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Development of biodegradable films with antioxidant properties based on polyesters containing α -tocopherol and olive leaf extract for food packaging applications

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Abstract

Biodegradable films with antioxidant properties based on Ecoflex® and Ecoflex®-polylactic acid (PLA) containing α -tocopherol and olive leaf extract were developed by blown film extrusion.

There was a good recovery of tocopherol from Ecoflex films (98-112%). Oleuropein and oleuroside were the main antioxidants detected in the studied olive leaf extract. A reduction of oleuropein content (21-33%) and an increase of oleuroside (14-31%) were observed in Ecoflex and Ecoflex/PLA films. All the films showed antioxidant capacity *in vitro*. The films containing tocopherol exhibited higher antioxidant activity than the films containing olive leaf extract.

The incorporation of both antioxidants gave a coloured taint to the films. The films containing olive leaf extract showed the highest colour changes (ΔE). Films containing 2.8% of antioxidants showed increased elongation at break (EB), however at higher antioxidant concentrations reductions of tensile strength and EB were observed. These results confirmed the feasibility to produce antioxidant films with Ecoflex and Ecoflex/PLA blend. However, the concentration of antioxidant that can be added to the films is constrained by the changes induced on the physical properties. The weak interaction between the antioxidants and the polymer matrix would provide a material suitable for food products with a short shelf life.

Key words: active packaging, biodegradable films, natural antioxidants, α -tocopherol, olive leaf extract

1. Introduction

The global production of plastics was around 280 Mt in 2011. In Europe, the total production of plastic reached 58 Mt in 2011, from which 25.1 Mt ended up in the waste stream (Anonymous, 2012). The resulting environmental impact of the high consumption of plastic materials in the food industry has encouraged special efforts from the packaging industry to develop biodegradable packaging materials. Most biodegradable polyesters have high potential to be used in packaging applications since they have desirable thermoplastic and biodegradable properties in addition to high strength, high modulus, and good processability (Rhim, 2007). Biodegradable polyesters can be divided into aliphatic and aromatic groups, with members of each group being derived from renewable and non-renewable sources (Sin, Rahmat, & Rahman, 2013). Ecoflex[®] produced by BASF is an example of a synthetic biodegradable copolyester obtained from fossil monomers 1,4-butanediol, adipic acid and terephthalic acid. Ecoflex[®] combines the good biodegradability known for aliphatic polyesters with the good mechanical properties of aromatic polyesters (Siegenthaler, Künkel, Skupin, & Yamamoto, 2012). Polylactic acid (PLA) is an aliphatic polyester derived from renewable sources, mainly starch and sugar (Lim, Auras, & Rubino, 2008). PLA is the biodegradable polymer present in the market in largest amounts. Its high stiffness and transparency make it a suitable material for manufacturing of plastic bottles, cups and rigid trays. However, PLA is too stiff for most flexible packaging applications (Jiang, Wolcott, & Zhang, 2006). A range of material properties can be obtained by blending stiff and brittle PLA with soft and flexible Ecoflex[®].

The main objective of food packaging is to protect food products, particularly from oxidative and microbial spoilage and to extend its shelf-life. Active packaging can enhance food protection through the addition of active substances such as antimicrobials and antioxidants (López-de-Dicastillo, Alonso, Catalá, Gavara, & Hernández-Muñoz, 2011; Marcos, Aymerich, Garriga, & Arnau, 2013; Pereira de Abreu, Paseiro Losada, Maroto, & Cruz, 2011; Unalan, Arcan, Korel, & Yemenicioglu, 2013). Antioxidant packaging can prevent food oxidation that can derive in the development of off-

flavours, colour and flavour changes, and nutritional losses. The addition of antioxidants in the packaging material provides advantages compared to direct addition to food, such as lower amounts of active substances required, activity focused on the product surface, controlled release to the food matrix, and elimination of additional steps on the production process needed for antioxidant addition (Bolumar, Andersen, & Orlien, 2011).

The growing demand by consumers for clean label food products with no added artificial additives has derived in an increased interest in the use of natural antioxidants as food additives. In this sense, some research efforts have focused on the addition of natural antioxidants such as phenolic compounds, acerola pulp, oregano and rosemary extracts to packaging materials (Camo, Lorés, Djenane, Beltrán, & Roncalés, 2011; Mastromatteo, Barbuzzi, Conte, & Del Nobile, 2009; Nerín et al., 2006; Souza et al., 2011).

The objective of the present study was to develop biodegradable films with antioxidant properties intended for packaging of clean label food products. With this purpose antioxidant biodegradable films based on Ecoflex[®] and Ecoflex[®]-polylactic acid (PLA) were obtained by film blowing extrusion. The antioxidants used for this study were α -tocopherol (T) and olive leaf extract (O), known by its good thermal stability (Peltzer, Wagner, & Jiménez, 2007; Salta, Mylona, Chiou, Boskou, & Andrikopoulos, 2007).

2. Materials and methods

2.1. Materials and reagents

Ecoflex[®] F Blend A 1200, Ecoflex[®] Batch AB1 and Ecoflex[®] Batch SL 1 were purchased from BASF (Ludwigshafen am Rhein, Germany). PLA resin Ingeo[™] 4060 was from Natureworks (Minnetonka, MN, USA). Olive leaf extract enriched in oleuropein (20.8%) was purchased from Monteloeder (Elche, Spain) and DL- α -tocopherol from Panreac Química SLU (Castellar del Vallès, Spain).

Ultrapure water was obtained with a Milli-Q Advantage System of Millipore Iberica (Madrid, Spain). Tetrahydrofuran (THF), acetonitrile (ACN), hexane, methanol (MeOH), acetone and 2- propanol were

HPLC-grade from J.T. Baker (Deventer, The Netherlands). Phosphoric acid, α -tocopherol (pure standard) and oleuropein (purity >98%) were purchased from Sigma-Aldrich (Madrid, Spain). Other reagents used were of analytical grade.

2.2. Masterbatch production

Ecoflex pellets were dried at 80°C for at least 4h prior to compounding. Masterbatches were prepared with a co-rotating twin screw extruder Berstorff ZE 40 A * 38 D (Berstorff GmbH, Hannover, Germany) coupled with an underwater pelletiser (Gala GmbH, Xanten, Germany).

Masterbatches were obtained by mixing Ecoflex® F Blend A 1200 with the antioxidants. Two different masterbatches were obtained: a masterbatch containing a final concentration of 10% of DL- α -tocopherol (MBT) and a masterbatch with a 10 % of olive leaf extract (MBO).

2.3. Melt flow index

Melt flow index (MFI) of virgin Ecoflex and the obtained masterbatches (MBT, MBO) were measured using a Zwick melt plastometer as per ASTM D-1238-10 standard (ASTM, 2010). The sample was heated in the plastometer for 5 min at 190°C to allow the resin to melt. A weight of 2.16 kg was placed on the plunger to force extrusion of the molten polymer through a die. The melt flow index (MFI) was measured as the mass in g of material that extruded from the die over a given period of 10 min. Three measurements of each sample were performed.

