DOCTORAL THESIS

ACTIVE FILMS FOR FOOD PACKAGING BASED ON POLY(LACTIC) ACID AND STARCH

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Instituto Universitario de Ingeniería de Alimentos
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Hacen constar que:

La memoria titulada **“ACTIVE FILMS FOR FOOD PACKAGING BASED ON POLY(LACTIC) ACID AND STARCH”** que presenta **Dª Justine Muller** para optar al grado de Doctor por la Universitat Politècnica de València, ha sido realizada en el Instituto de Ingeniería de Alimentos para el Desarrollo (IuIAD - UPV) bajo su dirección y que reúne las condiciones para ser defendida por su autora.

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À suivre...

ABSTRACT

Biodegradable active films based on poly(lactic) acid (PLA) and starch were developed and characterized as to their functional and structural properties, using the bilayer film strategy, in order to obtain materials that better meet the food packaging requirements. Plasticization of semicrystalline PLA was analysed by using three different biodegradable compounds to enhance the PLA mechanical performance. Likewise, cinnamaldehyde was incorporated to PLA-starch bilayer films and their antimicrobial properties and release kinetics in food simulants were analysed. Semicrystalline and amorphous PLA and cassava starch (S) were used to obtain the films. Semicrystalline PLA and S were processed by melt blending and compression moulding, whereas amorphous PLA films were obtained by casting the ethyl acetate solution of the polymer, with and without cinnamaldehyde. Bilayer films were obtained by compression moulding of amorphous PLA layers with starch sheets, while semicrystalline PLA sheets were also used in bilayers for comparison purposes.

The analyses of crystallization behaviour and glass transition of neat and plasticized semicrystalline PLA films revealed an increase in the crystallinity, along with a reduction in the glass transition temperature (T_g), in line with the rise in plasticizer concentration in every case. Despite the decrease in T_g, the tensile test did not reflect an enhancement of the film elongation capacity, in comparison to the non-plasticized polymer. Likewise, plasticizers did not reduce the film elastic modulus due to the greater induced crystallinity. On the basis of these results, a more extensible material, amorphous PLA, was considered to obtain PLA-starch bilayer films, taking advantage of the complementary barrier properties of both polymers and the high mechanical resistance of PLA.

Despite the lower ratio of PLA sheet in the starch bilayer assembly (about 1/3 of the film thickness), a great improvement in tensile and water vapour barrier properties was achieved with respect to the neat starch films, the films maintaining high transparency and oxygen permeability as low as that of starch films. When cinnamaldehyde was included in the cast PLA sheet, films became thinner due to the losses of the volatile active during processing, but the improvement in barrier properties was maintained, with lower mechanical resistance. Thermal analyses revealed diffusion of cinnamaldehyde or low molecular weight compounds from the amorphous PLA layer to the adhered sheets (starch or semicrystalline PLA) which contributed to their plasticization and reduced the PLA crystallization.

Antimicrobial activity of the cinnamaldehyde-loaded films against Gram- (*Escherichia coli*) and Gram+ (*Listeria innocua*) bacteria was tested through *in vitro* tests. Both amorphous PLA monolayers and starch-PLA bilayers were effective at controlling the bacterial growth, thus

indicating that the active amount released into the growth medium exceeded the minimum inhibitory concentration (MIC) of both bacteria, despite the losses of the active compound during the film processing. In contrast, PLA-cinnamaldehyde loaded PLA bilayer films did not show antimicrobial activity. The analysis of the release kinetics of cinnamaldehyde from the films to the different food simulants revealed differing behaviours of the active compound delivery, depending on both films and food simulants. PLA bilayer films exhibited the slowest cinnamaldehyde release and the predicted amount delivered into the aqueous culture medium did not reach the MIC, explaining the lack of antimicrobial effect of these films.

Therefore, the developed bilayer films based on amorphous PLA and starch represent an interesting strategy to obtain high barrier-highly resistant packaging films, with active properties when they include cinnamaldehyde as antimicrobial compound.

RESUMEN

Se han desarrollado films biodegradables activos a base de ácido poliláctico (PLA) y almidón y se han caracterizado en sus propiedades funcionales y estructurales. Se ha utilizado la estrategia de films bicapa para obtener materiales con propiedades más ajustadas a los requerimientos del envasado de alimentos. Se ha estudiado la plastificación del PLA semicristalino con tres compuestos biodegradables para mejorar la extensibilidad del PLA. Así mismo, se ha incorporado cinamaldehído a los films bicapa PLA-almidón y se ha analizado su capacidad antimicrobiana y su cinética de liberación en simulantes alimentarios. Se han utilizado PLA semicristalino y amorfo y almidón de yuca para la obtención de los films. El PLA semicristalino y el almidón se han procesado por mezclado en fundido y termocompresión, mientras que los films de PLA amorfo se han obtenido por extensión y secado de su disolución en acetato de etilo, con y sin cinamaldehído. Los films bicapa se han obtenido por termocompresión de las láminas de PLA amorfo con las de almidón, y también con las de PLA semicristalino para su comparación.

El análisis de la cristalización y transición vítrea del PLA semicristalino, con y sin plastificantes, reveló un incremento en la cristalinidad, junto a la reducción de la temperatura de transición vítrea (T_g), al aumentar el contenido en plastificante, en todos los casos. No obstante, a pesar del descenso en la T_g, no se obtuvo un incremento en la extensibilidad de los films. Así mismo, los plastificantes no redujeron el módulo de elasticidad debido al aumento de la cristalinidad. En base a estos resultados, se optó por un material más extensible, el PLA amorfo, para la obtención de los films bicapa con almidón, aprovechando la complementariedad de propiedades de barrera de ambos polímeros y la alta resistencia mecánica del PLA.

A pesar de la baja proporción de la capa de PLA en la bicapa (aproximadamente 1/3 del grosor del film), se obtuvo una gran mejora en las propiedades mecánicas y de barrera con respecto a los films de solo almidón; manteniendo una alta transparencia y una permeabilidad al oxígeno tan baja como la de los films de almidón. Cuando se incluyó cinamaldehído en la capa de PLA amorfo, los films fueron más finos debido a las pérdidas del volátil durante el procesado, pero se mantuvo la mejora en propiedades de barrera, con films un poco menos resistentes. El análisis térmico reveló la difusión del cinamaldehído u otros compuestos de bajo peso molecular desde la capa de PLA amorfo a las capas adheridas (almidón o PLA semicristalino), lo que contribuyó a su plastificación y redujo la cristalización del PLA.

La actividad antimicrobiana de los films con cinamaldehído contra bacterias Gram- (*Escherichia coli*) y Gram+ (*Listeria innocua*) se analizó mediante test *in vitro*. Tanto las monocapas de PLA amorfo como las bicapas de almidón-PLA fueron efectivas en el control

del crecimiento microbiano de ambas bacterias, lo que indicó que, a pesar de las pérdidas de cinamaldehído, la cantidad liberada al medio de cultivo superó la concentración mínima inhibitoria (MIC) de ambas bacterias. Por contra, la bicapa de PLA con cinamaldehído no mostró actividad antimicrobiana. El análisis de la cinética de liberación del cinamaldehído de los films en los diferentes simulantes reveló diferencias de comportamiento en la liberación del activo dependiendo del tipo de film y simulante. Las bicapas de PLA presentaron la liberación más lenta y la cantidad liberada predicha en medios acuosos, como él del cultivo, no alcanzó la MIC de las bacterias, lo que explicó la falta de actividad antimicrobiana observada para estos films.

Por lo tanto, los films bicapa desarrollados con PLA amorfo y almidón representan una estrategia interesante para obtener films de envasado de alta barrera y buena resistencia mecánica, con propiedades activas cuando incluyen cinamaldehído como compuesto antimicrobiano.

RESUM

S'han obtingut films films biodegradables actius de àcid polilàctic (PLA) i midó i s'han caracteritzat en les seues propietats funcionals i estructurals. S'ha utilitzat l'estratègia de films bicapa per obtindré materials amb propietats mes properes als requeriments de l'envasat d'aliments. S'ha estudiat la plastificació del PLA semicristal·lí amb tres compostos biodegradables per a millorar l'extensibilitat del PLA. Així mateix, s´ha incorporat cinamaldehido als films bicapa de PLA-midó i s'ha analitzat la seua capacitat antimicrobiana i la seua cinètica d'alliberació en simulants alimentaris. S'han utilitzat pla semicristal·lí i amorf i midó de iuca per a l'obtenció del films. El PLA semicristal·lí i el midó s´han processat mitjançant mescla en fos i termocompressió, mentre que els films de PLA amorf s´han obtingut mitjançant extensió i assecat de la seua dissolució en acetat de etilo, amb i sense cinamaldehido. Els films bicapa s'han obtingut per termocompressió de les lamines de PLA amorf junt les de midó, i també amb les de PLA semicristal·lí per a la seua comparació.

L'anàlisi de la cristal·lització i transició vitrea del PLA semicristal·lí, amb i sense plastificants, va revelar un increment en la cristall·initat, a mes de una reducció de la temperatura de transició vitrea (T_g), a l'augmentar el contingut en plastificant, en tots els casos. No obstant això, a pesar del descens en la T_g, no s'obtingué un increment en l'extensibilitat dels films. Així mateix, els plastificants no van reduir el mòdul d'elasticitat a causa de l'augment de la cristal·linitat. Basant-se en estos resultats, es va optar per un material més extensible, el PLA amorf, per a l'obtenció dels films bicapa amb midó, aprofitant la complementarietat de propietats de barrera d'ambdós polímers i l'alta resistència mecànica del PLA.

A pesar de la baixa proporció de la capa de PLA en la bicapa (aproximadament 1/3 del grossor del film), s'obtingué una gran millora en les propietats mecàniques i de barrera respecte als films de només midó; mantenint una alta transparència i una permeabilitat a l'oxigen tan baixa com els films midó. Quan es va incloure cinamaldehido en la capa de PLA amorf, els films van ser més fins a causa de les pèrdues del volàtil durant el processat, però es mantingué la millora en propietats de barrera, amb films un poc menys resistents. L'anàlisi tèrmic va revelar la difusió del cinamaldehido o altres compostos de baix pes molecular des de la capa de PLA amorf a les capes adherides (midó o PLA semicristal·lí), la qual cosa va contribuir a la seua plastificació i va reduir la cristal·lització del PLA.

L'activitat antimicrobiana dels films amb cinamaldehido contra bacteris Gram- (*Escherichia coli*) i Gram+ (*Listeria innocua*) es va analitzar per mitjà de test *in vitro*. Tant les monocapes de PLA amorf com les bicapes de midó-PLA van ser efectives en el control del creixement microbià d'ambdós bacteris, la qual cosa va indicar que, a pesar de les pèrdues de

cinamaldehido, la quantitat alliberada al mig de cultiu va superar la concentració mínima inhibitòria (MIC) d'ambdós bacteris. Per contra, la bicapa de PLA amb cinamaldehido no va mostrar activitat antimicrobiana. L'anàlisi de la cinètica d'alliberament del cinamaldehido dels films en els diferents simulants va revelar diferències de comportament en l'alliberament de l'actiu depenent del tipus de film i simulant. Les bicapes de PLA van presentar l'alliberament més lent i la quantitat alliberada predita en mitjans aquosos, com el del cultiu, no va aconseguir la MIC dels bacteris, la qual cosa va explicar la falta d'activitat antimicrobiana observada per a estos films.

Per tant, els films bicapa obtinguts amb PLA amorf i midó representen una estratègia interessant per a obtindre films d'envasament d'alta barrera i bona resistència mecànica, amb propietats actives quan inclouen cinamaldehido com antimicrobià.

PREFACE

DISSERTATION OUTLINE

This Doctoral Thesis is organized in five sections: **Introduction**, **Objectives**, **Chapters**, **General Discussion** and **Conclusions**. The **Introduction** section discusses the state of the art concerning the use of plastics in the food packaging area, focusing on the development of materials based on biopolymers, such as poly(lactic) acid and starch, with the incorporation of active compounds to obtain antimicrobial and/or antioxidant food packaging materials. Properties of cinnamaldehyde, used as antimicrobial compound in this study, have been discussed. The **Objectives** section presents the general and specific objectives of the Thesis. The obtained results are presented in three **Chapters**, corresponding to three scientific publications, which contains the usual sections: Introduction, Materials and Methods, Results and Discussion, and Conclusion. The most relevant results are analysed together in the **General Discussion** section, from a global perspective. Finally, the last section reports the most important **Conclusions** of the Thesis.

Both starch and PLA has been widely studied in order to obtain packaging films for food packaging applications. Nevertheless, they have different drawbacks related with this use. PLA films are very brittle and exhibit high oxygen permeability whereas starch films exhibit high water sensitivity and high water vapour permeability with low mechanical resistance. In contrast, PLA films have good water vapour barriers while they present high tensile strength, and starch constitute a very good barrier to oxygen and gases. Combination of both biopolymers could offer a packaging material with interesting properties due to their complementary characteristics. However, their lack of compatibility make their blend difficult, since blends yield heterogeneous film structures or they require compatibilizers, which can compromise their use for food applications. To avoid these problems, the starch-PLA combination has been designed as bilayer assemblies in this study, where the compatibility of the polymers is less critical since the starch-PLA contact only occurs at the layer interface. Likewise, the incorporation of an active compound (cinnamaldehyde) has also been considered in order to obtain antimicrobial films.

To improve the extensibility of semicrystalline PLA before the bilayer formation, plasticizing substances were incorporated and their effect on the film properties was analysed. **Chapter I** "Influence of plasticizers on thermal properties and crystallization behaviour of poly(lactic acid) films obtained by compression moulding" reports this study. Comparison of the neat and plasticized PLA films with different substances at different concentrations, in terms of structural, thermal, optical, mechanical and barrier properties, as well as to the overall migration of plasticizers into food simulants, was carried out. However, the used plasticizers were not effective at increasing the film stretchability. Therefore, a more extensible PLA material (amorphous PLA) was considered for the bilayer formation.

Chapter II is entitled "Poly(lactic) acid (PLA) and starch bilayer films, containing cinnamaldehyde, obtained by compression moulding". This chapter deals with the combination of amorphous PLA with starch through a bilayer assembly. Amorphous PLA films were obtained by casting of the ethyl acetate solutions, where the active compound could also incorporated. Starch films obtained by melt blending and compression moulding were adhered to cast PLA sheets by thermocompression. Semicrystalline PLA-amorphous PLA bilayers were also obtained and characterized for comparison purposes. The relevant functional and structural properties of bilayers were analysed. Starch-amorphous PLA sheets showed good adhesion properties, while mechanical and water vapour barrier properties of bilayers were greatly improved, with respects to neat starch films. Despite some losses of cinnamaldehyde that occurred during the film processing, the active compound had a plasticizing effect on both mono and bilayer films.

Finally, the same PLA-starch bilayer films were also analysed in terms of release kinetics of cinnamaldehyde from the films to four food simulants and the antimicrobial activity of the films was tested against Gram+ and Gram- bacteria. Results are presented in **Chapter III** "Antimicrobial properties and release of cinnamaldehyde in bilayer films based on polylactic acid (PLA) and starch". Films with cinnamaldehyde were effective at controlling the growth of the tested bacteria and the release kinetics revealed significant differences in the rate and ratio of the compound delivery depending on both the kind of film and food simulant, the results being coherent with the observed antimicrobial activity.

DISEMINATION OF RESULTS

INTERNATIONAL JOURNALS JCR

Published

"**Influence of plasticizers on thermal properties and crystallization behaviour of poly(lactic acid) films obtained by compression moulding**". Justine Muller, Alberto Jiménez, Chelo González-Martínez and Amparo Chiralt (2016) *Polymer International, 65*, 970-978.

Accepted

"**Poly(lactic) acid (PLA) and starch bilayer films, containing cinnamaldehyde, obtained by compression moulding**". Justine Muller, Chelo González-Martínez and Amparo Chiralt. *European Polymer Journal*.

Submitted

"**Antimicrobial properties and release of cinnamaldehyde in bilayer films based on polylactic acid (PLA) and starch**". Justine Muller, Alba Casado Quesada, Chelo González-Martínez and Amparo Chiralt. *European Polymer Journal*.

COMMUNICATIONS IN INTERNATIONAL CONGRESSES

"**Effect of different plasticizers on physical properties of poly(lactic) acid films**". Justine Muller, Alberto Jiménez, Chelo González-Martínez, Amparo Chiralt. 3rd international meeting on Material/Bioproduct Interaction, Matbim 2015. Zaragoza, Spain. *Poster.*

"**Influence of plasticizers on crystallization behaviour of poly(lactic) acid films**". Justine Muller, Alberto Jiménez, Chelo González-Martínez, Amparo Chiralt. 5th Intenational Conference on Biobased and Biodegradable Polymers, Biopol 2015. Donostia, Spain. *Poster.*

"**Antimicrobial properties and release of cinnamaldehyde in PLA films**". Justine Muller, Alba Casado Quesada, Chelo González-Martínez, Amparo Chiralt. 6th International Symposium on Food Packaging: Scientific Developments Supporting Safety and Innovation, ILSI 2016. Barcelona, Spain. *Poster.*

“**Functional properties of starch-PLA bilayer active films containing cinnamaldehyde**”. Justine Muller, Chelo González-Martínez, Amparo Chiralt. International conference on Food, FoodInnova 2017. Cesena, Italy. *Poster.*

COMMUNICATION IN SCIENTIFIC EVENTS

“**Films activos para el envasado de alimentos a base de almidón y ácido Poliláctico (PLA)**”. Justine Muller. II y III Encuentro de estudiantes de Doctorado de la UPV (2015-2016). Universitat Politècnica de València, Valencia, Spain. *Poster and oral communication.*

PREDOCTORAL STAYS AT FOREIGN INSTITUTIONS

“**Characterization of the nanostructure of PLA active film with cinnamaldehyde**”. Laboratoire d'Ingénierie et des Biomolécules (LIBio), Ecole d'Ingénieurs Agronomie & Industries alimentaires (ENSAIA), Université de Lorraine, Vandoeuvre-lès-Nancy, France. From November 2016 to February 2017, under the supervision of Elmira Arab-Tehrany.

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LIST OF ACRONYMS

ASTM: American Society for Testing and Materials

ATBC: Acetyl Tri-n-Butyl Citrate

ATP: Adenosine triphosphate

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CA: Citric Acid

CEO: Cinnamon Essential Oil

CFU: Colony Forming Unit

CH: Chitosan

CIN: Cinnamaldehyde

CNC: Cellulose nanocrystal

DCP: Dicumyl Peroxide

DNA: Deoxyribonucleic Acid

DSC: Differential Scanning Calorimetry

DTG: Thermal weight loss derivate

EFSA: European Food Safety Authority

EM: Elastic Modulus

EO: Essential Oil

EPO: Epoxized Palm Oil

ESO: Epoxized Soybean Oil

EU: European Union

EVA: Polyethylene-co-vinylacetate

ε: Elongation at break

FDA: American Food and Drug Administration

FESEM: Field Emission Scanning Electron Microscopy

FTIR: Fourrier Transform Infrared Spectroscopy

GRAS: Generally Recognized As Safe

HDI: Hexamethylenediisocyanate

HDPE: High Density Polyethylene

LDPE: Low Density Polyethylene

LSD: Least Significant Difference

M: Mould

M: PLA Monolayer film

MA: Maleic Anhydride

M_{cin}: PLA Monolayer film with cinnamaldehyde

MDI: Methylenediphenyl diisocyanate

MIC: Minimum Inhibitory Concentration

MMT: Montmorillonite

NaMMT: Sodium montmorillonite

OM: Overall Migration

OP: Oxygen Permeability

OTR: Oxygen Transmission Rate

PA: Polyacrylamide

PAA: Poly(acrylic) acid

PBAT: Polybutylene adipate-co-terephthalate

PBSA: Polybutylene succinate-co-adipate

PCL: Polycaprolactone
PE: Polyethylene
PEG: Polyethylene glycol
PET: Polyethylene terephthalate
PG: Propylene Glycol
PHA: Polyhydroxyalkanoates
PHB: Poly-β-hydroxybutyrate
PLA: Poly(lactic) acid
PLA-M: PLA-PLA bilayer film
PLA-M_{cin}: PLA-PLA bilayer film with cinnamaldehyde
PP: Polypropylene
PS: Polystyrene
PTMC: Poly(trimethylene carbonate)
PVA: Polyvinyl alcohol
PVC: Polyvinyl chloride
RH: Relative Humidity
ROP: Ring Opening Polymerisation
S: Starch
SA: Stearic Acid
SEM: Scanning Electron Microscopy
S-M: Starch-PLA bilayer film
S-M_{cin}: Starch-PLA bilayer film with cinnamaldehyde
SPI: Isolated Soy Protein
SPS: Sugar Palm Starch
t: time
TA: Glycerol triacetate
TBC: Tributyl Citrate
TBHQ: Tertbutylhydroquinone
T_c: Crystallization temperature
T_g: Glass transition temperature
TGA: Thermogravimetric Analysis
T_i: Transmisttance
T_m: Melting temperature
T_{max}: Maximum decomposition temperature
TOA: Tung Oil Anhydride
T_{onset}: Onset Temperature
TPS: Thermoplastic starch
TR: Transmission Rate
TS: Tensile Strength
TSA: Tryptone Soy Agar
TSB: Triptone Soy Broth
UV: UltraViolet
UV-VIS: UltraViolet-Visible Spectroscopy
WVP: Water Vapour Permeability
WVTR: Water Vapour Transmission Rate
X_c: Degree of crystallinity
XRD: X-Ray Diffraction
Y: Yeast
ΔH_0: Melting enthalpy of 100% crystalline PLA
ΔH_c: Crystallization enthalpy
ΔH_{ex}: Excess of enthalpy
ΔH_m: Melting enthalpy

INTRODUCTION

INTRODUCTION

1. Food packaging materials

1. 1. Environmental context

Over the last decades, environment has become one of the major global concerns, especially with the pollution, depletion of natural resources and environmental degradation. Consequently, research efforts are made progressively at different levels to face this problematic. A specific concern is the packaging field, which produces great amounts of non-degradable plastic wastes that are accumulated in critical areas in the planet, causing severe problems, and representing high recycling costs. Europe ranks second, along with North America, in the global production of plastic materials with 18.5% of the worldwide annual production (322 million tonnes in total in 2015), while China the first producer of plastics (only thermoplastics and polyurethanes). In Europe, packaging applications are the largest sector for the plastic industry since it represents 40% of the total plastic demand, which reached 49 million tonnes in 2015. (Plastic Europe, 2016).

Food packaging necessitates an important consumption of different materials, while of all the materials available to this end, plastics have increased exponentially over the past two decades, with an annual growth of approximately 5%. Indeed, plastic represents the second most used material for food packaging application, after paper and cardboard.

Figure 1 shows the market share of food packaging materials. Of the plastic materials, petroleum-based plastics, such as polyethylene (PE), polypropylene (PP), polystyrene (PS), are widely used as packaging materials. Despite the environmental issues, plastics packaging are very successful in the market, due to their great combination of flexibility (from film to rigid applications), strength, transparency, stability, impermeability and ease of sterilization, which make them suitable for food packaging. However, despite their good properties, their use and accumulation imply serious environmental problems, and a dependence on fossil fuels. 63% of the current plastic waste comes from packaging applications and it is estimated that less than 14% are recyclable. In 2014, more than 7.5 tonnes of plastic wastes were collected for recycling in Europe (Plastic Europe, 2016).

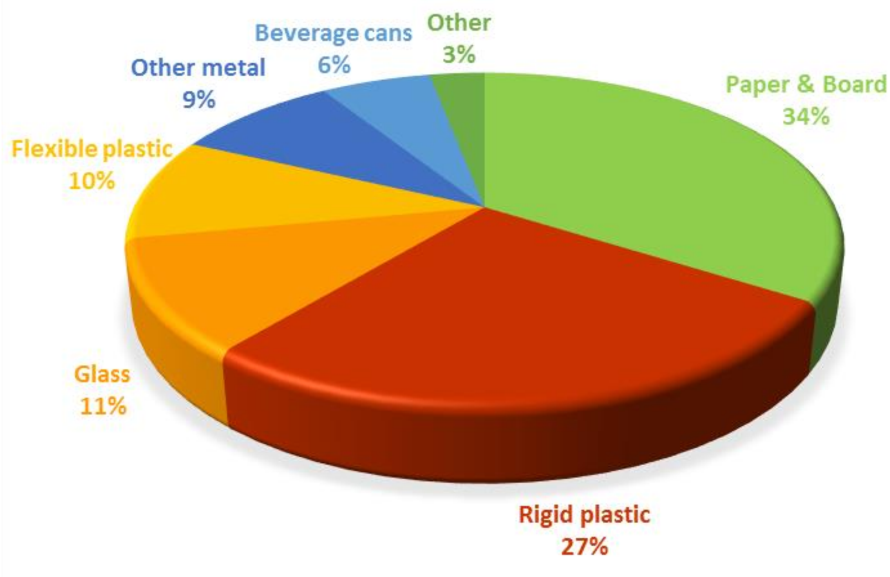

Figure 1. Market share of food packaging materials.
Source: Food Packaging Forum, 2016.

Considering that plastics is the most common primary packaging for food products, the allowed materials for food applications are regulated to ensure food safety, since they are in direct contact with food and beverage. The European Union (EU) has developed various regulations in this respect. The Framework Regulation EC 1935/2004 and the Commission Regulation EC 2015/174 set out the authorized materials and articles intended to come into contact with food. Further, EU Regulation EC 1895/2005 restricts the use of certain epoxy derivatives for the use as resins. Additives added to polymer materials also need to be authorized by the European Food Safety Authority (EFSA) and are available on the "Union's list of substances to be used in plastic food contact materials".

Among the different conventional plastics materials, high and low density polyethylene (HDPE and LDPE respectively), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS) and polyvinyl chloride (PVC) are the most common employed polymers in the food packaging area. Nevertheless, the final packaging is commonly made of several different polymers because of their different properties that make them more suitable for certain applications. Likewise, the technology of plastic bobbins on the form/fill/seal production lines ensures a high production rate, reducing the cost because of the few material loss and the logistic ahead (transport and stock of the PS bobbins) and guarantees the respect of hygiene norms. On another hand, since it is a very flexible but tough and easy processing material, it is also used for transport and distribution packaging, to ensure the integrity of the products or to group several unit products.

1. 2. Bio-packaging

To circumvent the growing plastic production and waste and thus the pollution problem, research has been focused on the development of alternative bio-packaging materials, derived from renewable sources, which are biodegradable or compostable. Biopolymers can be used to substitute non-biodegradable plastics with other more natural and eco-friendly materials, named bioplastics, reducing the environmental impact and oil-dependence. Bioplastics can be divided into three main categories, on the basis of their origin and biodegradable nature (Geueke, 2014):

1. Biobased-non-biodegradable (e.g. PET, PA);
2. Biobased-biodegradable (e.g. PLA, polyhydroxyalkanoates (PHA) or starch, other polysaccharides or proteins);
3. Fossil-based biodegradable (e.g. PCL).

Biodegradable plastics are degraded by microorganisms in their natural environment (soil, water) and can be obtained from organic and/or fossil resources. Bio-based plastics refer to polymers that are composed or derived of biological products from renewable resources such as grains, corn, potatoes, beet sugar, sugar cane or vegetable oils. Therefore, biopolymers are biodegradable, biobased or both and can be classified as those directly obtained from biomass (starch, cellulose, proteins), synthetic biopolymers from biomass or petrochemicals (PLA, PCL) or those obtained by microbial fermentation (PHA). The former are directly extracted from biological and natural resources and they are hydrophilic and somewhat crystalline in nature, making an excellent gas barrier. Biodegradable polyesters (synthetic or biosynthesized) are more hydrophobic and constitute better barriers to water vapour. In general, the functional properties of biopolymer-based materials in terms of their mechanical and barrier properties need to be adapted to food requirements, by using different strategies, such as physical or chemical modifications (crosslinking) or blending with other components, plasticizers or compatibilizers. Therefore, many studies have been carried out in order to obtain bioplastics with comparable functionalities as those of petrochemical polymers in order to respond to the environmental concern. PLA and starch, which have been used in the present study, have been extensively studied as potential replacers of non-degradable petrochemical polymers on the basis of their availability, adequate food contact properties and competitive cost. Their main characteristics are described below.

1. 3. Poly(lactic) acid

One of the most promising biopolymers to replace petrochemical plastics is poly(lactic) acid, a linear aliphatic thermoplastic polyester derived from lactic acid, which is obtained from the fermentation of 100% renewable and biodegradable plant sources such as corn or rice starches and sugar feed stocks. It can be produced by the chemical conversion of corn or other carbohydrate sources into dextrose. Dextrose is fermented to lactic acid followed by polycondensation of lactic acid monomers or lactide. However, the most common way to produce PLA is the Ring Opening Polymerization (ROP) of lactide monomer formed from lactic acid (Armentano *et al.*, 2013). **Figure 2** shows the cycle of PLA in nature while **Figure 3** presents the different ways to produce PLA. It was reported that the production of PLA resin pellets consumes from 25 to 55% less fossil energy than petroleum-based polymers (Xiao *et al.*, 2012).

Figure 2. PLA cycle in nature. Adapted from Xiao *et al.* (2012).

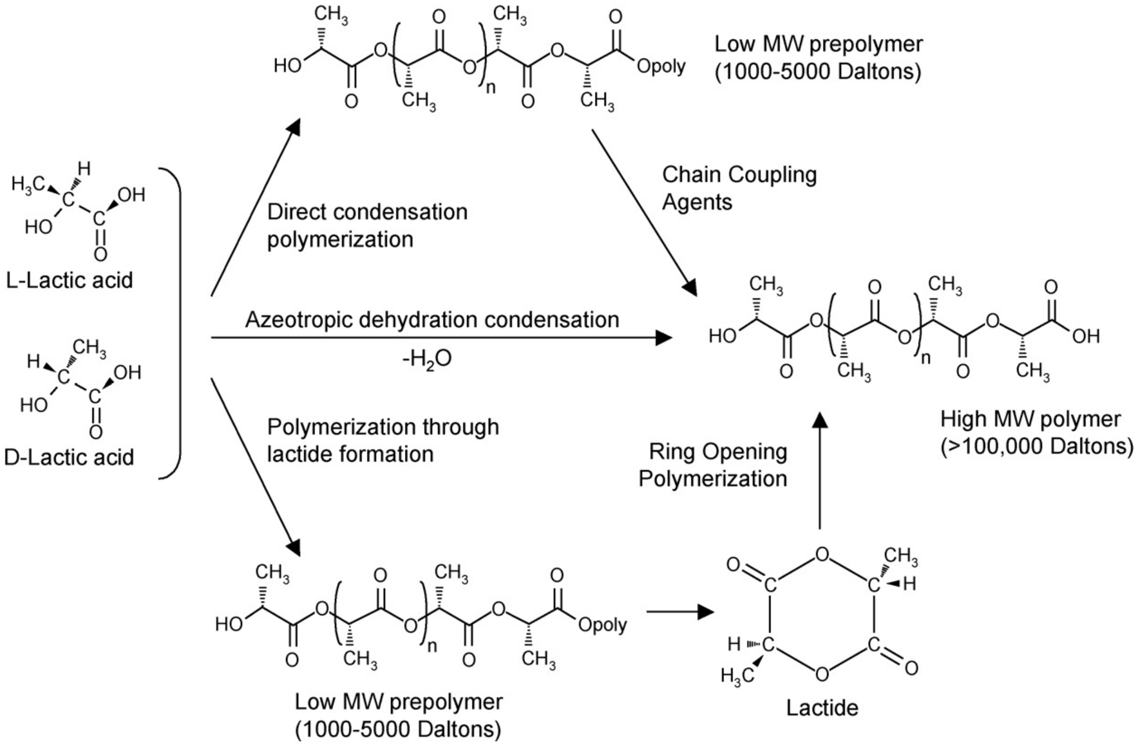

Figure 3. Different routes to produce PLA. Adapted from Auras *et al.* (2004).

1. 3. 1. General characteristics

Three different stereochemical forms exist for lactide: either L-, D- or both L, D-Lactide (Meso-Lactide) (**Figure 4**), having each one their own melting temperature. PLA is insoluble in water, ethanol, methanol and aliphatic hydrocarbons but it is soluble in chloroform, hot benzene, acetonitrile, which are toxic, but also in acetone, ethyl acetate and dichloromethane. Its degradation half-life goes from six months to two years, depending on its stereochemistry and molecular weight (Armentano *et al.*, 2013).

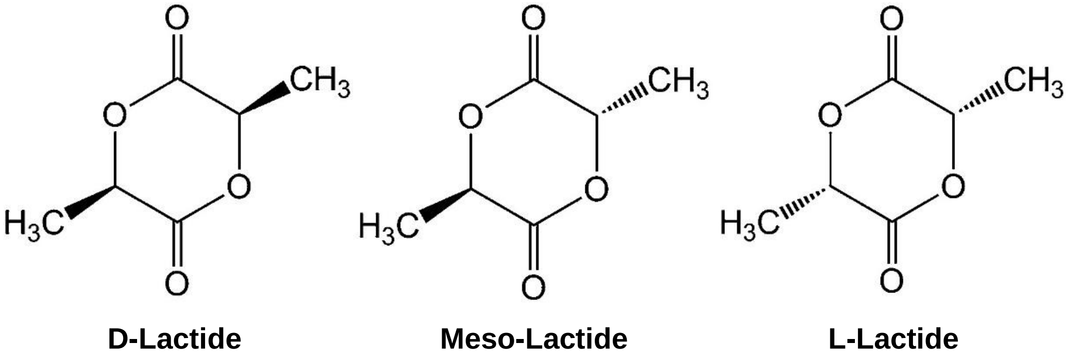

Figure 4. Stereochemical forms of lactide.

1. 3. 2. Advantages/Drawbacks

PLA exhibits many advantages; it is biodegradable, renewable and biocompatible and it has been approved by the American Food and Drug Administration (FDA) for direct contact with biological fluids. It is also highly transparent and has good water vapour barrier properties (Mattioli *et al.*, 2013; Rhim *et al.*, 2009), comparable to those of petroleum-based plastics, such as polyethylene terephthalate (PET) or polystyrene (PS). Because of emerging industrial production technologies, PLA is now very competitive in price. Nonetheless, PLA has limited gas barrier capacity due to its hydrophobic nature. Despite its strong resistance (tensile strength from 17 to 74 MPa) (Fortunati *et al.*, 2015; Gui *et al.*, 2012), it is very brittle, with less than 10% of elongation at break (Rasal *et al.*, 2008; Hiljanen-Vainio *et al.*, 1996).

