Hydroxytyrosol as Active Ingredient in Poly(vinyl alcohol) Films for Food Packaging Applications

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ABSTRACT: Hydroxytyrosol (HTyr), a biophenol found in extra-virgin olive oil or olive oil by-products, well known for its strong antioxidant activity, was used as active ingredient for poly(vinyl alcohol) (PVA) matrix to develop film formulations by solvent casting process. The effect of HTyr on the morphological, thermal stability, optical, mechanical and release properties of PVA were investigated, while water absorption capacity, migration with food stimulants, water vapor permeability and antioxidant properties were tested taking into account the final application as food packaging systems. Morphological investigations evidenced homogeneity of all PVA/HTyr films, while the presence of HTyr clearly accounted for an increase of the crystallinity degree of the polymer matrix and also for providing antioxidant activity of the final films. The successful development of novel, environmentally friendly, safe active food packaging materials as an alternative to that which is petroleum based represents solid ground for product improvement and future testing on food.

KEYWORDS: Biodegradable polymers, hydroxytyrosol, food active packaging, mechanical properties, antioxidant activity

1 INTRODUCTION

Active food packaging consists of the incorporation by design of active ingredients (AIs) into the material to achieve their release into food or for promoting the absorption of substances from the food or its surrounding environment [1]. This novel strategy is extremely promising, aiming to improve the primary role of traditional food packaging preservation and protection from external abiotic and biotic factors, thus guaranteeing food quality and safety [2]. A major objective in developing new functional materials is obtaining eco-friendly and biodegradable solutions for foodstuff active in extending product shelf life, which would lead to greater distribution opportunities and less food waste. Among the threats to fresh and processed food stands atmospheric air. Lipid oxidation is a major factor influencing food shelf-life, causing food products to turn rancid and encouraging anaerobic bacteria growth. Oxidation, due to the gas-like oxygen migration through the packaging, also promotes enzymatic browning and deterioration of flavor and vitamins, depleting food of its nutritional and economic value. In food packaging applications the gas barrier properties represent an important factor to control in order to maintain the safety and the quality of foodstuffs. The modulation and control of film barrier properties, in fact, permit an increase in the shelf life of fresh food products as required by the specific market and consumers [3].

To avoid or at least limit these detrimental effects, antioxidant agents, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been extensively proposed as additives in food packaging. But in recent times, concerns have been raised about their possible mutagenic and carcinogenic effects [4].

In search of safer solutions, AIs of natural origin and those derived from food processing and agricultural wastes represent an attractive alternative [5–16]. In this scenario, 2-(3,4-dihydroxyphenyl) ethanol (hydroxytyrosol HTyr), can play an important role as additive for active food packaging. HTyr is a phenolic component present in olive fruits (*Olea europaea* L.) mainly as oleuropein, a glucoside, which during the ripening and processing of olives undergoes hydrolytic cleavage by β-glucosidase [17]. *In-vivo* and *in-vitro* studies done by using HTyr in the free form reported a wide range of biological properties;

the prominent being antioxidant but also including hypoglycemic, antimicrobial, hypoglycemic, cardioprotective, antiproliferative, anti-platelet aggregation and anti-inflammatory activities [18–21]. To the best of our knowledge, in polymer chemistry, HTyr has been used only in combination with polypropylene as stabilizer [22, 23] and for the preparation of poly(ε-caprolactone)-based nanocomposites [24]. Moreover, we recently studied the effect of hydroxytyrosol methyl carbonate (HTyr-MC), a synthetic hydroxytyrosol-derived compound, on the final properties of a biodegradable thermoplastic matrix demonstrating good antioxidant activity induced by HTyr-MC and its nucleation action for the polymer. However, further studies about the mechanical response, for example, induced by similar active ingredients to the polymer, and useful for the final application of the produced formulations, are required and here considered [25].

Among biodegradable, biocompatible and non-toxic polymers accepted by the Food and Drug Administration (FDA) to realize flexible and safe packaging solutions, poly(vinyl alcohol) (PVA) is a good candidate and represents a sustainable alternative to traditional plastics such as polypropylene (PP), polyethylene (PE) and polystyrene (PS) [26–29]. Other characteristics, such as high polarity, strong solubility in water, good optical, physical and thermo-mechanical properties (thermal stability, strength, flexibility), make PVA one of the polymers of choice for the development of doped films loaded with hydrophilic ingredients for active food packaging applications [30, 31].

In this light, the objective of this work was to develop novel systems for antioxidant food packaging by using a safe polymer, such as PVA, as film matrix. Encouraged by our recent results [25], pure HTyr was successfully incorporated into PVA by solvent casting technique thanks to the strong hydrophilic character of both components. All novel formulations, namely PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10, were investigated in terms of optical, morphological, and thermal properties, whereas the mechanical properties were studied for these systems and compared to those of PVA. Finally, the water vapor permeability and antioxidant capacity of HTyr were tested in a hydrophilic food simulant after the release from PVA/HTyr binary films.

2 EXPERIMENTAL

2.1 Materials

Poly(vinyl alcohol) (99% hydrolyzed, M_W 124–146 kg mol^{-1}) and solvents were purchased from Sigma-Aldrich (Milan, Italy). Silica gel 60 F254 plates and silica gel 60 (230–400 mesh) were supplied by Merck (Germany). 1-Hydroxy-1-oxo-1H-1λ^5-benz[d][1,2]iodoxol-3-one (2-iodoxybenzoic acid, IBX) was prepared according to the procedure reported by Frigerio *et al*. [32].

2.2 HTyr Synthesis, Characterization and Stability

Pure hydroxytyrosol (HTyr) was synthetized by IBX oxidation of tyrosol, as already described [33, 34]. The structural assignment was confirmed by ^{1}H and ^{13}C NMR spectra (acetone-d$_6$, 99.9% in deuterium) using a 400 MHz Nuclear Magnetic Resonance spectrometer (Bruker, Germany). The stability of HTyr was evaluated by High Performance Liquid Chromatography (HPLC) (LC-20A instrument; Shimadzu, Kyoto, Japan) equipped with a UV detector selected at 280 nm and an LCMS-2010 with electrospray (ESI) interface (Shimadzu, Kyoto, Japan). Thermogravimetric measurements (TGA) were performed under nitrogen or air flow by using a Seiko Exstar 6300. Heating scans were performed from 30 to 700 °C at 10 °C min^{-1} and three different measurements were done.

2.3 Preparation of PVA and PVA/HTyr-based Films

The PVA systems combined with HTyr at 2, 5 and 10% wt/wt (PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10, respectively) were prepared by solvent casting technique. PVA (0.5 g) was dissolved in distilled water (10 mL) under magnetic stirring for 2 h at 80 °C. PVA aqueous solution was kept under magnetic stirrer to reach room temperature (RT). PVA/HTyr systems were obtained mixing PVA with a specific amount of the HTyr previously dispersed in distilled water. A magnetic stirrer at room temperature for 1 h and a sonication bath treatment for one additional hour was applied in order to obtain a uniform dispersion of the HTyr in the solvent. PVA solutions were mixed with the solution containing the active ingredient by magnetic stirrer (1 h at room temperature) and the solution was cast in a Teflon® mold and evaporated at RT. The films (of 90 mm diameter and 70–90 µm thick) were equilibrated for 7 days at 53% RH in desiccators, by using magnesium nitrate-6-hydrate oversaturated solution (Sigma-Aldrich, Milan, Italy) after the processing before the characterizations.