2.4. Film blown extrusion

Prior to extrusion, Ecoflex® F Blend A 1200, Ecoflex® Batch AB1 and Ecoflex® Batch SL 1 were dried at 80°C for at least 4h. PLA Ingeo™ 4060, oleuropein and tocopherol masterbatches were dried at 60°C for at least 4 h. Ecoflex, PLA, Ecoflex Batches and the masterbatches (MBT and MBO) were mixed using the needed concentrations to obtain control films with no added antioxidants (Ecoflex films and Ecoflex/ PLA blend films) and antioxidant films containing a range of tocopherol and olive leaf extract concentrations. Film formulations are shown in Table 1.

A single screw extruder Type Uni-Ex 1-45-25B (Battenfeld-cincinnati GmbH, Bad Oeynhausen, Germany) equipped with a circular opening die for blow extrusion was used. The extruder was equipped with a single screw of $D=45\text{mm}$ and $L/D=25:1$, where D and L are the diameter and the length of the screw, respectively. The barrel had three heating zones and a 50 kW motor. Extrusion conditions are shown in Table 2. Films of 36 cm width were obtained.

2.5. Chemical analyses of the films

Film samples were stored at room temperature until analysis to simulate industrial storage conditions. Portions of 100 mg of film were cut in small pieces for chemical analysis. Each sample was analysed in triplicate. All the procedures were carried out in subdued light.

2.5.1. Determination of α -tocopherol

Film samples were extracted in 3 ml hexane/2-propanol (3:2 v/v) in an ultrasonic bath for 15 min. The sample was centrifuged and the supernatant separated. The extractions were repeated twice in 6 ml of hexane/ethanol (1:2, v/v). The combined supernatants were evaporated to dryness and the residue was redissolved in 200 μl of 2-propanol and 800 μl of ACN. Twenty μl of the extract were diluted in ACN (1:10 v/v), filtered through polytetrafluoroethylene syringe filter (0.2 μm pore size) (Teknokroma, Barcelona, Spain) and injected into the HPLC-FLD system. Samples were analysed using an HP 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary gradient pump and a fluorescence detector. Separation was performed on a Luna C_{18} column (150 x 3.0 mm i.d., 3 μm particle diameter) from Phenomenex (Torrance, USA).

A linear gradient elution at a flow rate of 0.55 ml/min was carried out between mobile phase A (100 % ACN) and mobile phase B (ACN: THF; 80:20 v/v) by varying phase B percentage from 30 to 80% in 10 minutes. Fluorescent detector was set with $\lambda_{\text{exc}}=280\text{ nm}$ and $\lambda_{\text{em}}=330\text{ nm}$ and α -tocopherol was quantified according to a standard curve created with solutions containing a known amount of pure standard (0- 10 mg l^{-1}).

2.5.2. Determination and identification of oleuropein and oleurosides

Extraction of oleuropein was assayed with three different procedures, namely:

(a) Overnight extraction at room temperature in 4 ml of MeOH/water (95:5, v/v). Samples were then centrifuged at 4000 rpm during 5 min and the clear phase was made up to 15 ml with methanol.

(b) Extraction by sonication for 20 min in 4 ml MeOH/water (95:5, v/v). Samples were centrifuged and, after collecting the clear liquid phase, the solid was re-extracted with 4 ml acetone and sonicated. The clear liquid phases were combined and the final volume was made up to 15 ml with acetone.

(c) Same extraction procedure than (b), with an additional extraction in 4 ml of 100 % MeOH and subsequent sonication and centrifugation included before the last extraction with acetone. In this case, the three liquid phases were combined to a final volume of 20 ml.

In each case, one ml of the extract was evaporated to dryness with nitrogen and then re-dissolved in 2 ml of ultrapure water, pH was adjusted to 2.3 with 20 % o-phosphoric acid, filtered through a nylon syringe filter (0.2 μm) (Teknokroma, Barcelona, Spain) and then injected in the HPLC-DAD (40 μl).

Chromatographic separation was carried out with a Waters 1525 binary pump equipped with a 717 Plus autosampler, and a 2996 photodiode array detector (Water Corp., Milford, MA) at a flow rate of 1.1 ml/min. The column was a Waters C₁₈ Symmetry (250 x 3.9mm i.d. - particle diameter 5 μm) with a Sentry Guard C₁₈ Symmetry (20 mm x 3.9 mm i.d.). A linear gradient elution was carried out by varying the composition of mobile phase from initial 20 % phase A (MeOH adjusted to pH 2.5 with 20 % o-phosphoric acid) and 80 % B (water adjusted to pH 2.5 with 20 % o-phosphoric) to 30 % phase B in 30min. Quantification of oleuropein was made on the basis of the signal intensity acquired at 230 nm with a calibration curve (0-5 g l⁻¹) of olive leaf extract containing known amounts of oleuropein. Oleurosides were quantified as oleuropein equivalent on the basis of its response at 230 nm.

An Acquity UPLC® chromatographic system (Waters, Milford, MA) equipped with a Diode Array Detector and a Triple Quadrupole Mass Spectrometer (TQD) was used to confirm the identity of target compounds.

Chromatographic separation was carried out on a BEH Shield RP18 (150 mm x 1.0 i.d., 1.7 µm particle size) column at 35°C. A linear gradient elution was carried out by varying the composition of mobile phase from initial 100 % mobile phase A (water:ACN:formic acid 94.9:5.0:0.1, v/v/v) to 28 % mobile phase B (water:ACN:formic acid 39.9:60:0.1, v/v/v) in 25 min, with a flow rate of 0.130 ml/min. Samples were maintained at 4°C; the injected volume was 5 µl.

TQD was operated in negative electrospray ionization mode (ESI); the source temperature was fixed at 140°C, the capillary voltage was set at 2.5 kV and the desolvation temperature was set at 350°C. The cone gas (nitrogen) flow rate was 350 l/h. The collision gas used was argon, at 0.1 ml/min flow rate.

MS experiments were carried out in Scan Mode, in order to obtain m/z values of pseudomolecular ions (parent ions). MS² experiments were performed in Daughter Ions mode (cone voltage 30 V, collision energy 25 eV) to obtain the fragmentation patterns of pseudomolecular ions.

Thermal stability of oleuropein and oleuroside was checked by heating portions of 5 g of the olive leaf extract in a oven under two conditions:(i) 100°C overnight (16 h) and (ii) 165°C for 15 min. After thermal treatments, the samples were cooled at ambient temperature, suitably dissolved and diluted, and analyzed by UPLC-MS/MS.

2.5.3. Diffusion of antioxidants

A total immersion method was used to study the release of the antioxidants as suggested by Chen, Lee, Zhu and Yam (2012) with some modifications. Approximately 1 g of film samples were cut into discs and immersed in 100 ml of the food simulant (ethanol/water, 95:5) in 250 ml flasks. The flask was kept in an environmental chamber (Mettler GmbH, Schwabach, Germany) at 30°C. One millilitre of the food simulant was periodically sampled for quantification. The concentration of

antioxidants released into the simulant was quantified using a UV-vis spectrophotometer Shimadzu UV-1800 (Shimadzu Co., Kyoto, Japan) at 290 nm for α -tocopherol and at 280 nm for the olive leaf extract on the basis of standard curves (0-300 ppm). To eliminate the influence of other polymer additives, release studies were also conducted on control films (Ecoflex, Ecoflex/ PLA) and no significant absorbance at 280 and 290 nm was observed.