1. 3. 3. Processing

Due to its good thermal processability, PLA can be tailor-made for different fabrication processes, either injection moulding, sheet extrusion, blow moulding, thermoforming, film forming, or fiber spinning. However, depending on the process, some parameters must be controlled (D-isomer content, molecular weight distribution). L-, D- or meso-lactide stereoisomers can be incorporated into the polymer backbone, thereby obtaining specific PLA material and assigning it for specific applications. Although PLA is hydrophobic, the pellets must usually be dried from 60 to 100°C for several hours prior to processing in order to prevent excessive hydrolysis and modifications in physical properties of the polymer (Lim *et al.*, 2008).

The most common technique used to process PLA is extrusion. Indeed, an extruder enables the pellets to be mixed in a homogeneous way, under high temperature (Lim *et al.*, 2008). PLA can also be dissolved in chloroform (Fortunati *et al.*, 2015; González and Igarzabal, 2013; Jamshidian *et al.*, 2013, Qin *et al.*, 2015a) or other solvents such as dichloromethane (Ahmed *et al.*, 2016a), methylene chloride or acetonitrile (Hughes *et al.*, 2012), and cast to obtain films with high transparency and gloss.

1. 3. 4. Applications

Because of its bio-absorption characteristics and the fact that it used to be expensive, PLA was mainly studied for the biomedical uses, such as tissue engineering (Armentano *et al.*, 2013). Nonetheless, as a result of the new PLA production techniques and polymerization routes, the acquisition cost of high molecular weight PLA has now decreased. As a consequence of this price reduction, PLA is now more investigated for packaging applications and consumer goods. With comparable mechanical and optical properties as those of conventional plastics, PLA could possibly substitute LDPE and HDPE, as well as PS and PET for food packaging application to contain liquids (cups, bottles, bowls).

1. 3. 5. Modifications

Some factors limit the PLA application for packaging technologies and polymer modifications have been intended to adapt its properties to the packaging requirements. Since PLA-based materials are rigid and brittle, plasticizers have been added to enhance the mechanical performance of the PLA films.

Many studies have been made with different plasticizing agents, such as glycerol (Erdohan *et al.*, 2013), acetyl tri-n-butyl citrate (ATBC) (Baiardo *et al.*, 2003; Coltelli *et al.*, 2008), glycerol triacetate (TA) and tributyl citrate (TBC) (Grigale *et al.*, 2011), triacetine tributyl citrate, acetyl tributyl citrate, triethyl citrate and acetyl triethyl citrate (Ljungberg and Wesslén, 2002) or epoxized palm (EPO) and soybean (ESO) oils (Tee *et al.*, 2014). Several authors also reported results of PLA plasticized with polyethylene glycol (PEG) from molecular weight of 200 (Chieng *et al.*, 2013), 300 (Grigale *et al.*, 2011), 400 (Hughes *et al.*, 2012), 550 (Choi *et al.*, 2013), 1000 (Gui *et al.*, 2012, Pluta *et al.*, 2006) until 1500 and 10000 g.mol^{-1} (Baiardo *et al.*, 2003).

As plasticizers decreased the glass-transition temperature (T_g), a lower stress at yield and a higher elongation at break were observed in the different studies. Gas permeability being also a problem for PLA, Rocca-smith *et al.* (2016) applied a corona treatment to PLA films and observed modifications in both the surface and the bulk of the films. In particular, surface analysis revealed an increase of polarity and roughness. Some modifications of the mechanical properties and the crystallinity degree were also reported, resulting in films with improved gas (He, O_2, CO_2) barrier properties.

Another option considered to improve PLA properties is to combine it with other biopolymers by using an adequate compatibilizing strategy. Different PLA blends have been studied such as PLA-PHB/cellulose nanocrystals (CNCs) plasticized with 15 wt % of acetyl(tributylcitrate) (ATBC) (Arrieta *et al.*, 2015). CNCs permitted the improvement of the thermal stability of the PLA-PHB blends, and oxygen barrier and stretchability were achieved in PLA-PHB-CNCs-ATBC films.

Bonilla *et al.* (2013) added chitosan (CH) to PLA and obtained less rigid and less stretchable films, with no modification of PLA thermal properties. On another hand, chitosan incorporation led to higher water vapour permeability than that of neat PLA films and provided antimicrobial activity against total aerobial and coliform microorganisms.

PLA-Poly (butylene succinate-co-adipate) (PBSA) and Poly (butylene adipate-co-terephthalate) (PBAT) were obtained by Pivsa-Art *et al.* (2011). From the morphology study it was found that polymer blend of PLA-PBSA of 80-20 wt % shows good distribution of compositions and good mechanical properties after addition of PBAT at 10-30 wt %, with highest TS (40.71 Mpa) with 20 wt % PBAT, acting as an interfacial agent to improve the mechanical properties of the blends.

Finally, Qin *et al.* (2015b) developed PLA-PCL (70-30) by casting method and characterized their physicochemical properties, as well as their antimicrobial properties against button mushrooms (*Agaricus bisporus*) in the case of films carrying cinnamaldehyde. The CO_2 level inside the PLA-PCL films with cinnamaldehyde was lower than those inside the control and PLA-PCL films, but the O_2 level was similar in all packages. The films with 9 wt % CIN were the most effective in reducing microbial counts and preserving the colour of mushrooms and showed the highest water vapour permeability.

Other authors worked on PLA-starch blends. Indeed, starch is a good candidate to obtain PLA blend films since it is widely available and cheap with properties complementary to PLA. Nevertheless, the non-compatibility of both polymers requires the use of different compatibilizing strategies, as discussed in section **1.5.**

1. 4. Starch

Starch is a plant polysaccharide consisting of several glucose units joined by glycosidic bonds. It is found in cereals (30-70% of the dry matter), tubers (60-90%) and legumes (25-50%) (Wertz, 2011). It is one of the main sources of energy to sustain life and one of the most abundant biopolymers on earth, with cellulose and chitin, also being renewable, biodegradable and biocompatible.

Native starch is composed of two main macromolecular components, which are amylose and amylopectin. Amylose is a nearly linear polymer of α-1,4 anhydroglucose units that has excellent film forming ability, making for strong, isotropic, odourless, tasteless, and colourless films (Campos *et al.*, 2011). Meanwhile, amylopectin is a highly branched polymer of short α-1,4 chains linked by α-1,6 glucosidic branching points occurring every 25-30 glucose units (Durrani and Donald, 1995).

Figure 5 presents the amylose and amylopectin molecules and **Table 1** shows the ratio of amylose/amylopectin of different starches, depending on their source.

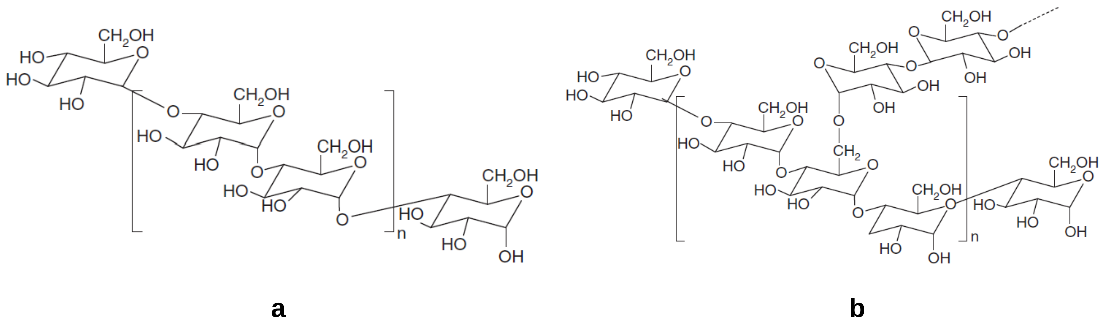

Figure 5. Amylose (**a**) and amylopectin (**b**) structures. Source: Carvalho *et al.* (2008).

Table 1. Amylose and amylopectin contents (%) in starch from different sources. Source: Carvalho *et al.* (2008).

Starch	Amylose (%)	Amylopectin (%)
Wheat	30	70
Corn	28	72
Potatoe	20	80
Rice	20-30	70-80
Cassava	16	84

1. 4. 1. Advantages/Drawbacks

Starch-based films have demonstrated several advantages such as their extensibility and oxygen barrier properties (Ortega-Toro *et al.*, 2014a; Acosta *et al.*, 2015). They also exhibit similar physical characteristics as those of the conventional packaging plastics in terms of transparency, odour and taste (Nísperos-Carriedo, 1994). Moreover, starch based films are reported to be nontoxic, contributing to their growing acceptance as potential packaging alternative (Chakraborty *et al.*, 2004). However, since it is highly hydrophilic, starch exhibits high water sensitivity and solubility and poor water vapour barrier capacity. It is a low resistance material, with a tensile strength lower than 5 MPa, depending on the moisture content (Ortega-Toro *et al.*, 2014b; Souza *et al.*, 2013, Acosta *et al.*, 2015; Versino *et al.*, 2015).

1. 4. 2. Processing

Starch-based films can be obtained by both thermoprocessing and casting techniques (Jiménez *et al.*, 2012; Acosta *et al.*, 2015; Cano *et al.*, 2016; Castillo *et al.*, 2013; García *et al.*, 2012; Ortega-Toro *et al.*, 2015). Films can only be obtained after gelatinization of the native starch to irreversibly disrupt the granules. Gelatinization can be carried out with an excess of water (> 90% w/w) (Carvalho *et al.*, 2008) in the case of films obtained by casting or it can also be achieved at low moisture content during a thermal processing. High-shear and high-pressure conditions are applied, in the presence of plasticizers, such as water or glycerol, which tear down the starch granules, allowing faster water transfer into the starch molecules and causing the breakage of the amylopectin matrix and releasing the amylose.

1. 4. 3. Applications

Many applications have been described for starch. Indeed, it can be converted into chemicals (ethanol, acetone, and organic acids) and used in the production of synthetic polymers, it can produce biopolymers through fermentative processes or it can be hydrolysed to provide monomers or oligomers. Likewise, it can be grafted with a variety of reagents to produce new derivative polymeric materials for different uses (Carvalho *et al.*, 2008). Starch has been used for pharmaceutical or cosmetic applications (Lafargue, 2007), as glue for paper and wood (Jarowenko, 1977) or gum for the textile industry (Daniel *et al.*, 1994; Whistler *et al.*, 1984). Native and modified starches play an increasingly important role in the food industry, modifying the physical properties of food products, such as sauces, soups, or meat products, mainly resulting in changes in texture, viscosity, adhesion, moisture retention, gel formation, and film formation (Thomas and Atwell, 1999). Thus, in food applications, starch is used as a thickener, binder or setting agent under its granular form or as a sweetener or binder under its hydrolysed form. Finally, with the emerging research in bioplastics and because of its outstanding characteristics, starch has become one of the best options for food packaging application.

1. 4. 4. Plasticization of starch

Thermoplastic starch, which is a rubbery material, is obtained by adding plasticizers to the native starch during its processing. Plasticizers can be fructose (Kalichevsky and Blanshard, 1993), sorbitol and maltitol (Mathew and Dufresne, 2002), glycols (Da roz *et al.*, 2006), ethanolamine (Huang *et al.*, 2005) or formamide (Ma and Yu, 2004), although most studies report water and glycerol as the best plasticizing agents. Glycerol is generally incorporated between 25% (Acosta *et al.*, 2015; Cano *et al.*, 2016) and 30% (w/w) (López *et al.*, 2014; Ortega-Toro 2014b). Perry and Donald. (2000) showed that glycerol alone can completely gelatinize starch and induce an increase in the gelatinization temperature of approximately 60°C, compared with the water-plasticized product.

1. 5. PLA-starch materials

To remedy the disadvantages of both PLA and starch previously described and to reduce the cost of the finished products, alternatives have been considered. Since both polymers show opposite mechanical and barrier properties, their combination could lead to films with improved functional properties. However, since starch is highly hydrophilic and PLA is hydrophobic, they are thermodynamically immiscible. The biggest issue with mixing both polymers relies on the phase separation. A solution is to modify either starch (Bastos *et al.*, 2009) or PLA (Turalija *et al.*, 2016) with plasma treatment, in order to change their hydrophobicity. Nonetheless, the strategy mostly investigated is PLA-starch blends.

1. 5. 1. Blend films

Table 2 summarizes some of the most recent studies on PLA-starch blends, with or without compatibilizers, which are incorporated to enhance the interfacial interactions between the two polymers. Several studies have been conducted regarding the combination of both materials by blending them with addition of different substances.

Xiong Zhu *et al.* (2013b) incorporated epoxidized soybean oil (ESO) in PLA-starch blends; with starch grafted with maleic anhydride (MA) to enhance its reactivity with ESO. Authors observed a marked increase of the elongation at break (from 6 to 140%) and impact strength of the PLA-ESO-S (80:10:10) films. Same authors (Xiong Zhu *et al.*, 2013c) also used tung oil anhydride (TOA), from 5 to 12 wt %, as a bio-based reactive plasticizer for PLA-starch blends via the ready reaction of MA and observed a better compatibility between the two polymers, as demonstrated the SEM micrographs and FTIR spectra, as well as a higher elongation at break (from 6 to 31%).

Several authors added methylenediphenyl diisocyanate (MDI) to the PLA-starch blend to enhance the compatibility of the two polymers (Acioli-Moura and Sun, 2008; Phetwarotai *et al.*, 2012; Wang *et al.*, 2002). They all found that this compound acted as a coupling agent, improving the interfacial interaction between the two materials, thus enhancing the mechanical properties of the PLA-starch blends. Wang *et al.* (2002) reported a smoother microstructure and higher tensile strength (68 MPa) for the PLA-S blend (55:45) with 0.5 wt % MDI. Acioli-Moura and Sun (2008) found a similar thermal decomposition profile of the blends, with or without MDI, although the compatibilizer led to a longer thermal endurance between 50 and 100°C. MDI incorporation also gave an improved elongation at break from 2.7 to 4.2%. MDI being a small molecule, composed of isocyanate groups, which are highly reactive with both hydroxyl and carboxyl groups to form urethane linkages, it is an effective compatibilizer for these two immiscible materials. Nevertheless, MDI is still recorded as a harmful substance by the EU Council Directive 67/548/EEC on Dangerous Substances and cannot be used for food packaging applications.

Several studies were also carried out using maleic anhydride, grafted either on PLA (Huneault and Li, 2007; Orozco *et al.*, 2009) and/or starch (Zuo *et al.*, 2014), in order to improve the interfacial adhesion between the polymer phases. Orozco *et al.* (2009) obtained PLA-g-MA copolymers using dicumyl peroxide (DCP) as an initiator of grafting and PLA-S copolymers were obtained by reactive blending, varying the starch composition from 0 to 60%. It was observed that MA had a plasticizing effect and enhanced the compatibility between PLA and starch, showing a stable and homogeneous interface with no stress fractures, holes nor cavities at the interface of the two polymers, as revealed by the SEM analysis. On another hand, Zuo *et al.* (2014) used the synthesis of MA esterified starches (1% MA) to blend it with PLA. The introduction of a hydrophobic ester bond in starch chains increased the polymer interfacial compatibility and led to an improvement of the PLA-S water resistance and tensile properties. Determined melt index showed that starch esterification also improved the melt flow properties of PLA-S composite material. MA was also grafted on both polymers by Hwang *et al.* (2013), in a mixer in the presence of DCP in a one-step reactive compatibilization process. PLA-S (80:20) with 2.0 phr of MA and 0.1 phr of DCP showed the best tensile properties and the reactive compatibilization significantly reduced combined molecular weight of the blend.

Le Bolay *et al.* (2012) investigated the co-grinding of starch-PLA in a tumbling ball mill without adding any compatibilizer or plasticizer. The hydrophilic behaviour of the blend decreased, as the interface between the matrix and the filler could be improved. Mechanical properties of the blends were improved, showing a higher ductility. Wokadala *et al.* (2014) also worked on PLA-S blends without compatibilizer but they proceeded to the butyl-etherification of waxy and high amylose starches to increase its hydrophobicity and compatibility with PLA. Although the polymer thermal stability decreased, the modified blend showed an improved mechanical performance, while SEM micrographs showed a more homogeneous structure with this starch modification. Besides, this study demonstrated that the amylose/amylopectin content of starch plays an important role on the tensile properties of the starch-PLA blend films. At higher starch levels, composites with butyl-etherified high amylose starch give a lower elongation at break and tensile strength compared to those with butyl-etherified waxy starch due to the tendency of amylose to self-aggregate.

Although relevant results showed that starch-PLA blending seems to be a good alternative to enhance the film properties of neat PLA or starch films, this requires the involvement of reactive processes, which, in turn, could lead to reactant residues in the films, which must be tested as to their food safety. In contrast, the strategy of the bi or multilayer films did not require such great efforts to enhance interfacial properties of the polymers since they only enter in contact at the layer interface. Moreover, in terms of barrier properties, multilayer assembly exhibits more advantages, since the complementary barrier capacity of each polymer can yield composite material with extremely high resistance to the mass transport of polar (water) and non-polar (gases) molecules, resulting in a very effective packaging material. Nevertheless, the good interfacial adhesion of the materials at the layers' contact must be ensured from the mechanical point of view. This aspect could be enhanced by using adequate adhesive materials, but studies on their migration are necessary, especially for food applications, where food safety can be compromised. Some studies carried out into biopolymer bilayer films are summarized in the next section.

Table 2. Examples of PLA-starch blends with or without incorporation of a compatibilizer.

PLA	Starch	Glycerol (w/w S)	PLA-S ratio	Compatibilizer	Compatibilizer content	Processing	Reference
120 kDa	Wheat	-	55-45	Methylene diphenyl diisocyanate (MDI)	0.05 wt %	Extrusion Compression moulding	Acioli-Moura & Sun, 2013
1.1-1.7% D-content	Potato	from 20 to 40	40-60	Sodium montmorillonite (NaMMT)	0.5-1.0 phr (/dry S)	Melt blending Compression moulding	Ayana *et al.*, 2014
2002D® 4% D-content 235 kDa	Wheat/Pea/Rice	from 30 to 39	83-27 40-60	a) Maleic anhydride (MA) b) 2,5-dimethyl-2,5-di-(*tert*-butylperoxy)-hexane	a) 2 wt % b) 0.1-0.25-0.5 wt %	Extrusion Injection moulding	Huneault & Li, 2007
4042D® 6% D-content 130 kDa	Corn	-	90-10 80-20 70-30	a) Dicumyl peroxide (DCP) b) MA	a) 0.1 phr b) 2 phr	Melt blending Compression moulding	Hwang *et al.*, 2013
2002D® 4% D-content 235 kDa	Cassava	-	90-10 70-30 50-50	Trimethoxy silane coupling agents	from 1 to 100% (w/w S)	Extrusion Blown moulding	Jariyasakoolroj & Chirachanchai, 2014
12% D-content 68 kDa	Waxy maize (99% amylopectin)	-	80-20	-	-	Co-grinding Compression moulding	Le Bolay *et al.*, 2012
n.r.	Potato		85-15 75-25 65-35 50-50 40-60	MA	n.r.	Melt blending Compression moulding	Orozco *et al.*, 2009
4042D® 6% D-content 130 kDa	Cassava/Corn	from 23 to 54	90-10 80-20 70-30 60-40	MDI PLA plasticizers: a) Propylene glycol (PG) b) Polyethylene glycol (PEG) 400 g.mol^{-1}	MDI: 1.25% (w/w S) a) b) from 5 to 20 wt %	Melt blending Compression moulding	Phetwarotai *et al.*, 2012
4% D-content 180 kDa	Corn	25	50-50	Anhydride functionalized polyester	1 wt %	Extrusion Injection moulding	Ren *et al.*, 2009
3251D®	Cassava	33	6.3-93.7 6.0-94.0	Adipate/citrate esters	0.7-1 wt %	Blown extrusion	Shirai *et al.*, 2013

125 kDa	Cassava	30	80-20	Citric acid (CA) Stearic acid (SA)	2% (w/w S)	Extrusion Compression moulding	Teixeira *et al.*, 2012
120 kDa	Wheat	-	80-20 70-30 55-45 50-50 40-60	MDI	0.5 wt %	Compression moulding	Wang *et al.*, 2002
12% D-content 160-220 kDa	Corn	40	50-50	CA	from 1 to 4% (w/w S)	Extrusion Compression moulding	Wang *et al.*, 2007
12% D-content 160-220 kDa	Corn	from 10 to 40	50-50	Formamide	from 10 to 40% (w/w S)	Extrusion Compression moulding	Wang *et al.*, 2008b
2002D® 4% D-content 235 kDa	Waxy maize (100% amylopectin)/ High amylose maize (70% amylose)	-	40-60 30-70 20-80 10-90	-	-	Butyl-etherification of starch Melt-blending Compression moulding	Wokadala *et al.*, 2014
4032D®	Corn	-	70-30	a) Hexamethylenediisocyanate (HDI) b) Castor oil	a) 5-8-11% (w/w S) b) 5 wt %	Extrusion Injection moulding	Xiong Zhu *et al.*, 2013a
4032D®	Corn	-	90-10 70-30	a) MA b) Epoxidized soybean oil (ESO)	a) 4,3-9-13,3% (w/w S) b) 10 wt %	Extrusion Injection moulding	Xiong Zhu *et al.*, 2013b
4032D®	Corn	-	70-30	Tung oil anhydride (TOA)	5-7-10-12 wt %	Extrusion Injection moulding	Xiong Zhu *et al.*, 2013c
4032D®	Corn	-	70-30	a) Epoxidized itaconic acid (EIA) b) Bio-based ether epoxidized cardanol	20% (w/w S)	Extrusion Injection moulding	Xiong Zhu *et al.*, 2014
4042D® 6% D-content 130 kDa	Cassava	33	70-30 50-50 30-70	Tween 60 Linoleic acid Zein	1.55% (w/w S)	Extrusion Injection moulding	Yokesahachart & Yoksan, 2011
Granular	Corn	50	60-40	MA	1% (w/w S)	Extrusion moulding	Zuo *et al.*, 2014b

PLA®: Natureworks supplier

n.r.: not reported

1. 5. 2. Bilayer film strategy

Table 3 summarizes a number of studies carried out into different materials assembled as bilayer films. Starch-PCL bilayer films were obtained by Ortega-Toro *et al.* (2015) by compression moulding. Before compression, starch layers (neat starch and starch with 5% PCL) were plasticized by the spraying of aqueous solutions of ascorbic acid or potassium sorbate, with the objective to improve the starch-PCL adhesion. Authors showed that all bilayers enhanced barrier properties to water vapour and oxygen (up to 96 and 99%, respectively), as compared to neat S and PCL films, respectively. Potassium sorbate was the most effective at obtaining a better interfacial adhesion, thus improving the barrier and mechanical properties of the films and providing them with antimicrobial activity.

Svagan *et al.* (2012) managed to improve the oxygen permeability of PLA films by adding chitosan and montmorillonite (MMT) clay, which has been reported as non-cytotoxic (Wang *et al.*, 2008a). Transmission electron microscopy revealed a well-ordered laminar structure in the deposited multilayer coatings, and the light transmittance of the films demonstrated the high optical clarity of the coated PLA. Bilayer films of the same materials could also be obtained with the objective of incorporating active compounds between the two layers.

Requena *et al.* (2016) added four active compounds (oregano and clove EOs and their respective main compound) at the interface of PHBV layers. Films with active agents were effective at controlling the bacterial growth of both *Escherichia coli* and *Listeria innocua*. Although tensile properties were not improved with respect to pure PHBV films, the active compounds yielded more transparent materials with better WVP.

Preparation and characterization of biodegradable bilayer films from isolated soy protein (SPI) and PLA were reported by González and Alvarez Igarzabal (2013). The tensile properties of the films were improved with incorporation of the PLA layer, whereas the water vapour permeability decreased as compared to SPI films. SPI-PLA bilayers were also obtained and characterized by Rhim *et al.* (2007), obtaining similar conclusions.

As for PLA-starch assemblies, Sanyang *et al.* (2016) developed sugar palm starch (SPS) and PLA bilayer cast films (with different PLA contents, from 20 to 50 wt %) and characterized their mechanical, thermal properties and water vapour barrier capacity. With a 1:1 PLA-starch ratio, tensile strength increased but elongation at break reduced drastically in comparison with neat starch films. However, incorporation of PLA layer significantly reduced the water vapour permeability, due to its hydrophobic resistance perpendicular to the transfer of water molecules. Film microstructure revealed a lack of strong interfacial adhesion between the starch and PLA cast layers.

Martucci and Ruseckaite (2010) obtained biodegradable three-layer films, with PLA as outer sheet and 30% glycerol plasticized gelatin as the inner layer. This assembly exhibited significantly improved water vapour barrier capacity and mechanical resistance.

The multilayer strategy is often used in the packaging industry and more and more of the food available in the stores comes in high-tech plastic packaging such as multilayer films, which ensure a longer-term preservation of the food than a monolayer structure.

Table 3. Recent studies on polymer bilayer films.

Polymers	Other compounds (Content)	Polymer ratio	Processing	Solvent for casting (Polymer content)	Reference
1. Corn starch (S) 2. Polycaprolactone (PCL)	S: Glycerol (30%)	S-PCL Or S (5% PCL)-PCL	Melt blending Compression moulding	-	Ortega-Toro *et al.*, 2015
1. Sugar palm starch (SPS) 2. PLA	SPS: Glycerol (30%)	SPS-PLA 50-50 60-40 70-30 80-20	Casting/Coating	1. SPS: Distilled water (8% w/w) 2. PLA: Chloroform (10% w/w)	Sanyang *et al.*, 2016
1. Soy protein (SPI) 2. PLA	SPI: Glycerol (50%)	SPI-PLA 60-40[a] 50-50[b]	Casting/Coating	1. SPI: Deionized water (0.75% w/w) 2. PLA: Chloroform (0.8[a] Or 1.2[b] % w/w)	González & Igarzabal, 2013
1. PLA 2. Chitosan (CH)	CH: Montmorillonite (MMT) (0.2 wt %)	-	1. PLA: Melt extrusion 2. Dipping of PLA film in CH/MMT solution	CH solution: 0.235% (v/v) Acetic acid (0.2 wt %) with MMT	Svagan *et al.*, 2012
1. CH 2. Poly(acrylic) acid (PAA)	PAA: Ammonium peroxydisulfate ($(NH_4)_2S_2O_8$) & Gallium nitrate ($(Ga5NO_3)_3$)	-	Electrosynthesis	-	Bonifacio *et al.*, 2017
1. Methylcellulose (MC) 2. Lipids: Paraffin oil/wax Or Hydrogenated Palm Oil (HPO) & Triolein	1. MC: PEG 400 (25%) 2. Lipids: Glycerol monostearate (GMS) (10%)	-	Layer chromatography spreading	MC: Water-ethyl alcohol (3:1 v/v)	Debeaufort *et al.*, 2000
1. FucoPol Polysaccharide 2. CH	1. Fucopol: Citric acid (CA) (50%) 2. CH: Glycerol (50%) & CA (50%)	-	Casting/Coating	1. FucoPol: Distilled water (1.5% w/w) 2. CH: 1 % w/w Acetic acid (1.5% w/w)	Ferreira *et al.*, 2016
1. Wheat gluten 2. Polyethylene (PE)	1. Wheat gluten: Glycerol (20%) 2. PE: Ethylene/acrylic ester/maleic anhydride terpolymer Or ethylene/glycidyl methacrylate copolymer	-	1. Wheat gluten: Casting 2. Bilayer films: Thermocompression	Wheat gluten: Absolute ethanol (varying contents), acetic acid & water	Irissin-Mangata *et al.*, 1999
1. PE (commercial) 2. PCL	PCL: Casein Or casein/ZnO nanoparticles (40%)	-	PCL coating on PE film	PCL: Tetrahydrofuran (10%)	Reščček *et al.*, 2015
Poly[(3-hydroxybutyrate)-*co*-(3-hydroxyvalerate)] (PHBV)	Oregano EO Or Carvacrol Or Clove EO Or Eugenol (15% w/w)	50-50	Melt blending Compression moulding	-	Requena *et al.*, 2016

2. Active packaging

The major driving forces for innovation in the food packaging technology have been the increase in consumer demand for minimally processed food, the change in retail and distribution practices associated with globalization, new distribution trends (such as Internet shopping), automatic handling systems at distribution centers, and stricter requirements regarding consumer health and safety. Active packaging technologies are being developed as a result of these driving forces to enhance safety or sensory properties, while maintaining the quality of the product. This is particularly important in the area of fresh and extended shelf life food while, in the area of active packaging, antimicrobial packaging has been identified as one of the most promising solutions (Vermeiren *et al.*, 1999; Suppakul *et al.*, 2003).

An active packaging is a mode of packaging in which the package, the product, and the environment interact to prolong shelf life or enhance safety or sensory properties, while maintaining the quality of the product (Suppakul *et al.*, 2003). Active packaging contains substances that absorb undesirable compounds in the food system, such as oxygen, ethylene, moisture, carbon dioxide or odors or that release beneficial compounds such as carbon dioxide, antimicrobial agents, antioxidants or flavors. A wide number of studies focusing on the development of active packaging materials have been carried out, by incorporating antimicrobials, antioxidants or different components that act as absorbers of undesirable compounds in the food or as releasers of beneficial compounds for food safety or quality.

As specific examples, different authors reported the incorporation of antioxidant agents into PLA films, such as α-tocopherol and resveratrol (Hwang *et al.*, 2012), natural (ascorbyl palmitate and α-tocopherol) and synthetic phenolic antioxidant substances (butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and tertbutylhydroquinone (TBHQ) (Jamshidian *et al.*, 2013). Byun *et al.* (2010) also observed the antioxidant activity of BHT/α-tocopherol combination, which was significantly more effective than when BHT was added alone.

The infectious sprouts are a public health issue, which implies the needs to intensify the strategies of preservation (Hyldgaard *et al.*, 2012). Therefore, antimicrobial packaging materials became of particular interest as a specific mode of action of active packaging. Antimicrobial materials interact with packaged food and headspace to reduce, delay, or even inhibit the growth of spoilage and pathogenic microorganisms. The antimicrobial agent can be physically separated by packaging materials (in sachets, dispensers, or capsules) or included in or coated onto the packaging material. Antimicrobial films can be classified as migrating and non-migrating systems. The former includes volatile antimicrobials that release to the headspace, acting on the surface microorganisms. In non-migrating systems, covalent immobilization of actives, such as antimicrobial polymers or enzymes in polymer matrices, limits their release to the food and their action occurs through their direct contact with the potentially contaminated surface or by adsorption of the electrostatically charged microbial cells. Materials with surfaces activated (for instance with amino groups) by using different chemical or physical methods have also been studied as antimicrobial materials. When the release of antimicrobial compounds occurs in the food, this can lead to an organoleptic impact and this aspect must be tested in order to guarantee adequate food quality (Article 3 of Regulation (EC) No 1935/2004).

The development of an active material implies the selection of the active compound, materials, and the design of the incorporation method, whose effectiveness must be demonstrated in the food system. Validation of the functionality of these materials in real foods and migration studies must be carried out. Likewise, the analysis of their stability throughout the storage time must also be tested.

Different approaches can be considered to incorporate active compounds into the food packaging material (Requena *et al.*, 2017). It can be carried out by direct addition into the polymer blend, trying to avoid high temperatures, which can degrade or volatilize the compound. This is a critical aspect since the melting temperatures of most of the polymers or biopolymers, reached during thermoprocessing, are relatively high and notable losses of actives during extrusion or melt blending steps can occur, depending on the compound (Joo *et al.*, 2012; Han, 2003). Encapsulating active agents in different carriers, prior to their incorporation, can contribute to mitigating this problem. To this aim, cyclodextrins, or different nanotechnology approaches using biopolymers have been used (Joo *et al.*, 2012; Wen *et al.*, 2016; Higueras *et al.*, 2013). Another option is to bond actives to different nano-matrices such as cellulose nanomaterials or nanoclays to limit their losses while allowing for a more controlled release (Castro *et al.*, 2016; Bidecci *et al.*, 2016; Wu *et al.*, 2015; Giménez *et al.*, 2012).

In addition, obtaining multilayer films is a common practice to optimize their function on the basis of the complementary properties of the different materials. Incorporation of active compounds at the interface of the layers, at the same time as the adhesives, could be an efficient method, since it does not involve high temperatures and the compounds can diffuse through the film layers to reach the target point in the food. Within the concept of multilayer films, the coating of thermo-processed layers with an active coating using techniques such as electrospinning or casting, also offers different alternatives for development of active packaging materials. Coating application requires a good extensibility and adhesion of the active material on the supporting polymer layer and so, an adequate selection of components.

The effectiveness of an antimicrobial film in the food systems depends on different factors: the antimicrobial compound and its particular interactions with the polymer matrix, the target microorganism and the corresponding minimally inhibitory concentration (MIC) required for the antimicrobial action; and the release kinetics of the active and its final concentration released from the film to the target point in the food (related to the corresponding partition coefficient). Likewise, particular interactions of the active with the food components will also greatly affect the antimicrobial effectiveness: complexation of the active with some food components would inhibit any antimicrobial action, although an effective release has occurred. Special nutrients of food for microorganisms also help for their resistance. Thus, different studies must be carried out to validate the developed active material: migration or release studies of the active into the food system, antimicrobial *in vitro* test against the target microorganisms and *in vivo* tests in real foods (Commission Directive 97/48/EC).

2. 1. Trends in the use of antimicrobial compounds

The current trends in the use of antimicrobials include the incorporation of natural, non-toxic compounds, such as essential oils or their main components (Atarés and Chiralt, 2016), plant extracts (Periago and Moezelaar, 2001; Periago *et al.*, 2001), peptides, some of them from microbial origin (Jenssen *et al.*, 2006; Yeaman and Yount., 2003) or polymer/enzymes with proven antimicrobial properties (Padgett *et al.*, 1998; Thallinger *et al.*, 2013). Synthetic antimicrobials such as different kinds of metallic nanoparticles or organic acids have also been extensively studied (Han and Floros, 1997; Ishitani, 1995). Even the incorporation of live microbial cells, bacteriocin producers, has been analysed (Sánchez-González *et al.*, 2013 and 2014). The incorporation of lactic acid bacteria in biopolymer films did not notably alter the films' functional properties as packaging material, while imparting antimicrobial activity through the cell production of bacteriocins. Sodium caseinate and methylcellulose films with glycerol proved to be effective carriers of *L. acidophilus* and *L. reuteri*, used as antimicrobial agents. In general, production of bacteriocins increased with the storage time and higher amounts were produced in polysaccharide than in protein films at short times.