2.4 Characterization of PVA and PVA/HTyr based Films

2.4.1 Thermal Characterization

Thermogravimetric measurements (TGA) (Seiko Exstar 6300) were performed in order to investigate the thermal stability of neat PVA and PVA/HTyr films. Heating scans under nitrogen atmosphere (250 mL min^{-1}) from 30 to 600 °C at 10 °C min^{-1} were performed for PVA and PVA/HTyr films. The first degradation temperature (Td_I), the second/maximum degradation rate temperature (Td_{IImax}) and its onset ($T_{onset\ main\ peak}$), the third (Td_{III}) and the residual mass at 600 °C were detected.

Moreover, TGA analysis, under oxidative atmosphere, was performed to test the effect of natural active ingredient as thermal stabilizer of PVA matrix. Specifically the following procedure was applied and five samples for each formulations were tested: heating scan up to 90 °C at 20 °C min^{-1} and isotherm step at 90 °C for 10 min under nitrogen flow (250 mL min^{-1}), switching to air atmosphere (50 mL min^{-1}) and heating scan up to 700 °C at 10 °C min^{-1}. The temperature at 5% wt loss ($T_{5\%}$), the first degradation temperature (Td_I), the second degradation temperature (Td_{II}), the third/maximum degradation rate temperature (Td_{IIIMAX}) and its onset ($T_{onset\ main\ peak}$), the fourth (Td_{IV}) and fifth degradation rate temperatures (Td_V) were measured from the weight loss and its derivative curves obtained from the thermogravimetric scan.

Differential scanning calorimeter (TA Instrument, Q200) measurements were performed from −25 to 240 °C, at 10 °C min^{-1}, carrying out two heating scans and one cooling scan. The glass transition temperature (T_g), melting temperature (T_m), crystallization temperature (T_c) and relative enthalpies were evaluated.

The crystallinity degree was calculated according to Equation 1:

$$\chi = \frac{1}{\left(w_f\right)}\left[\frac{\Delta H}{\Delta H_0}\right] \times 100 \qquad (1)$$

where ΔH is the enthalpy for crystallization or melting phenomena; ΔH_0 is the theoretical melting enthalpy value for a 100% PVA sample, taken as 161.6 J g^{-1}, and w_f is the weight fraction of PVA in the sample.

2.4.2 Morphological Investigation

Cryo-fractured surfaces of PVA and PVA/HTyr films were sputtered with gold and the microstructure was analyzed by field emission scanning electron microscopy (FESEM Supra 25-Zeiss). The transparency of PVA based films was analyzed by UV-Vis spectroscopy (Perkin Elmer Lambda 35) in the range of 250–900 nm. Three different specimens were cut and analyzed and the average value was reported as result.

2.4.3 Mechanical Characterization

The mechanical behavior of neat PVA and PVA/HTyr films was evaluated by tensile tests (rectangular probes (50 × 10 mm^2)) following UNI ISO 527, with a load cell of 500 N, an initial gauge length of 25 mm, a crosshead speed of 5 mm min^{-1} and at RT. Tensile strength (σ_b), elongation at break (ε_b), and Young's modulus (E) were expressed as average and calculated from the resulting stress-strain curves. The tests were done in at least five samples for each formulation.

2.4.4 Contact Angle and Water Absorption Capacity

Static water contact angle tests were performed at RT using a FTA 1000 dynamic contact angle analyzer. A droplet (20 µL) was delivered on the film surface and after 15 s the contact angle was recorded. The values are the mean of five independent measurements. The water absorption capacity of PVA-based systems was measured using rectangular probes (10 mm × 20 mm) in triplicate. The samples were first dried in a vacuum oven for 72 h at 40 °C, then cooled in a desiccator and immediately weighed at RT. The rectangular probes were immersed in a distilled water. The analysis was carried out in triplicate at 4, 20 and 37 °C in order to simulate the practical storage of packaged products. The samples were taken off of the container, and the water on the surface was removed by adsorbing it using filter paper, and then weighed. The water absorption capacity (WAC) was calculated according to Equation 2:

$$WAC\% = (W_A - W_I)/W_I \times 100 \qquad (2)$$

where W_A is the weight of the specimen at the adsorbing equilibrium and W_I is the initial dry weight of the specimen.

2.4.5 Overall Migration Tests

Overall migration tests were performed for each formualtion in triplicate according to the Commission Regulation EU No 10/2011 in ethanol 10% v/v water solution, the food simulant which represents foods having hydrophilic properties [35]. Rectangular strips of 10 cm^2 were immersed in a glass tube with 10 mL of food simulant. Samples were maintained for 10 days at 40 °C in a controlled chamber according to EN-1186

standard [36]. After the incubation period, the samples were removed and the simulant was evaporated to dryness. Furthermore, the residues were weighed with a balance (Sartorius ATILON, ±0.01 mg precision) in order to determine the overall migration value in mg kg^{-1} of simulant. The final migration values were expressed as the average and relative deviation.

2.4.6 Specific Migration Tests

Specific migration tests were carried out according to European Standard EN 13130-2005 [37] and European Commission Regulation 10/2011 [35] using ethanol 10% v/v water solution as food simulant. The samples of 12 cm^2 were immersed in 20 mL of simulant in triplicate at 40 °C in an oven (J.P. Selecta, Barcelona, Spain) and taken at 1, 3, 7, 10, 21 days. A blank test for the simulant was also included. The simulant was recovered after removal of samples and stored at –4 °C before the analysis.

2.4.7 Release Studies

Food simulant at different contact periods (1, 3, 7, 10 and 21 days) with PVA, PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 was analyzed in absorbance at 280 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The release was expressed as milligrams of HTyr per liter of food simulant, using a standard curve for HTyr (range: 20–650 μM).

2.4.8 Water Vapor Permeability Test

The barrier properties of PVA and PVA/HTyr systems were evaluated by water vapor permeability (WVP) test. WVP was performed following the gravimetric method ASTM E96-95 [38] at 25 °C and 53–100% RH gradient by using Payne permeability cups of 3.5 cm diameter. In the cup, deionized water was used to reach 100% RH, while an oversaturated magnesium nitrate hexahydrate saturated solution (MgN$_2$O$_6$) (Sigma-Aldrich®) was used to control the RH inside the chamber. The films were conditioned in a dessiccator before the characterization at 20 °C and 53% of relative humidity for one week. The WVP was measured according to Cano et al. [39]. For each formulation, WVP measurements were done in quadruplicate.

2.4.9 Antioxidant Activity

The antioxidant activity was determined using the ABTS method according to the literature [40] with few modifications. Briefly, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic) diammonium salt (ABTS) and potassium persulfate (K$_2$S$_2$O$_8$) were dissolved in phosphate buffer (5 mM NaH$_2$PO$_4$-H$_2$O and 5 mM Na$_2$HPO$_4$-2H$_2$O,

pH 7.4) at a final concentration of 7 mM and 2.5 mM, respectively. The solutions were kept in the dark for 12 h at room temperature to generate the radical cation (ABTS•$^+$). Then, they were diluted in ethanol to measure an absorbance of 0.70 ± 0.05 at 734 nm. Finally, 10 μL of each sample (food simulants at contact period 1, 3, 7, 10 and 21 days with PVA, PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 films) was mixed with 200 μL of ABTS•$^+$ solution in a 96-multiwell and the absorbance was recorded at 734 nm for 90 seconds. All samples were evaluated in triplicate. The reference was 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); the standard curve was recorded between 60–300 μM. The antioxidant capacities of food simulant solutions were referred to the Trolox equivalent antioxidant capacity (TEAC) and expressed as μmol/L of Trolox equivalents.

