EN-1186 standard [34] in contact with the simulant A, while in the case of simulant D the samples
231 were maintained in contact for 2 days at 20 °C. The simulant was then evaporated and residues

weighed with an analytical balance (Sartorius ATILON) with ± 0.01 mg precision. The migrated materials were expressed in mg kg^{-1} of the each simulant.

The moisture content (MC) of EVOH based formulations produced by solvent casting and extrusion was evaluated at 53% RH and 25 °C. Firstly, the different samples were dried in a vacuum oven at 40 °C for 72 h, after that the pre-dried formulations were placed in desiccators containing $\text{Mg}(\text{NO}_3)_2$ salts until reaching constant weight. Three replicates for each formulation for 1 and 5 weeks were analysed. MC was estimated according to the Eq.5.

$$MC(\%) = \frac{W_{\text{Final}} - W_{\text{Initial}}}{W_{\text{Final}}} * 100 \quad (\text{Eq. 5})$$

where W_{Final} is the weight of sample after 1 or 5 weeks at 53% RH and 25 °C and W_{Initial} is the initial weight of different samples dried in a vacuum oven at 40 °C for 72 h

2.4.6. Antibacterial and antifungal studies

Four strains of plant pathogens (bacteria and fungi) were used. Three known high virulent strains of plant pathogenic bacteria (*Pectobacterium carotovorum* susp. *odoriferum* (Pco –1115), *Erwinia carotovora* subsp. *carotovora* (Ecc – CFBP 2577), *Xanthomonas axonopodis* pv. *vesicatoria* (Xav – CFBP 3274) and one virulent strain of a phytopathogenic fungus, *Botrytis cinerea* (CBS 120091), were here evaluated for film antimicrobial assays. Specifically Pco, able to destroy, due to its enzymatic properties, external and internal tissues of several vegetables, such as lettuce, was selected and used [35]. Ecc is able to cause damages to fresh fruits and/or vegetables in open field and during post-harvest phases. In particular, this phyto bacterium is especially dangerous, due to its ability to develop severe soft rot, and then determine relevant economic losses on fresh vegetables and stored fruits [36]. Xav is a quarantine pest particularly dangerous in and out of EU respect to tomato and pepper. It is the causal agent of bacterial spot disease on plants, causing severe symptoms throughout the above-ground portion of the plant, including leaf spots, fruit spots and stem cankers [37]. Finally, *B. cinerea* is a fungus worldwide known, able to colonize host tissue by

a relevant mycelium development, causing extended gray mold, collapse of attacked tissue with consequent heavy damages and economical losses in post-harvest and commercialization phases of numerous vegetable and fruits, like kiwifruits and strawberry [38].

For antimicrobial assays, the reading of the optical density of the bacterial suspension to a wavelength equal to 590 nm was conducted for each bacterial strain, by means of a turbidimeter (Biolog Turbidimeter Mod. 21907). This reading allowed to make appropriate dilutions with sterile distilled water (SDW) necessary to obtain, for each bacterial suspension per isolate, a concentration of 1×10^8 colonies forming units (CFU/mL). The reading of the optical density was different for the strains; in fact it was equal to 0.22 nm for Xav, 0.20 nm for Ecc and of 0.18 nm for Pco, respectively. These values were determined by the development of a calibration curve. The obtained bacterial suspension at a concentration of 1×10^8 CFU/mL 1 mL was then 1/10 diluted with SDW in order to obtain a bacterial concentration of 1×10^7 CFU/mL. 1 mL of this suspension was taken and placed into microbiological tubes with 9 mL of Nutrient Broth (NB) 32%, to obtain a suspension of 10 mL with a final bacterial concentration of 1×10^6 CFU/mL. Eight microbiological tubes per thesis were previously prepared with 9 mL of NB 32%, in which a film of dimensions of 1 cm $\times$ 1 cm was immersed. Two microbiological tubes with microbial inoculation and without the films inside have been considered as positive control. The bacterial solutions at 1×10^6 CFU/mL concentration were incubated in an orbital shaker at a temperature of 27 ± 1 °C at 150 rpm. Subsequently, different samplings were performed after 1 h, 3 h, 12 h, 24 h and 48h. For each tube (3 per thesis) 1 mL of suspension was taken, after that its decimal dilutions (dd) were carried out. For each of them, 100 μ L of dd was plated into a Petri dish containing the growing media (3 plates per dd). The Petri dishes were then incubated for 24–48 h at a temperature of 27 ± 1 °C; subsequently, by using a stereomicroscope, they were observed and the developed bacterial colonies were counted. For the fungus *B. cinerea* the process was different; indeed, the inoculation of microbiological tubes was carried out by using a portion of fungal mycelium, which was grew for one week in a Petri dish containing Potato Dextrose Agar (PDA) medium. The portion used for

283 fungal inoculation had a circle shape of 0.5 mm radius. These tests provided 3 samplings at
284 predetermined intervals (1 day, 4 days and 7 days). Samples were then filtrated with ME 24 0.2 μ m
285 filter in Kitazato flasks using a vacuum pump. Filtrated samples were maintained in biochemical
286 oxygen demand (BOD) and then incubated at 50 °C and weighed daily [39]. Statistical analysis of
287 antimicrobial assays was performed according to the Tukey's honest significant difference (HSD)
288 test, using a significance level with $p=0.01$.

289

290 3. Results and Discussion

291 3.1. GA and UMB characterization

292 **Figure 1, Panel A** shows the results of AI powder characterizations, while **Figure 1, Panel B**
293 shows the results of characterizations made in the case of AIs solutions. Specifically, **Figure 1,**
294 **Panel A** shows the visual observation (chemical structure of the two AIs is also included), and
295 FESEM investigation of gallic acid (**Figure 1, Panel A a,c**, respectively) and umbelliferone
296 (**Figure 1, Panel A b,d**), respectively. GA is a white powder, while UMB denotes yellowish-white
297 crystals [40]. Both AIs in powders have an elongated shape similar to chopsticks, as revealed by
298 FESEM analysis. FESEM images show that GA powder is characterized by higher dimensions
299 respect to the UMB powder. GA powder has a length ranging from 50 μ m to 100 μ m and width
300 ranging from 10 μ m to 25 μ m (**Figure 1, Panel A c**), while UMB powder has a length ranging from
301 2 μ m to 10 μ m and width ranging from 1 μ m to 3 μ m (**Figure 1, Panel A d**).