The antimicrobial properties of plant essential oils (EOs) have been widely studied and tested both directly or when included in polymer matrices. EOs and several of their constituents represent a natural alternative to chemical food preservatives, due to their capacity to inhibit the growth of a wide variety of pathogenic and food-spoiling microorganisms. They are complex in composition, being a source of bioactive terpenoids and phenolic compounds, which exhibit antioxidant and antimicrobial properties (Atarés and Chiralt, 2016). Several antimicrobial mechanisms have been described to explain the action of EOs against

microorganisms, namely membrane destabilization and disruption, the inhibition of membrane localized metabolic events; damage in the membrane proteins; and the leakage of cytoplasmic constituents, metabolites and ions. Moreover, a synergistic effect between EOs and their major components has recurrently been observed in different studies. For instance, Ye *et al.* (2013) concluded that cinnamaldehyde and carvacrol show a high degree of antibacterial activity and display synergistic antimicrobial activity for a great number of bacteria tested.
However, the concentrations of the EOs or their individual constituents, required to inhibit bacteria in foods, are frequently higher than what is organoleptically acceptable. Moreover, the highly volatile nature of these compounds, along with their great sensitivity to oxidation, which makes them prone to deterioration, limits their free-form application onto the surface of food systems. This can be partially mitigated by their inclusion/encapsulation in different carriers or films. A wide variety of EOs has been tested in different carrying biopolymers, and the *in vitro* studies, using the agar diffusion method, have proved their efficiency as antimicrobials. They mainly act against different bacteria, although they also feature antifungal activity.

Taking these considerations into account, cinnamaldehyde has been considered in the present study to obtain PLA-starch active films and its relevant characteristics as active compound are described below on the basis of previous studies.

2. 2. Cinnamaldehyde (CIN) as active compound

Cinnamaldehyde, also called cinnamic aldehyde, is one of the major constituents of cinnamon bark oil (~ 60-90%) (Kim *et al.*, 2015; Parthasarathy *et al.*, 2008), among other phenolic terpenoid compounds. CIN is extracted from the rind of cinnamon (*Cinnamomum spp.*), which contains mucilage, tannins, sugar and resin that can be used as a spice or herb.

2. 2. 1. Antimicrobial properties

Cinnamaldehyde is a natural antimicrobial substance, classified as GRAS (Generally Recognized As Safe) by the FDA. It has high antibacterial, antifungal, antiinflammatory and antioxidant properties and it is widely used in the cosmetic, food and pharmaceutical industries. However, it exhibits a strong smell and high volatility (Sun *et al.*, 2015; Wang *et al.*, 2015; Xing *et al.*, 2014).

The antimicrobial activity of CIN is mainly due to the aldehyde groups of the molecule that are reactive and have the ability to cross over in a covalent way with the DNA and the proteins through the amine groups, interfering with its normal function (Feron *et al.*, 1991). Still, surveys made on the mechanism of action of CIN are not very conclusive. This limits its development and use as a food preservative, since there is not enough information to predict its effect on the different microorganisms and how it interacts with the different compounds in each food matrix (Hyldgaard *et al.*, 2012). Some authors establish at least three mechanisms of action:

1. At low concentrations, CIN could inhibit different enzymes that participate in the cytokinesis or in other less important functions of the cells.
2. At higher concentrations, it could act as an inhibitor of the ATPase (Kwon *et al.*, 2003; Denyer, 1990).
3. It has been proved that, at very high concentrations, CIN causes perturbations in the cytoplasmatic membrane of the cells, which is mainly the target of many antibacterial agents.

The interactions of the bacterial membrane with biocides often provokes fundamental changes in the structure and in the function of the membrane (Denyer, 1990). Several studies were carried out to determine the effects of CIN on the bacterial membranes (Shen *et al.*, 2015) and results show that CIN generally permeabilizes the internal membranes of *E. coli* and *Staphylococcus aureus*, and that the permeability rate correlates with the corresponding concentration of CIN. This conclusion is coherent with what Hammer and Hell (2012) describe, using the multiparametric flow cytometry to examine the changes in the membrane polarity and in the permeability in *S. aureus, Staphylococcus epidermidis* and *Enterococcus*. These results also prove that cells show an external modification, probably because CIN penetrates into the cell envelope, altering its structure. It has also been reported that CIN is an inhibitor of β-(1,3) glucan synthase and quitin synthase 1, which are important during the synthesis of enzymes in the cell wall of yeasts and moulds (Bang *et al.*, 2000). These alterations in the cell wall can be attributed to the lipophilic properties of the essential oil compounds that make it more permeable to the cell wall and to the cytoplasmic membrane and helps to the accumulation of polysaccharides under hydric stress conditions (Ultee *et al.*, 2002; Yamamoto-Ribeiro *et al.*, 2013). The interaction of the cinnamon essential oil (CEO) compounds with the cell wall contributes to inhibit the enzymatic reactions that regulate the synthesis of the cell wall and thus the integrity of the cell turns out affected (Maffei *et al.*, 2001; Rasooli *et al.*, 2006; Tyagi and Malik, 2010). As a consequence of the mechanism, the membrane fluidity worsens, changing the structure of several layers of phospholipids, fatty acids, proteins and polysaccharides, which leads to the filtration of the cytoplasmic contents (Rasooli *et al.*, 2006; Tyagi and Malik, 2010).

Nonetheless, one of the main obstacles for the use of essential oils is their negative organoleptic effect when they are added in the sufficient quantity to exert an antimicrobial effect, and the fact that some of their compounds, by themselves, do not have antimicrobial activity. As a potential solution, the use of more active blends based on the synergistic effect between several compounds has been proposed (Hyldgaard *et al.*, 2012). The studies of synergistic effects could provide a better use of the essential oil compounds taking advantage of the synergistic antibacterial activity of some blends, thus reducing the required concentrations to reach a determinate antimicrobial effect (Nazzaro *et al.*, 2013). Ye *et al.* (2013) showed that CIN and carvacrol, apart from exhibiting an antibacterial activity by themselves, have a high synergistic effect, which indicates that the combination of both compounds could be used as a non-synthetic preservative with lower dose, with an excellent bactericidal activity for food preservation.

As regards the antifungal effect, Sun *et al.* (2015) demonstrated the CIN's effectivity as an alternative compound to prevent the growth of *Aspergillus flavus* and the aflatoxin B1 (AFB1) production during the storage of grains, as well as in field applications. Then, it could be used as a natural preservative to control moulds and mycotoxins. Previous studies also showed that CEO and CIN turn out to be very effective against other moulds such as *Fusarium verticillioides* and *Aspergillus ochraceu*s (Hua *et al.*, 2014; Xing *et al.*, 2014).

On the other hand, the extended use of antibiotics has generated resistant bacteria to multiple drugs, while many bacteria can form biofilms less susceptible to antibiotics, which stick on different surfaces, such as polystyrene, glass or stainless steel from different environments (Goulter *et al.*, 2009; Patel *et al.*, 2011). Pathogen biofilms can be found in medical devices or food processing equipment, being a relevant source of contamination that requires methods of control. Kim *et al.* (2015) demonstrated that the use of cinnamon's rind oil and CIN decreased significantly the formation of biofilms in *E. coli* enterohemorrhagic O157:H7 and *P. aeruginosa.*

The application of CIN as food preservative requires a detailed knowledge of the minimal inhibitory concentration (MIC) against a determined target microorganism. This requires knowing the least concentration of active compound, which inhibits completely the growth of the microorganism. Various studies report specific MIC values of CIN for different bacteria, moulds and yeasts. **Table 4** summarizes these values for several microorganisms. Generally, values are highly dependent on the type of microorganism, and the high sensitivity on some bacteria such as *Enterococcus faecalis* or *Aeromonas hydrophila* and yeasts such as *Fusarium verticilloides*, can be emphasised.

Table 4. MIC values (mg/mL) of cinnamaldehyde for bacteria (Gram+ and Gram-), Moulds (M) and Yeasts (Y).

Gram+	MIC (mg/mL)	Gram-	MIC (mg/mL)	M and Y	MIC (mg/mL)
Listeria innocua	0.25 [7] 0.4 [10]	*Escherichia coli*	0.31 [4] 0.28 [5] 0.5 [6] 0.4 [8]	*Saccharomyces cerevisiae*	1.56 [1]
Listeria monocytogenes	6.25 [1] 0.5 [9]			*Zygosaccharomyces rouxii*	1.56 [1]
Staphylococcus aureus	0.31 [4] 0.37 [5] 0.25 [6] 0.156 [9]	*Aeromonas hydrophila*	0.78 [1] 0.31 [4]	*Torulaspora delbrueckii*	0.05 [2]
		Yokenella regensburgei	0.31 [4]	*Schizosaccharomyces pombe*	0.05 [2]
Bacilus cereus	0.312 [9]	*Enterobacter clocacae*	0.63 [4]	*Candida krusei*	0.05 [2]
Enterococcus faecalis	0.78 [1] 0.25 [6]	*Salmonella enteriditis*	0.31 [4]	*Zygosaccharomyces bailii*	0.05 [2]
Kokuria kristinae	0.31 [4]	*Pseudomonas aeruginosa*	1.0 [6]	*Fusarium verticilloides*	0.045 [3]
Staphylococcus intermedius	0.16 [4]			*Candida albicans*	0.312 [11]
Lactococcus garviceae	0.63 [4]			*Aspergillus flavus*	1.0 [12]
Streptococcus sanguinis	0.63 [4]			*Penicillium roqueforti*	1.0 [12]
Staphylococcus haemolyticus	0.16 [4]				

[1] Sanla-Ead *et al.* (2012); [2] Monu *et al.* (2016); [3] Xing *et al.* (2014); [4] Ye *et al.* (2013); [5] Becerril *et al.* (2007); [6] Chang *et al.* (2001); [7] Han *et al.* (2006); [8] Pei *et al.* (2009); [9] Shan *et al.* (2007); [10] (Thanh *et al.* (2013); [11] Rahemi *et al.* (2015); [12] Manso *et al.* (2015).

2. 2. 2. In vivo *studies in food systems*

In spite of the wide number of *in vitro* studies of the antimicrobial properties of essential oils, their inhibitory properties of the microbial growth in food matrices are less known. Specifically, in the case of yeasts, Monu *et al.* (2016) showed that CIN and cinnamon's rind oil were the most effective against yeasts in salad dressings, in comparison with other studied oils (clove, thyme, eugenol, carvacrol and timol oils). Romero-Gil *et al.* (2016) proved that this active compound also possesses a wide inhibitory effect in the growth of acid lactic bacteria, and could be employed as an alternative to other preservatives that are used in packaged olives.

Different studies have demonstrated that CIN could potentially be used, at different concentrations, as an effective antimicrobial agent against *E. coli* O157: H7 in apple juice and cider (Baskaran *et al.*, 2010), *Enterobacter sakazakii* in reconstituted milks for breastfed babies (Amalaradjou *et al.*, 2009), *E. coli* in bovine meat cakes (Amalaradjou *et al.*, 2010), *Salmonella spp* in tomato washing (alternative to sodium hypochlorite) (Mattson *et al.*, 2010) or mix of strains of bacteriophage and *E. coli* O157:H7 in green leaf vegetables (Viazis *et al.*, 2011). CEO or CIN also caused the growth inhibition of psychotropic strains of *Bacillus cereus* (EPSO-35As y el INRA TZ415) in minimally processed products, such as carrot (Hernández-Herrero *et al.*, 2008). Despite the effectiveness of CIN as an antimicrobial, its strong smell and high volatility are still important drawbacks (Wang *et al.*, 2015). This implies the necessity of carrying out additional tests on the quality of the final product and the sensorial impact of the compound when it is incorporated into food matrices (Amalaradjou *et al.*, 2009; Baskaran *et al.*, 2010; Hernández-Herrero *et al.*, 2008; Mattson *et al.*, 2010; Romero-Gil *et al.*, 2016).

2. 2. 3. Polymer matrices as cinnamaldehyde carriers

In terms of polymeric matrices used for active materials development of food packaging or coating, petroleum derivative plastic represents a serious environmental issue, since the majority of this plastic is not biodegradable and it can take from 100 to 450 years to degrade (Alvira, 2007). This is why developing these materials requires to attend to the consumers' needs in their new habits, such as synthetic chemical substances free food, sustainably processed. This generates interest for the research on developing new biodegradables active materials destined to food products and can increase the shelf life of such products. The incorporation of natural active substances like essentials oils in biodegradables films is currently an alternative way to prevent food damage mainly for it functional properties like is antioxidant and antimicrobial capacities (Corrales *et al.*, 2014: Lanciotti *et al.*, 2004).

It is important to underline that different polymers, nanoparticles and other materials have been used to encapsulate CIN, with antimicrobial goals. Cellulose (Sanla-Ead *et al.*, 2012), pectin (Otoni *et al.*, 2014), PLA (Ahmed *et al.*, 2016b; Makwana *et al.*, 2014; Qin *et al.*, 2015a), starch (Souza *et al.*, 2013), proteins (Balaguer *et al.*, 2013) or alginates (Rojas-Graü *et al.*, 2007) have been studied as CIN carriers.

In **Tables 5** and **6**, some studies into the antimicrobial effect of these materials are shown, carried out in both *in vitro* tests or applied on food matrices. All the films where CEO or CIN was incorporated were effective at controlling the growth of the tested microorganisms.

Table 5. Polymeric matrices used as carriers of CEO or CIN and *in vitro* tested microorganisms.

Polymeric matrix	CIN/CEO	% Active compound (g/100 g polymer)	Microorganism	Reference
PLA	CEO	25-50-75	*S. aureus* *C. jejuni*	Ahmed *et al.*, 2016a
Polypropylene (PP)	CEO	2-4-6	*A. flavus* *A. niger* *P. roqueforti* *P. expansum*	Manso *et al.*, 2015
Cassava starch	CEO	8-12-16	*E. amstelodami* *P. commune*	Souza *et al.*, 2013
Polyvinyl alcohol (PVA)-β-cyclodextrin	CEO	28.6-25-22.2	*S. aureus* *E. coli*	Wen *et al.*, 2016
PLA	CIN	2.5	*E. coli* *B. cereus*	Makwana *et al.*, 2014
PLA-poly(trimethylene carbonate) (PTMC)	CIN	3-6-9-12	*E. coli* *S. aureus*	Qin *et al.*, 2015a
Polycaprolactone (PCL)	CIN	10-20	*S. enterica* *L. monocytogenes*	Martinez-Abad *et al.*, 2013
Polyethylene-co-vinylacetate (EVA)	CIN	3.5-7	*L. monocytogenes* *E. coli* *S. aureus* *S. epidermidis*	Nostro *et al.*, 2012
Methylcellulose	CIN	8	*B. cereus* *E. faecalis* *L. monocytogenes* *M. luteus* *S. aureus* *A. hydrophila* *E. coli* *E. coli O157: H7* *P. aeruginosa* *S. enteritidis* *C. albicans* *S. cerevisiae* *Z. rouxii*	Sanla-Ead *et al.*, 2012
Pectin	CIN	66.7	*E. coli* *S. enterica* *L. monocytogenes* *S. aureus*	Otoni *et al.*, 2014

Table 6. Polymeric matrices used as carriers of CIN and *in vivo* tested microorganisms in different food products.

Matrix	**% CIN (g/100 g polymer)**	**Food product**	**Microorganism**	**Reference**
Gliadin	1.5-3-5	Bread and spreadable cheese	*in vitro* and *in vivo* tests: *P. expansum* *A. niger*	Balaguer *et al.*, 2013
PLA-PCL	3-9	Button mushrooms	Psychrophilic and mesophilic bacteria	Qin *et al.*, 2015b
Chitosan	0.1-0.25-0.5	Bread slices	*R. stolonifer*	Demitri *et al.*, 2016
Apple-coated PET	0.5-1.5-3	Chicken breast	*C. jejuni*	Mild *et al.*, 2011
Apple-coated PET	0.5-1.5-3	Meat and poultry products	*S. enterica* *E. coli O157:H7*	Ravinshankar *et al.*, 2009
Apple-Carrot-Hibiscus coated PET	0.5-1.5-3	Ham and Bologna	*L. monocytogenes*	Ravinshankar *et al.*, 2012

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OBJECTIVES

The **General Objective** of this Doctoral Thesis was to combine PLA and starch films for obtaining bilayers with optimized functional properties as food packaging material, and the incorporation of an active compound, cinnamaldehyde, in an effective way to promote its controlled release in food systems, conferring antimicrobial properties to the films. Then, the next specific objectives were intended.

Specific objectives

1. To develop PLA films by compression moulding, using different biodegradable compounds, with food contact properties, and to analyse the plasticizing behaviour of the films.

2. To develop PLA-starch bilayer films, with or without cinnamaldehyde, by compression moulding and to analyse their structural and functional properties.

3. To analyse the antimicrobial properties and release kinetics of cinnamaldehyde in PLA-starch bilayer films obtained by compression moulding.

CHAPTERS

CHAPTER I	Influence of plasticizers on thermal properties and crystallization behaviour of poly(lactic acid) films obtained by compression moulding
CHAPTER II	Poly(lactic) acid (PLA) and starch bilayer films, containing cinnamaldehyde, obtained by compression moulding
CHAPTER III	Antimicrobial properties and release of cinnamaldehyde in bilayer films based on poly(lactic) acid (PLA) and starch

CHAPTER I

Influence of plasticizers on thermal properties and crystallization behaviour of poly(lactic acid) films obtained by compression moulding

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ABSTRACT

Neat and plasticized poly(lactic acid) (PLA) films were obtained by compression moulding. Three different plasticizers, at concentrations of 5% and 10%, were used: poly(ethylene glycol) (PEG) 1000 and 4000 g/mol molecular weights and a commercial plasticizer, Palamoll®638. Crystallization behaviour and glass transition of PLA in the films were analysed using differential scanning calorimetry (DSC) and X-Ray diffraction. Films were also characterized in terms of structural, tensile, barrier and optical properties. The addition of the three plasticizers reduced the glass transition temperature (T_g) while promoting the crystallization of PLA in the compression moulded films, depending on their type and concentration. PEG 4000 enhanced crystallization more than PEG 1000 and Palamoll®638, the latter being the one which induced the lowest degree of crystallization. The T_g values of the PLA amorphous phase was reduced by the addition of plasticizers, proportionally to their concentration. Nevertheless, all of the plasticized films exhibited similar elastic modulus and were less resistant to break and less extensible due to the greater induced crystallinity. PEG 4000 drastically reduced the film's stretchability whereas this effect was less marked when Palamoll®638 was used.

Key words: Poly(lactic acid), plasticizer, compression moulded films, crystallization, glass transition temperature, stretchability.

1. INTRODUCTION

PLA is a linear aliphatic thermoplastic polyester derived from lactic acid and obtained from 100% renewable plant sources, such as corn starch or sugar. Best known for its biodegradability, PLA is a good alternative to replace petroleum-based polymers, such as polystyrene or polyethylene. Since the field of packaging is one of the major consumers of plastics, it is becoming more and more necessary to use biodegradable polymers to limit pollution associated with plastic wastes. PLA is biocompatible and has been approved by the Food and Drug Administration (FDA) for direct contact with biological fluids. It can be processed either by extrusion, injection moulding, film casting or fibre spinning (Byun *et al.*, 2010; Cai *et al.*, 1996; Hughes *et al.*, 2012; Casasola *et al.*, 2014). It also has good transparency, higher than that of polyethylene terephthalate (PET) or polystyrene (PS), and a high comparable mechanical strength. However, it is very brittle, with an elongation at break (ε) of less than 10% (Rasal *et al.*, 2008; Hiljanen-Vainio *et al.*, 1996), which restricts its application as films for the packaging industry. Plasticizers are widely used to improve the processability, flexibility and ductility of polymers. In the case of amorphous or semi-crystalline polymers, such as PLA, an efficient plasticizer not only has to reduce the glass transition temperature, but also to depress the melting point and the crystallinity. Considerable efforts have been made to improve the mechanical flexibility of PLA by blending it with compatible plasticizers or other soft polymers.

The use of various low molecular weight plasticizers, such as citrates or adipates (Coltelli *et al.*, 2008), leads to the migration of plasticizers within the PLA matrix, due to their high molecular mobility. To avoid this migration problem, many high molecular weight plasticizers including poly(ethylene glycol) (PEG), poly(propylene glycol), polyester diol or poly(diethylene adipate) have been used (Byun *et al.*, 2010; Choi *et al.*, 2013). The plasticizing properties of PEG, with different molecular weights, in PLA matrices have been extensively studied. A large amount of these compounds is usually needed in order to be effective at promoting film plasticity, which can overcome the miscibility limit (Pillin *et al.*, 2006), leading to phase separation, and negatively affecting the properties of the material. The aging of plasticized PLA also provoked the migration and phase separation of PEG (Gumus *et al.*, 2012). Hu *et al.* (2003) observed crystallization of PEG 8000 when it was added as plasticizer in PLA at 30 wt % in PLA matrices. This compound accelerated the PLA crystallization, depending on the cooling rate, and PEG crystallinity was proportional to PLA crystallinity. Sungsanit *et al.* (2012) also reported phase separation and crystallization of PEG 1000 above 15% in the PLA blends. The impact resistance and crystallinity of PLA films rose in line with the increase in plasticizer content, whereas both a drop in glass transition temperature and less stiff films were observed. Similar results were obtained by Chieng *et al.* (2013) using PEG 200 at different ratios in the

PLA blends. The plasticization of the PLA with PEG 200 effectively lowers T_g as well as the cold-crystallization temperature, in line with the increase in the plasticizer content. Baiardo *et al.* (2003) reported a very good correlation between mechanical properties of plasticized PLA and the T_g of the blend using PEGs with different molecular weights. The lower the T_g depression by the plasticizer, the more extensible the PLA material, although some deviations from the established tendencies were observed, which could be explained by the contribution of the degree of crystallinity of the samples. In this sense, it is remarkable that, whereas the thermal history of the samples was erased to obtain thermal parameters, tensile properties were analyzed in the samples with their own thermal history of unknown crystallinity. The contribution of the crystalline fraction to the mechanical properties of PLA has not been reported.

Other compounds, such as hazelnut and cocoa extract, can also act as plasticizers for PLA (Battegazzore *et al.*, 2014), as well as keratins and brown alpaca fibres (Fortunati *et al.*, 2015). Likewise, PLA was toughened by blending with a PEG-based polyester, poly (polyethylene glycol-co-citric acid) (Gui *et al.*, 2012), which greatly improved elongation at break and the impact resistance as compared with the same amount of the corresponding PEG.

The presence of compatible plasticizers enhances the free volume of the chains which induces molecular mobility. This reduces the glass transition temperature but it can also favour the crystallization process in semi-crystalline polymers. These two phenomena have opposing effects on the ductility of the polymer and can be dependent on the molecular structure of plasticizer, and the associated intrinsic viscosity, and the molecular interactions with the polymer chains. Likewise, the processing conditions, with different heating and cooling rates, determine the crystallinity degree of the polymer, which in turn affects the mechanical performance of the material.

In this study, two extensively studied plasticizers (PEG 1000, PEG 4000) and one new one (Palamoll®638), in two different ratios (5 and 10%), were melt blended with PLA to obtain films by compression moulding and their effect on the crystallization and thermal behaviour of the PLA during film processing was analysed, as well as their impact on the tensile and barrier properties of the obtained films. The relatively low amount of plasticizers was considered in order to avoid phase separation of these compounds in the films, as described in previous studies. The overall migration values of both un-plasticized and plasticized films in aqueous food simulants were also analysed for the purposes of determining the influence of plasticizers on the potential film compound release into food systems.

2. MATERIALS AND METHODS

2. 1. Materials

PLA resin (LL700, density of 1.25 g/cm^3) was purchased from Ercros (Spain). Poly(ethylene glycol) 1000 and 4000 g/mol molecular weights (PEG 1000 and PEG 4000) were obtained from Sigma–Aldrich (Germany). Palamoll®638 was purchased from BASF (Germany). This commercial polymeric plasticizer is derived from adipic acid, 1,2 propane diol and 1 octanol, with a high viscosity of 7000-9500 mPa.s (at 20°C), listed for food contact applications and it is practically insoluble in water and oils.

2. 2. Film preparation

PLA was first dried at 60°C for 24 h to remove residual water. PLA and plasticizers (10% w/w) were then hot-mixed in a two-roll mill (ModelLRM-M-100, Labtech Engineering, Thailand) at 200°C and 10 rpm for 20 min to ensure a good homogenization of plasticizers with the polymer. Nevertheless, this relatively long time could provoke chain scission in the polymer. The resulting paste was then compression moulded in a hydraulic press (Model LP20, Labtech Engineering, Thailand). 3 g of sample were placed onto steel sheets and pre-heated at 200°C for 4 min. Then, compression was performed at 200°C and 100 bars for 4 min, followed by a cooling cycle of 3 min until the temperature reached about 70°C. The obtained films were maintained in desiccators with silica-gel at room temperature until analyses were carried out.

2. 3. Film characterization

2. 3. 1. Film thickness

A hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure film thickness to the closest 0.001 mm, at 6 random positions around the film prior to mechanical test analyses.

2. 3. 2. Thermal properties

Differential scanning calorimetry analyses were carried out using a DSC (1 StareSystem, Mettler-Toledo, Inc., Switzerland). Small amounts (5-10 mg approx.) of samples were placed into aluminium pans (Seiko Instruments, P/N SSC000C008) and sealed. Samples were heated from room temperature to 200°C at 10K/min in order to analyse phase transitions in the PLA film. Samples were kept a 200°C for 5 min, cooled to -10°C at -50K/min, kept at -10°C for 5 min and heated again to 200°C at 10K/min; finally they were cooled to 25°C at -100K/min. An empty aluminium pan was used as reference. The same method was used to analyse melting properties of PEG 1000 and PEG 4000. Each sample was analysed in duplicate.

A thermo-gravimetric analyser (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland) was used to characterize thermal degradation. The analysis was performed from room temperature to 600°C at 10°C/min under a nitrogen flow (50 mL/min). Each sample was analysed in duplicate. The initial degradation temperature (T_{onset}), i.e. the temperature at which 5% mass loss is registered, was recorded. The temperature at which the maximum degradation rate was observed (T_{max}) was also determined. Each sample was analysed in duplicate.

2. 3. 3. Microstructure

The microstructural analysis of the cross-sections of the films was carried out by means of a scanning electron microscope (JEOL JSM-5410, Japan). Film pieces, 0.5 x 0.5 cm^2 in size, were cryofractured from films and fixed on copper stubs, gold coated, and observed using an accelerating voltage of 10 kV. Observations were taken in duplicate for each formulation.

2. 3. 4. X-ray diffraction

A diffractometer (XRD, Bruker AXS/D8 Advance) was used to record the X-ray diffraction patterns. All the samples were analysed at room temperature, between $2\theta = 5°$ and $2\theta = 30°$ using Kα Cu radiation (λ: 1.542 Å), 40 kV and 40 mA with a step size of 0.05°. For this analysis, samples were cut into 4 x 4 cm^2. Each sample was analysed in duplicate.

2. 3. 5. Tensile properties

A texture analyser (TA-XT plus, Stable Micro Systems, Surrey, United Kingdom) was used to measure the mechanical properties of films. Strips of films (25 mm wide and 100 mm long) were mounted in the tensile grips (A/TG model) and stretched at a rate of 50 mm/min until breaking. The elastic modulus (EM), tensile strength at break (TS) and percentage of elongation at break (ε) were determined from stress-strain curves, obtained from force-deformation data. The experiments were carried out at 25ºC and eight replicates were made for each film.

2. 3. 6. Water Vapour Permeability (WVP) and Oxygen Permeability (OP)

The WVP of films was measured using a modification of the ASTM E96-95 (1995) gravimetric method, using Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm diameter. For each formulation, the measurements were replicated four times and the WVP was calculated following the methodology described by Gennadios *et al.* (1994), at 25°C and a 53-100% relative humidity (RH) gradient, which was generated using an oversaturated $Mg(NO_3)_2$ solution and pure distilled water. To determine the WVP, the cups were weighed periodically for 4 days after the steady state was reached using an analytical balance (±0.00001 g). The slope obtained from the weight loss vs. time was used to calculate WVP, according to ASTM E96-95 (1995).

The OP of the films was determined by using the ASTM F1927 method (2014), using OXTRAN SL 2/21 MOCON equipment (U.S.A.). Measurements were carried in duplicate at 23°C and 50% RH. Film thickness was considered in all samples to obtain the oxygen permeability values.

2. 3. 7. Optical properties

The optical properties of the films were determined in film samples by means of a spectrocolorimeter (CM-3600d, MinoltaCo., Tokyo, Japan) with a 10 mm diameter window.

The internal transmittance (T_i) of the films was determined, as an indicator of the transparency of the films, by applying the Kubelka-Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra, following the methodology described by Pastor *et al.* (2010).

2. 3. 8. Ultraviolet–visible spectrophotometry (UV-VIS)

UV-VIS absorption spectra of the samples (4.5 × 1.2 cm^2) were recorded in the wavelength range of 250-700 nm using a Thermo Scientific (Evolution 201) UV–VIS spectrophotometer.

2. 3. 9. Overall migration (OM) in aqueous food simulants

Overall migration tests were carried out for plasticized and unplasticized films, following the current legislation (Commission Regulation 10/2011). Film samples with a total area of 15 cm^2 were immersed in 25 mL of different simulants: simulant A (ethanol 10% in water, simulating foods which are hydrophilic in nature) and simulant B (acetic acid 3% in water, simulating hydrophilic foods with pH below 4.5). All the samples were kept in contact with the simulants for 10 days at 20°C, to simulate any food contact under frozen or refrigerated conditions. After incubation, the film samples were removed from the simulants, which were evaporated to dryness. The migration value in each case was taken as the residue weight. The results were expressed as mg per dm^2 of film. All the tests were run in duplicate.

2. 3. 10. Statistical analysis

StaTgraphics Plus for Windows 5.1 (Manugistics Corp., Rockville, MD, U.S.A.) was used to carry out statistical analyses of data through an analysis of variance (ANOVA). Fisher's least significant difference (LSD) was used at the 95% level.

3. RESULTS AND DISCUSSION

3. 1. Characterization of amorphous and crystalline phases of the films

The degree of crystallization of compression moulded films with and without plasticizers was analysed from the first heating scan of DSC, taking the X-Ray diffraction pattern of the films into account. **Figure 1** shows the thermograms obtained for both neat PLA films and those containing 5% of plasticizers. Glass transition was observed in all cases, followed by a crystallization exotherm and the subsequent melting endotherm of the polymer. An excess enthalpy of relaxation (ΔH_{ex}) was observed as an endotherm at the glass transition. As observed by other authors for PLA (Cai *et al.*, 1996), this has been associated with the physical aging of the material, since ΔH_{ex} increases as the material ages. Likewise, a small exotherm prior to the melting endotherm was observed in all the samples, which is characteristic of a solid-solid transition (Zhang *et al.*, 2008). This corresponds to the disorder-to-order (α′-to-α) phase transition, in which the chain packing of the crystal lattice becomes more ordered. This thermal behaviour of the films indicates that a large part of the polymer is in an amorphous state after compression moulding under the conditions applied (fast cooling rate after thermocompression). Then, after glass transition, crystallization occurs when the temperature rises to nearly 100°C. Afterwards, the polymer melts at about 175ºC, but prior to that, the α’ crystal fraction crystallizes into the α form (small exotherm before melting).

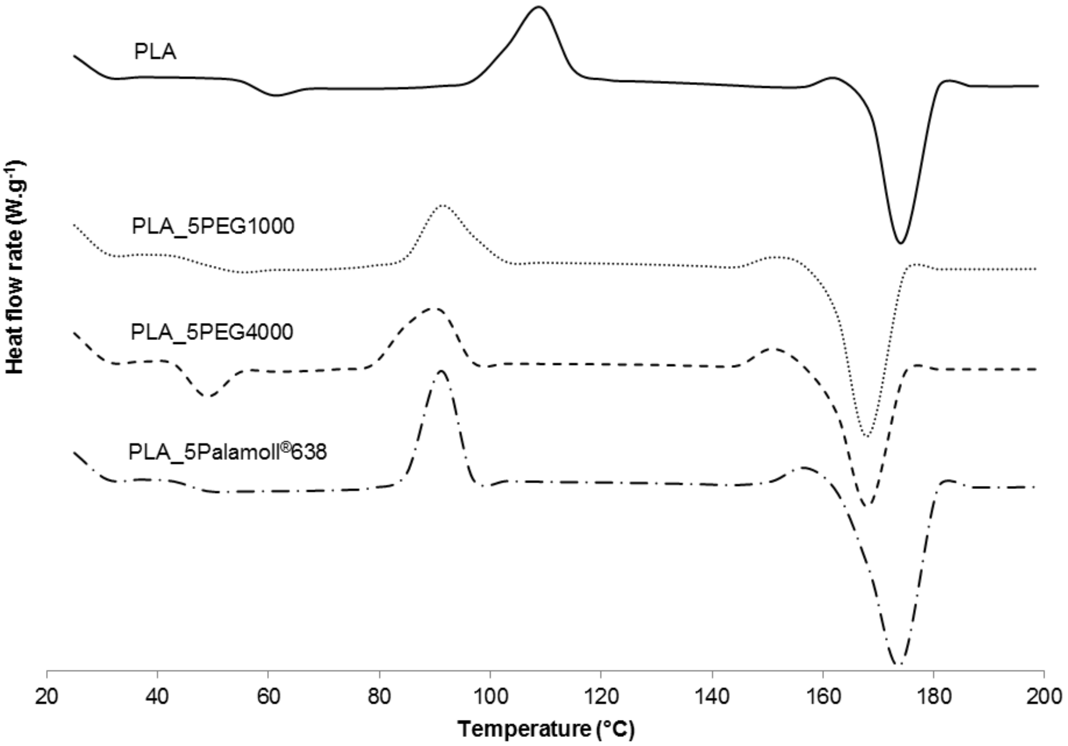


Figure 1. DSC thermograms of neat PLA and PLA with 5% of plasticizers at the first heating.

As shown in **Table 1**, the onset glass transition temperature (T_{g0}) of PLA significantly decreased after the addition of plasticizers, regardless of their type; the higher the concentration of plasticizer, the greater the decrease in T_{g0}. This indicates that the compounds used effectively plasticize the amorphous phase of the polymer, depending on their concentration, reducing the interchain forces of the polymer and increasing its free volume, thus enhancing the rubbery character. No notable differences in the plasticizing effect of the different compounds used were observed despite their different molecular weight, since a similar T_g decrease was obtained at a determined concentration of each compound. It is remarkable that, in no case did PEG crystallization occur in the films, as deduced from the DSC curves where no melting of PEG was observed. Melting properties of pure PEG 1000 and PEG 4000, determined by DSC, gave melting temperature values of 38°C and 56°C (within the range of the film analyses) with melting enthalpies of 181 and 210 J/g, respectively. Therefore, a good miscibility of the plasticizers and polymer, at the used ratios, was observed in all cases, exhibiting no phase separation.