2.4.10 Statistical Analysis

Statistical analysis of thermal studies (TGA in oxidative atmosphere and DSC), wettability, mechanical properties, overall migration data and water absorption capacity was performed through analysis of variance (ANOVA), using the Statgraphics Plus 5.1. program (Manugistics Corp., Rockville, MD). To differentiate the different systems, Fisher's least significant difference (LSD) was used at the 95% confidence level. Statistical analysis of release studies and antioxidant activity was performed by the one-way ANOVA, according to the Tukey's honestly honest significant difference (HSD) test using a significance level of $p \leq 0.05$.

3 RESULTS and DISCUSSION

3.1 Synthesis, Characterization and Stability of Hydroxytyrosol (HTyr)

With the aim to evaluate the effective role of HTyr in PVA-based films, this work was based on the use of pure HTyr, synthetized in our laboratories using a three-step procedure, already optimized [33, 34].

Generally recognized as safe (GRAS) authorization has been granted to HTyr by the Food and Drug Administration, while recent in-vitro studies demonstrated that HTyr is non-mutagenic and non-genotoxic at concentrations that far exceed those attainable after oral intake [40]. HTyr is present in olive fruits, the main food sources being olive oil and olive oil by-products. Among them, olive mill wastewater (OMWW) produced every year in large volumes in the Mediterranean area, contains a discrete amount of polyphenols including HTyr (up to 1 g L^{-1}) [41]. Lately, many efforts have been focused on developing

efficient techniques in order to optimize the extraction yields of polyphenols from these sources, OMWW in particular. They include solvent extractions, supercritical fluid separations and membrane separations used individually or in combination [41, 42]. All the above-mentioned techniques provide mixtures of phenolic compounds containing HTyr in different percentages.

As mentioned above, HTyr exhibits a strong antioxidant activity widely demonstrated by specific assays and this property is related to the presence of the catecholic moiety able to be converted into *o*-quinone after redox cycling in the presence of oxidants, i.e., air, hydrogen peroxide or free radicals [18, 43]. Before using HTyr for the production of PVA based films, the stability of HTyr at migration test experimental conditions (40 °C in the absence of air after 1, 7, 10 and

21 days) [35–37] and its thermal oxidative stability under nitrogen and air atmosphere were extensively investigated (Figure 1).

The stability of HTyr at migration test experimental conditions was evaluated by HPLC/LC-MS analysis. The experimental data evidenced that HTyr was stable, remaining almost unchanged for 7 days (Figures 1a,b); in the following days the amount of *o*-quinone gradually increases (Figure 1c) up to being equimolar to HTyr at day 21 (Figure 1d). These data demonstrated both the stability and the antioxidant efficiency of HTyr which, exercising its activity, gradually produced the corresponding *o*-quinone.

The thermal stability under inert and oxidative atmosphere was analyzed by thermogravimetric analysis (TGA). The experimental results showed that in
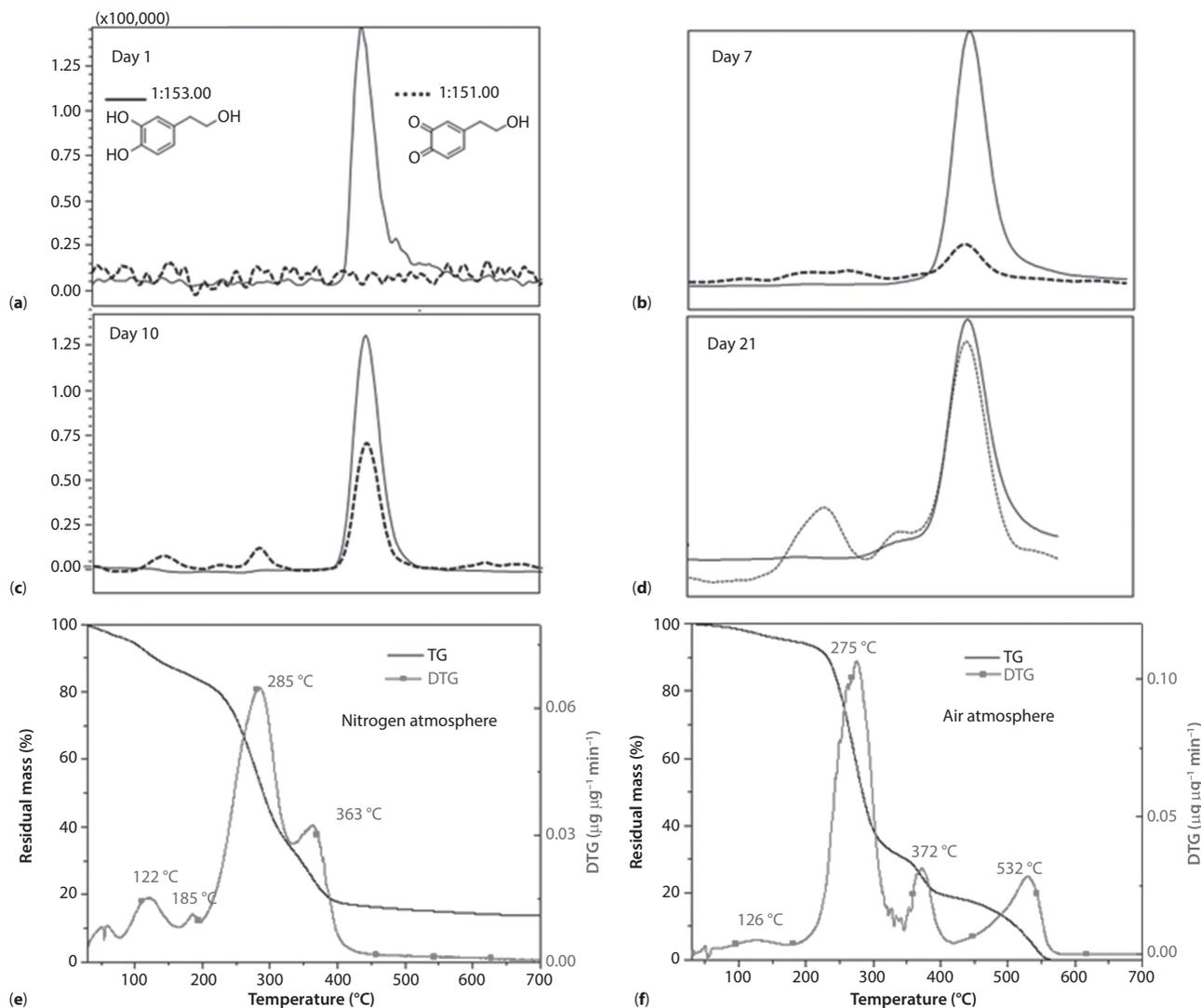

Figure 1 (**a–d**) HTyr stability under test migration conditions; (**e**) Thermogravimetric analysis of HTyr under nitrogen (**f**) and air flow.

both atmospheres, HTyr showed a multi-step degradation behavior. Specifically, under nitrogen atmosphere it exhibited five peaks of degradation (Figure 1e). The first degradation peak was centered at around 55 °C and showed a low intensity with a 5–6% weight loss; the second was centered at 122 °C with a 10–11% weight loss. The third degradation peak, at 185 °C, corresponding to the loss of volatile compounds [44], was characterized by a very low intensity. Finally, the main peak, centered at 285 °C, and the last degradation phenomenon at 363 °C were due to the decomposition of more stable compounds. Under oxidative atmosphere, HTyr showed four peaks of degradation (Figure 1f). The first peak, at 126 °C, showed a 4–5% weight loss, followed by the main peak centered at 275 °C. In this case, the main degradation peak was at a lower temperature with respect to the test performed under nitrogen atmosphere as a consequence of the oxidative environment [45]. The last two degradation peaks were centered at 372 °C and 532 °C, respectively. Finally, the residual mass measured at 700 °C of HTyr under inert and oxidative atmosphere was around 14% and lower than 1%, respectively.