The migration process is described by the kinetics of the diffusion of the migrant in the film and it is expressed by the diffusion coefficient (D). The one dimensional diffusion solution equation of Fick's second law for the studied antioxidants to an infinite volume solution was considered to determine D :

$$\frac{M_t}{M_{F,\infty}} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[\frac{-D(2n+1)^2 \pi^2 t}{l^2}\right]$$

where $M_t/M_{F,\infty}$ is the concentration of antioxidant diffused at time t , divided by the concentration of antioxidant diffused at equilibrium, l is the thickness of the film, and D is the diffusivity of the migrant into the film. This equation was selected according to Crank (1975) as the values of $Kp, s = C_{p,\infty}/C_{s,\infty}$ were lower than 1 in all cases (where $C_{p,\infty}$ and $C_{s,\infty}$ are the amount of antioxidant in the polymer and in the simulant at equilibrium, respectively).

2.5.4. Antioxidant capacity by the DPPH scavenging activity

The stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to measure the free radical scavenging capacity of the sample extracts as described by Wijngaard, Rossle, and Brunton (2009). Methanol extracts from the films were obtained as suggested by Byun et al. (2010). Briefly, film samples were extracted with 2 ml of methanol. The mixture was vigorously vortexed for 3 min and allowed to stand at room temperature for 3h. Then, it was vigorously vortexed for another 3 min and centrifuged at 2,300 rpm for 10 min. The supernatant obtained was analysed for DPPH radical scavenging activity. The reaction mixture consisted of 500 μ l of diluted sample (serial dilutions of the film extracts were prepared prior analysis) and 500 μ l of a freshly made DPPH methanolic solution

(0.05 mg/ml) and was prepared in 1.5 ml microcentrifuge tubes. The absorbance of the freshly prepared DPPH solution was measured prior to analysis and absorbance values were in the range 1.2-1.3. After vortexing, the tubes were left in the dark for 30 min at room temperature. The absorbance was then measured against methanol at 515 nm in 1 ml cuvettes using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Milton Keynes). As the DPPH is reduced by the antioxidants present in the sample, the solution colour fades in a way that is proportional to the antioxidant concentration. The sample concentration that causes a decrease in the initial DPPH concentration by 50% is defined as the IC50 and is used to calculate the antioxidant capacity. The IC50 of Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), a synthetic hydrophilic vitamin E analogue, is also calculated and the antioxidant capacity of the sample is then expressed as Trolox equivalent antioxidant capacity values (TEAC) using the formula $TEAC = (IC_{50}^{Trolox} / IC_{50}^{Sample}) \times 10^5$.

2.6. Physical analysis of the films

2.6.1. Film thickness

Film thickness was measured using a Digimatic micrometer (Mitutoyo, Japan). The value of film thickness was obtained by averaging 10 measurements.

2.6.2. Colour measurement

Instrumental colour measurement of films was performed using a Konica Chroma Meter CR-410 (Minolta, Osaka, Japan). C illuminant and 2° standard observer were chosen. L* (lightness), a* (redness, greenness), and b* (yellowness, blueness) colour values were determined in the 1976 CIELAB system. The chromameter was calibrated before each series of measurements using a white ceramic plate. The mean of 6 measurements was recorded for each film. Three different points from

each film type were tested. The total colour difference (ΔE) was determined as an estimate of colour changes: $\Delta E = [(L^*-L_0^*)^2 + (a^*-a_0^*)^2 + (b^*-b_0^*)^2]^{1/2} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

The colour values of control films (Ecoflex and Ecoflex/PLA) were used as reference values for ΔE calculation (L_0^* , a_0^* , b_0^*).

2.6.3. Mechanical properties

2.6.3.1. Tensile test

A MTS Texture Analyser (MTS Systems Corp., MN, USA) was used to measure the tensile properties of the films. Film strips of 8 × 3 cm were placed between grips placed at an initial separation of 40 mm and the crosshead speed was set at 100 mm/min. Tensile strength (TS) (MPa) and strain at break (SB_T) (%) were calculated from the load-deformation curves. Ten samples from three different points of each film were evaluated.

2.6.3.2. Puncture test

A TA.TX plus texture analyser (Stable Micro Systems, Surrey, UK) was used to measure the puncture properties of the films. Film samples of 3 × 3 cm were fixed in a 1 cm diameter film support rig. The samples were perforated with a penetration probe with a spherical end with a crosshead speed of 2 mm/s. Force at break (puncture force, PF) (N) and probe penetration (strain at break, SB_p) (mm) were calculated with the Exponent software (Stable Micro Systems, Surrey, UK) from the load-deformation curves. Five samples from three different points of each film were evaluated.

3. Statistical analysis

All statistical analyses were performed using SAS 9.2 software (Statistical Analytical Systems Institute, Cary, NC, USA). Two different models were applied. The first model applied to control films and films with low level of antioxidants included the film batch (control, α -tocopherol and olive leaf extract), the type of film (Ecoflex or Ecoflex/PLA), and their interaction as fixed effects. The second model

applied to Ecoflex films with added antioxidants included antioxidant concentration, type of antioxidant, and their interaction as fixed effects. Differences were assessed by the Tukey test ($p < 0.05$). Matlab (R2008b) software was used to determine the fit of the migration experimental data using a non-linear regression function. The estimation of the diffusivity coefficient (D) was obtained using a routine that implements a multidimensional unconstrained non-linear minimization. The minimum root mean square error of the model was chosen for function minimisation. The 95% confidence interval of D for each film type was calculated from the mean and standard deviation of the replicates.

4. Results and discussion

4.1. Melt flow index

The melt flow index (MFI) of Ecoflex® F Blend A 1200 was 2.99 ± 0.03 g/ 10 min. After masterbatch production, the MFI of 10% α -tocopherol masterbatch (TMB) and 10% olive leaf extract masterbatch (OMB) were 9.87 ± 0.3 and 8.57 ± 0.21 g/ 10 min, respectively. Antioxidants are usually used in polymer compounding as melt stabilizers preventing thermal degradation. As a consequence of polymer stabilisation addition of antioxidants may result in reductions in the MFI (Peltzer, Navarro, López, & Jiménez, 2010). In this sense, Al-Malaika, Ashley, and Issenhuth (1994) reported the effectivity of α -tocopherol, added at very low concentrations (100-300 ppm), as a melt stabiliser for LDPE (low density polyethylene). On the contrary, the addition of high concentrations of α -tocopherol in the present study resulted in an increase of the MFI. Therefore, addition of a high concentration of antioxidants to Ecoflex resin decreased the melt viscosity and increased the flowability of the resin. The results suggest that the polymer would have suffered some degree of degradation during the extrusion process.