Table 1. Thermal data obtained by DSC measurements (first heating scan) for PLA films with different contents of plasticizers. Mean values ±standard deviation.

Film	T_{g0}^{1} (°C)	T_c^{1} (°C)	ΔH_c^{1} (J/g PLA)	ΔH_{ex}^{1} (J/g PLA)	T_m^{1} (°C)	ΔH_m^{1} (J/g PLA)	X_{c1}^{1} (%)	X_{c2}^{1} (%)	X_c film (%)
PLA	55.6 ±0.4 ^{e}	108.0 ±0.1 ^{d}	47.0 ±2.0 ^{d}	1.4 ±0.1 ^{ab}	175.7 ±0.1 ^{d}	52.3 ±0.3 ^{a}	49.0 ±3.0 ^{d}	54.5 ±0.1 ^{a}	5 ±3 ^{a}
PLA_5PEG1000	48.5 ±0.4 ^{d}	93.7 ±0.1 ^{c}	37.8 ±0.2 ^{c}	3.8 ±0.3 ^{bc}	169.9 ±0.4 ^{b}	60.0 ±0.4 ^{c}	39.4 ±0.2 ^{c}	62.5 ±0.6 ^{c}	23 ±1 ^{b}
PLA_10PEG1000	38.0 ±5.0 ^{a}	87.0 ±3.0 ^{bc}	30.8 ±1.1 ^{bc}	3.0 ±2.0 ^{abc}	170.2 ±0.6 ^{b}	62.8 ±0.3 ^{c}	32.0 ±2.0 ^{bc}	65.4 ±0.5 ^{c}	33 ±2 ^{cd}
PLA_5PEG4000	45.4 ±0.2 ^{bcd}	87.5 ±0.4 ^{bc}	32.0 ±3.0 ^{bc}	4.3 ±0.5 ^{bc}	170.8 ±0.0 ^{b}	60.5 ±0.3 ^{c}	33.0 ±4.0 ^{bc}	63.0 ±0.5 ^{c}	30 ±3 ^{c}
PLA_10PEG4000	38.4 ±1.4 ^{ab}	75.0 ±3.0 ^{a}	17.0 ±4.0 ^{a}	5.0 ±1.2 ^{c}	168.1 ±0.3 ^{a}	61.0 ±2.0 ^{c}	17.0 ±6.0 ^{a}	63.0 ±3.0 ^{c}	46 ±2 ^{e}
PLA_5Palamoll®638	46.2 ±1.2 ^{cd}	91.8 ±1.4 ^{c}	37.0 ±2.0 ^{c}	0.3 ±0.1 ^{a}	174.1 ±0.3 ^{c}	56.7 ±0.5 ^{b}	38.0 ±3.0 ^{c}	59.0 ±0.8 ^{b}	21 ±2 ^{b}
PLA_10Palamoll®638	39.0 ±2.0 ^{abc}	81.0 ±4.0 ^{ab}	26.0 ±2.0 ^{b}	2.4 ±1.0 ^{abc}	173.4 ±0.2 ^{c}	62.9 ±0.5 ^{c}	27.0 ±3.0 ^{b}	65.5 ±0.7 ^{c}	38 ±2 ^{d}

X_{c1}: degree of crystallization estimated from crystallization exotherm = $\Delta H_c / \Delta H^0_m$

X_{c2}: degree of crystallization estimated from the melting endotherm = $\Delta H_m / \Delta H^0_m$

X_c film: deduced from the difference between X_{c2} and X_{c1}

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

Table 1 also gives the crystallization temperature (T_c), crystallization enthalpy (ΔH_c), melting temperature (T_m) and melting enthalpy (ΔH_m), determined from the respective exothermic and endothermic peaks of the registered first heating scan. Taking into account the temperature at which crystallization occurs in the heating step, α and α' crystals could be formed, according to previous studies about the influence of the temperature on PLA crystallization (Cocca *et al.*, 2011). Yasuniwa *et al.* (2006) carried out the isothermal crystallization of PLA at various crystallization temperatures and elucidated that the crystallization behaviour discretely changes at crystallization temperatures below and above 113 and 135°C, respectively and a double melting behaviour was observed when crystallization occurred between 113 and 135°C. The observed α′-to-α transition could be partly attributed to the crystalline forms present in the thermocompressed films, in line with the fast cooling applied. In fact, the X-ray diffraction patterns of all the samples (**Figure 2**) presented the main peak at 2θ = 16.5, which is characteristic of the α' crystals of the PLA, slightly displaced at low angle with respect to the α form (2θ = 16.8) (Yasuniwa *et al.*, 2008). A broad amorphous band can also be observed in every sample, in line with the presence of amorphous polymer regions in the films.

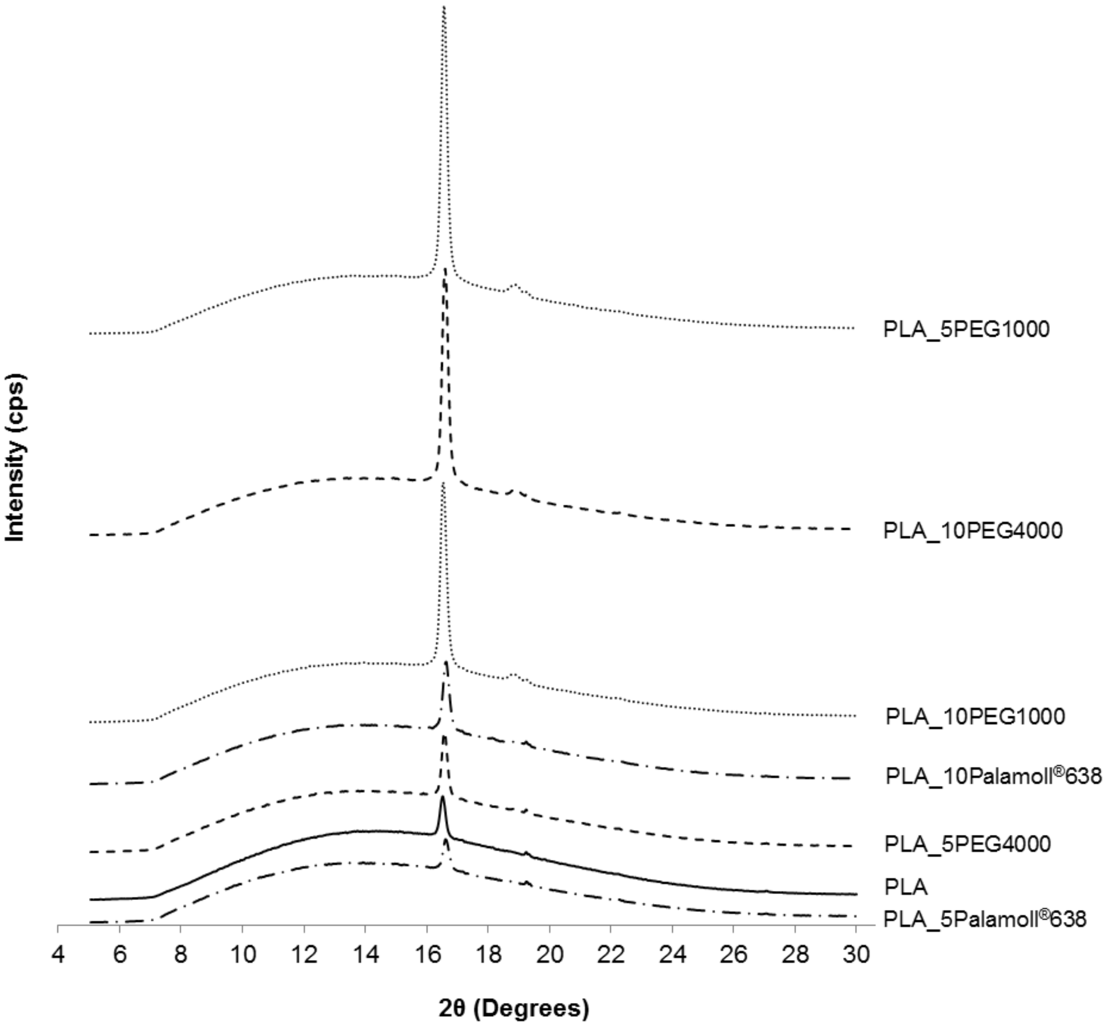


Figure 2. X-Ray diffraction patterns of neat and plasticized PLA films.

The degree of crystallinity of PLA (X_c film (%)) was estimated from the difference between the melting and crystallization enthalpies, by assuming that α crystals were formed, and considering the melting enthalpy of the fully α crystalline PLA phase (ΔH^0_m (α) = 96 J.g^{-1}), reported by Kalish *et al.* (2011). From this estimation (**Table 1**), a very low crystallinity degree (5%) was obtained in the PLA compression moulded films, which notably increased when plasticizers were incorporated. In general, the greater the amount of plasticizer, the higher the degree of crystallinity obtained in the film. Likewise, it is remarkable that PEG 4000 was significantly more effective than PEG 1000 and Palamoll®638 to promote PLA crystallization. The values obtained are coherent with the X-Ray diffraction patterns, which exhibited different relative intensities for the peak at 2θ = 16.5.

As concerns the melting temperature, films containing Palamoll®638 show a peak value that is very similar to that of the neat PLA film (175ºC) whereas significantly lower values were obtained for films with PEG 1000 and PEG 4000, which indicates that the latter plasticizers also interact with the PLA lattice, reducing the melting point of about 5ºC.

The previous thermal history of the films was erased in the second heating scan in the DSC analysis, but the thermal behaviour was similar to that obtained in the first scan, with the same events appearing on the thermograms, except for the relaxation endotherm at T_g which does not appear because of the newly crystallized polymer. The fast cooling rate applied during the cooling scan (50°C/min) in DSC allowed for reproducing thermal behaviour of the thermo-compressed films with their thermal history. The obtained values of T_g, T_c and T_m were similar to those obtained in the first heating scan, as well as the crystallization and melting enthalpy values (**Table 2**). The crystallization degree which occurred in the fast cooling scan was also estimated by the difference between exo and endo transitions and very small crystallization was determined for the neat PLA films. Nevertheless, crystallinity increased to a greater extent in this step when films contained plasticizers, in line with the promotion of molecular mobility. The higher the plasticizer concentration, the higher the crystallization rate. As observed in the first heating scan, PEG 4000 greatly enhanced PLA crystallization despite the scarce differences induced in the T_g compared to the other used plasticizers. The fact that plasticizers promote the crystalline form of thermocompressed PLA can be explained by the T_g reduction, which allows for enhanced molecular mobility at lower temperatures during the cooling step of the film, favouring the polymer chain rearrangement in crystalline forms. In fact, the crystallization temperature for all the plasticized films (**Tables 1-2**) was lower than for the neat PLA film and the reduction was in line with the T_g decrease, since crystallization occurred at 45-50°C above T_g in every case.

These results, valuable for lab scale production of films, could be far from those obtained at industrial level, mainly due to very different times of processing. In industry, the times of processing are considerably shorter and this will have an effect on film crystallinity. Nevertheless, a faster cooling of the polymer melt will lead to more amorphous material, since it is only above T_g that crystallization occurs extensively. The incorporation of plasticizers reduces the T_g and so, extends the time range for polymer crystallization during the cooling step, which suggests that a fast-cooling, quenching process must be implemented to optimize the plasticizer role.

Table 2. Thermal data obtained by DSC measurements (second heating scan) for PLA films with different contents of plasticizers. Mean values ±standard deviation.

Film	**T_{g0}^{2} (°C)**	**T_{c}^{2} (°C)**	**ΔH_{c}^{2} (J/g PLA)**	**ΔH_{ex}^{2} (J/g PLA)**	**T_{m}^{2} (°C)**	**ΔH_{m}^{2} (J/g PLA)**	**X_{c1}^{2} (%)**	**X_{c2}^{2} (%)**	**X_{c} cool (%)**
PLA	57.1 ±0.1 ^{d}	108.0 ±0.2 ^{d}	46.0 ±1.4 ^{d}	0.38 ±0.01 ^{c}	175.3 ±0.1 ^{d}	53.1 ±0.3 ^{a}	48.0 ±2.0 ^{d}	55.3 ±0.4 ^{a}	7 ±2 ^{a}
PLA_5PEG1000	45.8 ±0.2 ^{bc}	95.3 ±0.4 ^{bc}	39.4 ±0.7 ^{cd}	0.18 ±0.02 ^{b}	169.4 ±0.2 ^{b}	61.4 ±0.4 ^{cd}	41.0 ±0.8 ^{cd}	63.9 ±0.6 ^{c}	23 ±1 ^{b}
PLA_10PEG1000	36.0 ±5.0 ^{ab}	86.0 ±3.0 ^{a}	24.0 ±8.0 ^{b}	n.d.	169.1 ±0.8 ^{b}	64.4 ±0.9 ^{e}	25.0 ±8.0 ^{b}	67.1 ±0.9 ^{d}	42 +9 ^{d}
PLA_5PEG4000	42.0 ±0.9 ^{abc}	90.7 ±0.4 ^{abc}	34.0 ±3.0 ^{bc}	0.09 ±0.02 ^{a}	170.2 ±0.1 ^{b}	60.1 ±0.7 ^{bc}	36.0 ±5.0 ^{bc}	62.6 ±0.7 ^{bc}	27 ±4 ^{bc}
PLA_10PEG4000	34.0 ±5.0 ^{a}	86.0 ±6.0 ^{ab}	7.0 ±3.0 ^{a}	n.d.	167.5 ±0.5 ^{a}	63.0 ±2.0 ^{cde}	7.0 ±4.0 ^{a}	65.4 ±3.0 ^{cd}	58 ±7 ^{e}
PLA_5Palamoll®638	48.3 ±1.1 ^{cd}	98.7 ±0.9 ^{cd}	41.4 ±0.5 ^{cd}	0.18 ±0.01 ^{b}	173.7 ±0.4 ^{c}	57.9 ±0.2 ^{b}	43.1 ±0.7 ^{cd}	60.3 ±0.3 ^{b}	17 ±1 ^{ab}
PLA_10Palamoll®638	40.0 ±4.0 ^{abc}	87.0 ±5.0 ^{ab}	26.6 ±0.7 ^{b}	n.d.	173.0 ±0.2 ^{c}	63.2 ±0.5 ^{de}	27.7 ±0.7 ^{b}	65.8 ±0.7 ^{cd}	38 ±1 ^{cd}

n.d.: non detected

X_{c1}: degree of crystallization estimated from crystallization exotherm = ΔH_c / ΔH^0_m

X_{c2}: degree of crystallization estimated from the melting endotherm = ΔH_m / ΔH^0_m

X_c cool: occurred during the cooling step and deduced from the difference between X_{c2} and X_{c1}

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

Film microstructure (**Figure 3**) revealed a rather brittle fracture in both plasticized and unplasticized PLA films, exhibiting no plastic deformation, as correspond to the sample fracture conditions, below the T_g values. No phase separation of plasticizers was observed in any case, which confirms the good integration of the compounds into the polymer matrix. Nevertheless, some qualitative differences, reflected in a higher frequency of stripes and white dots, can be observed in the fractured surface of samples with the different plasticizers, which could be attributed to certain local plasticization effect, affecting the fracture behaviour.

The thermal stability of the PLA-based films is also coherent with the observed effect of plasticizers. The incorporation of plasticizers should reduce the thermal degradation temperature depending on how much they weaken the polymer network (Pillin *et al.*, 2006), but the increase in the degree of crystallization should promote the opposite effect due to the higher energy of the matrix. **Table 3** shows the obtained degradation temperatures (onset: T_{onset} and at the maximum rate: T_{max}) for the different films, where very small differences can be observed for the different samples. These range from 317 to 324°C (onset) and from 337 to 339°C (max) for all the samples. The opposite effect produced by the plasticizing of the amorphous region and the increase in crystallization only led to scarce changes in the thermo-stability of the films.

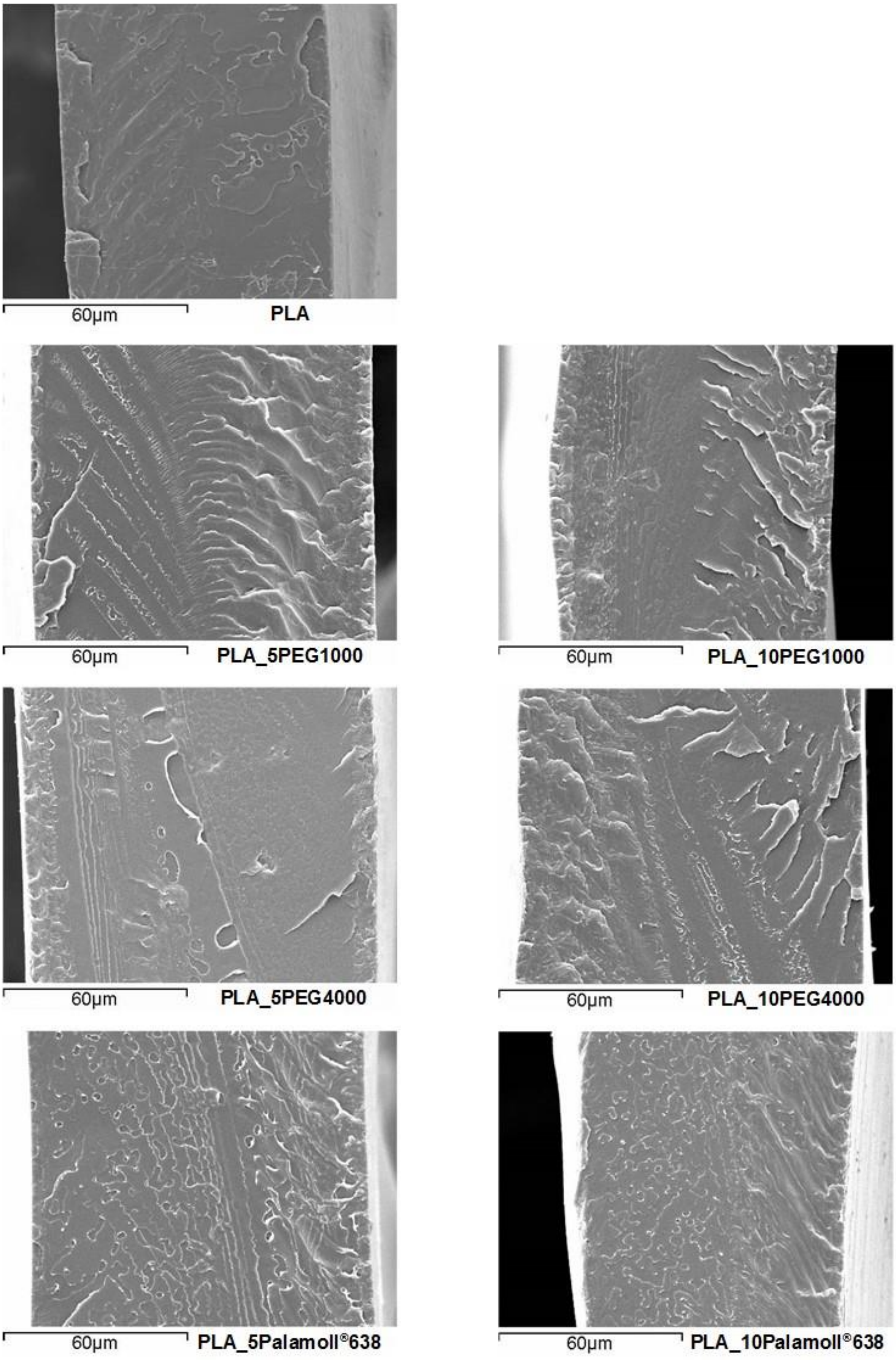


Figure 3. SEM images of the cross-sections of neat and plasticized PLA films.

Table 3. Thermal degradation temperatures (onset value and value at maximum degradation rate) of the different films obtained by TGA. Mean values ±standard deviation.

Film	T_{onset} (°C)	T_{max} (°C)
PLA	324.0 ±0.5 ^{e}	339.6 ±0.6 ^{c}
PLA_5PEG1000	320.4 ±0.2 ^{cd}	338.9 ±0.4 ^{bc}
PLA_10PEG1000	319.7 ±0.5 ^{bc}	337.3 ±0.2 ^{ab}
PLA_5PEG4000	318.8 ±0.3 ^{ab}	337.3 ±0.2 ^{ab}
PLA_10PEG4000	317.9 ±0.6 ^{a}	337.0 ±0.3 ^{a}
PLA_5Palamoll®638	321.5 ±0.1 ^{d}	338.8 ±0.3 ^{bc}
PLA_10Palamoll®638	321.3 ±0.6 ^{d}	338.8 ±1.0 ^{bc}

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

3. 2. Influence of the changes induced by plasticizers on the film physical properties

The tensile behaviour of the different PLA films can be observed in **Figure 4**. All of the plasticizers significantly reduced the film's stretchability, contrary to what was expected from the plasticization effect. The most marked reduction occurred for PEG 4000, whereas Palamoll®638 was the least effective.

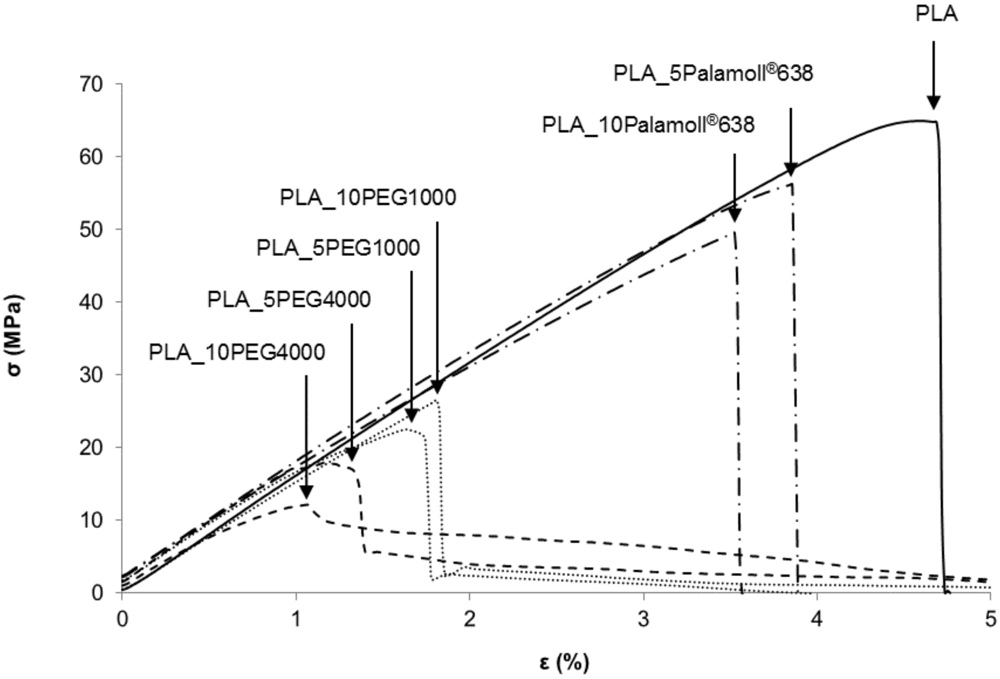


Figure 4. Tensile behaviour of neat and plasticized PLA films.

Table 4 shows the tensile parameters obtained for the different films, where similar values to those obtained by Wang *et al.* (2002) can be observed for un-plasticized PLA (about 5%). All the formulations exhibited values of the elastic modulus in the same range, but tensile and deformation at break were notably reduced by the addition of plasticizer. Of the plasticized films, that containing Palamoll®638 showed the greatest resistance to break while that containing PEG 4000 exhibited the lowest value. The plasticizer concentration hardly affected the tensile behaviour for a given compound, although a tendency to reduce the resistance to break when the concentration increased could be appreciated. This effect of the incorporated plasticizers can be explained by the reduction of the interchain attraction forces in the amorphous matrix, while a higher ratio of crystalline domains appeared when the plasticizer concentration increased. A different effect of each plasticizer suggests different molecular interactions between polymer chains and compound molecules.

The water vapour permeability of the PLA films slightly increased when plasticizers were added, which can be explained by the decrease in the glass transition temperature of the amorphous phase, which facilitates all of the diffusion controlled processes. However, the reduction in the film barrier capacity will be limited by the increase in the crystalline fraction in the films. On the contrary, OP decreased in plasticized films with 5% of PEG 1000, but increased in the other cases, even overcoming the upper sensitivity limit of the used equipment. This behaviour can be explained by the overall enhancement of the hydrophilic character of the film amorphous phase when PEG 1000 molecules are present at low concentration, although plasticization effect predominates at the highest concentration level. The promotion of hydrophilic character decreases the oxygen solubility in the amorphous phase, thus limiting their permeation capacity. In the case of PEG 4000 and Palamoll®638, more non-polar in nature, the OP was enhanced at the two used concentration levels.

Table 4. Thickness, tensile parameters (TS, EM, ε), water vapour permeability (WVP: m.g.h^{-1}.Pa^{-1}.m^{-2}), oxygen permeability (OP: cm^{3}.m^{-1}.s^{-1}.Pa^{-1}) and internal transmittance (T_i at 600 nm) of films. Mean values ±standard deviation.

Film	Thickness (mm)	TS (MPa)	EM (MPa)	ε (%)	WVP	OP x10^{12}	T_i (%)
PLA	0.136 ±0.008 d	62 ±2 ^{f}	1583 ±17 ^{cde}	4.4 ±0.2 ^{d}	0.093 ±0.003 ^{a}	2.44 ±0.14 ^{a}	88.96 ±0.02 ^{e}
PLA_5PEG1000	0.126 ±0.011 b	25 ±4 ^{c}	1530 ±50 ^{bc}	1.9 ±0.4 ^{b}	0.113 ±0.007 ^{ab}	1.90 ±0.01 ^{b}	88.79 ±0.03 ^{d}
PLA_10PEG1000	0.129 ±0.008 bc	27 ±3 ^{c}	1500 ±40 ^{ab}	1.9 ±0.1 ^{b}	0.127 ±0.011 ^{b}	> D.L.	88.54 ±0.06 ^{b}
PLA_5PEG4000	0.119 ±0.009 a	18 ±7 ^{b}	1620 ±30 ^{e}	1.2 ±0.5 ^{a}	0.115 ±0.010 ^{ab}	> D.L.	88.67 ±0.02 ^{c}
PLA_10PEG4000	0.117 ±0.010 a	10 ±3 ^{a}	1400 ±100 ^{a}	1.0 ±0.7 ^{a}	0.147 ±0.002 ^{c}	> D.L.	88.46 ±0.02 ^{a}
PLA_5Palamoll®638	0.132 ±0.007 cd	56 ±2 ^{e}	1600 ±70 ^{de}	3.9 ±0.1 ^{c}	0.103 ±0.009 ^{a}	2.90 ±0.60 ^{a}	88.85 ±0.04 ^{d}
PLA_10Palamoll®638	0.129 ±0.009 bc	51 ±3 ^{d}	1550 ±60 ^{bcd}	3.7 ±0.3 ^{c}	0.108 ±0.008 ^{a}	> D.L.	88.80 ±0.05 ^{d}

D.L.: 200 cm^{3}/m^{2}.day

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

On the other hand, all the films were highly transparent when exposed to wavelengths between 400 and 700 nm, as deduced from the high values of the internal transmittance shown in **Table 4**, and a very slight decrease in transparency with respect to the control film was provoked by the addition of plasticizers. Nevertheless, packaged food products are also submitted to UV irradiation, which may affect both the material and the foodstuff. Different published works have studied the behaviour of PLA films when exposed to UV-vis irradiation. Arrieta *et al.* (2015) found that cellulose nanoparticles were able to block the UV-light when added to PLA-PHB films whereas Cele *et al.* (2014) observed that clay nanoparticles reduced the transparency of films but no further discussion was considered in the UV range. For the obtained PLA films, a marked reduction in the UV light transmission was observed when they contained plasticizers as shown in **Figure 5**.

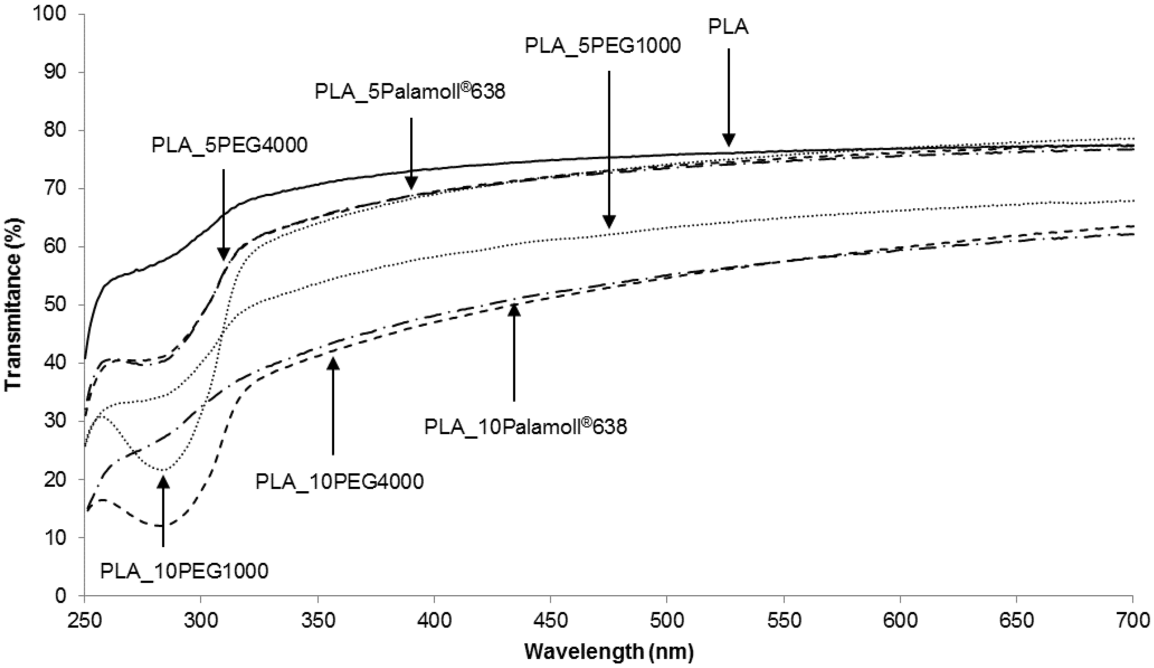
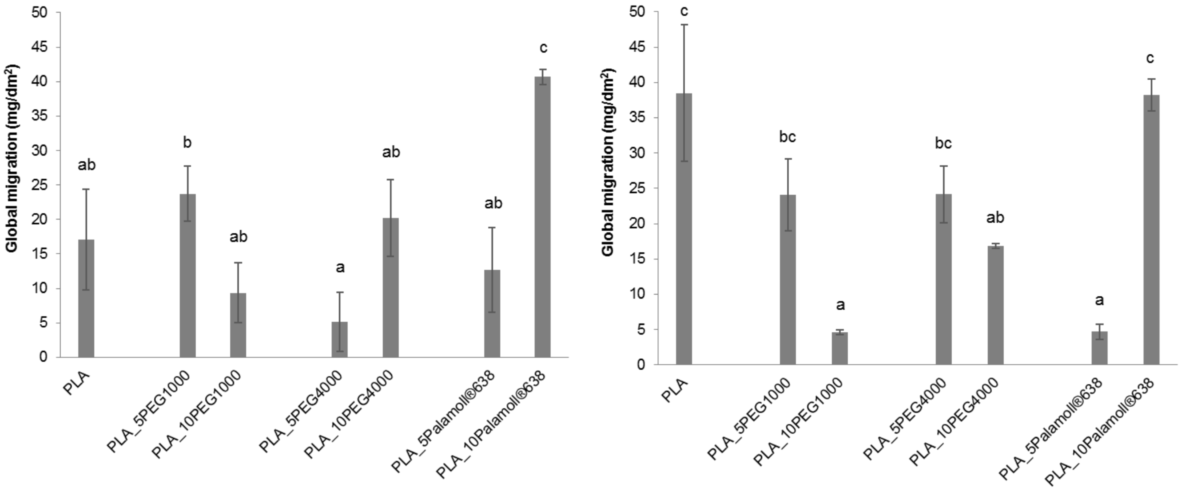


Figure 5. Transmittance of neat and plasticized PLA films in the UV-vis range.

The most significant differences in the film transmittance were found in the UV region, where Palamoll®638 and PEG containing films highly blocked the UV light. The main reduction of the transmittance started approximately at wavelengths below 340 nm, regardless of the formulation, in accordance with the observations of Cele *et al.* (2014). The highest blocking effect was found when the plasticizer content was 10%, thus indicating that this effect is directly related to the plasticizer content. Furthermore, both PEG were more effective at reducing the transmission of UV irradiation than Palamoll®638. The UV blocking effect of PEG also observed by Guha *et al.* (2004).

As concerns overall migration of the PLA films in aqueous food simulants, the values of the different samples showed great variability in both A (10% ethanol in water) and B (3% aqueous acetic acid) simulants. This suggests that different factors such as the sample crystallinity degree could affect the total migration, since crystalline zones are usually less soluble than amorphous ones. The relative chemical affinity of plasticizers with the polymer and solvent determines the migration of these compounds to the simulant. In this sense, different migration levels are expected depending on the crystallinity degree of the samples, the type of plasticizer, its amount in the film and the type of simulant.

In simulant A, the incorporation of PEG 4000 at 5% significantly decreased the OM levels of the films, whereas 10% Palamoll®638 greatly promoted it. In simulant B, unplasticized PLA exhibited higher migration, which can be attributed to the partial hydrolyses of the polyester and the release of more soluble monomers. However, the incorporation of 5% Palamoll®638 and 10% PEG (1000 and 4000) reduced the migration of the films. In general, the incorporation of the studied plasticizers in PLA matrices, obtained by compression moulding, did not enhance the migration of PLA in aqueous food simulants, except when Palamoll®638 was used at 10%, where total migration increased in simulant A. In contrast, in the other cases, the plasticizers did not significantly affect the migration capacity of the polymer or decrease it. It is remarkable that only some plasticized PLA films meet the requirements for total migration (10 mg/dm^2) established by the EU Commission Regulation 2015/174 in simulants A and B.



a **b**

Figure 6. Overall migration values for different films in aqueous food simulants (A: 10% Ethanol **(a)** and B: 3% acetic acid **(b)**). Different letters within the same figure indicate significant differences among formulations ($p<0.05$).

