UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Instituto Universitario de Ingeniería de Alimentos para el Desarrollo

UNIVERSITAT
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DOCTORAL THESIS

Biodegradable materials based on poly (vinyl alcohol) (PVA) and poly (lactic acid) (PLA) with antioxidant and antimicrobial activity for food packaging applications

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A mi amada madre Gloria

y a mis hermanos

Miguel Ángel y Juan Felipe

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ABSTRACT

The massive use of synthetic plastics and their environmental impact, as well as the need of improving food preservation, makes necessary the development of biodegradable alternatives for food active packaging, to extend the food shelf life. The use of active compounds from natural origin to obtain this kind of materials is a new trend based of the consumer demand for safer and healthier products. The present Doctoral Thesis, has been focused on the development of multilayer active packaging materials, based on biodegradable polymers with complementary properties, in order to obtain materials with adequate mechanical and barrier properties to meet food packaging requirements. To this end, poly (vinyl alcohol) (PVA) (with high oxygen barrier capacity), of different molecular weight and acetylation degree, and poly (lactic acid) (PLA) (with high water vapour barrier capacity) were considered. Likewise, carvacrol (liposomal encapsulated or not) and ferulic and cinnamic acid, were chosen as antimicrobial/antioxidant compounds, which were loaded into the PVA sheets.

The liposomal encapsulation of carvacrol, using different kinds of lecithin was evaluated in order to improve the carvacrol retention in the polymer matrix during the film formation step. Soy lecithin enriched in phosphatidylcholine was the most effective at maintaining the stability of the carvacrol emulsion during film formation, leading to its highest retention in the PVA films. Incorporation of carvacrol (lecithin encapsulated or not) at 5 or 10 %, with respect to the polymer, slightly modified the film microstructure and physical properties and polymer crystallinity and thermal behaviour. The fully hydrolysed PVA provided films with a better mechanical performance and oxygen barrier capacity, but with less carvacrol retention. In contrast, partially hydrolysed PVA gave rise to more homogenous films with a higher carvacrol content, while acetyl groups protect the polymer for thermodegradation, shifting the degradation temperature to temperatures above the melting point. This allows the production of films using the common industrial thermoplastic processing techniques. Therefore, partially acetylated PVA has a great potential for the production of active films with carvacrol, with a wider range of possibilities (casting or thermoprocessing) than fully hydrolysed PVA.

Cinnamic and ferulic acids, with lower potential sensory impact than of carvacrol, were also incorporated into partially and fully hydrolysed PVA, at 1 and 2 % with respect to the polymer, by casting of aqueous solutions containing glycerol (10%) to improve the acid solubility. Glycerol plasticized PVA films exhibited poorer barrier capacity than those non-plasticized.

The incorporation of ferulic acid promoted greater changes in the film properties than cinnamic acid, due to a crosslinking effect, which promoted crystallinity, stiffness and barrier capacity of the material. Glycerol plasticized films based on partially hydrolysed PVA with phenolic acids were also obtained by melt blending and compression moulding. Termoprocessed films were less stretchable and resistant to break and more permeable to oxygen and water vapor. *In vitro* studies demonstrated that cast and termoprocessed films containing phenolic acids exhibited antioxidant and antimicrobial activity, especially with ferulic acid.

Three-layer films composed of a central layer of PVA loaded with active compounds (carvacrol lecithin encapsulated carvacrol or ferulic acid) and two external layers of termoprocessed, PEG plasticised PLA, were obtained by thermo-compression, aimed to meet food packaging requirements. Thermocompression was effective for the interlaminar adhesion, providing the laminates with regular thickness layers. Multilayers exhibited mechanical performance close to the PLA sheets and high oxygen and water vapor barrier capacity. Nevertheless, functional properties in each laminate were modulated by the inter-sheet compound migration during thermocompression. All laminates with active compounds incorporated into the PVA sheet were effective at controlling microbial growth in packaged beef meet. Therefore, lamination of partially hydrolysed PVA and PLA films represents a successful strategy to obtain packaging materials with functional properties closer to those of some synthetic plastics commonly used in food packaging. Likewise, these materials incorporating carvacrol or ferulic acid could be used for packaging of foods highly susceptible to oxidative or microbiological degradative process to extend their shelf life.