4.2. Stability of natural antioxidants

4.2.1. *α-tocopherol*

There was a good recovery of α -tocopherol from Ecoflex films (Table 3). These results indicate that the added tocopherol was stable and fully extractable after masterbatch production and blown film extrusion. Schaich, Obinata, and Yam (2008) reported negligible tocopherol losses in linear low density polyethylene: high density polyethylene(LLDPE:HDPE) and LLDPE:PS (polystyrene) films extruded at 193-200°C, while LLDPE:PP (polypropylene) films extruded at higher temperatures (216-220°C) showed losses of about 18%. However, Peltzer, Wagner, & Jiménez (2007) stated that the stabilising performance of α -tocopherol is not affected by processing temperatures in the range of 200-220°C. In the present study, the Eco/ PLA films showed some reduction of tocopherol content (about 16%), although the extrusion temperatures did not differ from those used for Ecoflex film extrusion (range 138-170°C). Similarly, Manzanarez-López, Soto-Valdez, Auras, and Peralta (2011) reported losses of tocopherol about 15% during masterbatch production of PLA films by extrusion in a temperature range of 165-170°C. These results would suggest that the differences in tocopherol extraction between Ecoflex and Ecoflex/PLA films would rather be due to a stronger interaction of tocopherol with PLA compared to Ecoflex, than to thermal degradation of the antioxidant.

4.2.2. *Olive leaf extract*

The olive leaf extract used for film formation was rich in oleuropein (20.8 %). After film formation, the content of oleuropein in the films was assessed. The best results in terms of apparent recovery were obtained with the extraction procedure c, which included a double extraction step.

The chromatograms obtained from sample extracts showed two major peaks (Figure 1A), which were identified as oleuropein and oleuroside on the basis of their MS fragmentation (Figure 2). Oleuropein and oleuroside show a very similar structure, which differs only for a shift of the double bond on the side chain of the exocyclic ring (Figure 3).

Table 3 shows a loss of oleuropein (21-33%) and an increase in oleuroside content (14-31%) as a consequence of film processing. Due to the lack of bibliographic data on the thermal stability of

oleuropein, an *in vitro* thermal stability study was performed. No significant losses of oleuropein were observed when the olive leaf extract was treated at 100 °C overnight, while a 75 % loss was observed after treatment at 165°C for 1h (Figure 1B and C, respectively).

Laguerre et al. (2009) described the possible bioconversion of oleuropein into oleuroside during olive ripening, but no information has been found about the conversion of oleuropein into oleuroside as a consequence of heating.

The decrease of oleuropein levels and the increase of oleuroside could be the result of a thermodynamic controlled reaction as a consequence of the temperature increase during extrusion. *In vitro* assays suggest that the high temperature during extrusion could affect the stability of oleuropein. However, the influence of other factors such as the interaction with the plastic monomer, pressure increase and the presence of additives could also influence both stability and equilibrium of these compounds and would require further studies for confirmation. It should be highlighted that both oleuropein and oleuroside possess the same catecholic moiety and a similar antioxidant capacity (Laguerre et al., 2009) suggesting that the increase in oleuroside content would have mainly counteracted the loss of antioxidant capacity derived from oleuropein reduction derived from film processing.

4.3. Diffusion of antioxidants from Ecoflex and Ecoflex/PLA films

Figure 4 shows the diffusion of the antioxidants from Ecoflex and Ecoflex/PLA films into the food simulant (ethanol 95%) at 30°C. A slower release of α -tocopherol was observed in Ecoflex/PLA films (Figure 4a), where the equilibrium was reached after 4h, compared to Ecoflex films (Figure 4b), that arrived at the equilibrium after 31h (data not shown). These results would suggest a stronger interaction of α -tocopherol with the polymer matrix when PLA was present. The films containing olive leaf extract reached the equilibrium after 48h (data not shown), showing no differences on the diffusion behaviour among film types (Figures 4c and d).

Diffusion coefficients estimated according to Fick's second law are shown in Table 4. The D values of the studied antioxidants were in the range of 10^{-12} m²/s for both film types. Among α -tocopherol containing films, the D values were higher in Ecoflex films compared to Ecoflex/PLA films. No differences on the D values for olive leaf extract were observed between the two studied polymers. Several authors have reported slower diffusion of α -tocopherol from biodegradable and synthetic polymers (PLA, PLA/starch, LDPE), with values of the diffusion coefficient in the range of 10^{-13} - 10^{-15} (Graciano-Verdugo et al., 2010; Hwang et al., 2013; Manzanarez López et al., 2011).

The results observed in the present study suggest a weak interaction between the antioxidants and the polymer matrix resulting in a very fast migration of the antioxidants into the food simulant. Therefore, the use of this type of biodegradable antioxidant films would provide a short-term protection and it would be more suitable to avoid oxidation of food products with a short shelf life.

4.4. Antioxidant activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to measure the antioxidant capacity of the developed films. The results, expressed as Trolox equivalent antioxidant capacity values (TEAC), are shown in Tables 5 and 6. Control films (Ecoflex and Ecoflex/PLA blend) showed no DPPH radical scavenging activity, while antioxidant films showed high values of DPPH radical scavenging activity. These results confirmed antioxidant stability after film processing and the antioxidant capacity of the films *in vitro*.

The films containing α -tocopherol exhibited higher antioxidant activity than the films containing olive leaf extract for both polymer compositions (Ecoflex and Ecoflex/PLA blend, Table 5). No differences on DPPH radical scavenging capacity was observed among the two polymer compositions studied. Therefore, the antioxidant potential of Ecoflex/PLA films was not significantly reduced despite that a lower amount α -tocopherol was recovered from these films compared to Ecoflex films.

Finally, an increase of antioxidant capacity was observed with increasing antioxidant concentration although it was only significant for α -tocopherol (Table 6). At the same concentration, higher TEAC values were observed in films with α -tocopherol added.

4.5. Physical analysis of the films

4.5.1. Colour measurement

The visual examination of the films showed that Ecoflex films were white, opaque and matte and Ecoflex/PLA films were white, opaque and opalescent. Instrumental colour analysis of the films showed that Ecoflex/PLA presented higher lightness (L^*) values than films formed only with Ecoflex (Table 5). The incorporation of both antioxidants gave a coloured taint to the films. These appreciations were confirmed instrumentally with ΔE (total colour change) parameter (Table 5). The films containing olive leaf extract experienced bigger ΔE ($p < 0.01$) than those with added α -tocopherol. Among polymer types, the colour of Ecoflex/PLA films, with lower ΔE values ($p < 0.01$) than Ecoflex films, was less affected by the addition of the antioxidants.

The effect of antioxidant concentration on colour parameters is shown in Table 6. The presence of higher α -tocopherol concentration resulted in darker films ($p < 0.01$). Films containing antioxidants also showed higher a^* and b^* values with increasing concentration of antioxidant. Manzanarez-López et al. (2011) also reported increased yellowness in PLA films containing 2.58% of α -tocopherol although no changes on L^* and a^* coordinates were observed.

4.5.2. Mechanical properties

The addition of active substances to packaging films can modify the mechanical properties of the films (Marcos, Aymerich, Monfort, & Garriga, 2010). The tensile and puncture properties of the films were measured to assess the effect of the antioxidant.

The results of the tensile test expressed as tensile strength (TS) and strain at break (SB_r) are summarised in Tables 5 and 6. The range of values observed indicate that the studied films presented

moderate to good mechanical properties (considering TS and SB_T, respectively) according to Krochta and De Mulder-Johnston (1997) classification for biodegradable films.