302 **Figure 1, Panel B a** shows the visual observation of AIs in 1-propanol/water solution, both
303 solutions containing AIs are transparent. Specifically, GA solution was uncoloured while UMB
304 solution tends to yellow colour yellowish colour [40]. The different colour perceived by the human
305 eye is also confirmed by UV-Vis absorption results, reported in **Figure 1, Panel B b**. The
306 absorption spectrum of GA solution shows the main absorption band centred at 310 nm, while the
307 main absorption bands of UMB solution are centred at 320 nm and at 412 nm [41]. This result
308 justified the yellowish colour of UMB solution observed by the human eye and the deviation of film

309 colour to yellow for poly (vinyl alcohol-co-ethylene) films combined with umbelliferone respect to
310 neat sEVOH and eEVOH films.

311 The morphological investigation of AI solutions highlights that the procedure applied to obtain the
312 dispersion of active ingredients permitted to modify the dimension of initial powder (**Figure 1,**
313 **Panel B c,d**). GA structure appears individualized, with reduction in diameters and increase in
314 lengths after the evaporation on 1-propanol/water solution (**Figure 1 Panel B c, d**). In details,
315 FESEM images show that GA was organized as long (10-100 μm) filaments with diameter of 500-
316 550 nm (see the insert **Figure 1, Panel B c**). The FESEM image of UMB in solution shows that the
317 active ingredients after the solution process appear individualized with reduced dimensions respect
318 to the powder, being the width at a nano-scale level (around 500 nm) while the length ranged from
319 1 μm to 5 μm .

320 The thermal stability under inert atmosphere of AIs was analysed by thermogravimetric analysis
321 (TGA). The DTG curve of AIs show a multi-step degradation behaviour for GA [42], similar
322 behaviour has been observed in literature for hydroxytyrosol [9], while only one degradation step
323 is detected for UMB (**Figure 2**).

324 The first low intensity degradation peak for GA, (initial thermal decomposition temperature of 69
325 $^{\circ}\text{C}$) centred at 87 $^{\circ}\text{C}$, is due to the degradation of volatile compounds with low molecular weight
326 [42] and to the mass-loss event attributed to the water content [43]. It is supposed that this
327 evaporation at low temperatures was responsible for the unprocessability of poly (vinyl alcohol-co-
328 ethylene) based formulations combined with both concentration (5 and 15 wt%) of gallic acid
329 (eEVOH_5GA and eEVOH_15GA) (see **Figure 3 b**); the second and main peak, characterized by
330 high intensity, is centred at 270 $^{\circ}\text{C}$, while the third degradation peak at 310 $^{\circ}\text{C}$ is characterized by a
331 very low intensity and corresponds to loss of hydroxyl groups. The fourth peak is centred at 330 $^{\circ}\text{C}$,
332 characterized by very high intensity corresponds to the residual decomposition of gallic acid.
333 Finally, minor degradation phenomena of low intensity were observed at 358 $^{\circ}\text{C}$ and 466 $^{\circ}\text{C}$,

334 respectively. The both last two peaks correspond to the residual decomposition of gallic acid at high
335 temperature [43, 44].

336 The main degradation step of UMB is centred at 305 °C. In general, the coumarine (powder)
337 exhibits a single degradation step [45]. Comparing the onset temperature of main degradation peak
338 (T_{onset} main peak) of both active ingredients, it is possible to underline that UMB showed the T_{onset}
339 main peak at 203 °C, while the onset for GA moved at 223 °C.

340

341 **3.2. Transparency, colorimetric analysis and FESEM investigation of EVOH and EVOH_AIs** 342 **based systems**

343

344 Transparency and colour are the most important properties of the polymeric films, especially when
345 they were realized and used in food packaging sector. The aesthetic properties, in fact, can influence
346 the commercial success and consumer acceptance.

347 **Figure 3** and **Figure 4** show visual observations and UV-Vis characterizations, respectively, of
348 EVOH based formulations produced by solvent casting and extrusion. Specifically, **Figure 3 a** and
349 **Figure 4 a** show the transparency and the visual observation of sEVOH films, while the UV-Vis
350 curves and the photo of eEVOH films have been reported in **Figure 3 b** and **Figure 4 b**,
351 respectively.

352 sEVOH and eEVOH, as shown in **Figure 4 a,b** are transparent films, with a transmittance value of
353 94% at a visible wavelength of 700 nm. All the sEVOH systems, except sEVOH_15UMB, maintain
354 good levels of transparency (92%) from 450 to 900 nm. The presence of GA in sEVOH does not
355 influence the transparency values detected at 700 nm (**Figure 4a**), while the transparency of
356 sEVOH films combined with gallic acid at both concentrations (sEVOH_5GA and sEVOH_15GA)
357 is drastically reduced above 357 nm, being this behaviour induced by the presence of GA that
358 absorbs the light at 350 nm (**Figure 2 Panel B,b**).

359 The presence of UMB at high concentration (15 wt%) in sEVOH_15UMB induces a clear reduction
 360 in terms of transparency respect to sEVOH_5UMB. is related to the presence of Being the UMB
 361 slight soluble in aqueous solutions, the transparency of sEVOH_15UMB at 700 nm is, accordingly,
 362 around 80% and decreases drastically from 450 nm to 250 nm. The transparency reduction in
 363 sEVOH_5UMB and in sEVOH_15UMB at 420 nm (**Figure 4a**, see the arrows) is due to the light
 364 absorption of UMB (**Figure 2 Panel B, b**) [41]. In eEVOH based formulations, the presence of
 365 UMB does not reduce the transparency of eEVOH_5UMB and eEVOH_15UMB from 450 to 900
 366 nm. Also in the case of extruded films, the presence of UMB reduces the transparency of eEVOH
 367 film for wavelengths below 450 nm, the reduction level is centred at 420 °C (see arrow in **Figure**
 368 **4b**).

369 The colour and gloss parameters of EVOH based formulations are reported in **Table 2**. The
 370 colorimetric and gloss investigations have been performed to evaluate the influence of active
 371 ingredients on the optical properties of EVOH based formulations.