4. CONCLUSION

Although a low degree of crystallinity was obtained (5%) in the PLA films obtained by compression moulding, crystallization was promoted by the addition of plasticizers (PEG 1000, PEG 4000 and Palamoll®638); the greater their ratio, the higher the degree of crystallinity. All of the plasticizers reduced the glass transition temperature of the film's amorphous phase to a similar extent, proportionally to their concentration. Nevertheless, in no case did this imply an improved film ductility, since all of the plasticized films were less resistant to break and less extensible. The extent of the reduction in these parameters depended on the plasticizer used, Palamoll®638 being the one which promoted the smallest reduction in line with the lower degree of crystallization induced by this compound. So, the studied plasticizers, at 5-10 wt %, were not effective at reducing the film's brittleness, mainly due to the fact that they enhanced the polymer´s crystallization at the same time as they reduced the glass transition temperature of the amorphous PLA. Further studies with other potential plasticizing substances would be required, taking into account the potential antagonistic effect of plasticizers, increasing the molecular mobility of the polymer chains which, at the same time, can promote polymer crystallization, all of which affects the mechanical performance of materials based on PLA.

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CHAPTER II

Poly(lactic) acid (PLA) and starch bilayer films, containing cinnamaldehyde, obtained by compression moulding

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ABSTRACT

Bilayer films from thermoplastic starch and cast amorphous PLA were obtained by compression moulding, incorporating or not cinnamaldehyde in the PLA layer. Films were characterized as to their microstructure and barrier, tensile and optical properties, as well as thermal behaviour, X-Ray diffraction pattern and FTIR spectra. Bilayers using semicrystalline PLA, instead of starch, were also analysed for comparison purposes. Despite the lower ratio of cast PLA sheet in the bilayer assembly (about 1/3 of the film thickness), a great improvement in tensile and water vapour barrier properties was achieved with respect to the net starch films, the films maintaining high transparency and oxygen permeability as low as starch films. When cinnamaldehyde was included in the cast PLA sheet, films became thinner due to the losses of the volatile active during processing, but the improvement in barrier properties was maintained, with lower mechanical resistance. Thermal analyses revealed diffusion of cinnamaldehyde or low molecular weight compounds from cast PLA layer to the adhered sheets (starch or semicrystalline PLA) which contributed to plasticizing the amorphous regions and affected crystallization pattern of PLA, as also revealed by the X-Ray diffraction patterns. The obtained results offer an interesting option to obtain high barrier-highly resistant active films from thermoplastic starch and amorphous PLA, including cinnamaldehyde as active compound.

Key words: Bilayer films, starch, poly(lactic) acid (PLA), cinnamaldehyde, tensile properties, barrier properties.

1. INTRODUCTION

Although petroleum-based plastics are still extensively used in the food packaging area, they represent one of the biggest environmental issues as regards the exhaustion of natural resources and the accumulation of non-biodegradable materials. The development of more ecological alternatives, reducing the amount of materials used for packaging or recycling are necessary to respond to this environmental problem. In this sense, bioplastics, that are either biodegradable or bio-based polymers, represent an adequate alternative. Although biopolymers do not exhibit excellent functional properties in terms of mechanical performance, thermal stability or barrier properties, they have been the subject of an increasing number of studies for the purposes of improving them, thus obtaining a functionality comparable to that of petrochemical plastics. One of the most promising biopolymers is poly(lactic) acid since it exhibits many advantages; it is biodegradable, renewable and biocompatible and has been approved by the Food and Drug Administration (FDA) for direct contact with biological fluids. It is highly transparent and exhibits good water vapour barrier properties (Muller *et al.*, 2016), comparable to those of petroleum-based plastics, such as polyethylene terephthalate (PET) or polystyrene (PS). Nonetheless, PLA is highly hydrophobic, with limited gas barrier capacity and it is very brittle, with less than 10% of elongation at break (Rasal *et al.*, 2010; Hiljanen-Vainio *et al.*, 1996). In order to face these drawbacks, one of the many conceivable options is to combine it with other biopolymers with complementary properties. Starch is a good candidate since it is widely available and cheap, while its films are extensible with good oxygen barrier properties (Ortega-Toro *et al.*, 2016). However, starch films exhibit poor water vapour barrier capacity, being very water sensitive, and are not a resistant material, with relatively low tensile strength (Souza *et al.*, 2013; Acosta *et al.*, 2015; Versino *et al.*, 2015). Since PLA is highly hydrophobic and starch hydrophilic, it is difficult to blend both polymers without phase separation, so different strategies have been analysed to combine these materials. Either starch or PLA have been modified with plasma treatment (Bastos *et al.*, 2009; Turalija *et al.*, 2016) in order to modify their hydrophobicity. Different compatibilizers have also been used to enhance the interfacial interactions between both polymers (Xiong *et al.*, 2013a; 2013b; 2013c; Phetwarotai *et al.*, 2012). However, there have been few studies into the obtaining of PLA-starch bilayer films. Sanyang *et al.* (2016) characterized starch-PLA bilayers obtained by casting, with different ratios of both polymers, and found that these bilayers exhibit lower water vapour permeability and better mechanical performance than starch films. Indeed, since starch and PLA exhibit complementary properties in terms of the barrier capacity and the mechanical performance, their combination in bilayer assembly could offer adequate materials to meet the food packaging requirements. PLA exhibits good barrier capacity to water vapour and starch to oxygen. Although PLA is more brittle than starch, it exhibits a good resistance that can enhance the strength of PLA-starch bilayers. Besides, more and more of the food available in the stores comes in high-tech plastic packaging multilayer films, ensuring longer-term preservation of the food than a monolayer structure. Likewise, the incorporation of active compounds into starch-PLA bilayers could, moreover, yield an active biodegradable food packaging material with optimised functional properties. In this sense, cinnamaldehyde (CIN), one of the major constituents of cinnamon bark oil (~ 60-90%) is a good option, since it has been classified as GRAS (Generally Recognized As Safe) by the FDA and it possess high antibacterial, antifungal, antiinflammatory and antioxidant activity (Sun *et al.*, 2015; Wang *et al.*, 2016; Xing *et al.*, 2014). Several studies have demonstrated that CIN could be potentially used, in different concentrations, as an effective antimicrobial agent, as observed by Baskaran *et al.* (2010) for *E. coli* O157: H7 in apple juice and cider, by Amalaradjou *et al.* (2009) for *Enterobacter sakazakii* in reconstituted milks or by Amalaradjou *et al.* (2010) for *E. coli* in bovine meat pasties. CIN has also been included in different polymer matrices, such as PLA (Ahmed *et al.*, 2016b; Makwana *et al.*, 2014; Qin *et al.*, 2015a), starch (Souza *et al.*, 2013), proteins (Balaguer *et al.*, 2013) or alginates (Rojas-Graü *et al.*, 2007) to obtain active films.

Thus, the objective of this study was to analyse the functionality of starch-PLA bilayer films obtained by compression moulding as packaging material, and the effect of cinnamaldehyde incorporation on their properties. For comparison purposes, PLA-PLA bilayers were also studied. Likewise, microstructural and physical properties of the films were analysed as well as thermal behaviour and molecular interactions through FTIR spectra.

2. MATERIALS AND METHODS

2. 1. Materials

PLA resins: semicrystalline PLA LL700, density of 1.25 g/cm^3, purchased from Ercros (Spain) and amorphous PLA 4060D, density of 1.24 g/cm^3, for heat seal layer in coextruded oriented films, purchased from Natureworks (U.S.A) were used to obtain films by compression moulding and casting method respectively. Cassava starch was produced by Asia CO., LDT (Kalasin, Thailand) and purchased from Quimidroga S.A. (Barcelona, Spain) and glycerol used as a plasticizer for starch was provided by Panreac Química SLU (Castellar del Vallés, Barcelona, España). Ethyl acetate, used as solvent for amorphous PLA, was obtained from Indukern S.A. (Barcelona, Spain) and trans-cinnamaldehyde and Poly(ethylene glycol) (PEG 4000 g/mol) were provided by Sigma-Aldrich (Madrid, Spain). For the quantification of cinnamaldehyde, pure methanol, UV-IR-HPLC quality, was obtained from Panreac (Barcelona, Spain). Magnesium nitrate ($Mg(NO_3)_2$), used to reach a relative humidity (RH) of 53% for the starch film conditioning, was supplied by Vidra Foc S.A. (Barcelona, Spain) and phosphorus pentoxide (P_2O_5) was purchased from Panreac (Barcelona).

2. 2. Film preparation

Monolayer films of starch or semicrystalline PLA and amorphous PLA, with or without cinnamaldehyde, were obtained and combined to obtain S-PLA and PLA-PLA bilayer films.

2. 2. 1. PLA monolayers obtained by casting (M and M_{cin})

Amorphous PLA pellets were used to obtain PLA monolayers, with and without CIN, by casting method from the film forming dispersions. PLA pellets were first dried at 60°C overnight to remove residual water and then dissolved in ethyl acetate at 10% (w/w) for 4 hours while being stirred at room temperature. CIN-free layers (M) were obtained as control films and, in the case of active layers (M_{cin}), CIN was dispersed at 2.5%, which means a PLA:CIN ratio of 10:2.5. Dispersions were poured into 15 cm diameter Teflon plates at a solid surface density of 1.5 g/plate and solvent was evaporated overnight. Dried films were peeled off the casting surface and stored in a desiccator with silica gel (SiO_2) at room temperature until further analysis or use to obtain bilayer films. The nominal concentration of CIN in dried films was 20 g/100 g. Ethyl acetate and amorphous PLA were chosen to prepare cast monolayers because this polymer can be adequately dissolved at room temperature in this food compatible solvent, thus minimising the risks of the solvent use, while cinnamaldehyde can also be incorporated in the solvent-polymer binary system.

2. 2. 2. PLA monolayers obtained by compression moulding (PLA)

Semicrystalline PLA pellets were first dried at 60°C overnight and then hot-mixed in a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 200°C and 10 rpm for 10 min. The resulting paste (3 g sample) was then compression moulded in a hydraulic press (Model LP20, Labtech Engineering, Thailand). The steel sheets were pre-heated at 200°C for 4 min and compression was performed at 200°C and 100 bars for 4 min. A cooling cycle of 3 min until the temperature reached about 70°C was applied afterwards. The obtained films were maintained in a desiccator with SiO_2 at room temperature until further analysis or use to obtain bilayer films.

2. 2. 3. Cassava starch monolayers obtained by compression moulding (S)

Starch was first hand mixed with glycerol (30 g/100 g of starch), water (55 g/100 g of starch) and small amount of PEG (0,5 g/100 g starch). The dispersion was then melt blended in a two-roll mill at 160°C at 10 rpm for 25 min. The obtained thermoplastic starch was conditioned at 53% RH (in a desiccator containing oversaturated solution of $Mg(NO_3)_2$) at room temperature for 10 days. Starch films were also obtained by compression moulding. 4 g of sample (3.56 g dry solids) were placed onto steel sheets and pre-heated in a hydraulic press at 160°C for 1 min. Then, two step compression was performed at the same temperature; 50 bars for 2 min, plus 100 bars for 6 min. Afterwards, a cooling cycle of 3 min about 70°C was applied. The obtained films were maintained at 53% RH at room temperature until further analysis or use to obtain bilayer films.

2. 2. 4. Starch (S-M, S-M_{cin}) and PLA bilayer films (PLA-M, PLA-M_{cin})

Bilayer films were obtained through thermocompression of the different monolayers. Either compression moulded PLA or S films were hot pressed with cast PLA films with and without CIN. Films were heated at 160°C and 100 bars for 3 min and cooled down until 70°C in 3 min. The nominal mass fraction of cast PLA film in the bilayers was about 0.3. Films were maintained in a desiccator with SiO_2 at room temperature until further analysis.

2. 3. Film characterization

2. 3. 1. Quantification of cinnamaldehyde in the films

The final CIN content in the films was determined by methanol extraction and spectrophotometric quantification. Film samples (1 cm side) were submitted to constant stirring for 24 h at 20°C in 100 mL of methanol. Then, absorbance was measured at 286 nm (maximum of absorption of the cinnamaldehyde in methanol) using a UV-visible spectrophotometer (Thermo Scientific Evolution 201, EEUU). The extract of the corresponding control film without CIN was used as a blank in each case. The calibration curve was obtained from the absorbance measurements of standard solutions of CIN and was used to determine its concentration in the films.

2. 3. 2. Film thickness

A hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure film thickness to the closest 0.001mm, at six random positions around the film.

2. 3. 3. Microstructure

The microstructural analysis of the cross-sections of the films was carried out by means of a Field Emission Scanning Electron Microscope (FESEM) (ULTRA 55, ZEISS, Germany). Film pieces, 0.5 x 0.5 cm^2 in size, were cryofractured with liquid nitrogen and fixed on copper stubs, platinum coated, and observed using an accelerating voltage of 2 kV. Observations were taken in duplicate for each formulation.

2. 3. 4. Tensile properties

A texture analyser (TA-XT plus, Stable Micro Systems, Surrey, United Kingdom) was used to measure the mechanical properties of films. Strips of films (25 mm wide and 100 mm long) were mounted in the tensile grips (A/TG model) and stretched at a rate of 50 mm/min until breaking. The elastic modulus (EM), tensile strength at break (TS) and percentage of elongation at break (ε) were determined from stress-strain curves, obtained from force-deformation data. The experiments were carried out at 25ºC and eight replicates were made for each film, according to the ASTM D882 method (1992).

2. 3. 5. Barrier properties

The water vapour permeability (WVP) of the films was measured using a modification of the ASTM E96-95 gravimetric method (1995), using Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm diameter. For each formulation, the measurements were replicated four times and the WVP was calculated following the methodology described by Gennadios *et al.* (1994) at 25°C and a 53-100% RH gradient, which was generated using an oversaturated $Mg(NO_3)_2$ solution and pure distilled water, respectively. To determine the WVP, the cups were weighed periodically, using an analytical balance (±0.00001 g), until the steady state was reached (about 2 and 5 days for S and PLA films, respectively). The slope obtained from weight loss vs. time was used to calculate WVP, according to ASTM E96-95 (1995).

Oxygen permeability (OP) of the films was determined by using the ASTM F1927 (2014) method, using OXTRAN SL 1/50 MOCON equipment (U.S.A.). Measurements were taken in triplicate at 25ºC and 50% RH. Film thickness was considered in all samples to obtain the OP values.

2. 3. 6. Optical properties

The internal transmittance (T_i) of the films was determined by means of a spectrocolorimeter (CM-3600d, MinoltaCo., Tokyo, Japan) with a 10 mm diameter window. T_i was obtained as an indicator of the transparency of the films, by applying the Kubelka-Munk theory (Hutchings *et al.*, 1999) for multiple scattering to the reflection spectra, as described by Pastor *et al.* (2010). Four measurements per film formulation were carried out.

The gloss of the films was measured at a 60° incidence angle, using a flat surface gloss meter (Multi.Gloss 268, Minolta, Germany), according to the ASTM standard D523 (1999). Samples were measured over a black matte standard plate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a gloss value close to 100. Five replicates were made in three different films for each film formulation.

2. 3. 7. Thermal analysis

Differential scanning calorimetry analyses were carried out using a DSC (1 StareSystem, Mettler-Toledo, Inc., Switzerland). Samples (5–10 mg) were placed into aluminium pans (Seiko Instruments, P/N SSC000C008) and sealed. Samples were heated from room temperature to 200°C at 10 K/min in order to analyse phase transitions in the films. Samples were kept at 200°C for 5 min, cooled to -10°C at -50 K/min, kept at -10°C for 5 min and heated again to 200°C at 10 K/min; finally, they were cooled to 25°C at -100 K/min. An empty aluminium pan was used as reference. Each sample was analysed in duplicate.

A thermo-gravimetric analyser (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland) was used to characterize film thermal degradation. The analysis was performed from room temperature to 600°C at 10°C/min under a nitrogen flow (50 mL/min). The initial degradation temperature (T_{onset}), i.e. the temperature at which 5% mass loss is registered, was recorded. The temperature at which the maximum degradation rate was observed (T_{max}) was also determined. Each sample was analysed in duplicate.

2. 3. 8. X-Ray diffraction (XRD)

X-Ray diffraction analysis was performed on the films using a diffractometer (Bruker AXS/D8 Advance, Germany) at 40 kV and 40 mÅ . Scattered radiation was detected in an angular range of 5–50° (2θ), at a step size of 0.05.

2. 3. 9. Fourier Transform Infrared spectroscopy (FTIR)

The vibration mode of the chemical groups in the films was assessed through attenuated reflectance ATR-FTIR analysis (Bruker, Tensor 27, Germany) within a wave range of 4000-400 cm^{-1}. Samples were scanned 40 times with a resolution of 4 cm^{-1}. Analyses were performed in triplicate on monolayers and on both sides of bilayer films.

2. 3. 10. Molecular weight distribution in amorphous and semicrystalline PLA

In order to analyse differences in the properties of amorphous and semi-crystalline PLA, the polymer molecular weight distribution was analysed by using a Waters HPLC 1525 chromatograph equipped with a Waters 996 PDA detector by using a GPC column Tosoh Bioscience TSKGEL GMHHR-H. Polymer samples were dissolved at 0.5% in chloroform and eluted with this solvent at 1 mL/min. To obtain the calibration curve, polystyrene standards (TSK Standard, Tosoh Corporation, Japan) of known molecular weight were used. From the obtained chromatograms, Mw (weight average molecular weight), Mn (number average molecular weight), Mp (Molecular weight at the maximum peak) and PI (Polydispersity index) were determined.

2. 3. 11. Statistical analysis

StaTgraphics Plus for Windows 5.1 (Manugistics Corp., Rockville, MD, U.S.A.) was used to carry out statistical analyses of data through an analysis of variance (ANOVA). Fisher’s least significant difference (LSD) was used at the 95% level.

3. RESULTS AND DISCUSSIONS

3. 1. Film microstructure and cinnamaldehyde retention

The cross-section micrographs of monolayers and bilayers are showed in **Figures 1** and **2**, respectively. The starch micrograph did not show any granules, indicating the complete gelatinization of the polymer during thermal processing. Likewise, amorphous and semicrystalline PLA had a different appearance. Amorphous films exhibited the rubbery fracture, reflected in a higher frequency of stripes, whereas a rather brittle fracture was observed for semicrystalline PLA, exhibiting no plastic deformation. The incorporation of CIN to the PLA amorphous layer did not provoke appreciable structural changes.

In the bilayer films, the two different layers of amorphous PLA and starch or semicrystalline PLA are clearly appreciable, as is the good adhesion of respective monolayers. No layer detachment was observed for either S-M or PLA-M films, whether or not they contained CIN. This indicates a good compatibility of both S and semicrystalline PLA with the amorphous PLA which, in turn, showed a very different molecular weight distribution. Semicrystalline PLA had a monomodal distribution with Mp=134,778 D (Mw= 590,062 D, Mn= 43,997 D, PI: 13.4), whereas the amorphous polymer exhibited a bimodal distribution with Mp_1= 80,016 D and Mp_2= 220 D; the latter peak was attributed to the plastizicing fraction in the sample. Therefore, the amorphous polymer had lower molecular weight (Mw= 106,226 D, Mn= 58,432 D, PI: 1.8) and contained 40% of a low molecular weight fraction (Mw= 275 D, Mn= 54 D, PI: 5), which exerts the plastizicing effect, thus contributing to the amorphous nature of the polymer. This low molecular weight fraction could positively affect the good layer adhesion at the interface of the bilayer films.

Noticeable differences in the thickness of the layers can be observed in the micrographs for both mono and bilayer films, as also shown in **Table 1**. Every film with CIN was thinner than the corresponding CIN-free films. This difference points to the losses of the volatile compound both during the film preparation by casting and also during the thermocompression step of bilayers. In fact, a weight loss of 28.5% and 4.4%, respectively for starch and PLA bilayers with CIN was observed during thermocompression. A part of the mass loss of starch bilayers must be attributed to the water evaporation of starch conditioned sheet (11% water content). The thickness reduction in M_{cin} layer with respect to the M sample was about 9%, which would correspond to losses of about 45% in the incorporated CIN during the casting-drying process. The ethanol extraction of CIN from this film only yielded 11.7±0.8 g/100 g film, as opposed to the nominal concentration of 20%, which represents losses in the same range as those estimated from the thickness reduction.

Likewise, the thickness of both bilayer sheets was reduced during thermocompression to a different extent, depending on the film. In fact, the overall measured thickness for bilayers was lower than the theoretical value (sum of each layer thickness). Two factors may contribute to this reduction: 1) flow of the sheets under pressure and heat in every case and 2) the losses of CIN when it was present in the films. The thickness reduction of each sheet in bilayers was estimated from the FESEM micrographs. For S sheets, these reductions were 4 and 14%, when adhered to M and M_{cin} layers, respectively. For semicrystalline PLA sheets, these were 19 and 23% when adhered to M and M_{cin} layers, respectively. On the other hand, M and M_{cin} sheets were reduced by 40 and 64%, respectively, in S bilayers and by 13 and 32%, respectively, in PLA bilayers. This indicates that the sheet flow was higher in the amorphous PLA layer, with or without CIN, followed by the semicrystalline PLA layer. CIN incorporation promoted the flow of both S and semicrystalline PLA layers, which suggests the CIN diffusion into these matrices from M_{cin} produced a plastizicing effect. The great thickness reduction of the PLA amorphous layer without CIN indicates the great flowability of this plastiziced material. When it contained CIN, a greater thickness reduction was observed, due to both the enhancement of the material flowability by the CIN plasticization and to the additional losses of this compound during the thermocompression step. The greater reduction in M or M_{cin} thickness that occurred when in contact with the starch was remarkable (40%, without CIN), which points to additional plasticization efects associated with the migration of some compounds from the starch layer to the M layer. The ethanol- extracted CIN from S and PLA bilayers yields 2.7 and 4.0 g/100 g films, respectively. In contrast, the nominal CIN contents, estimated from the sheet weight ratio in the bilayer films, were 8.1 and 7.6 g/100 g film, respectively. This indicates that greater losses of CIN occurred in the starch bilayers during thermocompression, which can be attributed to the steam drag effect associated with the water evaporation in the conditioned starch layer.

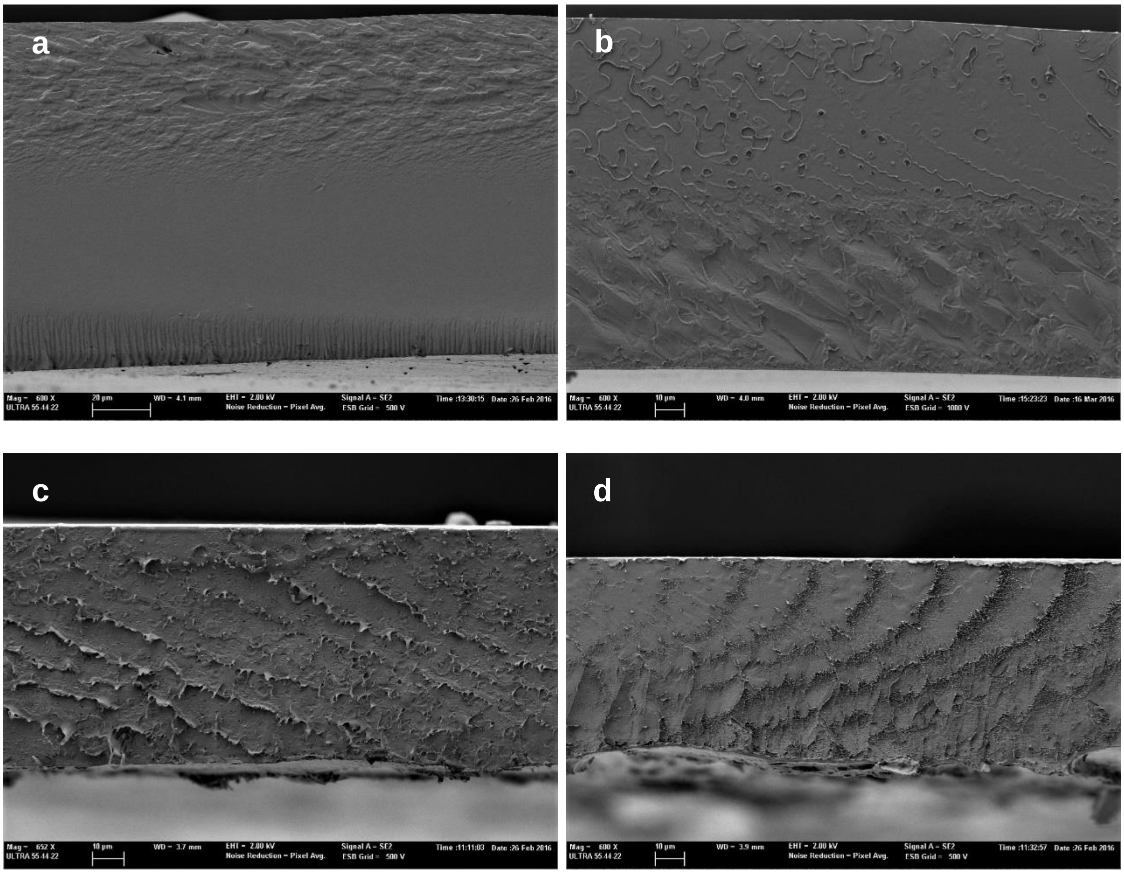


Figure 1. FESEM micrographs of cassava starch (S) (**a**) and PLA (**b**) monolayer films obtained by compression moulding and PLA films without (**c**) and with (**d**) cinnamaldehyde, obtained by the casting of ethyl acetate solutions.

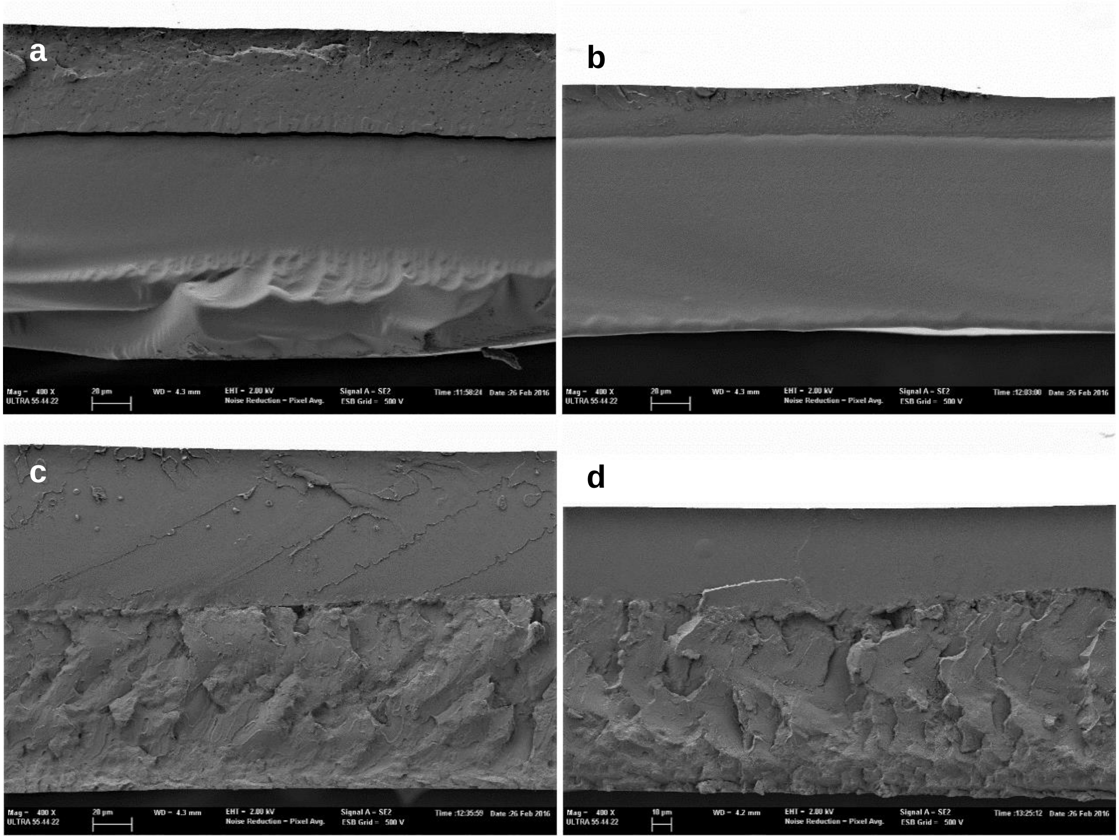


Figure 2. FESEM of the starch (top) and PLA (bottom) bilayer films without (left) and with cinnamaldehyde (right) obtained by compression moulding.

Monolayers M and M_{cin} appear at the top of bilayers.

3. 2. Physical properties

Table 1 shows the tensile parameters of starch and PLA mono and bilayers: tensile strength (TS), Young modulus (EM) and percentage of elongation at break (ε). It is noticeable that amorphous PLA (M) is much more extensible than crystalline PLA, with Young's modulus reduced by five. This different behaviour is mainly due to both the differences in the polymer's molecular weight and the plasticizer effect, previously commented on. The processing method may also affect the film tensile behaviour. Indeed, films obtained by casting are more stretchable than those that are thermo-processed. Different authors (Choi *et al.*, 2013; Coltelli *et al.*, 2008; Gui *et al.*, 2012; Pillin *et al.*, 2006) reported Young's modulus of 1300-3000 MPa for thermo-processed PLA as opposed to 600-950 MPa for cast PLA films (Sanyang *et al.*, 2016; Ahmed *et al.*, 2016b; Fortunati *et al.*, 2012 and 2015). Both amorphous and semicrystalline PLA were stiffer and more resistant to fracture than starch films, although the latter was more extensible than semicrystalline PLA. CIN incorporation in amorphous PLA caused a drop in both film stiffness and resistance to break in agreement with the weakening effect in the polymer matrix associated with the CIN interactions with the PLA chains, which limits the inter-chain forces.

When combining both types of PLA in bilayers, films exhibited a similar extensibility to semicrystalline PLA films, regardless of the presence of CIN, since this layer limits the extension capacity of bilayers. Nevertheless, the combination slightly reduced film stiffness, especially when CIN was present, while reducing resistance to break (by half) with respect to the semicrystalline PLA film. The greater reduction in the film stiffness when CIN was present could be due to the CIN diffusion into the semicrystalline layer, which weakened the PLA matrix by interfering with the chain associations. The reduction in the film's resistance to break (to a similar extent regardless of the presence of CIN) could be due to the rupture at the layer's interface level, where adhesion forces control the bilayer resistance.

For starch bilayers, a notable increase in the film stiffness was observed with respect to the starch monolayer when there was not CIN in the bilayer, with no significant changes induced in the resistance and elongation at break. Nevertheless, when M_{cin} bilayer was considered, no changes in tensile behaviour were observed with respect to the starch monolayer. This indicates that, with the presence of CIN, the starch-PLA bilayer was not notably reinforced, as compared to net starch layers. In general, CIN incorporation slightly promoted the films' extensibility, while reducing the film's stiffness in both mono and bilayers. This points to its plasticizing effect, as reported by Ahmed *et al.* (2016b).

Table 1. Thickness, tensile parameters (TS, EM, ε), water vapour (WVP: $m.g.h^{-1}.Pa^{-1}.m^{-2}$) and oxygen permeability ($cm^3.m^{-1}.s^{-1}.Pa^{-1}$), internal transmittance (T_i at 600 nm) and gloss (at 60° incidence angle) of films. Mean values ±standard deviation. In bilayers, the mean gloss values for both sides of the films are shown, since no significant differences were detected between faces.

Film	Thickness (mm)	TS (MPa)	EM (MPa)	ε (%)	WVP	OP x 10^{12}	T_i (%)	Gloss 60°
S	0.155 ±0.008 ^{d}	4.1 ±0.4 ^{ab}	51 ±13 ^{a}	75 ±7 ^{c}	10.2 ±0.2 ^{c}	0.123 ±0.017 ^{a}	86.00 ±0.05 ^{a}	29 ±1 ^{b}
PLA	0.141 ±0.012 ^{c}	49 ±6 ^{e}	1599 ±64 ^{e}	3.5 ±0.5 ^{a}	0.088 ±0.008 ^{a}	15 ±1 ^{b}	88.88 ±0.02 ^{f}	22 ±3 ^{a}
M	0.085 ±0.006 ^{b}	71 ±6 ^{f}	298 ±86 ^{b}	174 ±5 ^{d}	0.132 ±0.009 ^{a}	41 ±7 ^{c}	88.1 ±0.4 ^{e}	75 ±3 ^{d}
M_{cin}	0.077 ±0.013 ^{a}	44 ±5 ^{d}	11 ±3 ^{a}	184 ±3 ^{d}	0.122 ±0.013 ^{a}	3.8 ±0.2 ^{a}	87.7 ±0.3 ^{cd}	75 ±5 ^{d}
S-M	0.205 ±0.013 ^{g}	6.7 ±1.4 ^{b}	248 ±65 ^{b}	52 ±19 ^{b}	0.43 ±0.05 ^{b}	0.18 ±0.09 ^{a}	86.56 ±0.02 ^{b}	30 ±2 ^{bc}
S-M_{cin}	0.183 ±0.012 ^{e}	2.3 ±0.6 ^{a}	26 ±11 ^{a}	62 ±19 ^{b}	0.49 ±0.02 ^{b}	0.15 ±0.03 ^{a}	86.84 ±0.06 ^{b}	24 ±2 ^{a}
PLA-M	0.197 ±0.012 ^{f}	25 ±2 ^{c}	1487 ±44 ^{d}	1.9 ±0.3 ^{a}	0.097 ±0.009 ^{a}	4.0 ±0.7 ^{a}	87.4 ±0.2 ^{c}	32 ±2 ^{c}
PLA-M_{cin}	0.188 ±0.013 ^{e}	24 ±4 ^{c}	1211 ±52 ^{c}	2.3 ±0.5 ^{a}	0.061 ±0.006 ^{a}	4 ±2 ^{a}	87.9 ±0.2 ^{de}	31 ±2 ^{bc}

Different superscript letters within the same column indicate significant differences between formulations ($p<0.05$).

As concerns the barrier properties, starch monolayers showed the highest WVP and the lowest OP as reported by several authors (Souza *et al.*, 2013; Shirai *et al.*, 2013; Ortega-Toro *et al.*, 2014). Amorphous PLA films exhibited less barrier capacity than semicrystalline PLA, both for water vapour and oxygen; the latter was greatly enhanced by CIN incorporation. This effect may be due to the antioxidant properties of CIN which provide it with an oxygen scavenging capacity, thus reducing OP, as previously observed for other antioxidants included in polymer films (Bonilla *et al.*, 2013).