3.2 Characterization of PVA/HTyr Films

PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 systems were successfully prepared by eco-friendly and cheap solvent casting in water technique, loading PVA matrix with HTyr at 2, 5 and 10% wt/wt; the morphological, thermal and optical properties of the multifunctional films were deeply investigated and discussed, while mechanical responses were here considered in order to evaluate the effect of the active ingredient on this important final characteristic of the proposed formulations. Moreover, water absorption capacity and antioxidant migration properties were tested and discussed taking into account the final application as food packaging materials.

3.2.1 Morphological and Transparency Properties

The incorporation of the AI into a film to generate active packaging polymer-based systems could modify the overall film structure. Properties of the resulting film indeed depend on the binary combination and ratio between the polymer and the AI, hence besides evidence of bioactivity, prior detailed studies on the film performance should be conducted to elucidate the influence of the AI on the material. Morphological aspects of the cryo-cross sections of pure PVA and PVA/HTyr films were investigated by FESEM in order to evaluate the influence of the different amounts of HTyr on the morphology of the PVA matrix

(Figures 2a–d). As previously observed, the fractured surfaces of neat PVA film appeared smooth and uniform [29, 44]. No particular alterations were induced by the different amounts of HTyr added into the PVA matrix. The FESEM images underlined that no separation phases or holes were present on the fractured surfaces of the PVA/HTyr formulations, highlighting that homogeneous dispersal of the antioxidant agent in the polymeric compound was obtained for all the novel formulations during the processing procedure. The solvent casting technique in water guaranteed, in fact, a good dispersion of the hydrophilic AI and a consequent homogeneous aspect of the final PVA/HTyr formulations.

The PVA and PVA/HTyr films were also analyzed by UV-Vis spectrophotometry (Figure 2e). The results from visual and UV-Vis investigations confirmed the efficiency of the processing procedure of PVA and PVA/HTyr, as previously observed by FESEM investigation. The spectrum transparency curves established that PVA was a transparent matrix (transmittance of 94% at a visible wavelength of 600 nm), while the transparency values at the same wavelength for PVA/HTyr systems decreased progressively, increasing the amount of the AI 92, 91 and 89% for PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 respectively. The transparency behavior rapidly decreased from 600 nm to lower wavelength and all PVA/HTyr systems showed a visible absorption band centered at 280 nm due to the presence of HTyr. The transparency properties obtained for PVA/HTyr systems highlighted the possibility of using these formulations to realize transparent packaging in the wavelength range of 600–900 nm and with a light barrier between 250–600 nm. This different transparency vs. wavelength permits modulation of the employment of packaging films in different applications in relation to specific requests, such as the increase of food shelf life, or simply to shield against a specific wavelength. Visual observations (Figure 2f) confirmed the UV-Vis results, clearly showing the transparent nature of PVA. Moreover, color distribution of the binary films appeared uniform in all the sample sizes, highlighting that the active ingredient was homogenously dispersed in the PVA matrix and asserting the success of the solvent casting applied process.

3.2.2 Thermal Properties

Thermal properties of PVA and PVA/HTyr films were evaluated by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) under nitrogen atmosphere in order to evaluate the effect of HTyr in terms of presence and content. The effect of the different content on the thermal degradative properties of PVA matrix was investigated by TGA (Table 1).
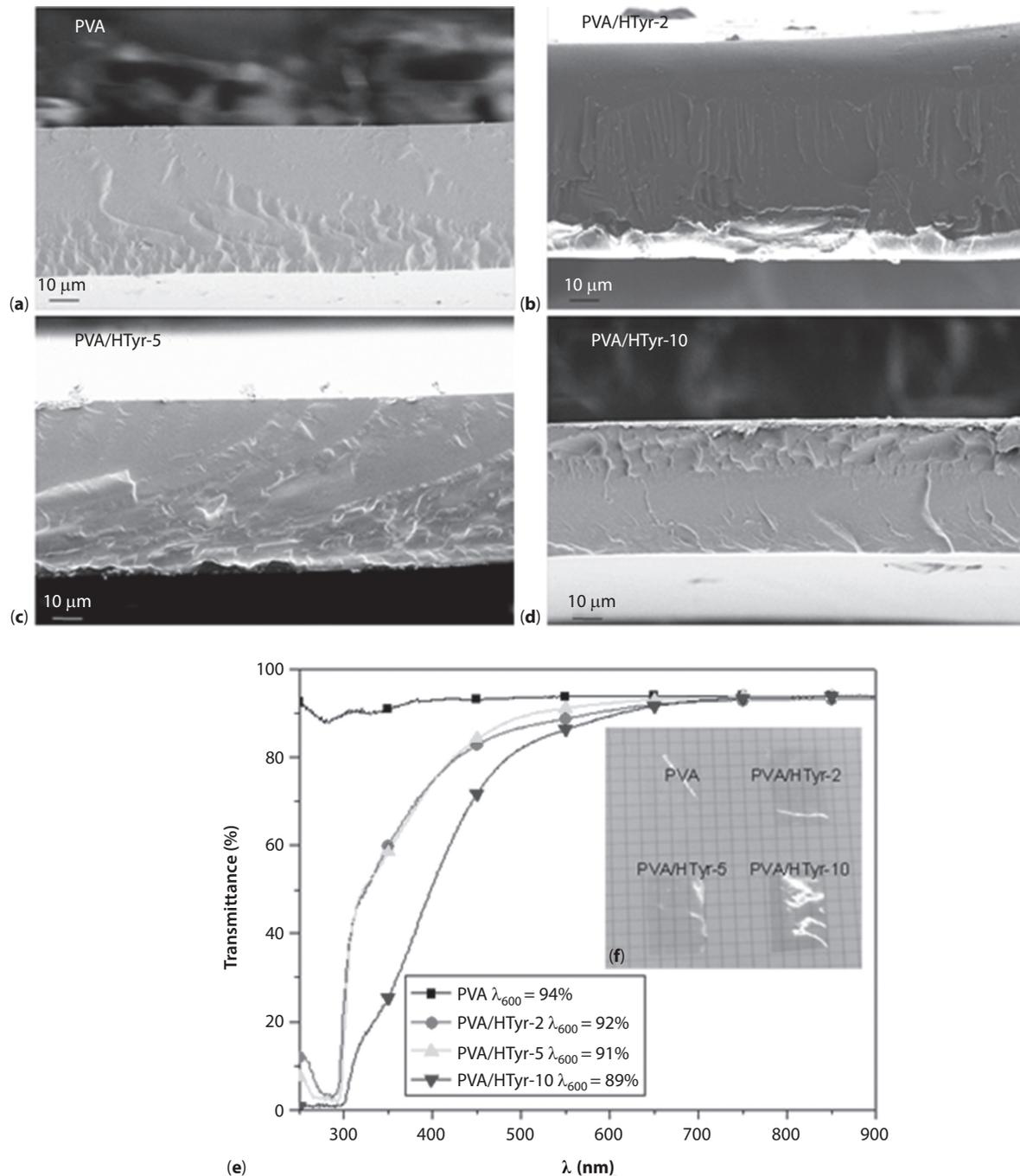
Figure 2 (**a–d**) Microstructure of fractured surfaces of PVA and PVA/HTyr films; (**e**) UV-Vis measurement results; (**f**) Visual observation of produced films.