372 sEVOH and eEVOH films have been characterized by high and similar lightness value
 373 ($L^*=98.73\pm0.16$) and ($L^*=98.42\pm0.10$), respectively for cast and extruded samples. Moreover, no
 374 significant variations have been registered between neat sEVOH and eEVOH films in terms of
 375 a^* and b^* coordinates. All of the samples are transparent and homogeneous, as the luminosity values
 376 (L) have shown, similar observation have been done for EVOH combined with catechin in
 377 accordance to the literature [46]. This behaviour highlights how the presence of GA does not affect
 378 the colour of different formulations. For this formulation, in fact similar colour parameters
 379 (L , a^* and b^*) have been registered (**Table 2**). This behaviour can be ascribed to the transparent
 380 presence of GA that in 1-propanol/water solution, as analysed by visual observation (**Figure 2**
 381 **Panel B a**) and by UV-Vis analysis from 900 nm to 450 nm (**Figure 2 Panel B b**).

382 The presence of UMB in sEVOH based formulations induces a colour variation respect to the white
 383 control and neat sEVOH film, as expected observing the visual images of UMB powder (**Figure 2**
 384 **Panel A b**) and solution (**Figure 2 Panel B a**).

385 Positive values have been registered in sEVOH films combined with UMB for b^* parameter,
386 indicating a slight deviation towards brown hues, yellowish and desert sand colour (reflected by the
387 rise in the a^* and b^*). The film colours have been induced by the colour of umbelliferone, in
388 agreement with visual observations and UV–Vis analysis (**Figure 3 a** and **Figure 4 a**). The highest
389 ΔE^* value for sEVOH films has been obtained for sEVOH_15UMB based system
390 ($\Delta E^*=14.96\pm0.39$).

391 A similar behaviour has been found also for eEVOH based films. The presence of UMB in eEVOH
392 films induced a clear variation of colour respect to neat film. Even in the case of extruded films, the
393 maximum colour differences have been registered for eEVOH_15UMB ($\Delta E^*=10.89\pm0.06$). For
394 extruded films containing UMB, the colour parameters have in general lower values, with respect of
395 ones registered for sEVOH films combined with UMB. This result could be considered due to the
396 melting of UMB during the extrusion process.

397 The gloss values of EVOH and EVOH based formulations are summarized in **Table 2**. The
398 presence of GA in sEVOH films induces a slight increase of gloss and surface finish also at high
399 content respect to neat film (gloss: sEVOH= $(158\pm2)^\circ$, sEVOH_5GA= $(200\pm4)^\circ$,
400 sEVOH_15GA= $(187\pm2)^\circ$). The presence of UMB in eEVOH films induces a slight reduction of
401 gloss and surface finish (gloss: sEVOH_5UMB= $(138\pm2)^\circ$, sEVOH_15UMB= $(46\pm3)^\circ$), and this
402 result could be correlated to the presence UMB at micron scale dimension. The gloss in eEVOH
403 films increases adding the UMB in the films also at high concentration, being again this
404 phenomenon due to the melting of UMB gloss: eEVOH= $(206\pm5)^\circ$, eEVOH_5UMB= $(268\pm1)^\circ$,
405 eEVOH_15UMB= $(262\pm3)^\circ$).

406 **Figure 5** shows the microstructure of the fractured surfaces of EVOH based films produced by
407 solvent casting and extrusion, analysed by FESEM. The FESEM study was carried out to evaluate
408 the effect, on microstructure, of AIs and different process techniques used to produce the different
409 EVOH based formulations.

sEVOH film fractured surface appeared homogeneous, uniform and smooth (**Figure 5 a,b**) [47], typically found for thermoplastic and well processed semi-crystalline polymers and copolymers [18]. This behaviour highlights the good film-forming properties and processability of EVOH films during solvent casting process. The presence the AIs (GA and UMB) in cast based formulations does not induce alterations in terms of uniformity and homogeneity on the fractured surfaces of sEVOH_5GA (**Figure 5c**), sEVOH_15GA (**Figure 5d**) and sEVOH_5UMB (**Figure 5e**) respect to neat sEVOH film, expect in the presence of 15 wt% of UMB in the copolymer matrix (see **Figure 5f and Figure 5f insert**). GA in copolymer matrix processed by solvent casting results well dispersed, due to its high solubility in water solution [48]. The FESEM image of sEVOH_15 UMB, especially at higher magnification, (**Figure 5f insert**, see the arrows) shows the presence of UMB, not properly solubilized in 1-propanol/water solution during the film-forming production. This effect is due to the presence of UMB that is slightly soluble in aqueous solutions [27, 40]. The insolubility of UMB in 1-propanol/water solution has been also demonstrated by the FESEM investigation of the AI itself (**Figure 2, Panel B, d**).

Similar results obtained for EVOH based films produced by solvent casting (sEVOH) have been also observed for EVOH based formulations produced by extrusion (eEVOH) (**Figure 5 g-l**).

Neat eEVOH film fractured surface appears homogeneous, uniform and smooth (see **Figure 5 g**) as previously observed for sEVOH film. The addition of UMB does not affect the homogeneity and uniformity of eEVOH fractured surface by using both concentrations (5 and 15 wt%) of UMB, as already observed applying solvent casting process to produce sEVOH_15UMB. This behaviour can be related to the melting temperature of UMB powders, that was at approximately 224-227 °C [27]. The melting temperature of UMB is, in fact, in the same range of the temperature profile (200-210-220 °C) selected and optimized to produce eEVOH films.

3.3. Thermal characterizations of EVOH and EVOH_AI based systems

435 In order to evaluate the effect of AIs and the influence of different applied processes on the final
 436 thermal properties of realized films, thermogravimetric analysis (TGA) and differential scanning
 437 calorimetry (DSC) under inert atmosphere of EVOH based formulations produced by solvent
 438 casting and extrusion techniques have been considered.

439 **Figure 6** shows the derivative curves (DTG) of sEVOH (**Figure 6a**) and eEVOH (**Figure 6b**). Neat
 440 EVOH and EVOH based films are characterized by the presence of a multi-step degradation
 441 behaviour.