In starch bilayer films, WVP was greatly reduced with respect to starch films, as observed by Sanyang *et al.* (2016) for starch-PLA bilayers, while OP had similar values to those of the starch monolayer. In PLA bilayers, the WVP was similar to that of the semicrystalline PLA film, while OP was significantly reduced. The incorporation of CIN did not notably change the WVP or OP in the bilayer films, despite the observed OP decrease in M_{cin}. The obtained results showed a great improvement in the barrier properties of starch-PLA bilayers, which represented a 96% decrease in WVP with respect to the net starch films and a 99% decrease in OP with respect to the amorphous PLA films. Then, the assembly of starch and amorphous PLA exhibited very good barrier capacity for both water vapour and oxygen, which confers them adequate functionality for food packaging applications, meeting the requirements of a wide range of food products (Schmid *et al.*, 2012). Amorphous-semicrystalline PLA assembly exhibited good water vapour barrier capacity, but higher oxygen permeability. The incorporation of CIN into the amorphous PLA sheet in bilayers did not introduce WVP changes since it did not affect this property in M_{cin}; however, the oxygen scavenging effect observed in M_{cin} was not reflected in bilayers, probably due to the great reduction in OP induced by the PLA assembly. This could be explained by the formation of a less oxygen-permeable zone at the interface associated with strong polymer interactions, as observed in FESEM images.

As concerns the optical properties of the films, very similar values of Gloss and transparency were observed for the different mono and bilayers, except for cast PLA monolayers that exhibited higher gloss (**Table 1**). Gloss is directly related with the surface roughness of the films, whereas transparency, quantified by the internal transmittance (T_i), depends on the internal microstructure of the matrix and the distribution of the components, which produced more or less light scattering. The surfaced roughness can be greatly influenced by the film processing and composition. Indeed, semicrystalline PLA and starch films, obtained by compression moulding, had the lowest gloss value. In the case of monolayers, CIN incorporation did not affect the gloss as the films already had a very smooth surface. Nevertheless, this gloss was greatly reduced after thermocompression in bilayers, where no significant differences were observed between the gloss values on each side of the films.

The internal transmittance of the films was high in every case, between 86 and 89%, which reveals a high degree of transparency and homogeneity. CIN incorporation barely changed T_i in starch bilayers but slightly decreased it in monolayers, while it was noticeable that the M_{cin} films were more yellowish than M films. It was shown that crystalline PLA films obtained by compression moulding were the most transparent, with a T_i comparable to that of synthetic polymers, such as poly(ethylene terephthalate) (PET) and poly(styrene) (PS) (Auras *et al.*, 2004). As for the PLA bilayers, a slight decrease in the film transparency was noticeable in comparison with the monolayers, coinciding with their greater film thickness.

3. 3. Thermal properties

Figure 3 shows the DSC thermograms of the different PLA-based films during the second heating scan. The absence of crystallization and melting in M and M_{cin} films is noticeable, which is due to the amorphous character of the polymer, only exhibiting glass transition. Nevertheless, for semicrystalline PLA films in both mono and bilayers, a crystallization exotherm was observed after the glass transition, followed by the subsequent melting at about 170°C, depending on the film. An excess enthalpy of relaxation (ΔH_{ex}) during the glass transition was also observed, as reported by other authors (Cai *et al.*, 1996; Acioli-Moura and Sun, 2008). Likewise, another small exotherm prior to the melting endotherm appeared in mono and bilayer semicrystalline PLA films. This is associated with the α'-α phase transition at ~160-165°C, as reported by several authors (Muller *et al.*, 2016; Yasuniwa *et al.*, 2008).

Table 2 shows the temperature and enthalpy of the different thermal events observed in every mono and bilayers during the second heating step, where the previous thermal history of the samples has been deleted and all samples were submitted to the same heating-cooling cycles. The glass transition temperature (T_g) of starch was in the range previously reported by other authors (Ortega-Toro *et al.*, 2015) and it decreased ($\Delta T_g \approx 27°C$) in S-M_{cin} bilayers, thus indicating the CIN diffusion into the starch layer and its plasticizing effect, as previously pointed out from FESEM observations. This CIN plasticizing effect was also observed in amorphous PLA monolayer ($\Delta T_g \approx 7°C$) and in PLA bilayers ($\Delta T_g \approx 16°C$). A similar effect of CIN was observed by Ahmed *et al.* (2016a) in PLA/PEG blend films and by Qin *et al.* (2015) in PLA/poly(trimethylene carbonate) films. Semicrystalline PLA had a higher T_g value than the amorphous one, according to the higher molecular weight of the semicrystalline product. However, in PLA bilayers, only one glass transition was appreciated to be closer to that of the semicrystalline fraction, according to its higher mass fraction in bilayer films and the potential diffusion of the low molecular plasticizing fraction of the amorphous product, which could also plasticize the semicrystalline layer. Indeed, the crystallization of PLA was also affected by its assembly with the amorphous PLA monolayer, mainly when this contained CIN. The crystallization temperature (T_c) drops by about 3 and 14°C in PLA bilayers without and with CIN, respectively, while the crystallinity degree (X_c) of PLA in the films decreased from about 27 to 8 and 5% in PLA bilayers, without and with CIN respectively. This behaviour also agrees with a greater plasticization of semicrystalline PLA in bilayer films due to the diffusion of either low molecular compounds from amorphous PLA layer or CIN. After glass transition, the decrease in T_c during the heating step of bilayers points to a faster crystallization, due to the fact that there is greater molecular mobility in the polymer in the more plasticized system. However, the melting behaviour of PLA in bilayers points to a less cohesive crystalline zone (lower T_m and ΔH_m), which suggests that the migrated compounds from the amorphous layer interfered with the crystalline arrangement, leading to an apparently lower degree of crystallinity (X_c) in the semicrystalline layers.

Thus, the thermal analysis of the films revealed that CIN diffuses into the starch and PLA sheets in bilayer films, significantly decreasing the T_g of both starch and semicrystalline PLA and also enhancing the PLA crystallization kinetics (lower T_c), but reducing the final degree of crystallinity in the semicrystalline layer.

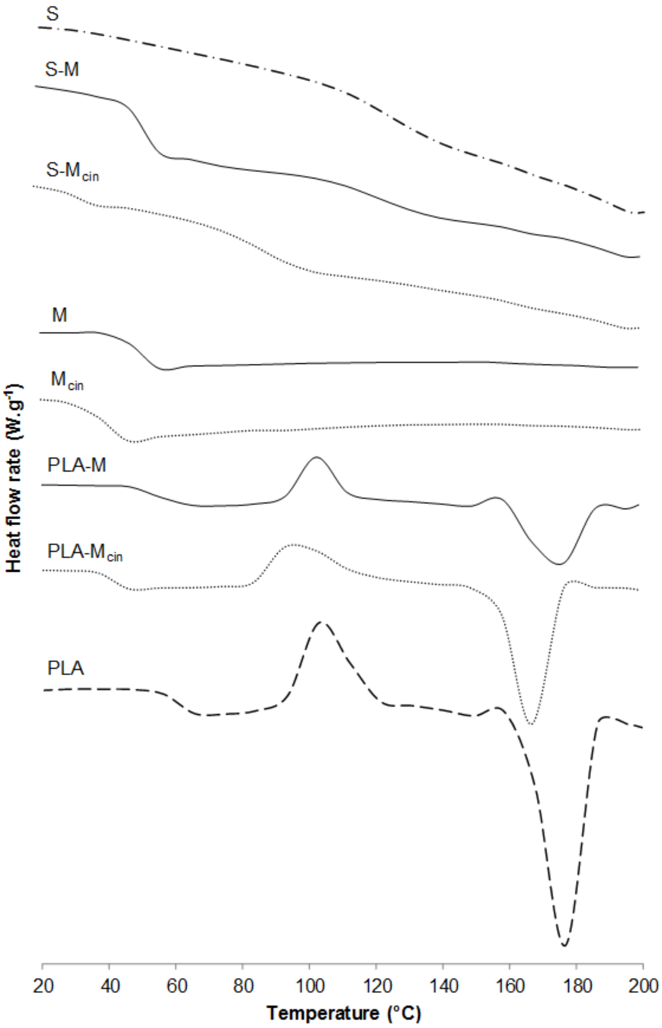


Figure 3. DSC thermograms of monolayers (S, PLA, M, M$_{cin}$) and bilayers (S-M, S-M$_{cin}$, PLA-M, PLA-M$_{cin}$) from the second heating step.

Table 2. Thermal properties of films obtained by DSC (second heating scan). Mean values ±standard deviation. PLA enthalpy values are expressed per g of semicrystalline PLA in bilayers.

Film	T_g S (°C)	T_g PLA (°C)	ΔH_{ex} (J/g PLA)	T_c (°C)	ΔH_c (J/g PLA)	T_m (°C)	ΔH_m (J/g PLA)	X_c (%)
S	120.4 ±0.3 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PLA	n.d.	58.7 ±0.2 ^{d}	0.28 ±0.08 ^{a}	107.0 ±0.2 ^{b}	31 ±1 ^{c}	174.8 ±0.2 ^{b}	57.1 ±0.4 ^{b}	27 ±1 ^{b}
M	n.d.	44 ±5 ^{b}	0.8 ±0.3 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
M_{cin}	n.d.	37 ±1 ^{ab}	0.8 ±0.2 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
S-M	123 ±3 ^{b}	50.62 ±0.06 ^{c}	0.22 ±0.02 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.
S-M_{cin}	93 ±3 ^{a}	31 ±1 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PLA-M	n.d.	55.34 ±0.04 ^{cd}	0.14 ±0.02 ^{a}	103.8 ±0.2 ^{b}	13 ±1 ^{a}	174.6 ±0.4 ^{b}	20.8 ±0.5 ^{a}	8 ±1 ^{a}
PLA-M_{cin}	n.d.	39 ±5 ^{b}	0.24 ±0.05 ^{a}	93 ±3 ^{a}	18.6 ±0.2 ^{b}	165 ±4 ^{a}	24 ±2 ^{a}	5 ±2 ^{a}

$X_c = (\Delta H_m - \Delta H_c) / \Delta H_0$; with ΔH_0 = 96 J/g crystalline PLA (value from Kalish *et al.* (2011)

n.d.: Non-detected

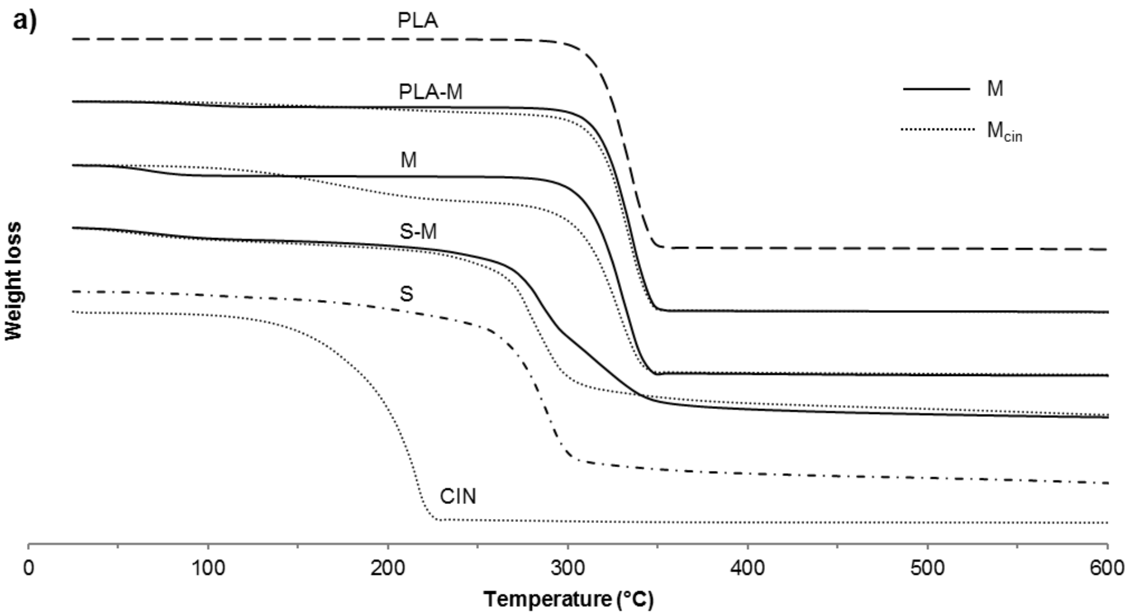
Different superscript letters within the same column indicate significant differences between formulations ($p<0.05$).

Thermogravimetric analysis (TGA) also revealed differences in polymer thermodegradation associated with the compression moulding of bilayers and the presence of CIN. **Table 4** presents the onset (T_{onset}) and maximum degradation rate (T_{max}) temperature values of films, while the TGA curves from 25 to 600°C are shown in **Figure 4.a)**.The T_{onset} of semicrystalline PLA was found to be around 320°C, with a total weight loss in a single step (maximum at 333°C), as observed by Muller *et al.* (2016), this polymer being the most thermally stable of the studied matrices. In all of the other films, two weight losses could be observed. The first one corresponds to water (in the case of S mono and bilayers) and/or CIN losses (in the films carrying the active compound), whereas the second most intense weight loss, occurring in a temperature range of 264-320°C (depending on the film), corresponds to the polymer matrix decomposition. A slight weight loss (5.2%) is also noticeable between 40 and 85°C in the amorphous PLA film (M), which can be associated with the degradation/vaporization of some molecular weight compounds present in the polymer, which were delayed in the M_{cin} films (~100°C), thus indicating interactions between CIN and these compounds. In fact, a DSC analysis of M_{cin} exhibited a small, broad exothermic peak between 70-200°C, which could be associated with the enthalpy reaction of CIN with some low molecular weight compounds present in the amorphous PLA. This peak did not appear in the second heating scan nor in bilayer films containing CIN, because the previous thermal treatment would already have promoted this reaction. In every case, the incorporation of CIN led to a progressive, slow weight loss from about 120°C, which was more marked in M_{cin} samples with the highest mass fraction of CIN. This also affected the T_{onset} of films, with PLA monolayers exhibiting the most significant difference, from 316 to 305°C. Starch films were the least thermally stable in comparison with amorphous and semicrystalline PLA; the degradation step took place between 275 and 310°C, as reported by other authors (Jaramillo *et al.*, 2016; Sanyang *et al.*, 2016). Then, due to starch being less thermally stable, the bilayers showed lower T_{onset} (268°C) and exhibited two degradation steps, as shown more clearly in **Figure 4.b)**, through the first derivative of the TGA curves. S-M bilayers presented two well-defined peaks, related to the first degradation of the starch fraction and the subsequent degradation of the PLA fraction. However, when CIN was present, the second degradation step appeared as a shoulder, according to the lower ratio of the PLA layer, as deduced from FESEM images. No different steps were observed either for amorphous or semicrystalline PLA bilayers in line with the similar degradation behaviour of both amorphous and semicrystalline PLA.

Table 3. Thermal degradation temperatures (onset value T_{onset} and value at maximum degradation rate T_{max}) of the different films and of cinnamaldehyde (CIN) obtained by TGA. Mean values ±standard deviation.

Film	**T_{onset} (°C)**	**T_{max} (°C)**	**Weight loss (%)**
S	275 ±3 ^{c}	285.0 ±0.2 ^{bc}	91 ±3 ^{a}
PLA	320.0 ±0.3 ^{ef}	333.0 ±0.2 ^{f}	100.0 ±0.0 ^{b}
M	316 ±2 ^{e}	329.7 ±0.5 ^{de}	100.0 ±0.0 ^{b}
M_{cin}	208 ±3 ^{a}	217.9 ±0.2 ^{a}	100.0 ±0.0 ^{b}
CIN	305 ±3 ^{d}	328 ±2 ^{d}	100.0 ±0.0 ^{b}
S-M	268 ±2 ^{b}	286.8 ±0.8 ^{c}	90.4 ±0.5 ^{a}
S-M_{cin}	264 ±2 ^{b}	284 ±2 ^{b}	89.8 ±0.9 ^{a}
PLA-M	320.1 ±0.5 ^{f}	335.3 ±0.4 ^{g}	100.0 ±0.0 ^{b}
PLA-M_{cin}	316.4 ±0.9 ^{ef}	331.5 ±0.9 ^{ef}	100.0 ±0.0 ^{b}

Different superscript letters within the same column indicate significant differences between formulations ($p<0.05$).



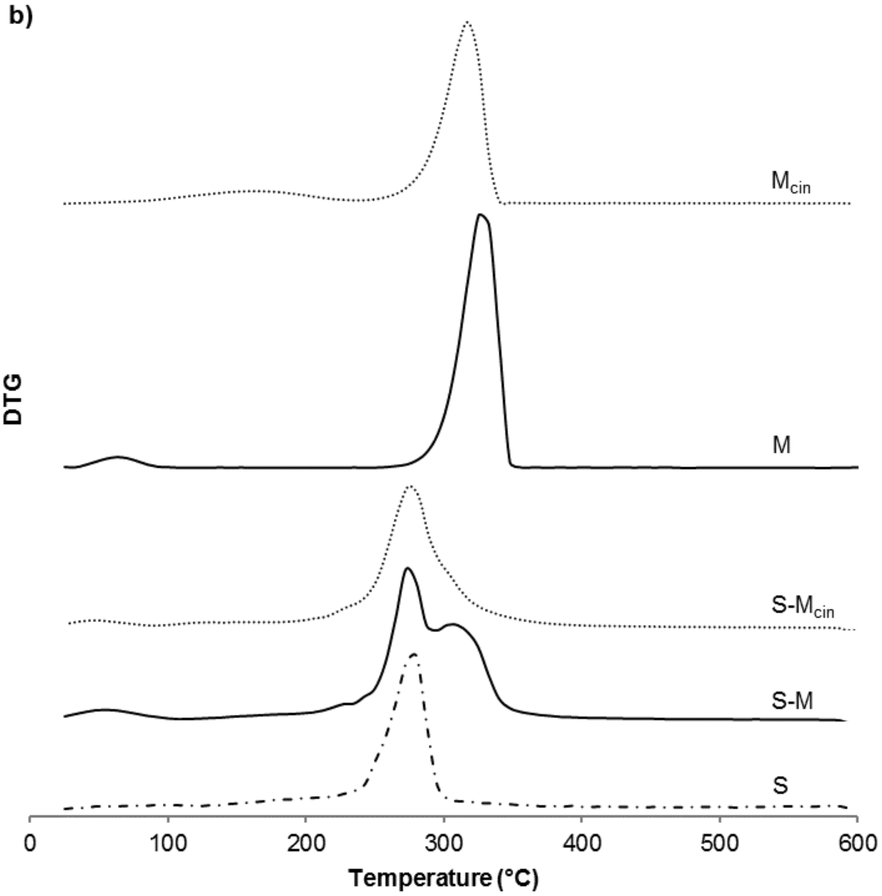


Figure 4. a) Weight loss curves of mono and bilayer films with (dashed lines) and without CIN (continuous line) from TGA analysis, from 25 to 600°C, also showing the pure CIN thermal weight loss. **b)** Derivative curves of starch (S) and amorphous PLA (M) mono and bilayer films with (M_{cin}; S-M_{cin}) and without (S, M; S-M) cinnamaldehyde.

3. 4. Crystallization pattern in the films by X-Ray diffraction analysis

Figure 5 shows the XRD patterns of film samples. The absence of crystalline peaks for amorphous PLA films is remarkable, regardless of the incorporation of CIN (M, M_{cin}), as observed by Gorrasi *et al.* (2013) for amorphous PLA. On the other hand, semicrystalline PLA (PLA) showed the characteristic sharp peak at 2θ = 17°, associated with the helical 10_3 chain of PLA (α-form crystals) (Hughes *et al.*, 2012). On the contrary, neat starch (S) presented a large amorphous scattering background with small diffraction peaks at 2θ = 12, 13.5, 18.3, 20 and 20.7°, characteristic of a semicrystalline polymer with a low degree of crystallinity. In thermoplastic corn starch with glycerol, Shi *et al.* (2007) found two peaks at 13.5 and 20.9° and assigned them to a V-type structure, which is formed by the crystallization of amylose in single helices involving glycerol or lipids. It can be divided into two subtypes, named V_a (anhydrous) with peaks at 13.2° and 20.6° and V_h (hydrated) with peaks at 12.6° and 19.4° (Corradini *et al.*, 2007). Yang *et al.* (2006) stressed that V_h-style crystallinity is induced by thermal processing where the strong interaction between hydroxyl groups of starch molecules was substituted by the hydrogen bonds formed between the plasticizer and starch during thermoplastic processing. The peak at 18° is associated with the intermediate form between V_h and V_a crystallinity (hydrated and anhydrous lattices, respectively), according to Zobel *et al.* (1967). So, for the obtained thermo-processed starch films, both V_h and V_a crystallinity was observed. The melting of the crystalline starch fraction was observed in the first heating scan of DSC between 110-200°C as a broad endotherm with a temperature peak at about 150°C, as reported by López *et al.* (2014), coherent with the coexistence of several crystalline forms.

In the X-Ray diffractogram of starch bilayers, a similar dispersion band as that observed in the starch monolayer can be seen, indicating a similar crystallization pattern for the polymer regardless of the adhesion to the amorphous PLA sheet. XRD patterns of PLA bilayers exhibited some difference with respect to semicrystalline PLA, indicating the presence of different crystalline phases. Indeed, a new peak at 2θ = 19° was recorded; revealing the presence of α'-form crystals (Kalish *et al.*, 2011). This peak showed lower intensity in the PLA-M_{cin} film, while the amorphous response was intensified. These results suggest that the diffusion of low molecular weight compounds or CIN from the amorphous PLA layer to the semicrystalline PLA during thermocompression slightly modified the crystallization pattern of PLA due to the promotion of chain interactions with these compounds. Thus, more unstable crystal forms (α') or crystallization inhibition was observed in PLA bilayers as deduced from the crystallization enthalpy values obtained by DSC.

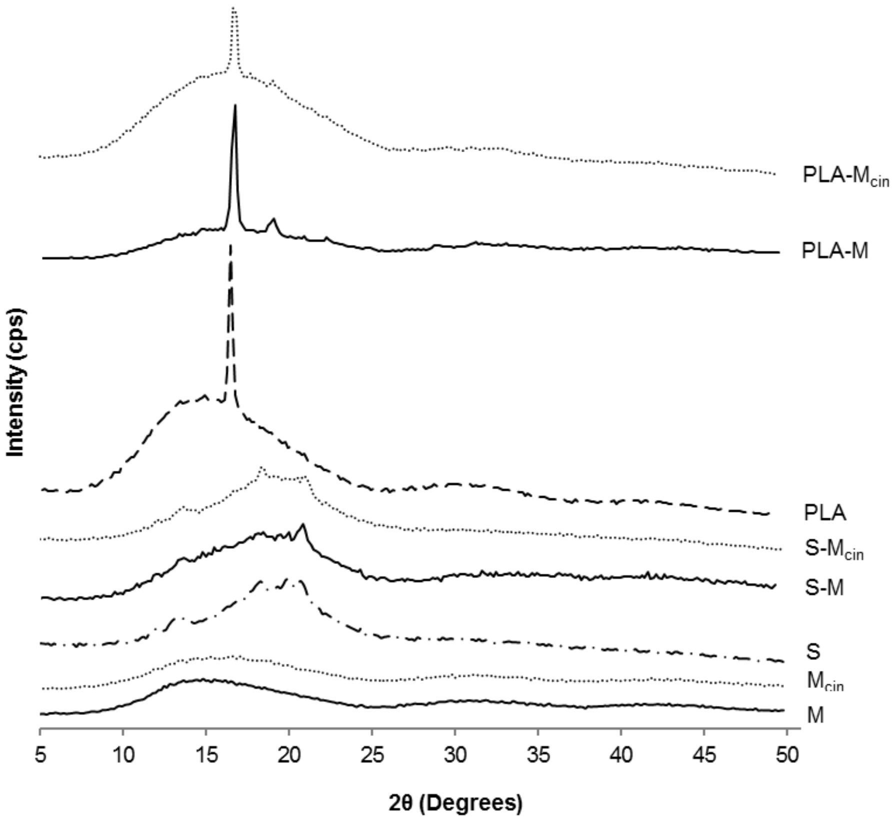


Figure 5. X-Ray Diffraction patterns of starch and PLA mono and bilayer films, with and without cinnamaldehyde.

3. 5. FTIR spectroscopy

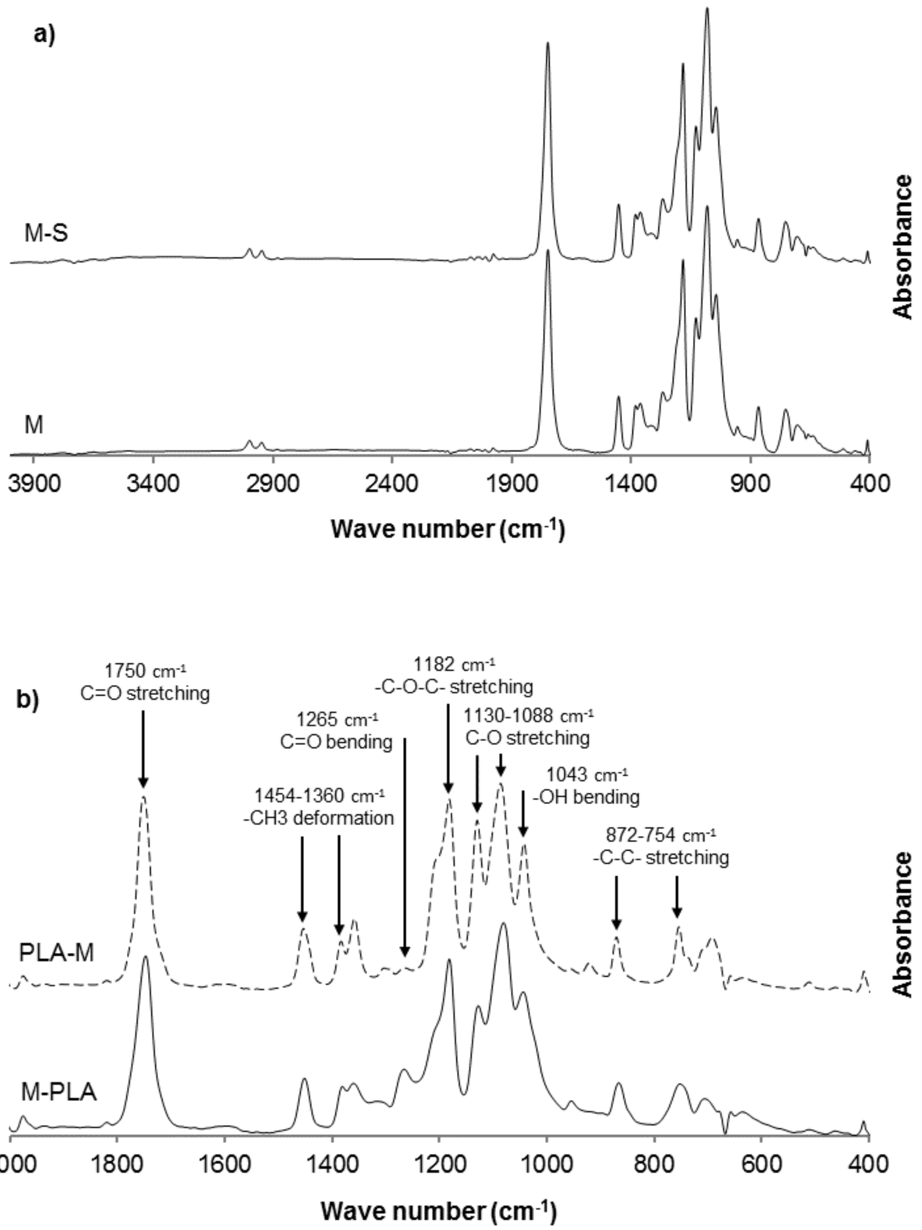
FTIR analysis was carried out on the films in order to identify interactions between film components, especially when CIN was incorporated. First, it is noticeable that the FTIR patterns of the different replicates of each film formulation exhibited great reproducibility, which indicates a high degree of homogeneity in the films. **Figure 6** shows the different spectra, adequately grouped to better analyse the different features. The total correspondence of the spectra of M and M-S (obtained from the M layer) samples is remarkable (**Figure 6.a)**), revealing no changes at molecular level in amorphous PLA during compression moulding with the starch layer. This points to the absence of S-PLA grafting at the interface, although this process would only involve molecules at the interface, which could not give enough signal intensity. All of the characteristic peaks of PLA were observed in amorphous and semicrystalline PLA spectra in both monolayers (**Figure 6.a)**) and bilayers (**Figure 6.b)**). The main peaks correspond to the -C=O stretching vibration of the ester group of the polymer (1750 cm^{-1}), the asymmetric and symmetric $-CH_3$ deformation vibrations (1454 cm^{-1} and 1360 cm^{-1}, respectively), the C=O bending (1265 cm^{-1}), the -C-O-C- stretching of the ester groups (1182 cm^{-1}), the C-O stretching (1130 and 1088 cm^{-1}) and the -OH bending (1043 cm^{-1}). A peak at

872 cm^{-1}, ascribed to the -C-C- stretching of the amorphous phase was observed in both PLA films. Nonetheless, the peak at 754 cm^{-1}, associated with the -C-C- stretching in the crystalline phase was only seen in the semicrystalline PLA spectrum.

Figure 6.c) shows the FTIR spectra of starch bilayer films from both starch (S-M) and amorphous PLA (M-S) layers. The starch layer spectrum is dominated by a broad band at 3300 cm^{-1}, which is assigned to the stretching vibration modes of -OH groups belonging to starch, glycerol and adsorbed water (Fama *et al.*, 2010). Other characteristic bands of starch can be identified at 2928 cm^{-1} (C-H stretching), 1650 cm^{-1} (-OH bending) and 1078-930 cm^{-1} (C-O stretching), while the peaks at 854, 760 and 570 cm^{-1} are assigned to swing vibrational absorption peaks of the C-H bond of starch (Castillo *et al.*, 2013; Hu *et al.*, 2013). The characteristic absorption peaks of starch were not modified when starch was adhered to M or M_{cin} layers. This indicates that the potential interactions between starch and PLA chains at the interface are not revealed in the starch FTIR spectrum, nor did the CIN diffusion into the starch sheet affected the vibration modes of its chain chemical bonds.

As concerns the active layers, **Figure 6.d)** presents the spectra of the amorphous PLA monolayer (M) together with the spectra of M_{cin} samples from both mono (M_{cin}) and bilayers (M_{cin}-S and M_{cin}-PLA). The spectrum of M_{cin} films showed the characteristic peaks of CIN at 1680 and 1630 cm^{-1}, associated with the stretching vibration of the aldehyde carbonyl group (C=O) and the unsaturated vibration peak of the benzene ring, respectively, as observed by other authors (Martínez-Abad *et al.*, 2013; Wen *et al.*, 2016). Another weak intensity peak was seen at 1522 cm^{-1}, corresponding to the aromatic domain of CIN (Ahmed *et al.*, 2016a). The same peaks also appeared in M_{cin}-PLA and M_{cin}-S films, with no change in their intensity, revealing that CIN was efficiently included into the bilayers through the amorphous PLA sheet. No significant peak displacement was observed for CIN signals in the films with respect to the pure compound (as described by other authors, Martinez-Abad *et al.* (2013) and Wen *et al.* (2016), which indicates that CIN interactions with the polymer are not intense enough to modify the molecular group vibration pattern. Then, Van der Waals and hydrophobic interactions between CIN and PLA chains could be assumed.

The FTIR spectra of the films revealed the major presence of CIN in the amorphous PLA layer in which it was initially included and, although the diffusion of this compound into the starch or semicrystalline PLA sheets could occur, it was not quantitative enough to be evidenced through the reflectance FTIR spectra of the assembled layer. Likewise, the interactions between PLA chains and CIN were not intense enough to modify the typical vibration modes of the respective molecular bonds.


a)
M-S
M
Absorbance
3900
3400
2900
2400
1900
1400
900
400
Wave number (cm-1)
b)
1750 cm-1
C=O stretching
1182 cm-1
-C-O-C- stretching
1265 cm-1
C=O bending
1130-1088 cm-1
C-O stretching
1043 cm-1
-OH bending
1454-1360 cm-1
-CH3 deformation
872-754 cm-1
-C-C- stretching
PLA-M
M-PLA
Absorbance
2000
1800
1600
1400
1200
1000
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600
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Wave number (cm-1)

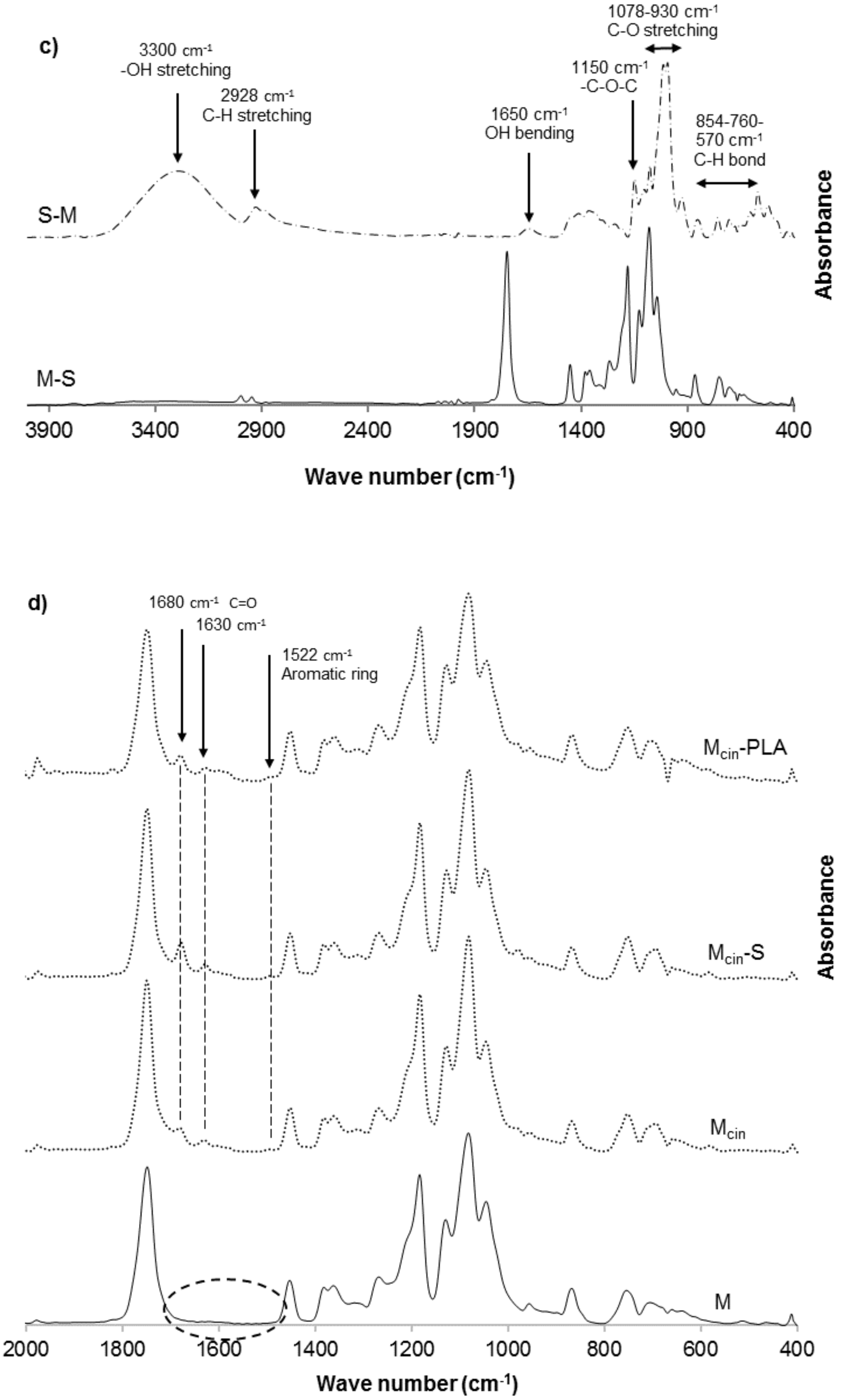

Figure 6. FTIR spectra of film samples **a)** Amorphous cast PLA (M) and starch bilayer films (M-S) from the M layer. **b)** PLA bilayer films from both semicrystalline (PLA-M) and amorphous (M-PLA) layers. **c)** Starch bilayer films from both starch (S-M) and amorphous PLA (M-S) layers. **d)** Amorphous PLA films with and without CIN in monolayers (M and M_{cin}) and with CIN in bilayers (M_{cin}-S and M_{cin}-PLA).