The main differences were obtained when comparing the mechanical properties among the two different materials used, Ecoflex and Ecoflex/PLA blend.

Tensile and puncture tests showed higher strength at break and lower elongation at break for Ecoflex/ PLA films (Table 5). Ecoflex is a tear-resistant and flexible polymer with mechanical properties similar to polyethylene (Siegenthaler et al., 2012). The addition of a 20% of PLA provided increased resistance and stiffness to Ecoflex films.

The inclusion of a low level (2.8%) of antioxidants (tocopherol or oleuropein) in Ecoflex films induced a significant increase of both SB_T and SB_P (Table 5). The tensile properties (TS and SB_T) of Ecoflex/PLA blend films were increased due to the addition of 2.8 % of tocopherol ($p < 0.01$), while the puncture properties were not altered by the addition of 2.8% of any antioxidant. Similarly, Jamshidian et al.(2012) reported increased elongation at break in PLA films containing 2 % of tocopherol. The authors related the plasticizing effect of tocopherol with the decrease of the glass transition temperature of the resulting film. Byun et al. (2010) showed that the incorporation of tocopherol in a PLA film containing a 10% PEG did not change the TS, but increased the elongation. However, the effect of tocopherol without PEG was not studied. On the contrary, no effect ($p > 0.05$) of the addition of 2.8% of olive leaf extract on the mechanical properties of Ecoflex/PLA films was observed.

No significant differences on the mechanical properties of Ecoflex films between films containing tocopherol and films containing olive leaf extract were observed at higher concentrations of antioxidant (Table 6). A decrease of the mechanical parameters studied was observed with increasing concentration of antioxidant for both T and O films.

5. Conclusions

This study showed that α -tocopherol and olive leaf extract were stable during film extrusion of Ecoflex and Ecoflex/PLA blend and the resulting films showed antioxidant capacity *in vitro*. However,

the concentration of antioxidant that can be added to the films is constrained by the changes induced on the physical properties. The weak interaction between the antioxidants and the polymer matrix would provide a material suitable for food products with a short shelf life.

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Figure 1. Chromatographic profiles (at 280 nm) corresponding to: a) olive leaf extract, b) olive leaf extract treated 100°C overnight and c) olive leaf extract treated at 165°C for 15 min.

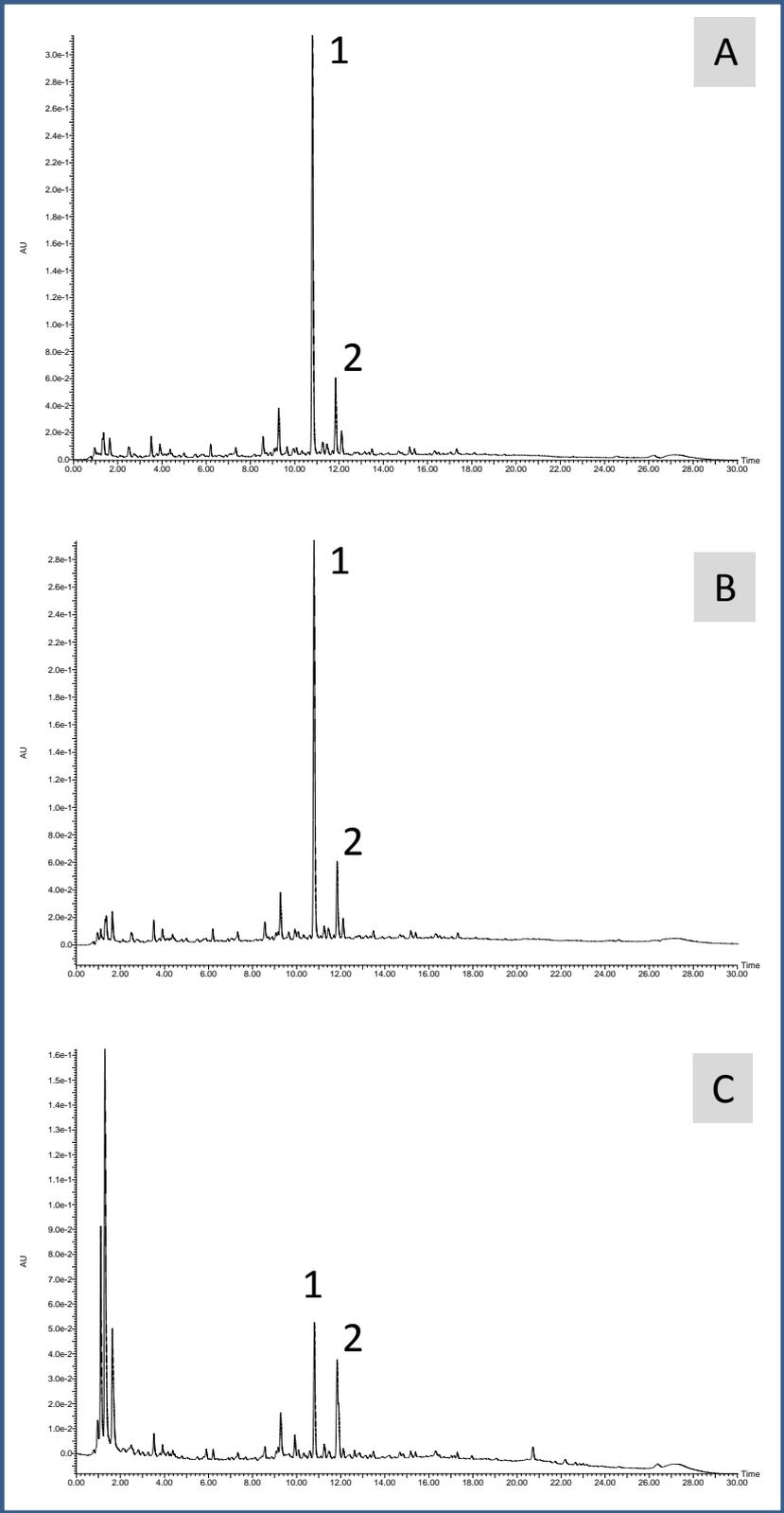


Figure 2. Mass spectra and MS/MS fragmentation supporting the identification of oleuropein and oleurosides.