442 Neat EVOH systems show two main degradation steps, the first one above 400 °C at 370-390 °C,
 443 attributed to the major component fraction, poly(vinyl alcohol) [4, 49]. The second one at higher
 444 temperature at around 450 °C [49] could be attributed to ethylene presence [49]. In cast films, the
 445 peak centred at low temperature (about 110-120 °C) is found only for cast films and corresponds to
 446 the evaporation of residual solvent (1-propanol/water) [50]. The peak at approximately 250 °C has
 447 been observed only for sEVOH films combined with GA, in fact the intensity increased with
 448 increasing content of GA in the copolymer matrix (**Figure 6 a**) and could be essentially correlated
 449 to the thermal degradation of GA. In the presence of both AIs, in cast films, thermal stability is
 450 improved, since a shift of the onset degradation temperature of this main peak ($T_{onset\ main\ peak}$) at
 451 higher temperature is observed ($T_{onset\ main\ peak\ sEVOH}=278\text{ °C}$, $T_{onset\ main\ peak\ sEVOH_5GA}=322\text{ °C}$,
 452 $T_{onset\ main\ peak\ sEVOH_15GA}=322\text{ °C}$, $T_{onset\ main\ peak\ sEVOH_5UMB}=315\text{ °C}$ and $T_{onset\ main\ peak\ sEVOH_15UMB}=316\text{ °C}$).
 453

454 The presence of AI in sEVOH based film increases the thermal stability of copolymer matrix, the
 455 effect is clearly visible also by using the low content of AI (5 wt%). The addition of GA and UMB
 456 shifts, to higher temperatures, the main degradation step of about 20 °C respect to neat sEVOH film
 457 (sEVOH $T_{max\ main\ peak}=372\text{ °C}$) (**Figure 6 a**). Similar phenomenon has been observed in literature by
 458 de Dicastillo and co-authors (2011) combining green tea extract in ethylene vinyl alcohol
 459 copolymer [4] and combining other phenol extracts in thermoplastic matrices [9, 18].

460 In extruded films, the main degradation peak of eEVOH film is centred at 375 °C. The neat EVOH
 461 films produced by solvent casting and extrusion have the main degradation peak at same
 462 temperature value, this phenomenon underlines that the different process techniques do not
 463 influence the thermal stability. However, the presence of UMB in eEVOH based films does not
 464 induced a high shift of main degradation temperature to higher values ($T_{main\ peak\ eEVOH}=375\ ^\circ C$,
 465 $T_{main\ peak\ eEVOH_5\ UMB}=380\ ^\circ C$ and $T_{main\ peak\ eEVOH_15\ UMB}=380\ ^\circ C$) as previously
 466 observed for sEVOH. A similar phenomenon with a shift to higher values of $T_{onset\ main\ peak}$ (onset
 467 degradation temperature of main peak) induced by the presence of AI in sEVOH films is observed
 468 also for eEVOH combined with UMB. The presence of UMB in eEVOH films induces a shift to
 469 higher temperature for $T_{onset\ main\ peak}$ at around of 20 °C ($T_{onset\ main\ peak\ eEVOH}=278\ ^\circ C$, $T_{onset\ main\ peak\ eEVOH_5UMB}=315\ ^\circ C$ and $T_{onset\ main\ peak\ eEVOH_15UMB}=315\ ^\circ C$).

471 Thermal properties of cast and extruded EVOH based formulations films have been also analysed
 472 by DSC analysis in order to evaluate the effect of AI presence and content on the crystallization and
 473 melting phenomena of copolymer matrix. **Table 3** summarizes the glass transition, crystallization
 474 and melting phenomena of sEVOH and eEVOH based formulations during the cooling and the
 475 second heating scan, while the thermograms for the second heating scan are reported in **Figure 7**
 476 **a,b**. **Figure 7 a** shows the thermograms of sEVOH based formulations, while **Figure 6 b** shows the
 477 thermograms of eEVOH based formulations.

478 The incorporation of AIs induces a change in the thermal properties of the different EVOH based
 479 formulations produced by solvent casting and extrusion techniques. The glass transition temperature
 480 (T_g) decreases increasing the amount of AI in the neat sEVOH and/or eEVOH during the cooling
 481 and second heating scan (**Table 3**), as already observed in literature [50]. No evident variations
 482 have been found in terms of glass transition temperature, when a comparison of the data obtained
 483 for the same formulation produced by solvent casting and extrusion techniques was done. Data
 484 obtained from cooling scan highlight the presence of two crystallizations peaks, the first one at low
 485 temperature at around 105 °C for neat sEVOH and eEVOH films, and the second one centred at

high temperature (160 °C for both neat sEVOH and eEVOH films). In general, during the cooling scan, the crystallization temperature and enthalpy decrease increasing the concentration of AIs in the matrix. As a consequence of cooling enthalpy decrease, even the crystallization degrees result reduced, the lower value of X_c for sEVOH based formulations has been registered for sEVOH_15GA film ($X_c=(24.5\pm0.5)\%$), while the lower value of crystallization degree has been measured for eEVOH_15UMB film ($X_c=(29.4\pm2.1)\%$) (**Table 3**). During the second heating scan, a general decrease of the T_m and the ΔH_m values has been registered in the presence of AIs, specifically these values decreased increasing the content of active ingredients in sEVOH and eEVOH based films (**Table 3 and Figure 7 a,b**) [50]. Summering, no particular variations have been observed from the second heating scan for the same formulations produced applying the two different processing techniques.

497

498

3.4. Mechanical characterizations of EVOH and EVOH_AI based systems

The presence of AIs in EVOH matrix and the effect of different applied process for the production of the different EVOH based systems, were investigated in terms of mechanical properties by means of tensile tests. Results are summarized in **Table 4**, while the stress-strain curves are reported in **Figure 9**. Specifically, **Figure 9 a** shows the stress-strain curves of EVOH films produced by solvent casting, while **Figure 9 b** shows the stress-strain curves of EVOH films produced by extrusion.