Table 1 Thermogravimetric analysis of PVA and PVA/HTyr systems under inert atmosphere.

Film	Td_I (°C)	$T_{onset\ main\ peak}$ (°C)	Td_{IImax} (°C)	Td_{III} (°C)	Residual mass at 600 °C
PVA	84	202	247	428	7
PVA/HTyr-2	101	224	269	429	19
PVA/HTyr-5	102	232	271	439	17
PVA/HTyr-10	100	225	265	412	18

The PVA and PVA/HTyr films were characterized by the presence of a multi-step degradation behavior. The first weight loss at low temperature centered around 80–110 °C (Td_I) corresponds to the evaporation of the water or the weight loss of low molecular weight compounds [44], $T_{onset\ main\ peak}$ shifted to higher temperature after adding the AI in the polymeric matrix and this effect was more evident upon increasing the contents; this phenomenon underlined that the active ingredient has an important effect on the PVA, improving thermal stability. The second and the third degradation temperatures correspond to the degradation of neat PVA [46–48]. In particular, the second/main degradation step of PVA film corresponds to the removal of residual acetate groups due to the incomplete hydrolysis of PVA that remains in the chains [49]. The presence of HTyr in the PVA matrix also shifted the main temperature (Td_{IImax}) to higher values with more evidence using 10 wt% of AI (265 °C for PVA/HTyr-10 versus 247 °C for neat PVA film), and this effect could be related to the higher thermal stability induced by the presence of HTyr that showed a main degradation temperature at around 285 °C under nitrogen atmosphere, as reported in Figure 3a and previously discussed. The third degradation step (Td_{III}), due to the cyclization reaction and continual elimination of residual acetate groups [50], was poorly affected by the presence and content of the AI. However, an increase in the residual mass values for PVA/HTyr formulations (around 20%) with respect to the polymer matrix (around 7%) was registered and related to the AI's presence. Results were in accord with the high values registered for HTyr during TGA analysis (Figure 1a).

Thermal properties of PVA and PVA/HTyr films were also analyzed by DSC analysis in order to evaluate the effect of HTyr on the melting and crystallization phenomena of polymeric matrix. Table 2 summarizes the glass transition, crystallization and melting phenomena of PVA and PVA/HTyr films during the cooling and the second heating scan. Glass transition temperatures (T_g) calculated during the cooling and the second heating scan, remain similar for all the systems with no evident shifts due to the presence of active ingredient. Both during the cooling and the second heating scan, the crystallinity degree of PVA increased with the addition of HTyr. The highest crystallinity degree values during the cooling scan were obtained for PVA/HTyr-2 and PVA/HTyr-5, while a slight decrease in the crystallinity degree (X_c) was registered for PVA/HTyr-10 formulation. A similar behavior was also detected during the second heating scan with evident increase in the X_m values registered for all HTyr-based formulations. Data highlighted that HTyr favors the crystallization phenomena of PVA/HTyr-based films [24] and this effect was also enhanced with respect to our previous studies about PVA/hydroxytyrosol methyl carbonate (HTyr-MC)-based formulations where the active compound was added in the same amount as in PVA matrix [25].
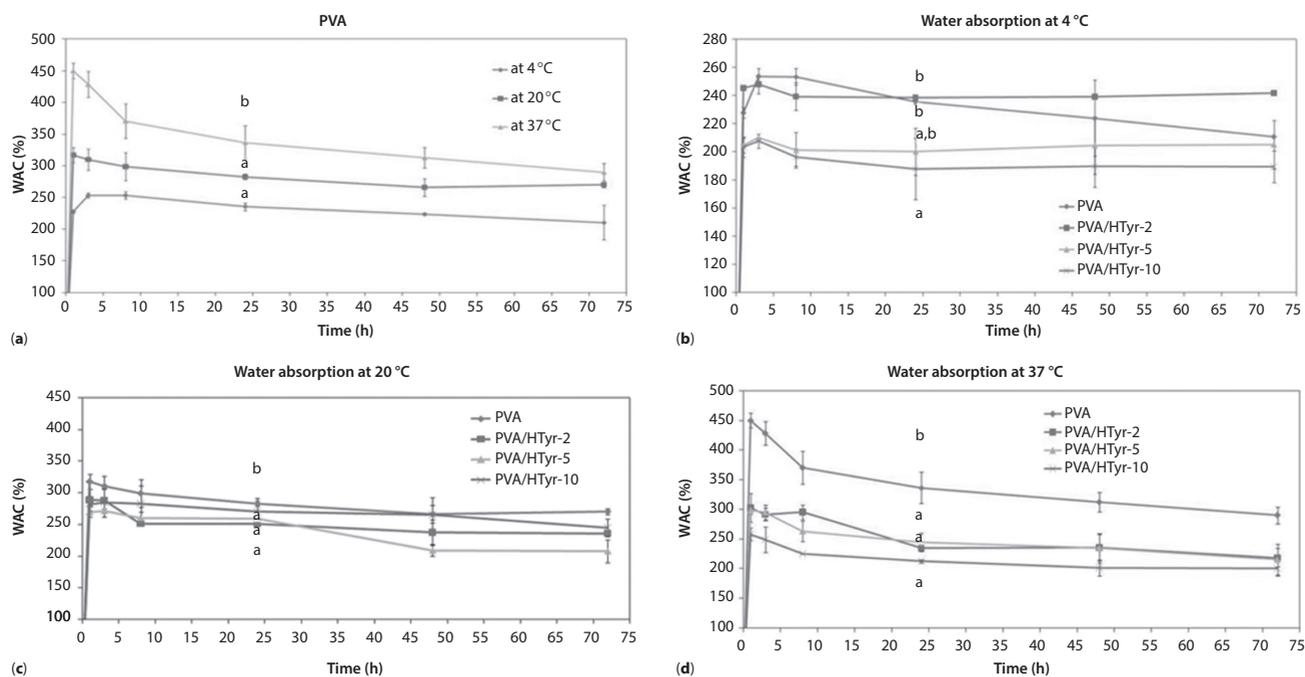

Figure 3 Water absorption capacity of PVA and PVA/HTyr films at 4, 20 and 37 °C. Different letters indicate significant statistical difference ($p < 0.05$) after 24 h of incubation in water – WAC saturation limit.

Table 2 Differential Scanning Calorimetry (DSC) data for PVA and PVA/HTyr films.

Film	*Cooling scan*			
	T_g *(°C)*	ΔH_c *(J g^{-1})*	T_c *(°C)*	X_c *(%)*
PVA	70.3 ± 0.8[a]	24.3 ± 1.3[a]	168.3 ± 1.6[a]	15.0 ± 0.9[a]
PVA/HTyr-2	68.9 ± 2.2[a]	51.2 ± 3.5[b]	194.3 ± 1.6[b]	31.1 ± 2.2[c]
PVA/HTyr-5	71.2 ± 0.6[a]	51.9 ± 1.7[b]	196.1 ± 1.9[b]	30.5 ± 1.0[c]
PVA/HTyr-10	72.4 ± 1.3[a]	46.6 ± 1.9[b]	195.1 ± 0.7[b]	26.0 ± 1.1[b]
	Second heating scan			
	T_g *(°C)*	ΔH_m *(J g^{-1})*	T_m *(°C)*	X_m *(%)*
PVA	84.1 ± 1.9[b]	12.6 ± 1.5[a]	206.1 ± 3.4[a]	7.8 ± 0.9[a]
PVA/HTyr-2	80.1 ± 1.2[ab]	39.0 ± 4.3[b]	220.3 ± 1.3[b]	23.7 ± 2.6[b]
PVA/HTyr-5	79.9 ± 1.1[ab]	46.5 ± 2.6[b]	222.1 ± 1.1[b]	27.3 ± 1.5[b]
PVA/HTyr-10	79.1 ± 1.9[a]	44.7 ± 3.4[b]	222.6 ± 0.7[b]	24.9 ± 1.9[b]

Different letters in the same column indicate significant differences among formulations (p < 0.05)

Table 3 Mechanical, wettability, barrier properties and overall migration of PVA and PVA/HTyr films.