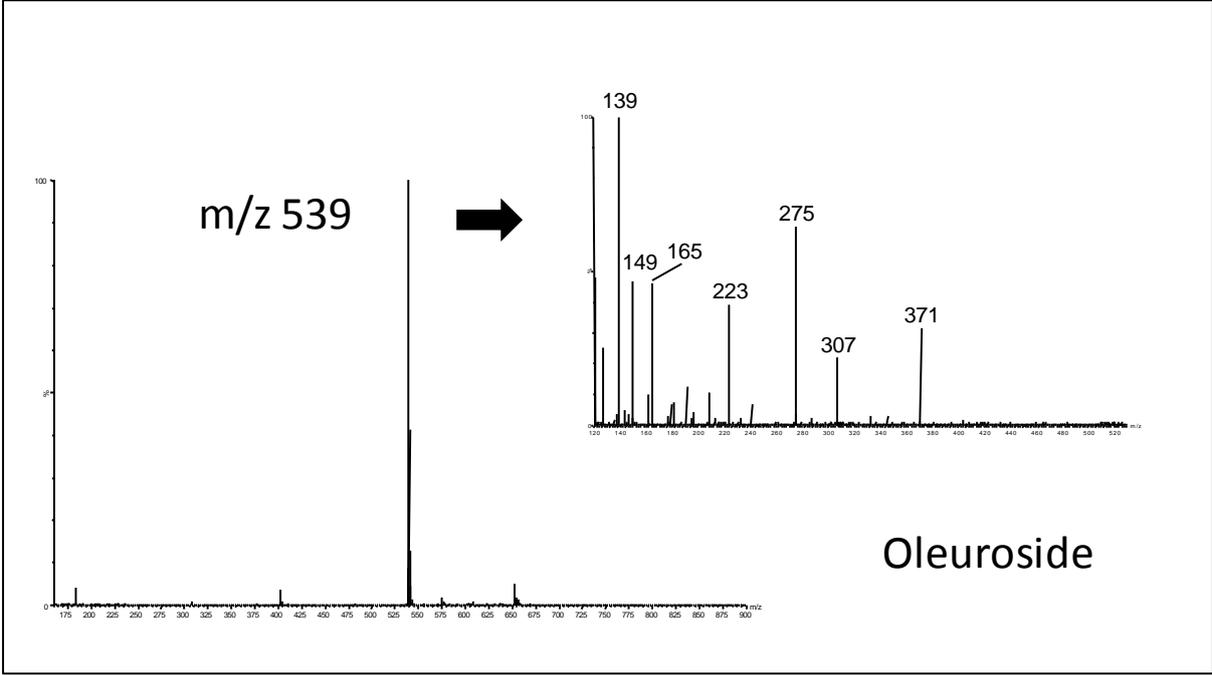
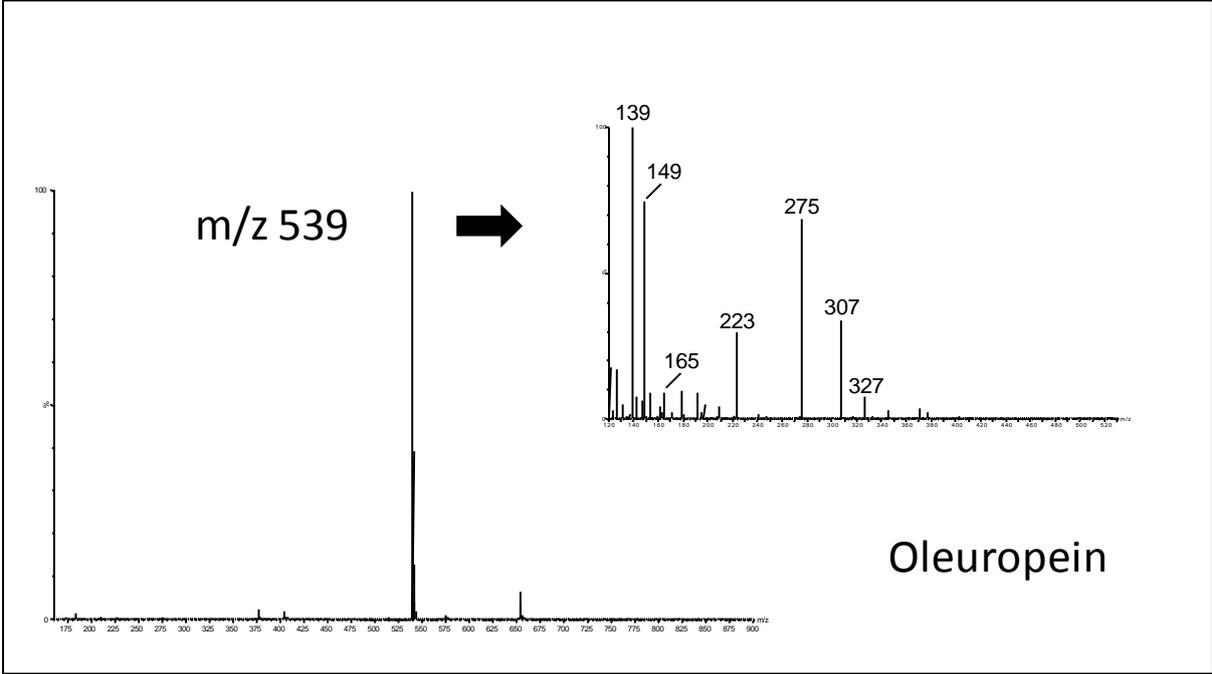


Figure 3. Chemical structure of oleuropein and oleurosides.

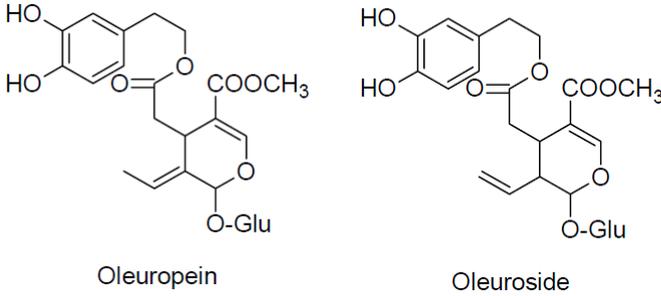
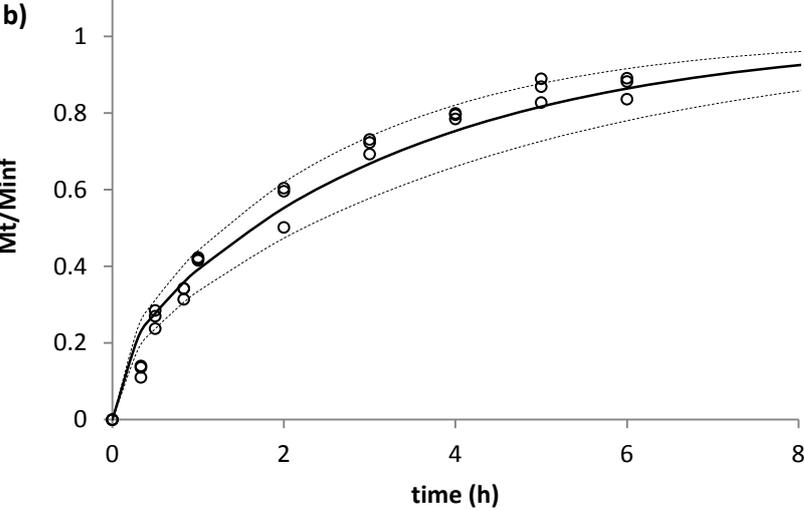
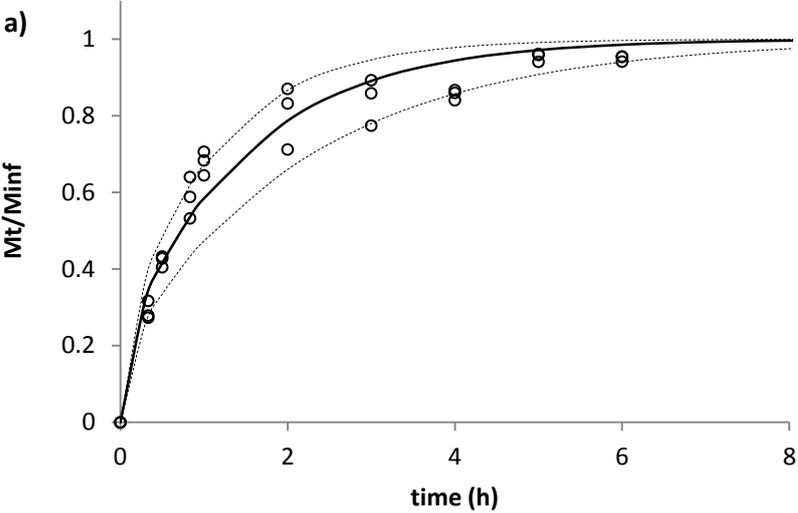


Figure 4. Diffusion of α -tocopherol from Ecoflex (a) and Ecoflex/PLA films (b) and of olive leaf extract from Ecoflex (c) and Ecoflex/PLA (d) films according to the second Fick's law. (o) observed values, () estimated values, () confidence-intervals.