The selection of different process techniques used to realize the different EVOH based formulations induces a clear difference in terms of mechanical performance (**Table 4**). Focusing on the elongation at break and on Young's modulus values obtained for all EVOH based formulations, it is relevant to observe that the films show an evident ductile behaviour. Analysing and comparing the data of eEVOH films respect to the sEVOH films, it is possible to see that extruded films have higher values of Young's modulus (E_{Young}) and lower values of elongation at break (ϵ_b). The main

512 reason of this different mechanical behaviour is strictly related to the processing methodology used
 513 to realize the different formulations. This phenomenon is also related to the presence of moisture
 514 content absorbed in the copolymer material, that behaves as a plasticizer, as previously observed in
 515 literature using different polymers and copolymers [16].
 516 Blanchard and co-authors (2017) investigated the effect of moisture content on the mechanical
 517 properties of EVOH copolymers, and they observed that the plasticization phenomenon of EVOH
 518 (32 %) increased increasing the amount of adsorbed water molecules in the material [16].
 519 Neat sEVOH shows tensile strength of ($\sigma_B=45\pm4$) MPa, elongation at break of ($\epsilon_B=265\pm28$) % and
 520 Young's modulus of ($E_{Young}=440\pm65$) MPa, while eEVOH shows tensile strength of ($\sigma_B=67\pm7$)
 521 MPa, elongation at break of ($\epsilon_B=226\pm25$) % and Young's modulus of ($E_{Young}=2028\pm115$) MPa.
 522 The presence of 5wt% of GA in sEVOH films permits to improve Young's modulus
 523 ($E_{Young}=560\pm65$)MPa with respect of neat copolymer, maintaining at the same time similar values
 524 of tensile strength and elongation at break respect to sEVOH film (**Table 4**). The presence of 15 wt
 525 % GA in sEVOH_15GA induces a slight reduction of E_{Young} (315 ± 53) MPa and ϵ_B (220 ± 26) with
 526 respect of sEVOH and sEVOH_5GA.
 527 The presence of umbelliferone, mainly used as antioxidant and antimicrobial additive in this
 528 research activity at 5 wt%, does not affect the mechanical response of EVOH copolymer processed
 529 by solvent casting (sEVOH_5UMB), except a slight reduction registered for elastic modulus. The
 530 presence of 15 wt% UMB in sEVOH_15UMB acts as a defect, the mechanical properties have been
 531 reduced respect to sEVOH film, essentially a reduction of tensile test at break and elastic modulus
 532 (sEVOH_15UMB: $\sigma_B=(32\pm4)$ MPa, $\epsilon_B=(260\pm30)$ % and $E_{Young}=(190\pm30)$ MPa) have been
 533 observed. This phenomenon is due to the presence of the AI powder that was not soluble in the 1-
 534 propanol / water solution used to dissolve the EVOH copolymers (**Figure 2, Panel B b**).
 535 Furthermore, the presence of UMB in powder has been also detected by FESEM investigations of
 536 sEVOH_15UMB fractured surface (**Figure 5f**). The presence of UMB in eEVOH at both the

concentrations reduces the ductile behaviour of neat copolymer film processed by extrusion (eEVOH) (**Table 4** and **Figure 9 b**).

Specifically, the presence of UMB induces a reduction of elongation at break and an increase of tensile strength respect to neat eEVOH film.

eEVOH_5UMB shows tensile strength of ($\sigma_B=102\pm16$) MPa, elongation at break of ($\epsilon_B=131\pm24$) % and Young's modulus of ($E_{Young}=2565\pm49$) MPa, while eEVOH_15UMB shows tensile strength of ($\sigma_B=89\pm10$) MPa, elongation at break of ($\epsilon_B=153\pm28$) % and Young's modulus of ($E_{Young}=2053\pm69$) MPa.

Concluding, sEVOH_5GA and eEVOH_15UMB show the best mechanical performance in terms of elastic and plastic mechanical response.

3.5. Antiradical activity, overall migration test and moisture content of EVOH_AI based systems

The main important properties requested to polymeric devices applied in food packaging applications and used in direct contact with food products are the antiradical activities, moisture content and overall migration.

Antiradical activity is a property induced by the presence of active compounds in polymeric systems, specifically antioxidant action of polymeric materials is based on a migration process into the food products of active ingredients incorporated in polymeric packaging [10].

The continue demand of intelligent and active food packaging had promoted the development and use of active ingredients into packaging devices. The antioxidant properties of EVOH and EVOH_AI based films produced by using solvent casting and extrusion techniques were evaluated by the scavenging activity of methanol migration extracts against the DPPH radical. This study is an indirect test used to evaluate the antiradical activity of the produced films.

Table 5 summarizes the antioxidant behaviour of migrating substances in methanol for 24 h for different EVOH based formulations. sEVOH and eEVOH have been selected as control for solvent

casting and extruded produced systems, respectively. GA and UMB intercept the free radical chain of oxidation by donating hydrogen from the phenolic hydroxyl groups, in this way there is the formation of a stable end product, which does not initiate or propagate oxidation of the lipids [51]. The incorporation of GA and UMB in sEVOH films induces a clear effect of RSA % [24, 25, 27]: the highest effect is induced by presence of GA respect to UMB (RSA (%): sEVOH_5 GA=(95.0±2.0) %; sEVOH_15 GA=(93.2±0.3) %; sEVOH_5 UMB=(20.3±3.2) % and sEVOH_15 UMB=(75.2±0.8) %). The highest RSA% has been registered for sEVOH films combined with GA respect to sEVOH films combined with UMB. This behaviour is due to the different chemical composition of the two different selected AIs [24, 25, 27]. However, the presence of highest content of GA (15 wt%) in sEVOH_15GA does not induce a higher RSA% respect to sEVOH_5GA, being this phenomenon due to the fact that the lowest concentration is able to induce the highest level of antioxidant activity (**Table 5**). The incorporation of UMB in eEVOH films induces a similar behaviour, as previously considered in sEVOH films. The highest antioxidant effect is observed for eEVOH_15 UMB RSA=(62.1±2.9) %.

Overall migration test was performed, in accordance with the Commission Regulation EU 10/2011 [33], to study if the different process used and the addition of AIs to produce EVOH based films could influence the migration level, results are shown in **Figure 9**. Specifically, this test permits to simulate and to evaluate the migration of polymeric materials to the foodstuffs and to determine if the produced packaging could be used in contact with food products directly. In this research activity, the test was performed by using two different food simulants, ethanol 10 (v/v) % and isooctane (A and substitutive D, respectively). The selection of both simulants was of great importance to analyse the behaviour of polymeric films in contact with two different types of foodstuffs. The simulants A and D have been used to simulate the behaviour of polymeric films in contact with aqueous based foods and with fatty foods, respectively.