4. CONCLUSION

Amorphous PLA-starch bilayer films were successfully obtained by compression moulding of cast PLA layers containing or not cinnamaldehyde, and thermoplastic starch films, with very good adhesion at the interface. Despite the lower ratio of PLA sheet in the bilayer assembly (1/3 of the film thickness), a great improvement in the tensile and water vapour barrier properties was achieved with respect to the net starch films, the films maintaining high transparency and an oxygen permeability as low as that of net starch films. When cinnamaldehyde was included in the cast PLA sheet, films became thinner due to the losses of the volatile active during processing. Nevertheless, the improvement in the barrier properties was maintained but films exhibited lower mechanical resistance. The fitting of the cast layer thickness using a constant polymer surface density (g polymer/cm^2) could mitigate this problem. PLA bilayers also exhibited lower oxygen permeability than the respective PLA monolayers, which could be associated with the good interfacial adhesion that created additional barrier efficiency. Thermal analysis revealed the diffusion of cinnamaldehyde or low molecular weight compounds from the cast PLA layer to the other sheets (starch or semicrystalline PLA) which contributed to plasticizing the amorphous regions and affected the crystallization pattern of PLA, as also revealed by the X-Ray diffraction analyses. Nevertheless, FTIR spectra did not evidence quantitative amounts of the active in these layers, probably due to the high dilution level of active in these thicker sheets of bilayer. The obtained results offer an interesting option to obtain high barrier-highly resistant active films from thermoplastic starch and amorphous PLA, including cinnamaldehyde in the ethyl acetate solution of the polyester; this solvent being food compatible, which reduces the risks associated with solvent residues and allows for the cold dissolving of amorphous PLA.

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CHAPTER III

Antimicrobial properties and release of cinnamaldehyde in bilayer films based on polylactic acid (PLA) and starch

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ABSTRACT

Cinnamaldehyde (CIN) loaded amorphous PLA films were obtained by casting, using ethyl acetate as solvent. Likewise, bilayer films were obtained by thermocompression of the PLA active layer and compression moulded cassava starch (S) films or semicrystalline PLA films. Starch-PLA laminated materials were considered to improve the barrier capacity (high oxygen barrier through the starch layer and high water vapour capacity through the polyester layer), while CIN incorporation confers antimicrobial activity on the films. The PLA bilayers were obtained for comparison purposes. The antimicrobial activity of the CIN loaded PLA films and S bilayer films was proved against *Escherichia coli* and *Listeria Innocua* through *in vitro* tests, which indicates that the active amount released into the growth medium exceeded the minimum inhibitory concentration (MIC) of both bacteria. The release kinetics of the active compound in different food simulants demonstrated that a part of CIN was tightly bonded to the PLA matrix, whereas the free compound diffused more easily through the starch layer, making S bilayers more active against the bacteria when the starch layer was in direct contact with the culture medium. CIN entrapped in PLA bilayers did not exhibited any antibacterial effect due to its release inhibition, associated to its bonding within the PLA matrix and the lower degree of relaxation in the semicrystalline PLA layer in contact with the food simulants.

Key words: PLA, cassava starch, cinnamaldehyde, compression moulding, antimicrobial activity, release kinetics.

1. INTRODUCTION

Antimicrobial biodegradable packaging materials are of great interest for the food industry and consumers not only as a means of enhancing food preservation and shelf life extension but also for the purposes of reducing the environmental problems caused by synthetic plastic packaging. Natural, non-toxic antimicrobials were also required in order to enhance the product health properties, avoiding the toxicity of other antimicrobials. Therefore, the development of biodegradable antimicrobial materials for food packaging applications, using natural antimicrobial substances, such as essential oils or their active compounds, is a current alternative for food preservation and shelf life extension (Corrales *et al.*, 2014: Lanciotti *et al.*, 2004).

Cinnamaldehyde, one of the major constituents of cinnamon bark oil (~ 60-90%) (Kim *et al.*, 2015; Parthasarathy *et al.*, 2008) is a phenolic terpenoid classified as GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration), with high antibacterial, antifungal, antiinflammatory and antioxidant activity. It is widely used in cosmetic, food and pharmaceutical industries since it is a natural antimicrobial substance (Sun *et al.*, 2015; Wang *et al.*, 2016; Xing *et al.*, 2014). Different studies analysed the effects of CIN on the bacterial membranes, showing that CIN permeabilizes the internal membranes of different bacteria, such as *E. coli* and *Staphylococcus aureus* (Shen *et al.*, 2015) or *Staphylococcus epidermidis* and *Enterococcus* (Hammer and Heel, 2012), altering their structure. It has also been reported that CIN is an inhibitor of β-(1,3) glucan synthase and quitin synthase 1, which are important during the synthesis of enzymes in the cell wall of yeasts and moulds (Bang *et al.*, 2000).

Several studies have demonstrated that CIN could be potentially used, in different concentrations, as an effective antimicrobial agent. This was observed for *E. coli* O157: H7 in apple juice and cider (Baskaran *et al.*, 2010), for *Enterobacter sakazakii* in reconstituted milks for breastfed babies (Amalaradjou *et al.*, 2009), for *E. coli* in bovine meat pasties (Amalaradjou *et al.*, 2010), for *Salmonella spp* in tomato washing (alternative to sodium hypochlorite) (Mattson *et al.*, 2010) or for a mix of strains of bacteriophage and *E. coli* O157:H7 in leafy green vegetables (Viazis *et al.*, 2011). Cinnamon essential oil or CIN also caused the inhibition of the growth of psychotropic strains of *Bacillus cereus* (EPSO-35As y el INRA TZ415) in minimally processed products, such as carrot (Hernández-Herrero *et al.*, 2008). CIN has also been incorporated into different polymer matrices to obtain antimicrobial packaging materials or food coatings. Polymers, such as cellulose (Sanla-Ead *et al.*, 2012), pectin (Otoni *et al.*, 2014), PLA (Ahmed *et al.*, 2016; Makwana *et al.*, 2015; Qin *et al.*, 2015), starch (Souza *et al.*, 2013), proteins (Balaguer *et al.*, 2013) or alginates (Rojas-Graü *et al.*, 2007), have been used as CIN carriers. Studies about the antimicrobial effect of the active films, *in vitro* as well as in food matrices, showed antimicrobial activity against different microorganisms. Nevertheless, the antimicrobial action depended not only on the active compound and microorganism, but also on the effective release of antimicrobial compounds onto the contaminated product where microbial growth occurs.

Both thermoplastic starch and PLA are biodegradable polymers with easy availability and relatively low cost whose bilayer combination could enhance the film functional properties, taking advantage of their complementary barrier capacity. Starch exhibits very good barrier capacity to oxygen (Ortega-Toro *et al.*, 2015) and PLA to water vapour (Muller *et al.*, 2016). Likewise, mechanical resistance of PLA is higher than that of starch, while being more brittle, and their combination could improve the film mechanical behaviour (Muller *et al.*, 2016). On the other hand, due to its highly hydrophilic nature, starch film properties greatly change in contact with high moisture foods and its application onto a more hydrophobic layer, such as a PLA layer, could improve its performance as a food coating. Sanyang *et al.* (2016) characterized sugar palm starch-PLA bilayers obtained by casting, and found that the films exhibit reduced water vapour permeability and enhanced mechanical properties in comparison with starch films. The incorporation of an active compound into starch-PLA bilayers could, moreover allow an active, biodegradable food packaging material with optimised functional properties to be obtained.

The objective of this study was to obtain bilayer films with PLA and starch, incorporating cinnamaldehyde as antimicrobial compound into the PLA layer prepared by casting, analysing both the release kinetics of CIN in different food simulants and the *in vitro* antimicrobial properties against Gram+ (*E. coli*) and Gram- (*L. innocua*) bacteria. For comparison purposes, the study of these properties in CIN loaded monolayers and PLA-CIN loaded PLA bilayers was also carried out.

2. MATERIALS AND METHODS

2. 1. Materials

PLA resins: Crystalline PLA LL700, density of 1.25 g/cm^3, purchased from Ercros (Spain) and amorphous PLA 4060D, density of 1.24 g/cm^3, for heat seal layer in coextruded oriented films, purchased from Natureworks (U.S.A) were used to obtain films by compression moulding and casting, respectively. Cassava starch was produced by Asia CO., LDT (Kalasin, Thailand) and purchased from Quimidroga S.A. (Barcelona, Spain) and glycerol, used as a plasticizer for starch, was provided by Panreac Química SLU (Castellar del Vallés, Barcelona, España). Ethyl acetate, used as solvent for amorphous PLA, was obtained from Indukern S.A. (Barcelona, Spain) and trans-cinnamaldehyde and Poly(ethylene glycol) (PEG 4000 g/mol) were provided by Sigma-Aldrich (Madrid, Spain). Magnesium nitrate ($Mg(NO_3)_2$), used so starch films reach a relative humidity (RH) of 53%, was supplied by Vidra Foc S.A. (Barcelona, Spain) and phosphorus pentoxide (P_2O_5), used to generate 0% RH, was purchased from Panreac (Barcelona). As regards the quantification and kinetics release of cinnamaldehyde, pure methanol, ethanol, acetic acid and isooctane, UV-IR-HPLC quality, were obtained from Panreac (Barcelona, Spain). For the microbiological analysis, stock cultures of *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) were purchased from the *Colección Española de cultivos tipo* (CECT; Valencia, España) and culture media: Tryptic Soy Broth (TSB), Trypcasein Soy Agar (TSA), buffered peptone Water ISO were supplied by Scharlau (Barcelona, España).

2. 2. Film preparation

Monolayer films of starch and PLA, with or without cinnamaldehyde, were obtained and combined to obtain S-PLA and PLA-PLA bilayer films.

2. 2. 1. PLA monolayers obtained by casting (M and M_{cin})

Amorphous PLA pellets were used to obtain active PLA monolayers with CIN. Films were prepared by means of the solvent casting procedure after the preparation of film forming dispersions. PLA pellets were first dried at 60°C overnight to remove residual water and then dissolved in ethyl acetate at 10% (w/w), for 4 hours while being stirred. CIN-free layers (M) were obtained as control films and, in the case of active layers (M_{cin}), CIN was dispersed at 2.5%, which means a PLA:CIN ratio of 10:2.5. The resulting dispersions were poured into Teflon plates of 15 cm diameter at a surface density of solids of 1.5 g/plate and the solvent was evaporated overnight. Consequently, dried films were peeled off the casting surface and stored in a desiccator with silica gel (SiO_2) at room temperature until further analysis or use to obtain bilayer films. Then, a nominal concentration of CIN of 20 g/100 g of dried film can be assumed.

2. 2. 2. PLA monolayers obtained by compression moulding (PLA)

Crystalline PLA pellets were first dried at 60°C overnight and then hot-mixed in a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 200°C and 10 rpm for 10 min. The resulting paste was then compression moulded in a hydraulic press (Model LP20, Labtech Engineering, Thailand). 3 g of sample were placed onto steel sheets and pre-heated at 200°C for 4 min. Then, compression was performed at 200°C and 100 bars for 4 min, followed by a cooling cycle of 3 min until the temperature reached about 70°C. The obtained films were maintained in a desiccator with SiO_2 at room temperature until their use to obtain bilayer films.

2. 2. 3. Cassava starch monolayers obtained by compression moulding (S)

Starch was first hand mixed with glycerol (30 g/100 g of starch), water (55 g/100 g of starch) and 0.5 g of PEG. The dispersion was then hot-mixed in a two-roll mill at 160°C at 10 rpm for 25 min. The resulting thermoplastic starch was stored in a desiccator with an oversaturated solution of $Mg(NO_3)_2$ at room temperature for 10 days, in order to reach 53% RH. S films were then obtained the same way as compression moulded PLA films. 4 g of sample were placed onto steel sheets and pre-heated in a hydraulic press at 160°C for 1 min. Then, compression was performed at the same temperature and 50 bars for 2 min, then 100 bars for 6 min, followed by a cooling cycle of 3 min until the temperature reached about 70°C. The obtained films were maintained in a desiccator with an oversaturated solution of $Mg(NO_3)_2$ at room temperature until their use to obtain bilayer films.

2. 2. 4. Cassava starch (S-M and S-M_{cin}) and PLA bilayer films (PLA-M and PLA-M_{cin})

Bilayer films were obtained through thermocompression of the different monolayers. Either compression moulded PLA or S films were hot pressed with PLA films obtained by casting with and without CIN. Films were hot pressed at 160°C and 100 bars for 3 min and cooled down until reaching a temperature of 70°C. Films were maintained in a desiccator with SiO_2 at room temperature until analyses were carried out.

2. 3. Film characterization

2. 3. 1. Concentration of cinnamaldehyde in the films

Samples containing cinnamaldehyde (M_{cin}, S-M_{cin} and PLA-M_{cin}) were analysed as to their content by methanol extraction and spectrophotometric determination. Square samples of each film (about 1 cm side) were weighed and extracted in 100 mL of methanol under constant stirring for 24 h (previously determined maximum time of extraction) at 20°C. Then, absorbance was measured using a UV-visible spectrophotometer (Thermo Scientific Evolution 201, EEUU) at a wavelength of 286 nm (maximum of absorption of the cinnamaldehyde in methanol). The extract of the corresponding control film without cinnamaldehyde was used as a blank in each case. The calibration curve was obtained from the absorbance measurements of standard solutions of cinnamaldehyde and was used to determine the concentration of cinnamaldehyde in the films.

2. 3. 2. In vitro *antimicrobial activity of the films*

L. innocua and *E. coli*, provided with 30% glycerol, were regenerated in Triptic Soy Broth (TSB) and were incubated at 37°C for 24 h. A 10-µL aliquot from this culture was then transferred to 10 mL of TSB and grown again at 37°C for 24 h. 10 g of TSA were poured into petri dishes of 5.5 cm diameter and used as a model solid food system (TSA-NaCl). After the culture medium solidified, 100 µL of the appropriate dilution of the TSB culture was inoculated on the surface of the agar plate in order to obtain an inoculum of 10^7CFU/g. Then, circles of the different active films (5.5 cm diameter) sterilized with UV light for 15 min, were placed in contact with the agar. The corresponding films without CIN were used as control samples. The plates were incubated at 10°C for 13 days. Inoculum controls (C) with no film were also analysed. The bacterial counts of each sample were carried out in duplicate at 0, 2, 6, 9 and 13 days. To this end, 10 g of sample were diluted in 90 mL of peptone water and were homogenized in a stomacher (IUL, Spain) for 3 min. Serial dilutions were then made and 1 mL of each adequate dilution was inoculated in petri dishes with 15 mL of TSA. Theses plates were incubated at 37°C for 24 h. In the case of the bilayer films, the antimicrobial test was carried out by placing both sides of the film in contact with the bacteria (for example S-M_{cin} or M_{cin}-S for the starch or active layer in contact with the bacteria, respectively). Every manipulation was performed in a sterile environment underneath a laminar flow cabinet (Telstar, Bio II Advance, Barcelona, España).

2. 3. 3. Release kinetics of cinnamaldehyde in food simulants

The release kinetics of the active compound from the films into four food simulants was analysed according to the Commission regulation (EU) 2015/174 amending and correcting

Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food. The four simulants were 10% ethanol (v/v) (A), 3% acetic acid (w/v) (B), 50% ethanol (v/v) (D1) and isooctane (D2). Film samples (about 100 mg depending on both film and food simulant) were cut, weighed and placed in contact with 100 mL of each simulant at 20°C, under constant stirring. The absorbance of the solutions was measured throughout time by means of spectrophotometry at the wavelength corresponding to the maximum of absorption of cinnamaldehyde in each simulant (between 280 and 286 nm, depending on the solvent). The calibration curves were obtained in each simulant. Each film sample was analysed in triplicate and the corresponding films without cinnamaldehyde, submitted to the same release process, were used to obtain the blank solution for the absorbance measurements. The amount of CIN delivered at each time (M_t) was fitted to Peleg's model (1980) (**equation 1**), and the parameters k_1 (inverse of the initial release rate) and k_2 (inverse of the asymptotic release) were obtained. The delivered amount at equilibrium (M_∞) was deduced from k_2 (**equation 2**).

$$\frac{t}{M_t} = k_1 + k_2 t \qquad \textbf{(1)}$$

$$M_\infty = \frac{1}{k_2} \qquad \textbf{(2)}$$

Fick's second law was considered to model the diffusion process of CIN from the films towards the food simulants. Film samples can be considered as infinite plane sheets where the active compound diffuses only in an axial direction, with the characteristic half thickness dimension. The diffusional long-time equation for an infinite plane sheet (Crank, 1975) with ten terms was used to determine the values of diffusion coefficient (D) of CIN in the different solvents (**equation 3**), by using the Solver tool (Microsoft Excel 2016®) to optimize the D values, by minimizing the Sum of Squared Errors (SSE), and considering the following boundary conditions:

$$t = 0 \qquad 0 < x < L \qquad c = c_0$$

$$t > 0 \qquad x = 0 \qquad x = L \qquad c = 0$$

$$M_t = M_\infty \left(\frac{8}{\pi^2} \sum_{n=0}^{\infty} \left[\frac{1}{(2n+1)^2} exp\left\{\frac{-\pi^2 D (2n+1)^2 t}{L^2}\right\}\right]\right) \qquad \textbf{(3)}$$

where:

M_t is the mass of compound released at time t

M_∞ is the mass of compound released at equilibrium

L: half thickness of film

The thickness of films was measured at six random positions using a hand-held digital micrometer (Electronic Digital Micrometer, Comecta SA, Barcelona, Spain).

2. 3. 4. Statistical analysis

StaTgraphics Plus for Windows 5.1 (Manugistics Corp., Rockville, MD, U.S.A.) was used to carry out a statistical analysis of data through an analysis of variance (ANOVA). Fisher's least significant difference (LSD) was used at the 95% level.

3. RESULTS AND DISCUSSION

Table 1 gives the values of the concentration of CIN in the active films, both the nominal value and that quantified through extraction with methanol. The surface concentration was also shown, this being equivalent to the nominal value, but greatly reduced when the extracted amount was considered. The extraction method gave values below those corresponding to the quantity incorporated into the PLA monolayer (11.7 g against 20 g per 100 g of film). Nevertheless, the final weight of the monolayers did not reflect notable losses of CIN by evaporation, taking into account the solid mass of the cast film forming solution for each film. Then, the 58.5% extraction with methanol should only correspond to the extractable compound from PLA, whereas a part of CIN remained bonded to the polymer matrix, despite that fact that the compound solubility in methanol was not exceeded. The extractable amount in the bilayer films was also lower than the theoretical one, which was estimated from the mass fraction of each layer in the laminated film (33.7 and 53.4% for S bilayer and PLA bilayer films, respectively). In that case, a significant difference between the weight of the films before and after the thermocompression process was observed, especially for the starch bilayers. The weight losses in the starch bilayer and PLA bilayer films during thermocompression were 28.5 and 4.4%, respectively, which could be associated with the water loss in the S layer and the CIN losses in both cases. During the heating and compression of the bilayers, diffusion of the compound could occur to the CIN-free layer. Likewise, the polymer radial flow could imply a lower concentration of the compound per surface unit. The radial flow was especially relevant in the starch layer, which in turn dehydrates, promoting a steam drag effect of the CIN. On the other hand, the compound diffusion to the CIN-free PLA layer could imply a tighter bonding of this active to the PLA chains, reducing the amount of compound available to be effectively released into the solvent. All of these factors could greatly reduce the CIN methanol extraction. Nevertheless, it could be assumed that the bilayer films have a higher content of CIN than that obtained from the methanol extraction, although the CIN-polymer interactions could affect its deliver capacity into the medium and, thus, its antimicrobial potential.

Table 1. Film thickness, concentrations (C) of theoretical and extractable CIN in mono (M_{cin}) and bilayer films with starch (S-M_{cin}) and PLA (PLA-M_{cin}) and percentage of CIN extraction in methanol.

Film	Thickness (μm)	Theoretical C		Extractable C		
		(g/100 g film)	(mg/cm^2)	(g/100 g film)	(mg/cm^2)	Extraction %
M_{cin}	77 ±13	20	1.7	11.7 ±0.8	1.0	58.5
S-M_{cin}	183 ±12	8.1	1.7	2.7 ±0.2	0.6	33.7
PLA-M_{cin}	188 ±13	7.6	1.7	4.0 ±0.1	0.9	53.4

3. 1. Antimicrobial properties of the films

The antimicrobial properties of the monolayer and bilayer films were analysed against Gram+ (*L. innocua*) and Gram- bacteria (*E. coli*). In the case of bilayer films, the film contact with the agar plate containing the bacteria was through both the PLA active layer with CIN and the PLA or S layer, into which CIN was not directly incorporated but where the compound diffusion could have occurred. **Figure 1** shows microbial counts for *E. coli* and *L. innocua* for the different samples and controls (the corresponding films without CIN (M, M-S and M-PLA) and the uncoated plate (C)) as a function of incubation time. For monolayer films with CIN (M_{cin}) applied on plates with *E. coli* (**a**), a bacteriostatic effect was observed during the first 6 days, in comparison with the controls; however, from 6 days onwards, a bactericidal effect occurred, with a reduction of nearly 4 Log (CFU/g) with respect to the initial counts at 13 days of incubation. In the case of *L. innocua*, an inhibition of the growth of the bacterium was observed during the first 9 days but afterwards, very similar counts to those of the control were obtained (**b**). That could be explained by the progressive evaporation of the active compound during the incubation time, reaching levels lower than the minimum inhibitory concentration (MIC) for this bacterium, or also to the adaptation of the bacterium, becoming less sensitive to the active agent.

In the case of the S bilayer films, a growth inhibition of both bacteria (**c-d**) was observed throughout the incubation time, even when films were in contact with the starch layer (S-M_{cin}) where no active was incorporated. This indicates that CIN effectively diffuses from the PLA to the starch layer and then through the starch layer to the agar medium. The latter was probably enhanced by the swelling of the S layer in contact with the wet medium, which favours CIN migration to the plate surface and resulted in greater antimicrobial effectiveness. In fact, for *E. coli*, a bactericidal effect was observed throughout the incubation time when the starch layer was in contact with the medium (S-M_{cin}), whereas the active compound had a bacteriostatic effect when the contact occurred through the M_{cin} layer (**c**). Coherent behaviour was observed for *L. innocua*, where the S layer contact also conferred greater antibacterial effect than that with the M_{cin} layer. In the last case, cell growth was observed at the end of the incubation time, as observed for the M_{cin} monolayer. This could be associated with an insufficient release of CIN from the PLA layer, which allows the bacterial adaptation, recovering the growth capacity at long contact times. This was also confirmed by the counts obtained for PLA bilayers, where no growth inhibition of either bacteria was observed for either contact type, the PLA or M_{cin} layer (**e-f**). The lack of antibacterial activity when the M_{cin} layer was in contact with the agar medium, despite the initial load of CIN, could be explained by the CIN diffusion and partition between the two PLA layers and its binding to the polymer matrix with the subsequent loss of availability for the antibacterial action.

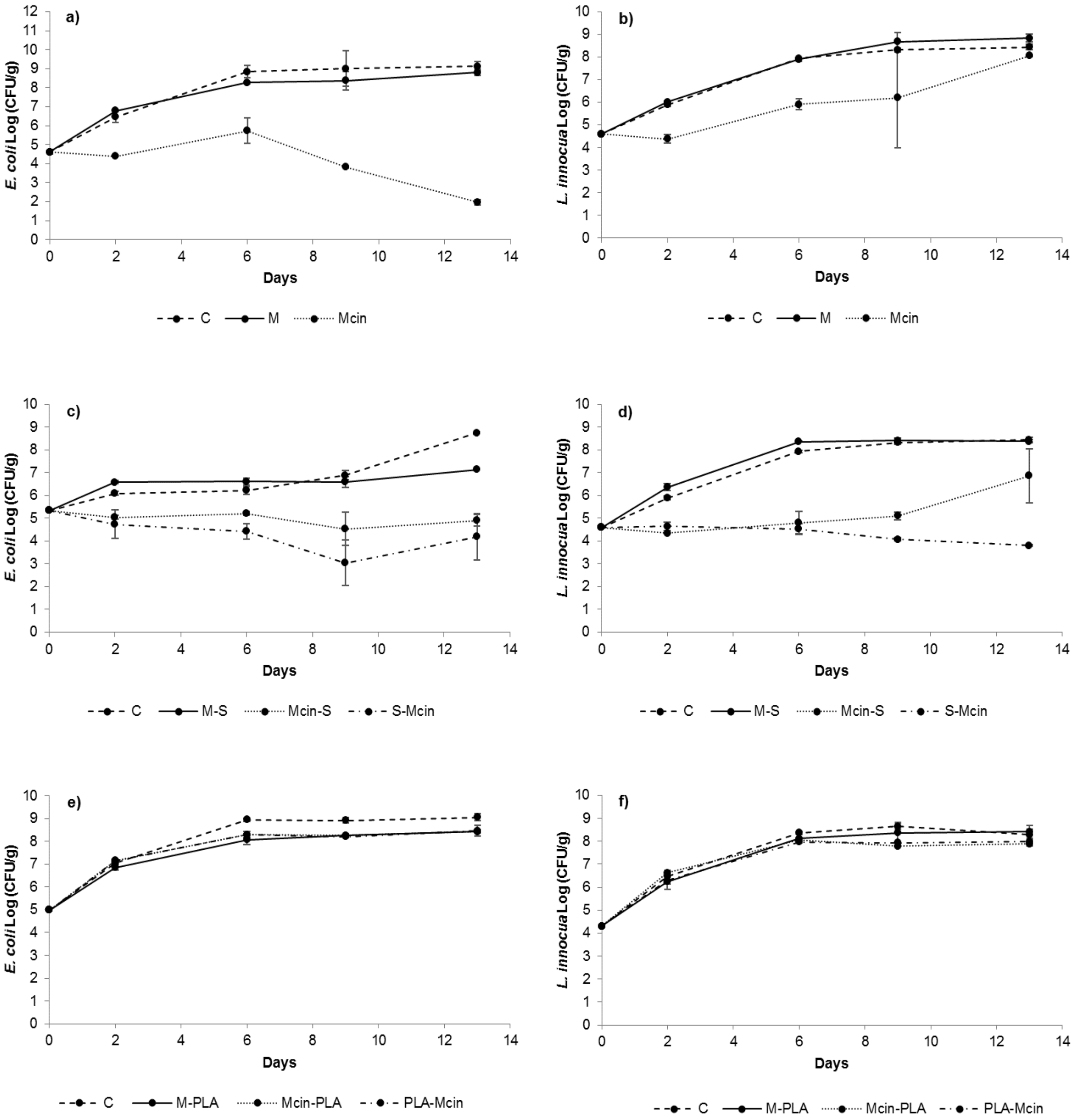

Figure 1. Microbial growth at the different incubation times for *E. coli* and *L. innocua* in samples with monolayer PLA films (**a-b**) and bilayer films with S (**c-d**) and PLA (**e-f**), in comparison with their respective control films without CIN and with the inoculum controls (C). For the bilayer films, count values are given for the culture medium in contact with the starch layer (S-M_{cin} data), compression moulded PLA layer (PLA-M_{cin} data) and CIN-loaded PLA layer (M_{cin}-S or M_{cin}-PLA data).

The results obtained suggest that CIN is tightly bonded to the PLA matrix, which limits its release rate into the medium and so its antibacterial capacity. In the PLA monolayer, the amount of CIN incorporated seems to be enough to deliver a CIN concentration above the MIC of both bacteria, but when this amount was distributed in a thicker PLA bilayer, not enough active compound is released to affect bacterial growth. In contrast, the S layer offers much less resistance to CIN migration, thus enhancing the antimicrobial capacity of the bilayer films. In that sense, since starch is a hydrophilic material, its hydration in contact with the medium would favour the molecular mobility and thus the diffusion of CIN into the medium. To corroborate this hypothesis, the next section analyses the release kinetics of CIN from mono and bilayer films in different food simulants.

3. 2. Release kinetics of cinnamaldehyde

The release kinetics of the active compound into food simulants of differing polarities was analysed. Aqueous (A: 10% ethanol (v/v) and B: 3% acetic acid (w/v)) and less polar simulants (D1: 50% ethanol (v/v) and D2: isooctane) were considered. The mass of CIN delivered at different contact times with simulants has been determined and **Figure 2** shows the mean values, referred to the maximum value (at equilibrium) for each case. This ratio represents the fraction of active released at each time with respect to the final amount delivered at equilibrium in each simulant. **Table 2** shows the maximum values (M_∞), referred per mass unit of the initial film, which were estimated by applying Peleg's model to the experimental data for the CIN release. The values of $1/k_1$ parameter, related to the release rate, were also included in **Table 2**. A good fit of the model was achieved in every case ($R^2 > 0.97$). Both the deliver rate and asymptotic value were greatly affected by the polarity of the food simulant and the kind of film, yielding different values depending on both factors. For every kind of film, the fastest release of CIN was observed in 50% ethanol (simulant D1) (c), whereas the slowest deliver occurred in the non-polar solvent (D2: isooctane) (e), as previously observed by several authors for other essential oil compounds in polymer matrices (Narayanan and Ramana, 2013; Sánchez-González *et al.*, 2011; Petchwattana *et al.*, 2015; Tawakkal *et al.*, 2016; Requena *et al.*, 2017). However, although the different films were observed to exhibit great differences in the CIN release rate for a determined simulant, the PLA bilayer (PLA-M_{cin}) exhibited the slowest release rate in every case.

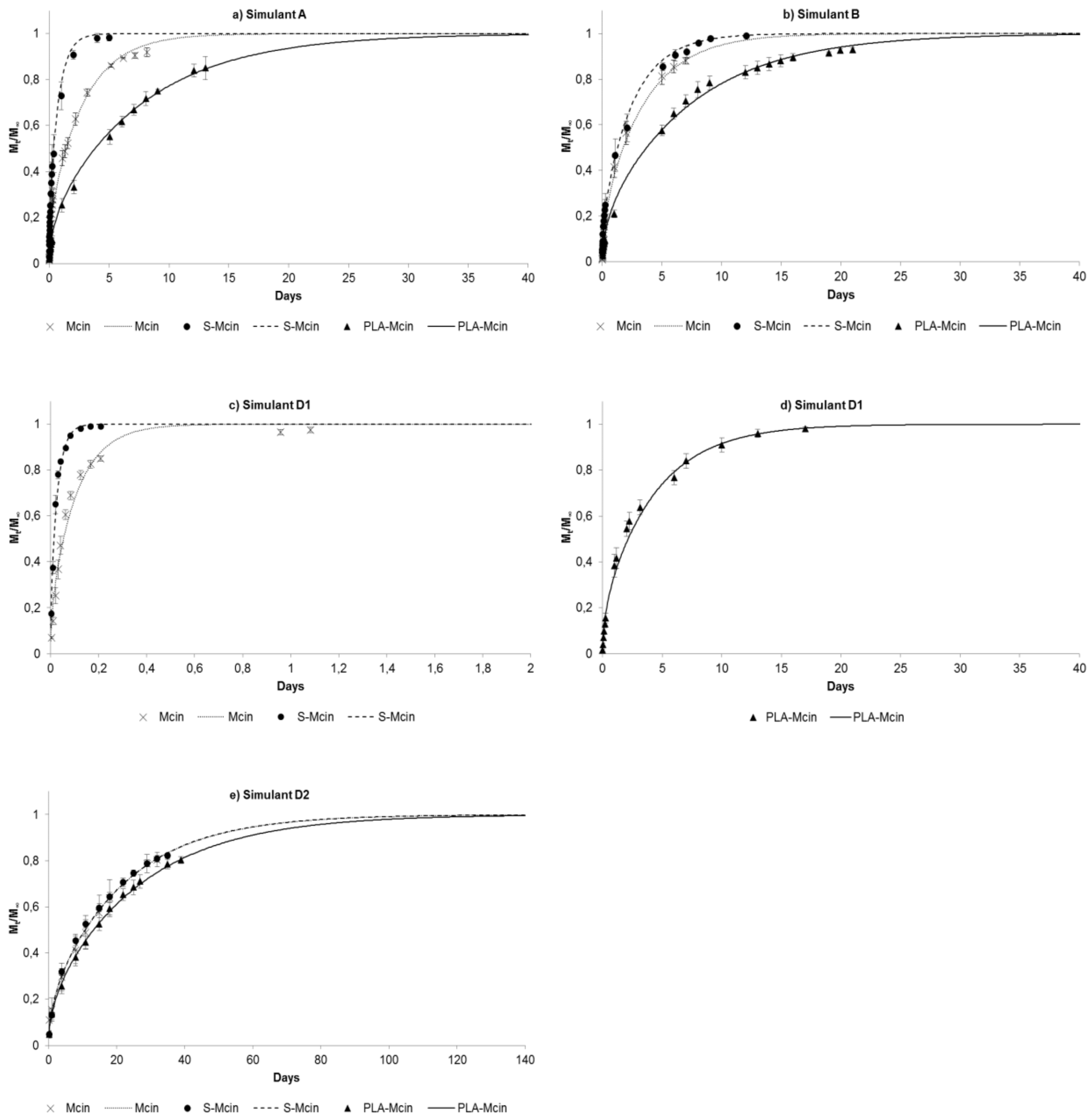

Figure 2. Ratio of the active compound released in each food simulant, with respect to the equilibrium value, as a function of contact time (points) and Fick's fitted model (lines). A: 10% ethanol (v/v), B: 3% acetic acid (w/v), D1: 50% ethanol (v/v), and D2: isooctane.