Film	*Mechanical properties*			*Wettability*	*WVP (100-53% RH)*	*Overall migration*
	σ_b*(MPa)*	ε_b*(%)*	E_{Young}*(MPa)*	*(°)*	*(g mm kPa^{-1} h^{-1} m^{-2})*	*(mg kg^{-1})*
PVA	70 ± 10[bc]	200 ± 40[a]	300 ± 80[b]	50 ± 2[b]	3.24 ± 0.40[c]	2.42 ± 0.13[a]
PVA/HTyr-2	45 ± 10[a]	180 ± 20[a]	550 ± 100[c]	50 ± 5[b]	1.50 ± 0.27[a]	2.50 ± 0.16[a]
PVA/HTyr-5	80 ± 15[c]	300 ± 50[b]	300 ± 50[b]	47 ± 8[b]	1.55 ± 0.51[a]	2.49 ± 0.14[a]
PVA/HTyr-10	60 ± 5[ab]	310 ± 20[b]	130 ± 15[a]	28 ± 5[a]	2.11 ± 0.15[b]	2.51 ± 0.17[a]

Different letters in the same column indicate significant differences among formulations (p < 0.05)

This behavior was also confirmed by the observation of crystallization and melting temperature values. A significant shift of about 15–30 °C of the crystallization temperatures (T_c) to higher values was registered for all PVA/HTyr systems with respect to the neat PVA ($T_c = 168.3 ± 1.6$ °C) with a consequent increase of the ΔH_c for the PVA/HTyr systems of about 20–25 (J g^{-1}) with respect to the neat polymer. A similar behavior was also detected for the melting phenomena during heating scan. The melting temperatures and enthalpies (T_m and ΔH_m) increased, in fact, proportionally with HTyr content. Specifically, the parameters T_m and ΔH_m increased about 20 °C and 30 J g^{-1}, respectively, for PVA/HTyr-5. These effects demonstrated that HTyr was able to act as nucleating agent, positively affecting the final thermal response of PVA matrix and also, in a more evident manner, with respect to other hydroxytyrosol-derived compounds recently studied in similar formulations [25], suggesting the effectiveness of these active ingredients as additive for the selected polymer matrix.

3.2.3 Mechanical Properties

The mechanical responses of the active ingredients-based PVA films were here investigated taking into

account the final application in the industrial sector for which the mechanical characteristics are crucial issues. The performed tensile test permitted evaluation of the influence of the AI on the PVA mechanical properties and the experimental results are reported in Table 3. The elongation at break of PVA neat film was around 200 ± 40%, underlining the ductile nature of the selected polymer matrix as previously observed in the literature [31, 51]. The addition of different amounts of HTyr to PVA induced a modification in the plastic response of PVA matrix. The deformation at break for PVA/HTyr-5 and PVA/HTyr-10 films, in fact, was increased respectively to about 150% and 155%, compared to the neat PVA, due to the presence of the AI as a result of a plasticizing effect, as previously observed for poly(ε-caprolactone)/HTyr-based films [24] and for other formulations based on biodegradable polymers [poly(lactic acid) and biopolyester] loaded with different AIs [52, 53]. On the contrary, the deformation at break value was maintained by adding a 2% wt of HTyr in the PVA matrix. Sometimes the AI included in the polymeric matrix could negatively affect the mechanical properties, reducing the performance of neat polymer due to poor miscibility and interaction of the AI [54]. In any case, with the exception of

PVA/HTyr-2 system, the values of tensile tests in the present work underlined that the addition of HTyr improved the internal movement and modulated the ductile response of PVA film features, which is particularly interesting in light of products related to packaging materials [24]. Moreover, the results from mechanical characterization underlined that the Young's modulus values increased for PVA/HTyr-2. This phenomenon was related to the increase of crystallinity degree (Table 3) observed for PVA/HTyr-2 and PVA/HTyr-5, while a predominant plasticizer effect was detected with higher content of HTyr (10 wt%); in fact, PVA/HTyr-10 showed a reduction of crystallinity (with respect to the other PVA/HTyr systems) of the highest elongation at break and of the lowest Young's modulus values. These results suggested the possibility to select different contents of HTyr in order to modulate the final desired properties. At the same time our findings demonstrated that PVA/HTyr-5 could represent the ideal formulation, compromising between elastic and plastic response of the film, which are indeed both useful characteristics for packaging material.

3.2.4 Contact Angle Measurements, Water Vapor Permeability and Water Absorption Study

A preliminary analysis of the surface wettability of PVA and PVA/HTyr was performed by water contact angle measurements in order to evaluate the influence of the presence and different contents of HTyr in PVA matrix. The results, summarized in Table 3, underlined that wettability was not affected by the presence of 2 wt% and 5 wt% of HTyr in PVA matrix due to the crystalline nature of the PVA/HTyr-2- and PVA/HTyr-5-based systems. On the contrary, a reduction in water contact angle values was measured for PVA/HTyr-10 [(28 ± 5)°] with respect to PVA neat film [(50 ± 2)°]. This behavior could be ascribed to an increase in the ductile nature previously observed for PVA/HTyr-10-based system [24].

The WVP of PVA and PVA/HTyr was measured at 25 °C and 100–53% RH and the results are summarized in Table 3. The data underlined the positive effect of the HTyr presence in the reduction of PVA permeability since all the studied formulations showed a significant reduction of the WVP values ($p < 0.05$). The best performances were obtained for PVA/HTyr-2 and PVA/HTyr-5, with a reduction of about 50–55% in WVP values, while a reduction of 35% was registered for PVA/HTyr-10. This behavior could be ascribed to the reported increase in the crystalline nature of PVA/HTyr (Table 2) induced by the presence of the active ingredient, which was particularly remarkable when 2 wt% or 5 wt% of HTyr was added to the PVA matrix [24].

Water absorption capacity (WAC) of PVA-based films was investigated at 4, 20, and 37 °C in order to reproduce the different conditions and temperatures food products are exposed to during transport and storage. Since it is directly linked to food quality and safety, WAC is one of the most important properties to study as far as biodegradable packages are concerned. The amount of water absorbed on the package material can, in fact, influence the microorganism growth and lead to an increased deterioration of the contained food products, thus decreasing their shelf life. PVA and PVA/HTyr films were analyzed, comparing their WAC at different temperatures, in order to evaluate if water diffusion kinetics can be influenced by storage conditions. This study also aimed to clarify how the different concentrations of active HTyr could influence WAC of PVA/HTyr films with respect to PVA neat system.