The overall migration levels using simulants A for all EVOH based formulations produced by solvent casting (**Figure 9 a**) and extrusion (**Figure 9 b**) were, as expected, up the migration

589 permitted limits (60 mg kg^{-1} of simulant), as established by the current European legislation [33],
 590 the migration level increased increasing the AI in the copolymer matrix.

591 Different behaviour was, on the other hand, registered by using simulants D: in this case, the overall
 592 migration levels for all EVOH based formulations produced by solvent casting and extrusion are
 593 below the migration permitted limits (60 mg kg^{-1} of simulant), as established by the current
 594 European legislation [33]. Specifically, after 2 days of incubation at 20°C (simulant D conditions)
 595 no polymeric migration level has been registered for neat sEVOH (**Figure 9 a**) and eEVOH (**Figure**
 596 **9 b**) films [52]. In general, the migration level increases increasing the AI content in the copolymer
 597 matrix. The presence of GA in sEVOH induces a slight increase of migration levels respect to
 598 sEVOH films (sEVOH_5GA= $(1.4\pm0.4) \text{ mg kg}^{-1}$ and sEVOH_15GA= $(2.0\pm0.4) \text{ mg kg}^{-1}$).

599 Similar migration values for EVOH films processed by solvent casting and extrusion, combined
 600 with the same content of UMB, have been registered. Moreover, the maximum migration levels is
 601 detected combining the copolymer matrix with 15 wt% of UMB (sEVOH_15UMB= $(4.8\pm0.6) \text{ mg}$
 602 kg^{-1} and eEVOH_15UMB= $(4.3\pm0.4) \text{ mg kg}^{-1}$), while combining EVOH with 5 wt% of UMB the
 603 same value for both processing techniques (sEVOH_5UMB=eEVOH_5UMB= $(3.8\pm0.4) \text{ mg kg}^{-1}$)
 604 has been registered (**Figure 8**).

605 The analysis demonstrated the possible practical application of the produced films in the food
 606 packaging sector in contact with fatty food product, otherwise EVOH based films could be used to
 607 cover fresh food with high content of water only after making a multilayer layer based system and
 608 putting in contact to fresh food hydrophobic polymers, as polypropylene (PP) [52].

609 **Table 6** shows the moisture content (MC) of EVOH and EVOH_AI based formulations produced
 610 by solvent casting and extrusion after 1 and 5 storage weeks at 53% RH and 25°C . This test is of
 611 great importance in food packaging sector, it permits to evaluate the moisture content that could be
 612 absorbed in different environmental condition (temperature and relative humidity). This study is of
 613 fundamental importance to determine the MC. The increase of MC causes a significant loss in terms

614 of thermal, mechanical and barrier properties at high relative humidity. The absorption of water
615 induces an increase of the copolymer free volume [16].

616 The MC % of the sEVOH film is (1.49 ± 0.10) % and (1.53 ± 0.14) %, while for eEVOH film was
617 (2.34 ± 0.23) % and (2.32 ± 0.12) %, after 1 or 5 week of storage, respectively for each formulation.
618 The obtained data indicated that the equilibrium was reached after only 1 week of storage [53]. The
619 addition of AIs in EVOH based films induces a slight increase of MC. Regarding the sEVOH films,
620 the addition of GA induces a slight increase of moisture content respect to the presence of UMB in
621 sEVOH films. This behaviour is due to the presence of GA, that showed an affinity to solubilize in
622 aqueous solutions [48]. In general, it is possible to underline that the films produced by solvent
623 casting are characterized by lower values of MC respect to extruded films. This behaviour is due to
624 two different phenomena that could influence the MC absorption. The first phenomenon can be
625 related to the microstructures of EVOH based films (**Figure 5**). Specifically, eEVOH films show
626 some irregularities respect to sEVOH formulations (see the arrows in **Figure 5 g-l**) on the surfaces
627 of different extruded films facilitating the moisture absorption. The second phenomenon that
628 justifies the higher absorption of moisture is the lower crystallinity degrees at second heating scan
629 (X_m) for eEVOH based formulations. (**Table 3**).

630 After 5 weeks of storage, the MC values for all different produced formulations tended to reduce or
631 remain unchanged respect to the values obtained after 1 week of storage (**Table 6**), this behaviour
632 can be ascribed to saturation phenomena, similar results are obtained in literature by using
633 poly(vinyl alcohol) [50].

634

635 **3.6. Antibacterial and antifungal properties of EVOH_AI based systems**

636 The antimicrobial test of neat EVOH and EVOH based formulations loaded with 5% wt of gallic
637 acid and umbelliferone processed by solvent casting and with 5 and 15% wt of umbelliferone
638 processed by melt compounding approach, have been conducted respect to three high virulent
639 strains of bacteria (*Xanthomonas axonopodis* pv. *vesicatoria* (Xav – CFBP 3274); *Pectobacterium*

640 *carotovorum* susp. *odoriferum* (Pco – CFBP 1878T), and *Erwinia carotovora* subsp. *carotovora*
641 (Ecc – CFBP 2577),) and one virulent strain of a fungal plant pathogen, *Botrytis cinerea* (CBS
642 120091), and the results are summarized in **Figure 10 a-d**.

643 The efficacy of 5%wt of umbelliferone in cast film is clearly evident against both Xav and Pco
644 multiplication just after 18 h (**Figure 10 a,b**) whereas both gallic acid and umbelliferone based
645 formulation produced by solvent casting show a significant influence in the reduction of bacterial
646 growth after 48h. On the contrary, solvent cast films, loaded with both gallic acid and umbelliferone
647 at 5%wt, do not highlight significant reductions on Ecc growth (**Figure 10 c**) and a similar result is
648 obtained, for these formulations, against *B. cinerea* growth, with only a significant low reduction of
649 fungal growth after 7 day exerted by sEVOH_5GA cast film (**Figure 10 d**).

650 A different behaviour has been detected for extruded based formulations loaded with two different
651 concentrations of umbelliferone. Growth curves with extruded films reveal a non-susceptibility of
652 Xav at both tested concentrations (5% and 15%wt) (**Figure 10 a**), whereas a reduction of Ecc
653 multiplication and a significant activity (reduction of 1.5 Log CFU/mL) against Pco is recorded
654 only for the EVOH loaded with the highest concentration of umbelliferone (**Figure 10 b,c**). Finally,
655 all extruded films reveal an interesting activity to reduce the fungal growth after 7 days (**Figure 10**
656 **d**).