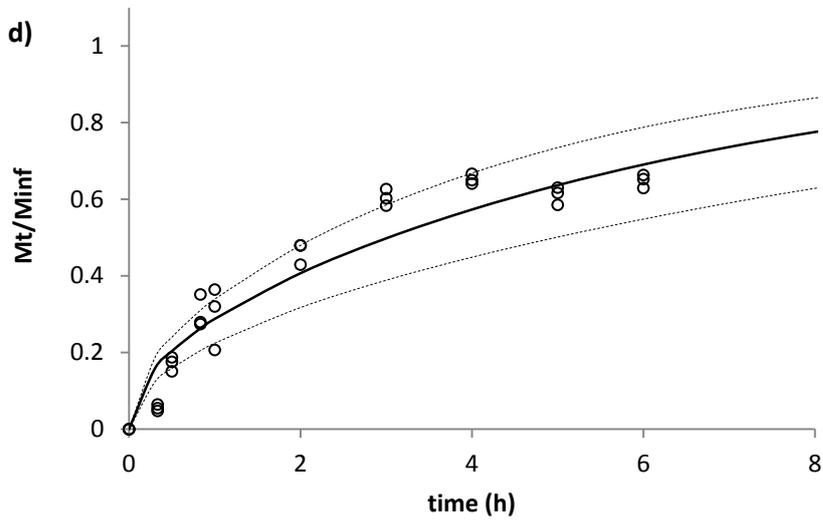
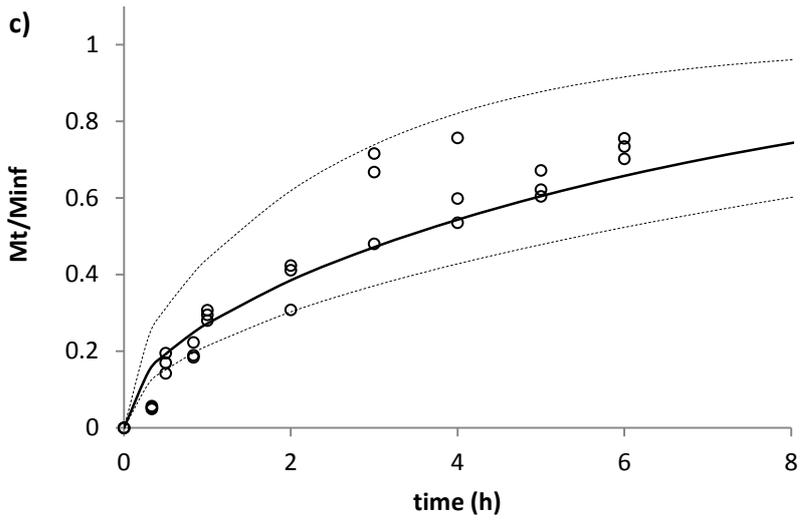


Table 1. Film compositions

Batch	Formula ¹
Eco	94% Ecoflex
Eco low-T	28.2 % TMB + 65.8 % Ecoflex
Eco med-T	56.4 % TMB + 37.6 % Ecoflex
Eco high-T	94 % TMB
Eco low-O	28.2 % OMB+ 65.8 % Ecoflex
Eco med-O	56.4 % OMB + 37.6 % Ecoflex
Eco high-O	74 % OMB + 20 % Ecoflex
Eco/PLA	74 % Ecoflex+20 % PLA
Eco/PLA low-T	28.2 % TMB+ 45 % Ecoflex + 20 % PLA
Eco/PLA low-O	28.2 % OMB + 45.8 % Ecoflex + 20 % PLA

Ecoflex: Ecoflex® F Blend A 1200; PLA: polylactic acid resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; med: antioxidant concentration of 5.64%; high: antioxidant concentration of 9.4 and 7.4% for T and O, respectively; T: α -tocopherol; O: olive leaf extract; TMB: 10% α -tocopherol masterbatch; OMB: 10% olive leaf extract masterbatch.¹ All film formulations contained: 4% Ecoflex® Batch AB1 + 2% Ecoflex® Batch SL 1.

Table 2. Processing conditions during film blown extrusion

Batch	Extruder temperature profile	Extruder pressure (bar)	Screw amperage (A)	Screw speed (rpm)	Feed rate (kg/h)	Line speed (m/min)	Blower set point/ air temperature (°C)
Eco	145/165/155/155/155	360	55	85	17.5	5	4/25
Eco low-T	145/165/155/155/155	325	50	51	17.8	5	4/25
Eco med-T	145/165/155/155/155	261	45	68	17.8	5	4/25
Eco high-T	145/165/155/155/155	180	40	100	18.3	5	4/25
Eco low-O	145/165/155/155/155	311	55	50	18.1	5	3.5/25
Eco med-O	138/150/142/145/145	344	60	50	18.5	5	3/25
Eco high-O	135/150/142/145/145	304	58	59	18	5	3/25
Eco/PLA	145/165/155/155/155	384	65	70	17.9	5	4/25
Eco/PLA low-T	145/165/155/155/155	315	50	40	18.2	5	3.5/25
Eco/PLA low-O	138/155/145/145/145	373	70	45	17.8	5	3/25

Eco: Ecoflex® F Blend A 1200; PLA: polylactic acid resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; med: antioxidant concentration of 5.64%; high: antioxidant concentration of 9.4 and 7.4% for T and O, respectively; T: α-tocopherol; O: olive leaf extract.