For all novel formulations, WAC reached the maximum values at all the observed temperatures (4, 20, and 37 °C) after 3 h of incubation, while all the tested materials showed a saturation limit during the first 24 h, underlining a fast absorption mechanism due to the hydrophilic nature of the selected matrix (Figure 3) [55]. Moreover, a clear effect of the incubation temperatures on the WAC of PVA is reported in Figure 3a. Data revealed that WAC of PVA was higher at 37 °C for all the considered incubation times [55]. This result can be related to higher polymeric chain mobility at 37 °C, since it is closer to the glass transition temperature of the film [T_g = (70.3 ± 0.8) °C]. The addition of the AI was able to reduce WAC behavior more clearly for PVA/HTyr-5 and PVA/HTyr-10 (see the behavior at cooling scan in Table 2), which can be ascribed to the higher values of crystallinity degree measured for these formulations. Finally, PVA/HTyr films (Figure 3b–d) showed an increase in WAC values with the incubation temperatures, underlining, also in this case, that test conditions facilitate the swelling phenomenon (especially at 37 °C closer to the glass transition temperature) typical of the PVA matrix.

3.2.5 Thermo-Oxidative Property Study

The effect of HTyr as thermo-oxidative stabilizer for PVA and PVA/HTyr films was evaluated by thermogravimetric analysis in air atmosphere according to the procedure reported in the literature [6] and the results are reported in Table 4. PVA neat film showed a thermal-oxidation degradative behavior characterized by four different steps [56], while the degradative phenomena for PVA/HTyr occurred in five steps (Figure S1). The first degradative rate temperature (Td_I) and the correlated weight loss correspond to the physically adsorbed water [57]. The second degradation temperature (Td_{II}) was present with a low

Table 4 TGA measurements under oxidative atmosphere for PVA and PVA/HTyr films.

Films	$T_{5\%}$	$T_{onset\ main\ peak}$	Td_I	Td_{II}	Td_{IIImax}	Td_{IV}	Td_V
PVA	223 ± 3[a]	200 ± 1[a]	115 ± 2[a]	–	252 ± 4[a]	414 ± 4[a]	498 ± 4[a]
PVA/HTyr-2	239 ± 3[b]	226 ± 2[b]	120 ± 2[b]	220 ± 2[a]	274 ± 3[b]	417 ± 3[a]	501 ± 8[a]
PVA/HTyr-5	248 ± 2[c]	228 ± 4[c]	125 ± 1[c]	218 ± 1[a]	284 ± 1[bc]	448 ± 5[c]	527 ± 5[b]
PVA/HTyr-10	257 ± 5[d]	234 ± 3[d]	126 ± 2[c]	220 ± 1[a]	288 ± 5[c]	435 ± 1[b]	511 ± 7[a]

Different letters in the same column indicate significant differences among formulations (p < 0.05).

intensity only in PVA/HTyr and it was due to the presence of HTyr (see Figure 1b). The maximum degradation temperature (Td_{IIIMAX}) of PVA film corresponds to the partial dehydratation of PVA accompanied by polyene formation [58]. This peak shifted to higher values in the presence of HTyr in PVA matrix and the effect was strongly related to the AI content. The highest values of maximum degradation temperature was, in fact, registered for PVA/HTyr-10 (288 °C). The fourth degradative step (Td_{IV}) consisted of the polyene decomposition followed by its cyclization and the condensation of polyaromatic structures [59, 60], while the last degradation steps (Td_V) represented the thermo-oxidation of carbonized residue. The presence of HTyr at the highest values (5 and 10 wt%) induced a shift to higher temperatures, underlining the stabilization effect exerted by HTyr on the thermo-oxidative process of the PVA matrix.

3.2.6 Overall Migration Tests

Migration tests are employed to determine the quantity of food packaging materials, mainly additives, that is able to migrate or to be transferred to wrapped food through direct contact. In the case of active packaging the release of AIs (e.g., antioxidants or antimicrobials) into the food is a prerequisite for promoting an extended product shelf life [61]. European Union Regulation no. 10/2011 defined the limits at which additives can migrate into food during the storage period of foodstuff; in this light, the present study is focused on antioxidants obtainable from natural sources that could limit the oxidation of food, meanwhile maintaining migration values below the limits set by the current European legislation [35].

Overall migration tests with food simulant of PVA/HTyr films were carried out and the values are shown in Table 3. The migrated values for all the studied formulations were lower than the migration limits for food contact materials, 60 mg kg⁻¹ simulant, established by the current European legislation [35], demonstrating the possible practical application of the produced films in contact with the foods. Specifically, after 10 days of incubation at 40 °C in ethanol 10% (v/v), the migrated value of PVA film was around 2.42 mg kg⁻¹, while the

higher level was detected for PVA/HTyr-10 formulation (2.51 mg kg⁻¹). However, no significant differences were detected in the overall migration levels between PVA and PVA/HTyr-based formulations due to the predominant effect of the migrated polymer into food simulant with respect to the HTyr migrated quantity. For this reason, specific migration tests were also performed and discussed below.

3.2.7 Specific Migration Tests

The final aim of an active packaging is providing something more than a barrier to detrimental factors; packaging needs to play an active role in preserving food, hence proof of functional activity is a requirement to correctly define a packaging film as active. In the case of an antioxidant additive intended to be extruded from films, the activity of the system is controlled by the release of the active compound itself. In this context, specific HTyr migration tests were carried out in order to verify that HTyr, successfully incorporated into PVA film matrix, could be indeed released into the food, and to define a release profile for each of the three typologies of films. In fact, the presence of an additive in the film matrix, even at high concentrations, does not necessarily mean high migration values [62]. Diffusion of AIs from polymeric matrices depends on several parameters, such as their content in packaging film, chemical nature, food characteristics, temperature, and time period over which duration of contact occurs. Given the hydrophilic properties of both the PVA and HTyr, to test films release performance under the best theoretical conditions, the food simulant representing the characteristics of fresh food with high water content, such as fruit and vegetables, was employed. Then, PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 binary films were exposed to the selected food simulant (ethanol 10% v/v) for 21 days according to the European Commission Regulation 20/2011 [35–37]. At days 1, 3, 7, 10 and 21, samples were collected and HTyr quantified in the ethanolic solutions by UV spectrophotometric analysis (Figure 4a).