657 The antimicrobial assays underlined that umbelliferone resulted the most effective active agent
658 against the considered strains, both bacteria and fungus, and this effect is related to the capacity of
659 this molecule to inhibit bacterial biofilm development [54] and to its negative effects on different
660 fungal structure development (i.e.: hyphae distorted) [55].

661 Mechanisms of activity of these films should be furtherly studied in deep, clarifying their
662 performance respect to different microorganisms (bacteria and fungi) and their mechanisms to cause
663 damages on fresh agro-production. In any case, the fact that selected natural active ingredients
664 (gallic acid and umbelliferone) resulted able to contrast the multiplication of dangerous
665 microorganisms like those selected, offers the opportunity to develop innovative and green

666 approaches to reduce damages caused by these dangerous microorganisms during post-harvest
667 phases.

668

669 **4. Conclusions**

670 This research activity considered the effect of solvent casting and extrusion process techniques on
671 EVOH based films to be used in food packaging applications. Active properties were offered by
672 gallic acid and umbelliferone, with improved antioxidant and antimicrobial properties, of great
673 importance in food packaging systems. EVOH based systems combined with umbelliferone were
674 successfully prepared by solvent casting (sEVOH) and extrusion (eEVOH), while EVOH based
675 films were combined with GA produced applying solvent casting techniques. The presence of AIs
676 influenced the thermal properties of EVOH based systems, in details the thermal degradation
677 stability increased adding GA or UMB to EVOH matrix, while the crystallinity degrees decreased
678 in active EVOH based formulations.

679 No particular alterations in terms of mechanical performance (strength and elastic modulus) in the
680 presence of AIs for cast and extruded films were noted, while a reduction in terms of elongation at
681 break was registered for extruded films combined with UMB. The registered migration levels were
682 below the permitted limits, by confirming the possible use of produced films in contact with fatty
683 foods. The use of AIs induced antioxidant activities, inhibitions on bacterial and antifungal growth,
684 suggested the practical and possible applications in food packaging industrial sectors to realize
685 active systems with enhanced antioxidant/antimicrobial properties respect to neat EVOH.

686 The results of functional characterization of proposed active formulations confirmed the possibility
687 to use these innovative solutions in the food quality preservation during transportation and storage.

688

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690

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693 antibatteriche e/o antiossidanti indotte da molecole bioattive naturali. (Founded by Fondazione
694 CARIT, Italy).

695

696

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851

852 **Figure and Table Captions**

853 **Figure 1:** Panel A AIs powders: Visual images(chemical structure, see insert of visual image of
854 AIs)and FESEM investigation of gallic acid (a,c respectively) and umbelliferone (b,d respectively).

855 Panel B AIs solutions: Visual images (a) and UV-Vis analysis (b) of AIs in 1-propanol/water
856 solution, FESEM investigation of gallic acid (c) and umbelliferone (d) in 1-propanol/water solution.

857 **Figure 2:** Derivative curves (DTG) of gallic acid and umbelliferone in powder.

858 **Figure 3:** Visual images of EVOH based formulations produced by solvent casting (a) and
859 extrusion (b).

860 **Figure 4:** UV-Vis analysis of EVOH based formulations produced by solvent casting (a) and
861 extrusion (b).

862 **Figure 5:** FESEM investigation of fractured surface of EVOH based formulations.

863 **Figure 6:** Derivative curves (DTG) of EVOH based formulations produced by solvent casting (a)
864 and extrusion (b).

865 **Figure 7:** DSC thermograms (second heating scan) of EVOH based formulations produced by
866 solvent casting (a) and extrusion (b).

867 **Figure 8:** Stress-strain curves of EVOH based formulations produced by solvent casting (a) and
868 extrusion (b).

869 **Figure 9:** Overall migration in ethanol 10 (v/v)% and isooctane of EVOH based formulations
870 produced by solvent casting (a) and extrusion (b).

871 **Figure 10:**Antibacterial activities respect to *Xanthomonas axonopodis* pv. *vesicatoria* (Xav - CFBP
872 3274) (a), *Pectobacterium carotovorum* susp. *odoriferum* (Pco – 1115) (b), *Erwinia carotovora*
873 subsp. *carotovora* (Ecc – CFBP 2577) (c) and antifungal test respect to *Botrytis cinerea* (CBS
874 120091) (d) for EVOH based systems produced by solvent casting and extrusion. For each
875 evaluation date, columns with different letters are significantly different according to Tukey's HSD
876 test ($p=0.01$). Significant differences among the bars are marked with different letters; if there is no
877 significant difference among the bars they get the same letter.

878

879 **Table 1:** Material formulations of EVOH based films produced by solvent casting and extrusion.

880 **Table 2:** Colour coordinates of EVOH based systems produced by solvent casting and extrusion.

881 **Table 3:** Differential scanning calorimetry (DSC) data of EVOH based systems produced by
882 solvent casting and extrusion.

883 **Table 4:** Mechanical properties of EVOH based systems produced by solvent casting and extrusion.

884 **Table 5:** Radical scavenging activity (RSA) of EVOH based systems produced by solvent casting
885 and extrusion.

886 **Table 6:** Moisture content (*MC*) of EVOH based systems produced by solvent casting and
887 extrusion.

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Table 1: Material formulations of EVOH based films produced by solvent casting and extrusion.

<i>Formulations</i>			
EVOH (wt %)	AI (wt %)	<i>Solvent casting</i>	<i>Extrusion</i>
100	-	<i>sEVOH</i>	<i>eEVOH</i>
95	5	<i>sEVOH_5GA</i>	<i>eEVOH_5GA</i> *
85	15	<i>sEVOH_15GA</i>	<i>eEVOH_15GA</i> *
95	5	<i>sEVOH_5UMB</i>	<i>eEVOH_5UMB</i> *
85	15	<i>sEVOH_15UMB</i>	<i>eEVOH_15UMB</i> *

* GA or UMB were added after 2 min of EVOH mixing. eEVOH_AI were mixed for 1 min.

Table 2: Colour coordinates of EVOH based systems produced by solvent casting and extrusion.