As concerns the maximum active compound delivered, **Table 2** also shows the maximum release ratio (M_∞/M_0) for each compound, related with its partition coefficient (defined as the mass of active released at equilibrium in the simulant (M_∞) with respect to the corresponding residual mass of the active in the film (M_0-M_∞)). This ratio was referred to the M_0 value of the theoretical amount incorporated into the films and also to the amount determined by methanol extraction. The highest M_∞/M_0 values occurred in the starch bilayer (S-M_{cin}) and PLA monolayer samples (M_{cin}) in almost every simulant, except in D1, where no significant differences were observed for the different films when considering the extractable CIN in methanol. In this case, a total release can be assumed, considering the extractable CIN in methanol of the films although only about 50% of the incorporated compound was delivered from the PLA mono and bilayers. The starch bilayers exhibited the greatest variability in this parameter, thus indicating more stochastic losses of the active compound during the bilayer processing, which could be due to the coupled effects of water loss from the S layer and the potential steam drag of CIN. In the non-polar simulant (D2), a very small release of CIN occurred in every case, with the PLA bilayer being the film that most strongly retains the active compound.

Table 2. Parameters of Peleg's model: amount of active compound released at equilibrium in the simulant (M_∞) and its release rate ($1/k_1$), and maximum release ratio (M_∞/M_0): mass of active delivered at equilibrium in the simulant related to the initial mass of the active (theoretical and extractable in methanol). Values of the Diffusion coefficient (D) were also included. Mean values ±standard deviation.

Simulant	**Film**	**$1/k_2 = M_\infty$ (g CIN/100 g film)**	**$1/k_1$ (µg/min)**	**M_∞/M_0 theoretical (%)**	**M_∞/M_0 extractable in methanol (%)**	**D x10^{11} (cm^2/s)**
A	M_{cin}	9.7 ±0.3 ^{e}	4.3 ±0.6 ^{ab}	48 ±2 ^{cd}	83 ±3 ^{c}	2.4 ±0.2 ^{a}
	S-M_{cin}	3.1 ±0.7 ^{c}	4.5 ±0.9 ^{ab}	35 ±8 ^{b}	113 ±25 ^{e}	53 ±20 ^{bc}
	PLA-M_{cin}	0.57 ±0.12 ^{ab}	0.9 ±0.4 ^{a}	7.5 ±0.4 ^{a}	14.2 ±0.7 ^{ab}	5 ±1 ^{a}
B	M_{cin}	9.0 ±0.2 ^{e}	4.0 ±0.8 ^{ab}	45 ±1 ^{c}	77 ±2 ^{c}	1.9 ±0.3 ^{a}
	S-M_{cin}	2.6 ±0.6 ^{c}	1.6 ±0.3 ^{a}	29 ±7 ^{b}	95 ±22 ^{cde}	15 ±4 ^{ab}
	PLA-M_{cin}	0.278 ±0.012 ^{a}	0.47 ±0.07 ^{a}	3.7 ±0.2 ^{a}	6.9 ±0.3 ^{ab}	5.4 ±0.5 ^{a}
D1	M_{cin}	10.9 ±0.2 ^{f}	97 ±8 ^{d}	55 ±1 ^{d}	93 ±2 ^{cde}	63 ±5 ^{c}
	S-M_{cin}	2.3 ±0.9 ^{c}	49 ±12 ^{c}	27 ±10 ^{b}	86 ±33 ^{cd}	1378 ±106 ^{d}
	PLA-M_{cin}	4.3 ±0.7 ^{d}	8.9 ±0.6 ^{b}	56 ±9 ^{d}	107 ±17 ^{de}	9 ±2 ^{a}
D2	M_{cin}	1.11 ±0.03 ^{b}	0.18 ±0.05 ^{a}	5.5 ±0.2 ^{a}	9.5 ±0.3 ^{ab}	0.31 ±0.08 ^{a}
	S-M_{cin}	0.7 ±0.2 ^{ab}	0.11 ±0.04 ^{a}	8 ±3 ^{a}	26 ±9 ^{b}	1.78 ±0.12 ^{a}
	PLA-M_{cin}	0.046 ±0.006 ^{a}	0.014 ±0.002 ^{a}	0.61 ±0.08 ^{a}	1.2 ±0.2 ^{a}	1.7 ±0.2 ^{a}

Different superscript letters within the same column indicate significant differences among samples (films or simulants ($p<0.05$)).

As regards the active agent release process, three steps can be assumed: a) the solvent diffusion into the polymer matrix, b) the network relaxation in line with solvation and plasticization and c) the diffusion of the compound through the relaxed polymer network until the thermodynamic equilibrium between phases (polymer/food system) is reached. The compound affinity with the solvated polymer and its solubility in the liquid food system will determine the partition coefficient at equilibrium for the delivered compound. Thus, the compound diffusion through the matrix will be affected by the solvent impregnation into the polymer network and the interactions established among the components. Considering bilayer films, two polymer matrices were involved in the film (amorphous PLA and starch or crystalline PLA, in S-M_{cin} or PLA-M_{cin} samples respectively). Therefore, the two layers could be expected to behave differently. Nevertheless, there was an overall tendency observed when bilayer films were submitted to the release studies. Previous studies into similar compounds in different polymer matrices revealed no coupling of the polymer relaxation and compound diffusion steps. So, the diffusional mechanism controlled the compound release in different polymer films. This was the case of thymol in poly(butylene succinate) (Petchwattana *et al.*, 2015) and PLA (Tawakkal *et al.*, 2016) or lemongrass essential oil in sodium alginate films (Matiacevich *et al.*, 2016). Taking these considerations into account, the apparent diffusion coefficient of the different mono and bilayer films were obtained.

Figure 2 shows Fick's model fitted to the experimental points in each simulant. A good fit of the model was observed in every case (SSE < 0.076) as well as the different pattern of the curves depending on the film and the simulant. In each simulant, the time required to reach the equilibrium value greatly varied from film to film: from a few hours in the case of the starch bilayer (S-M_{cin}) to several days in that of the PLA bilayer (PLA-M_{cin}). This agrees with that aforementioned CIN bonding to the PLA chains, which greatly retards the CIN deliver into the food simulants, regardless of their polarity. The differences were more marked for simulant D1 (50% ethanol), where a much faster release was observed for both monolayer and S bilayer films (**c**), in comparison with PLA bilayers (**d**). The decrease in the simulant polarity, related to the rise in the ethanol ratio, boosted the CIN release rate of both the PLA monolayer and starch bilayer, probably due to the greater affinity of CIN with the solvent and the greater relaxation of the PLA layer in contact with this ethanol-rich solvent. Finally, the three kinds of films exhibited very similar behaviour in isooctane (D2), with a very slow release until equilibrium is reached at longer contact times (nearly 120 days).

The values of the apparent diffusion coefficient (D) in each film and simulant are shown in **Table 2**, where both the polymer matrix and solvent influence can be observed. As the solvent penetrates the polymer matrix to a different extent, providing it with different relaxation effects, the compound diffusion was affected not only by the kind of polymer but also by the relaxation induced by the different solvents. In every case, D values were higher for the S bilayer, thus reflecting the poor binding capacity of starch chains to bond CIN, which can be released more easily. No significant differences in D values were observed in either film for the two most aqueous simulants (A and B). Nevertheless, the increase in the ethanol ratio in simulant D1 enhanced the CIN diffusion in every film, which can be attributed to a greater relaxation of the PLA matrix in both mono and bilayers. Finally, the lowest values of D were obtained in simulant

D2 due to both the low solvent chemical affinity with CIN and the polymers, which will imply low relaxation effects in the film matrices. It is remarkable that the D value in the PLA bilayer was significantly lower than in the PLA monolayer when simulant D1 was used, which can be attributed to the lower relaxation of the crystalline PLA layer in contact with the ethanol-rich simulant.

The obtained results confirm the great retention of CIN in the PLA matrix, which can be explained by the enhanced compound-chain interactions with great chemical affinity. This will limit both its release ratio and rate in food systems, which could compromise the CIN antimicrobial action. This retention seems to be enhanced after thermocompression in bilayer films, as also revealed by the antimicrobial test. In the S bilayer, the thermocompression step seems to involve the CIN diffusion into the starch layer, where no CIN binding occurred due to the poorer polymer-compound chemical affinity, and the migrated CIN could effectively be delivered into the simulants. This migration also occurred for the crystalline PLA layer in the PLA bilayer films, which contributes to the compound binding. The different relaxation of crystalline and amorphous PLA films in contact with the medium affected the CIN diffusion, limiting its release when it is entrapped in the more crystalline layer.

3. 3. Active release prediction during the plate incubation time

Based on the kinetics analysis, the active agent release concentration *vs*. time was predicted in the plates in which the antimicrobial test was performed, assuming bulk diffusion into the culture medium and assuming simulant A (10% ethanol) behaviour for the medium. **Figure 3** shows the predicted CIN concentration (mg/mL TSA medium) when each one of the films was placed in contact with the plate as a function of time. The CIN MIC range reported by several authors for *E. coli* and *L. innocua* (Pei *et al.*, 2009; Becerril *et al.*, 2007; Chang *et al.*, 2001; Han *et al.*, 2006: Thanh *et al.*, 2013) was reflected in the plot (shadow areas). As observed in the **Figure 3**, different CIN concentrations will be present in the plate at the different incubation times. The starch bilayer released the active compound faster, whose concentration reached the maximum level after about two days of contact, whereas the CIN release was slower from the PLA monolayer active films. For both films, the MIC value was exceeded, which explains the effective antimicrobial action observed and commented on previously. Nevertheless, the CIN delivered from the PLA bilayer film did not exceed the MIC value throughout the incubation time (13 days), which explains the lack of antibacterial action found in the antimicrobial test. Despite the fact that release kinetics provided information about the overall release from both sides of the films, the comparative analysis of both bilayers allows information to be obtained about the different release kinetics of starch and semicrystalline PLA layers. This information is completely coherent with the antimicrobial response obtained from the three types of films.

When the S bilayer is considered, CIN migration to the culture medium is promoted by the diffusion of the compound into the starch layer where the compound is delivered faster. When this diffusion occurred into the semicrystalline PLA layer, CIN was closely retained in the polymer network, which became less relaxed in contact with the medium, both factors inhibiting the compound release. The initially free CIN in the amorphous PLA layer will be greatly reduced by its distribution and partial bonding to the new PLA layer, when the bilayer is formed by thermocompression, and its availability to be delivered into the agar medium becomes more limited, not reaching the MIC value for the tested bacteria when the films are in contact with the plates.

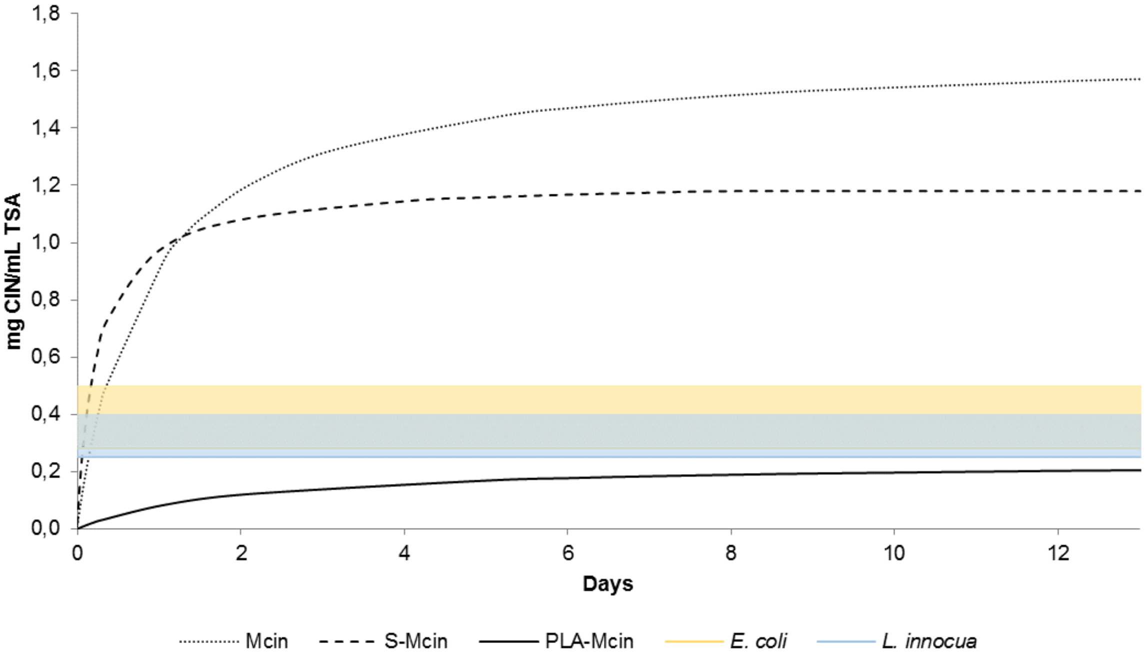

Figure 3. Predicted concentration of CIN released throughout the incubation time into the TSA culture medium for the three different types of film, considering simulant A. Shaded areas correspond to the range of the MIC of CIN against *E. coli* (yellow) and *L. innocua* (blue).

So, the kinetics analysis of the CIN release from the different films helps to explain the different antimicrobial effects of the compound, according to its different retention ratios in the polymer matrix.

The obtained results show great differences in the active compound release properties, affected by the polymer-retention capacity of the active compounds and the polymer-medium interactions, which greatly affect its relaxation capacity and compound diffusion. Crystalline PLA offers great resistance to CIN release, so limiting its antimicrobial action in active packaging materials. However, amorphous PLA and starch delivered CIN faster and at a higher ratio, which favoured the antibacterial activity. Of both polymers, starch seems both to retain CIN to a lesser extent and provide a higher CIN content to the culture medium, thus being more effective at controlling bacterial growth.

4. CONCLUSION

Antimicrobial films based on starch and PLA could be obtained by the thermocompression of a CIN loaded amorphous PLA layer and compression moulded thermoplastic starch. Films exhibited antibacterial effect in *in vitro* tests against *E. coli* and *L. innocua*, when the culture medium was in contact with both starch and PLA layers, being more effective in the case of S contact, despite the fact that CIN was loaded into the PLA layer. Both the antimicrobial test and CIN release kinetics suggest that a part of the incorporated CIN was tightly bonded to the PLA matrix and the free CIN diffuses into the initially free layer in the bilayer films. In the starch bilayers, CIN migrates into the food system faster through the starch side due to its fewer interactions with the starch chains and the fact that the polymer swells in contact with the wet medium, which favours compound diffusion. When bilayers were formed with CIN loaded amorphous PLA and a semicrystalline PLA layer, CIN migration was greatly inhibited, in line with the increase in the bonded/free CIN ratio and the milder solvent relaxation effects in the crystalline regions of the film. This implied a lack of antimicrobial action of CIN in these bilayers, where the released active compound did not exceed the MIC of both tested bacteria. Therefore, the lamination of CIN loaded amorphous PLA with thermoplastic starch represents a good strategy to obtain antimicrobial films for food applications, which can be applied through starch or PLA food contact, depending on the interest in a determined kind of food. In both cases, antimicrobial activity can be expected, while films show complementary barrier properties associated with the combination of both materials; the films facing the oxygen through the starch layer, whereas the PLA layer would provide good water vapour barrier capacity. Nevertheless, *in vivo* tests are required to assess the antimicrobial and functional effectiveness of these bilayer films in real foods.

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GENERAL DISCUSSION

Development of eco-friendly alternatives such as bio-based and/or biodegradable food packaging materials is one of the current research trends to face the environmental problems associated to the high consumption of non-biodegradable plastics. Active packaging materials also constitute a current trend with the objective to enlarge the food shelf life and better maintain their quality and safety. Among biopolymers, PLA is one of the most investigated because of its outstanding optical, mechanical and water vapour barrier properties. Indeed, PLA is highly transparent and very resistant but it is very brittle and exhibits poor oxygen barrier capacity. Starch is another good candidate for development of biomaterials, since it is widely available and cheap, and it also presents good functional properties, such its high oxygen barrier capacity. Since these two polymers exhibit complementary functional properties, they could be used in a bilayer assembly in order to obtain films with improved performance. Antimicrobial/antioxidant compounds from natural origin are currently demanded to preserve foods, avoiding the potential toxicity of some synthetic products. In the present Doctoral Thesis, the development of active biomaterials for food packaging was addressed by using PLA and starch, with the incorporation of cinnamaldehyde, one of the cinnamon essential oil compound with known antimicrobial activity. For this purpose, different processing techniques were used. First, semicrystalline PLA films were obtained by compression moulding, adding three different plasticizers at two different concentrations in order to obtain a more extensible material. Due to the low effectiveness of plasticizers at increasing the stretchability of semicrystalline PLA, a more extensible amorphous PLA (with lower molecular weight and containing a fraction of low molecular weight components) was used to the combination with starch. Amorphous PLA films were obtained by casting of its ethyl acetate solutions, with the objective to incorporate the active compound. Then, cassava starch films, obtained by melt blending and compression moulding, were adhered to the amorphous PLA sheets, with or without cinnamaldehyde, by compression moulding to obtain starch-PLA bilayers. These were also obtained with semicrystalline PLA and the amorphous sheets (PLA bilayers) for comparison purposes. The functional properties of the obtained materials as packaging films were analysed from different point of views, including structural characteristics, thermal behaviour and molecular interactions.

One of the most important parameters for food packaging is the barrier capacity of the materials for water vapour and gases, such as oxygen. Most of plastics are relatively permeable to these small molecules, which may move from the internal or external environment through the polymer package wall, resulting in possible negative changes in the product quality and shelf life. Indeed, the oxygen barrier performance of a film is critical because oxygen can promote several oxidation mechanisms which highly modify food quality.

In **Figure 1**, the barrier properties of the studied film formulations can be observed, in terms of the water vapour and oxygen transmission rates (WVTR vs. OTR map), together with the values of some synthetic polymers and the range for some food packaging requirements (Schmid *et al.*, 2012). The transmission rate (TR) is described as the quantity of permeant passing through a film, per unit of area and time, at steady state and it is related to the permeability values. All of the studied film formulations, as well as some synthetic plastics, have been located on the map for a comparison purpose, with the values normalized for the same thickness (100 μm). Neat PLA films, either the semicrystalline compression moulded or cast amorphous polymer, showed barrier properties very close from those of PS. Indeed, they exhibited high OTR values while they had lower WTR values than starch films, which is highly permeable to water vapour. Combination of both polymers in a bilayer assembly let to a high decrease of both TR values, thus considerably improving the barrier properties of the combined materials, which reached a very close position in the map to the meat packaging requirements. PLA bilayer barrier properties were also improved, with values of OTR highly reduced from 1383 cm^3/m^2.day of the neat PLA to 65 cm^3/m^2.day of the PLA bilayer films. The PLA bilayer assembly also permitted to slightly reduce the WVTR, with respect to neat PLA, allowing for approaching the packaging requirements of different foods. Cinnamaldehyde incorporation also played an importance role at decreasing OTR values of PLA monolayers and starch-PLA bilayers, although it did not have a significant effect on PLA bilayer films. However, cinnamaldehyde barely changed the water vapour permeability of both mono and bilayers. All plasticized PLA films showed WVTR values in the same range, from 22 to 34 g/m^2.day, while the incorporation of Palamoll®638, regardless its ratio, and 5% of PEG 1000 allowed for a high decrease in the OTR values of semicrystalline PLA. It is remarkable that OTR values of PLA films plasticized with PEG 4000 and 10% of PEG 1000 were very high and exceeded the detection limit of the used equipment (200 cm^3/m^2.day).

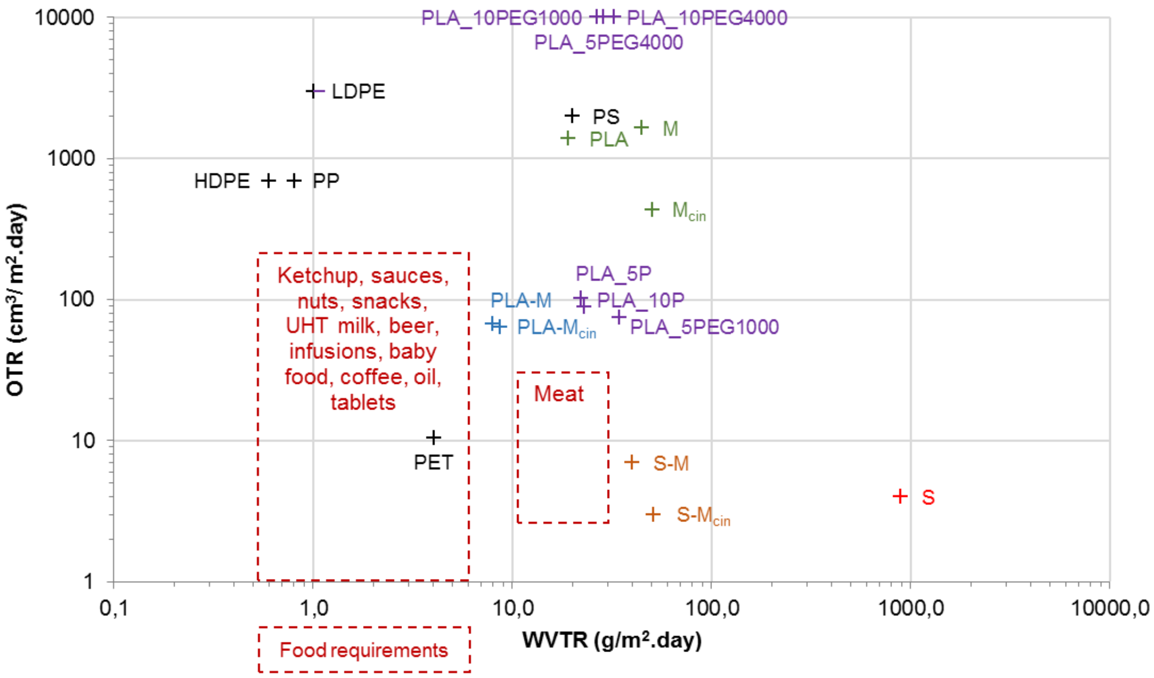

Figure 1. Map of barrier properties showing the location of the different film formulations and some synthetic plastics commonly used in food packaging (HDPE, LDPE, PET, PP, PS). Barrier capacity requirements for some foods are also indicated, according to Schmid *et al.*, (2012)

On another hand, mechanical performance of plastic films is also a critical issue in food packaging applications. Indeed, materials must have a high resistance to ensure food mechanical protection and preservation. Nonetheless, the extensibility requirements depend on the final product and its processing. **Figure 2** shows the map of tensile properties (the stress-strain values) of the different film formulations, along with those of some synthetic plastics commonly used in food packaging (Plackett, 2012).

As in the map of barrier properties, a remarkable difference in the mechanical performance of the films, depending on the material used can be observed. The amorphous cast PLA films exhibited a high extensibility whereas semicrystalline thermoprocessed PLA films were very brittle. However, they were the most resistant materials, with the highest TS values and the amorphous material exhibited similar mechanical performance as that of PET and PP. When amorphous PLA layer was thermocompressed with semicrystalline PLA, bilayer films showed deformability values even lower than those of neat semicrystalline PLA and they also were less resistant. As regards the starch films, both mono and bilayer films showed good extensibility, although they had the lowest resistance, from 7 to 2 MPa for bilayers without and with cinnamaldehyde, respectively. Thus, cinnamaldehyde incorporation led to a decrease in the resistance of both amorphous PLA monolayers and starch bilayer films. As concerns the effect of plasticizers on the resistance and elongation capacity of semicrystalline PLA films, Palamoll®638 did not have a significant effect, whereas PEG 1000 and PEG 4000) led to less resistant and less extensible films (values in the range of those of PS), although they still exhibited higher resistance than that of starch films.

The obtained results revealed that the plasticizers used did not have a positive effect on semicrystalline PLA films, neither as to the barrier nor as the mechanical properties of the films. Starch bilayer films exhibited good barrier properties, in comparison with neat starch films but they still showed limited mechanical resistance, which could be enhanced by using a thicker PLA sheet in the bilayer. PLA bilayers presented good barrier capacity with good mechanical resistance but they were very brittle.

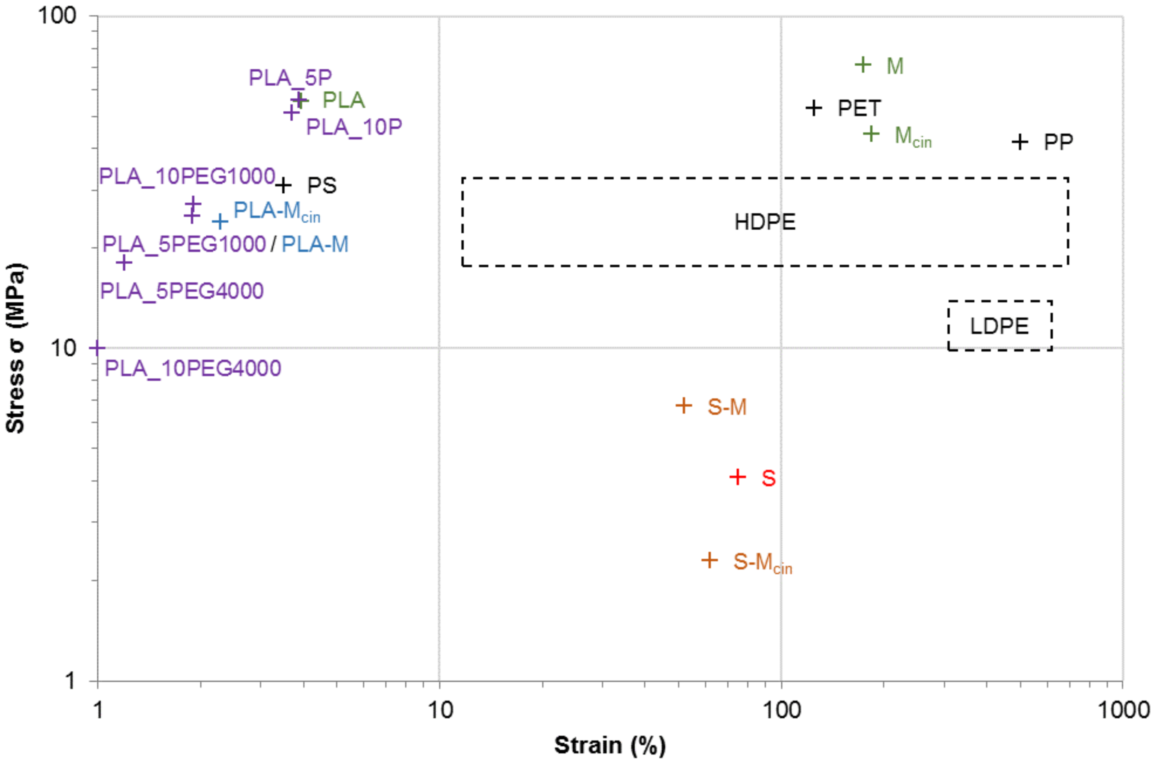

Figure 2. Map of tensile properties of the film formulations and some synthetic plastics used in food packaging.

As concerns antimicrobial properties of the films containing cinnamaldehyde, the incorporated amount of the active in the cast amorphous PLA layer was effective at controlling the growth of *E. coli* and *L. innocua*, both from the PLA monolayers and from the starch-PLA bilayers. Despite the fact that losses of the active occurred during either casting process or bilayer thermocompression, the released amounts of the compound from these films into the culture medium exceeded the MIC of both bacteria, causing their growth inhibition. From PLA bilayers, the release of the compound was very slow and the total amount released was more limited than that required for its antimicrobial activity. The release kinetics of cinnamaldehyde in the different food simulants was affected by either the kind of film or the polarity of simulant. For PLA bilayers, the greatest delivery occurred into the intermediate polarity system (50% ethanol), whereas it was much more limited in the more polar aqueous systems (10% ethanol), that could simulate the culture medium for the antibacterial tests. On the basis of the kinetics model, the predicted delivered amount on cinnamaldehyde in this aqueous medium did not exceed the MIC of the bacteria for PLA bilayer films, although it was greater than the MIC value for PLA monolayers and starch bilayer, where higher release was reached in this system. This indicates that the amount of free cinnamaldehyde in PLA bilayer, able to be released to the culture, was not enough to have antimicrobial effect and a part of the compound introduced in the amorphous PLA monolayer diffused to the semicrystalline sheet, becoming bonded to polymer matrix. Indeed, crystallization of semicrystalline PLA was affected by the compound diffusion into this layer (lower melting temperature of the crystalline zones, which are at lower ratio in the film). This bonding effect did not occurred in amorphous PLA or starch-PLA bilayers, where 80-100% of the total cinnamaldehyde present was delivered in the 10% ethanol aqueous medium. In this sense, it is remarkable that starch-PLA bilayers were effective as antimicrobial films through the contact of both the starch and PLA faces with the medium, thus indicating that the active migration occurred through both adhered sheets.

Therefore, the lamination of cinnamaldehyde loaded amorphous PLA with thermoplastic starch represents a good strategy to obtain antimicrobial films for food applications, which can be applied through starch or PLA food contact, depending on the interest in a determined kind of food. In both cases, antimicrobial activity can be expected, while films show adequate barrier properties and reasonable mechanical performance associated with the combination of both materials. Nevertheless, *in vivo* tests are required to assess the antimicrobial and functional effectiveness of these bilayer films in real foods.

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CONCLUSIONS

1. Although a low degree of crystallinity was obtained (5%) in the PLA films obtained by compression moulding, crystallization was promoted by the addition of plasticizers (PEG 1000, PEG 4000 and Palamoll®638); the greater their ratio, the higher the degree of crystallinity. All of the plasticizers reduced the glass transition temperature of the film's amorphous phase to a similar extent, proportionally to their concentration. Nevertheless, in no case did this imply an improved film ductility, since all of the plasticized films were less resistant to break and less extensible. The extent of the reduction in these parameters depended on the plasticizer used, Palamoll®638 being the one which promoted the smallest reduction in line with the lower degree of crystallization induced by this compound. So, the studied plasticizers, at 5-10 wt %, were not effective at reducing the film's brittleness, mainly due to the fact that they enhanced the polymer´s crystallization at the same time as they reduced the glass transition temperature of the amorphous PLA. Further studies with other potential plasticizing substances would be required, taking into account the potential antagonistic effect of plasticizers, increasing the molecular mobility of the polymer chains which, at the same time, can promote polymer crystallization, all of which affects the mechanical performance of materials based on PLA.

2. Amorphous PLA-starch bilayer films were successfully obtained by compression moulding of cast PLA layers containing or not cinnamaldehyde, and thermoplastic starch films, with very good adhesion at the interface. Despite the lower ratio of PLA sheet in the bilayer assembly (1/3 of the film thickness), a great improvement in the tensile and water vapour barrier properties was achieved with respect to the net starch films, the films maintaining high transparency and an oxygen permeability as low as that of net starch films. When cinnamaldehyde was included in the cast PLA sheet, films became thinner due to the losses of the volatile active during processing. Nevertheless, the improvement in the barrier properties was maintained but films exhibited lower mechanical resistance. The fitting of the cast layer thickness using a constant polymer surface density (g polymer/cm^2) could mitigate this problem. PLA bilayers also exhibited lower oxygen permeability than the respective PLA monolayers, which could be associated with the good interfacial adhesion that created additional barrier efficiency. Thermal analysis revealed the diffusion of cinnamaldehyde or low molecular weight compounds from the cast PLA layer to the other sheets (starch or semicrystalline PLA) which contributed to plasticizing the amorphous regions and affected the crystallization pattern of PLA, as also revealed by the X-Ray diffraction analyses. Nevertheless, FTIR spectra did not evidence quantitative amounts of the active in these layers, probably due to the high dilution level of active in these thicker sheets of bilayer. The obtained results offer an interesting option to obtain high barrier-highly resistant active films from thermoplastic starch and amorphous PLA, including cinnamaldehyde in the ethyl acetate solution of the polyester; this solvent being food compatible, which reduces the risks associated with solvent residues and allows for the cold dissolving of amorphous PLA.

3. Antimicrobial films based on starch and PLA could be obtained by the thermocompression of a CIN loaded amorphous PLA layer and compression moulded thermoplastic starch. Films exhibited antibacterial effect in *in vitro* tests against *E. coli* and *L. innocua*, when the culture medium was in contact with both starch and PLA layers, being more effective in the case of S contact, despite the fact that CIN was loaded into the PLA layer. Both the antimicrobial test and CIN release kinetics suggest that a part of the incorporated CIN was tightly bonded to the PLA matrix and the free CIN diffuses into the initially free layer in the bilayer films. In the starch bilayers, CIN migrates into the food system faster through the starch side due to its fewer interactions with the starch chains and the fact that the polymer swells in contact with the wet medium, which favours compound diffusion. When bilayers were formed with CIN loaded amorphous PLA and a semicrystalline PLA layer, CIN migration was greatly inhibited, in line with the increase in the bonded/free CIN ratio and the milder solvent relaxation effects in the crystalline regions of the film. This implied a lack of antimicrobial action of CIN in these bilayers, where the released active compound did not exceed the MIC of both tested bacteria. Therefore, the lamination of CIN loaded amorphous PLA with thermoplastic starch represents a good strategy to obtain antimicrobial films for food applications, which can be applied through starch or PLA food contact, depending on the interest in a determined kind of food. In both cases, antimicrobial activity can be expected, while films show complementary barrier properties associated with the combination of both materials; the films facing the oxygen through the starch layer, whereas the PLA layer would provide good water vapour barrier capacity. Nevertheless, *in vivo* tests are required to assess the antimicrobial and functional effectiveness of these bilayer films in real foods.