In all samples HTyr was analyzed in quantities well correlated with the different initial concentrations present in the films. Release from PVA/HTyr formulations
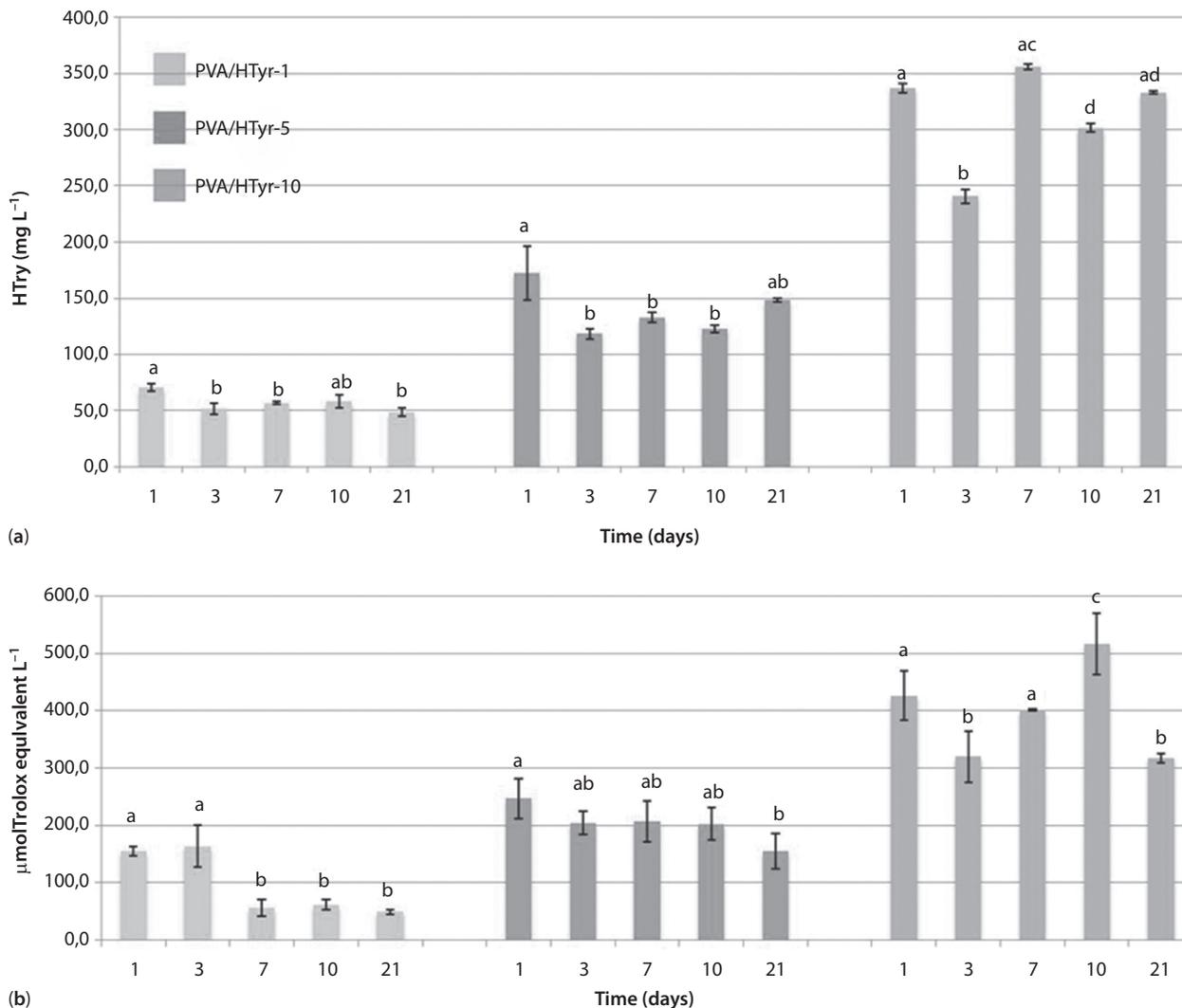
Figure 4 (**a**) Specific migration tests (release) of HTyr from PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 films; (**b**) Antioxidant activity of food simulant solutions from PVA/HTyr binary films studied by the ABTS assay. Different letters in the figure indicate statistical differences among formulations ($p < 0.05$).

reached 57, 55 and 63% of the initially incorporated HTyr from PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 systems, respectively. Beside some variability among samples of the same group, in all typologies of the PVA/HTyr films the release of HTyr reached its maximum immediately at day 1, and in all cases the migration profiles clearly delineated a very quick and significant release (in accord with results from water absorption test). Although the release mechanisms can be controlled by diffusion and/or dissolution (erosion), depending on the polymer properties, the additive distribution, loading and solubility, the reported profile clearly demonstrated that migration is the mechanism of release over the analyzed 21 days. In fact, after day 1, concentration did not sensibly grow over time, meaning that the diffusion that has

taken place at day 1 is the only mechanism of release. Nevertheless, it would be very interesting to determine how, at later time points, processes leading to polymer degradation will contribute to the release of the HTyr still retained in the film matrix (PVA/HTyr-2, 43%; PVA/HTyr-5, 45%; PVA/HTyr-10, 37%).

3.2.8 Antioxidant Activity

Antioxidant activity provided by novel PVA/HTyr binary films exposed to migration tests was determinated by measuring the radical scavenging ability of ethanolic food simulants, since scavenging of radicals is one of the main antioxidant mechanisms. Among the *in-vitro* assays reported in the literature, the ABTS assay was the test of choice commonly used to evaluate

the antioxidant activity of HTyr, hydrophilic foods and antioxidants released from films [62]. As shown in Figure 4b, a relevant antioxidant activity was clearly detected in all tested samples of the food stimulant and even the binary film at the lowest concentration exhibited appreciable values of antioxidant capacity. In agreement with the release studies, all samples already showed peak activity at day 1. However, instead of persisting over the course of the 21 days, antioxidant activity tends to decrease over time. As a matter of fact, in all typologies of films, activity at day 1 is significantly higher than at day 21 ($p < 0.05$, TEAC values: 154.4 +/− 8.3 *vs* 49.5 +/− 3.9; 246.9 +/− 34.8 *vs* 155.2 +/− 22.1; 425.7 +/− 42.6 *vs* 316.7 +/− 7.4 for samples from PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10 films, respectively). This reduction is well in agreement with the stability studies we performed with HTyr in food stimulant (Figures 1a–d). In fact, in the course of 21 days at 40 °C, HTyr oxidized as a result of exposure to oxygen, converting into the *o*-quinone which has indeed lost its antioxidant activity.

Some studies have demonstrated that casting processes produce films with a reduced loss of antioxidants, with respect to the nominal content, due to a less aggressive processing and manufacture conditions [62]. Our study fits well in those cases, the water-based casting conditions employed are extremely mild and, as we demonstrated, totally compatible with the thermal stability of HTyr (Figures 1e,f). Other studies have revealed that the maximum antioxidant activity was reached at certain concentrations and a further increase would lead to a decrease in activity [62, 63]. Although we tested up to 10% w/w of HTyr, which is indeed a considerable amount of AI, activity studies did not reveal any reduction, but instead a significant ($p < 0.05$) increase, as clearly shown at day 1, exhibiting TEAC values of 425.7 +/− 42.6 for PVA/HTyr-10 and of 246.9 +/− 34.8 and 154.4 +/− 8.3 for PVA/HTyr-5 and -2 respectively.

4 CONCLUSIONS

Novel pure HTyr-based films, namely PVA/HTyr-2, PVA/HTyr-5 and PVA/HTyr-10, were successfully developed using solvent casting by adding to PVA different amounts of pure HTyr (2, 5 and 10 wt%, respectively), a non-genotoxic and non-mutagenic phenolic antioxidant found in extra-virgin olive oil and olive oil by-products. All formulations were deeply investigated in terms of morphological, thermal, optical and mechanical properties. Water absorption and antioxidant effects were also investigated. Experimental data showed that pure HTyr, at all percentages, did not alter the homogeneity of the resulting films and induced a double positive effect on the PVA matrix: the

first related to the structural, mechanical and thermal properties, the second to the functional activity. As a matter of fact, HTyr also increased the thermal-oxidation stability and crystallinity degree of PVA in a more evident manner with respect to the previously found and published responses of hydroxytyrosol-derived compound-based formulations [25], while its mechanical response with a plasticizing effect and the positive effect on the final gas barrier properties of the produced PVA based formulations was here reported and found for the first time. The mechanical data underlined that the best performances were detected for the PVA/HTyr-5 film. In addition, the PVA/HTyr systems behaved as active films, releasing functional antioxidant HTyr into the hydrophilic food simulant. On the basis of the discussed results, we can assert that PVA/HTyr films, PVA/HTyr-5 in particular, are very promising novel active food packaging, which are sustainable alternatives to petroleum-based materials.

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