Table 3: Antioxidant content of films with added α -tocopherol (a) and olive leaf extract (b and c)

a) α -tocopherol content

Batch	Theoretical (mg/g film)	Experimental		
		mg/g film	mg/cm ² film	Recovery %
Eco low-T	28.20	27.59 ± 2.23	0.99 ± 0.08	97.9 ± 7.9
Eco med-T	56.40	66.64 ± 7.53	2.18 ± 0.25	112.0 ± 6.4
Eco high-T	94.00	98.50 ± 9.41	2.95 ± 0.28	101.5 ± 6.6
Eco/PLA low-T	28.20	23.50 ± 0.09	0.85 ± 0.03	83.4 ± 3.2

Eco: Ecoflex® F Blend A 1200; PLA: polylactic resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; med: antioxidant concentration of 5.64%; high: antioxidant concentration of 9.4%; T: α -tocopherol.

b) Oleuropein content

Batch	Theoretical (mg/g film)	Experimental		
		mg/g film	mg/cm ² film	Recovery %
Eco low-O	5.64	4.47 ± 0.48	0.16 ± 0.02	79.3 ± 8.6
Eco med-O	11.28	7.81 ± 0.92	0.24 ± 0.03	69.2 ± 8.2
Eco high-O	14.80	9.90 ± 0.78	0.30 ± 0.02	66.9 ± 5.3
Eco/PLA low-O	5.64	4.11 ± 0.38	0.13 ± 0.01	72.9 ± 6.8

Eco: Ecoflex® F Blend A 1200; PLA: polylactic resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; med: antioxidant concentration of 5.64%; high: antioxidant concentration of 7.4%; O: olive leaf extract.

c) Oleuroside content

Batch	Theoretical ¹ (mg/g film)	Experimental		
		mg/g film	mg/cm ² film	Recovery %
Eco low-O	1.02	1.34 ± 0.06	0.05 ± 0.00	130.8 ± 6.1
Eco med-O	2.04	2.51 ± 0.06	0.08 ± 0.00	122.7 ± 2.8
Eco high-O	2.67	3.06 ± 0.25	0.10 ± 0.01	114.4 ± 9.3
Eco/PLA low-O	1.02	1.30 ± 0.01	0.04 ± 0.00	127.2 ± 0.9

Eco: Ecoflex® F Blend A 1200; PLA: polylactic resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; med: antioxidant concentration of 5.64%; high: antioxidant concentration of 7.4%; O: olive leaf extract.¹Estimated according to the real oleuroside content (3.6%) quantified in the olive leaf extract.

Table 4. Diffusion coefficient ($D \times 10^{-12} \text{ m}^2/\text{s}$) of antioxidants from Ecoflex and Ecoflex/PLA films.

	Estimated	95% confidence interval ¹
Eco low-T	9.83	(8.07, 11.59)
Eco/PLA low T	4.58	(3.95, 5.21)
Eco low-O	1.96	(1.58, 2.34)
Eco/PLA low-O	2.19	(1.74, 2.62)

Eco: Ecoflex® F Blend A 1200; PLA: polylactic resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; T: α -tocopherol; O: olive leaf extract; ¹(min, max)

Table 5. Properties of control and films with low concentration of antioxidants added obtained with Ecoflex and Ecoflex/ PLA blend.

	Ecoflex			Ecoflex/PLA			Root MSE
	Control	Low-T	Low-O	Control	Low-T	Low-O	
L*	85.72 ^{a,B}	83.96 ^{b,B}	71.66 ^{c,B}	86.77 ^{a,A}	85.01 ^{b,A}	74.09 ^{c,A}	0.19
a*	-0.25 ^b	-1.22 ^c	2.34 ^{a,A}	-0.29 ^b	-1.06 ^c	1.52 ^{a,B}	0.11
b*	-2.52 ^c	6.30 ^{b,A}	35.26 ^{a,A}	-2.28 ^c	5.34 ^{b,B}	32.25 ^{a,B}	0.26
ΔE	-	9.04 ^{b,A}	40.40 ^{a,A}	-	7.86 ^{b,B}	36.83 ^{a,B}	0.37
TEAC	-	424.65 ^a	130.97 ^b	-	448.49 ^a	100.20 ^b	13.13
PF (N)	13.15 ^B	13.98	13.91 ^B	17.98 ^A	16.21	17.01 ^A	0.85
SB _p (mm)	15.16 ^{b,A}	16.80 ^{a,A}	16.75 ^{a,A}	11.44 ^B	11.37 ^B	11.13 ^B	0.34
TS (MPa)	18.52 ^B	20.19 ^B	21.30	25.20 ^{b,A}	34.71 ^{a,A}	23.25 ^b	1.55
SB _T (%)	429.50 ^{b,A}	491.53 ^a	496.10 ^{a,A}	337.83 ^{b,B}	469.87 ^a	389.17 ^{b,B}	23.06

Ecoflex: Ecoflex® F Blend A 1200; PLA: polylactic acid resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; T: α-tocopherol; O: olive leaf extract; Root MSE: root mean square error. L*: lightness; a*: redness; b*: yellowness; ΔE: total colour difference; TEAC: total antioxidant capacity expressed as Trolox equivalent antioxidant capacity; PF: puncture force; SB_p: strain at break measured with puncture test; TS: tensile strength; SB_T: strain at break measured with tensile test. Different lower case letters within a row within each type of film (Ecoflex and Ecoflex/PLA) indicate significant differences between control and films with antioxidants added. Different upper case letters within a row within a type of antioxidant indicate significant differences between film types (p<0.01).

Table 6. Properties of Ecoflex films with α -tocopherol and olive leaf extract added

	Film with α -tocopherol			Film with olive leaf extract			Root MSE
	Low	Medium	High	Low	Medium	High	
L*	83.96 ^A	83.41 ^A	83.31 ^A	71.66 ^{a,B}	68.63 ^{b,B}	67.14 ^{c,B}	0.25
a*	-1.22 ^{b,B}	-1.56 ^{ab,B}	-1.75 ^{a,B}	2.34 ^{c,A}	3.37 ^{b,A}	3.95 ^{a,A}	0.16
b*	6.30 ^{b,B}	8.64 ^{a,B}	9.38 ^{a,B}	35.26 ^{b,A}	37.60 ^{a,A}	38.10 ^{a,A}	0.40
ΔE	9.04	11.47	12.23	40.40	43.76	44.86	0.46
TEAC	424.65 ^{c,A}	709.92 ^{b,A}	1480.43 ^{a,A}	130.97 ^B	267.32 ^B	267.64 ^B	52.78
PF (N)	13.98	14.03	13.23	13.95 ^a	12.90 ^{ab}	12.23 ^b	0.62
SB _p (mm)	16.80 ^a	14.00 ^{ab}	12.23 ^b	16.75	15.44	16.64	1.06
TS (MPa)	20.19 ^a	15.34 ^b	13.88 ^b	21.30 ^a	16.48 ^b	17.47 ^b	0.98
SB _T (%)	491.53 ^a	432.07 ^b	459.20 ^{ab}	496.10 ^a	418.93 ^b	466.33 ^{ab}	17.55

Ecoflex: Ecoflex® F Blend A 1200; PLA: polylactic acid resin Ingeo™ 4060; low: antioxidant concentration of 2.82%; medium: antioxidant concentration of 5.64%; high: antioxidant concentration of 9.4 and 7.4% for α -tocopherol and olive leaf extract, respectively. Root MSE: root mean square error. L*: lightness; a*: redness; b*: yellowness; ΔE : total colour difference; TEAC: total antioxidant capacity expressed as Trolox equivalent antioxidant capacity; PF: puncture force; SB_p: strain at break measured with puncture test; TS: tensile strength; SB_T: strain at break measured with tensile test. Different lower case letters within a row within each type of film with added antioxidant indicate significant differences between antioxidant concentrations. Different upper case letters within a row within an antioxidant concentration indicate significant differences between type of antioxidant ($p < 0.01$).