<i>Formulations</i>	<i>L*</i>	<i>a*</i>	<i>b*</i>	<i>ΔE*</i>	<i>Gloss (°)</i>
<i>White Control</i>	99.47±0.00	-0.08±0.01	-0.08±0.01	-	121±0
<i>Solvent casting</i>					
<i>sEVOH</i>	98.73±0.16	-0.05±0.00	0.12±0.00	0.77±0.15	158±2
<i>sEVOH_5GA</i>	98.85±0.08	-0.11±0.00	0.21±0.02	0.68±0.08	200±4
<i>sEVOH_15GA</i>	98.61±0.08	-0.15±0.00	0.33±0.03	0.96±0.08	187±2
<i>sEVOH_5UMB</i>	95.11±0.33	0.44±0.03	6.34±0.46	7.78±0.52	138±2
<i>sEVOH_15UMB</i>	92.39±0.15	1.18±13.04	13.04±0.38	14.96±0.39	46±3
<i>Extrusion</i>					
<i>eEVOH</i>	98.42±0.10	-0.07±0.12	0.98±0.08	1.50±0.13	206±5
<i>eEVOH_5UMB</i>	96.44±0.07	-0.05±0.00	5.67±0.11	6.50±0.13	268±1
<i>eEVOH_15UMB</i>	95.24±0.04	-0.27±0.02	9.96±0.05	10.89±0.06	262±3

Table 3: Differential scanning calorimetry (DSC) data of EVOH based systems produced by solvent casting and extrusion.

<i>Formulations</i>	<i>Cooling scan</i>						<i>Second heating scan</i>			
	$T_g(^{\circ}\text{C})$	$\Delta H'_c(\text{J g}^{-1})$	$T'_c(^{\circ}\text{C})$	$\Delta H''_c(\text{J g}^{-1})$	$T''_c(^{\circ}\text{C})$	$X_c(\%)$	$T_g(^{\circ}\text{C})$	$\Delta H_m(\text{J g}^{-1})$	$T_m(^{\circ}\text{C})$	$X_m(\%)$
<i>Solvent casting</i>										
<i>sEVOH</i>	61.0±0.7	2.2±0.2	104.9±0.4	71.8±0.4	159.9±0.2	36.6±0.3	64.6±0.3	83.2±0.1	182.9±0.1	41.1±0.1
<i>sEVOH_5GA</i>	59.3±2.0	1.5±0.2	94.9±0.2	58.0±1.7	154.1±0.1	30.9±0.8	62.0±0.4	67.1±0.2	179.1±0.4	34.9±0.1
<i>sEVOH_15GA</i>	57.4±2.4	-	-	42.1±0.9	134.9±0.6	24.5±0.5	61.0±1.8	50.1±4.0	167.7±0.8	29.1±2.3
<i>sEVOH_5UMB</i>	51.8±0.7	1.3±0.4	98.8±1.6	57.1±0.3	154.7±0.1	30.4±0.1	55.5±0.8	68.9±2.8	178.6±0.1	35.8±1.5
<i>sEVOH_15UMB</i>	46.8±0.7	0.9±0.1	85.0±0.3	47.5±0.6	144.9±0.4	28.1±0.4	45.8±0.5	60.6±3.3	171.3±0.1	35.2±1.9
<i>Extrusion</i>										
<i>eEVOH</i>	57.3±0.1	1.7±0.2	105.5±0.1	62.4±1.4	160.6±0.4	31.7±0.8	65.7±2.0	75.1 ± 1.1	182.5 ± 0.1	37.1±0.6
<i>eEVOH_5UMB</i>	49.6±0.2	1.6±0.1	98.6±0.8	57.5±1.0	155.8±0.1	30.7±0.5	54.7±0.3	63.1 ± 2.0	178.4 ± 0.4	32.8±1.0
<i>eEVOH_15UMB</i>	42.1±0.3	0.5±0.1	87.9±0.4	50.1±3.5	149.1±0.2	29.4±2.1	47.8±1.6	56.1 ± 0.9	174.0 ± 0.1	32.6±0.5

Table 4: Mechanical properties of EVOH based systems produced by solvent casting and extrusion

<i>Formulations</i>	$\sigma_b(\text{MPa})$	$\varepsilon_b(\%)$	$E_{\text{Young}}(\text{MPa})$
<i>Solvent casting</i>			
<i>sEVOH</i>	45±4	265±28	440±65
<i>sEVOH_5GA</i>	44±7	240±38	560±45
<i>sEVOH_15GA</i>	42±1	220±26	315±53
<i>sEVOH_5UMB</i>	50±6	300±60	320±60
<i>sEVOH_15UMB</i>	32±4	260±30	190±30
<i>Extrusion</i>			
<i>eEVOH</i>	67±7	226±25	2028±115
<i>eEVOH_5UMB</i>	102±16	131±24	2565±49
<i>eEVOH_15UMB</i>	89±10	153±28	2053±69

Table 5: Radical scavenging activity (RSA) of EVOH based systems produced by solvent casting and extrusion.

<i>Formulations</i>	<i>DPPH scavenging activity, RSA (%)</i>
<i>Solvent casting</i>	
<i>sEVOH</i>	0
<i>sEVOH_5 GA</i>	95.0±2.0
<i>sEVOH_15 GA</i>	93.2±0.3
<i>sEVOH_5 UMB</i>	20.3±3.2
<i>sEVOH_15 UMB</i>	75.2±0.8
<i>Extrusion</i>	
<i>eEVOH</i>	0
<i>eEVOH_5UMB</i>	46.7±1.8
<i>eEVOH_15UMB</i>	62.1±2.9

Table6: Moisture content (MC) of EVOH based systems produced by solvent casting and extrusion.

<i>Formulations</i>	<i>MC (%) @ 1 Week</i>	<i>MC (%) @ 5 Week</i>
<i>Solvent casting</i>		
<i>sEVOH</i>	1.49±0.10	1.53±0.14
<i>sEVOH_5GA</i>	1.92±0.03	1.94±0.09
<i>sEVOH_15GA</i>	1.91±0.13	1.94±0.13
<i>sEVOH_5UMB</i>	1.71±0.06	1.72±0.10
<i>sEVOH_15UMB</i>	1.62±0.10	1.62±0.14
<i>Extrusion</i>		
<i>eEVOH</i>	2.34±0.23	2.32±0.12
<i>eEVOH_5UMB</i>	2.30±0.17	2.42±0.28
<i>eEVOH_15UMB</i>	2.29±0.17	2.33±0.24