RESUMEN

El uso masivo de plásticos sintéticos y su impacto medioambiental, así como la necesidad de mejorar la conservación de los alimentos, hace necesario el desarrollo de alternativas biodegradables para el envasado activo de los alimentos, con el fin de alargar su vida útil. El uso de compuestos activos de origen natural para la obtención de este tipo de materiales es una nueva tendencia basada en la demanda del consumidor de productos más seguros y saludables. La presente Tesis Doctoral, se ha centrado en el desarrollo de materiales multicapa activos para el envasado, basados en polímeros biodegradables con propiedades complementarias, con el fin de obtener materiales con propiedades mecánicas y de barrera adecuadas para cumplir con los requisitos del envasado alimentario. Para ello, se han considerado el poli (vinil alcohol) (PVA) (con alta capacidad de barrera al oxígeno), de diferente peso molecular y grado de hidrolisis, y el poli (ácido láctico) PLA (con alta capacidad de barrera al vapor de agua). Asimismo, se eligieron el carvacrol (encapsulado o no en liposomas) y los ácidos ferúlico y cinámico, como compuestos antimicrobianos/antioxidantes, que se cargaron en los films de PVA.

Se estudio la encapsulación del carvacrol en liposomas de diferentes tipos de lecitina para mejorar la retención del carvacrol en la matriz polimérica durante la etapa de formación del film. La lecitina de soja enriquecida en fosfatidilcolina fue la más eficaz para mantener la estabilidad de la emulsión de carvacrol durante la formación del film, dando lugar a su mayor retención en el mismo. La incorporación de carvacrol (encapsulado o no en lecitina) al 5 o al 10 %, con respecto al polímero, modificó ligeramente la microestructura y las propiedades físicas de la película, así como la cristalinidad y el comportamiento térmico del polímero. El PVA totalmente hidrolizado proporcionó películas con un mejor rendimiento mecánico y capacidad de barrera al oxígeno, pero con menor retención de carvacrol. Por el contrario, el PVA parcialmente hidrolizado dio lugar a películas más homogéneas, con un mayor contenido de carvacrol, mientras que los grupos acetilos protegieron al polímero de la termodegradación, desplazando la temperatura de degradación a temperaturas por encima del punto de fusión. Esto permite la obtención de films mediante las técnicas habituales del procesado termoplástico industrial. Por lo tanto, el PVA parcialmente acetilado tiene un gran potencial para la producción de films activos con carvacrol, con una más amplia gama de posibilidades (casting o termoprocesado) que el PVA totalmente hidrolizado.

Se incorporaron también ácido cinámico y ferúlico, con un potencial impacto sensorial menor que el del carvacrol, al PVA parcial y totalmente hidrolizado, al 1 y al 2% con respecto al polímero, mediante casting de las soluciones acuosas con glicerol (10%) para mejorar la solubilidad de los ácidos.

Los films de PVA plastificados con glicerol mostraron una menor capacidad de barrera que los no plastificados. La incorporación del ácido ferúlico promovió mayores cambios en las propiedades de los films que el ácido cinámico, debido a un efecto de reticulación, que promovió la cristalinidad, la rigidez y la capacidad de barrera del material. También se obtuvieron películas plastificadas con glicerol a base de PVA parcialmente hidrolizado con ácidos fenólicos, mediante mezclado en fundido y moldeo por compresión. Los films termoprocesados fueron menos extensibles y resistentes a la rotura y más permeables al oxígeno y al vapor de agua. Estudios *in vitro* demostraron que los films con ácidos fenólicos obtenidos por casting o termoprocesado presentaron actividad antioxidante y antimicrobiana, especialmente con ácido ferúlico.

Con el objetivo de cumplir los requisitos de envasado de alimentos, se obtuvieron films tri-capa por termocompresión, compuestos por una capa central de PVA, cargada con compuestos activos (carvacrol encapsulado en lecitina o ácido ferúlico), y dos capas externas de PLA termoprocesado, plastificado con PEG. La termocompresión fue eficaz para la adhesión interlaminar, proporcionando capas de espesor regular a los laminados. Las multicapas mostraron un rendimiento mecánico próximo al de los films de PLA y una elevada capacidad de barrera al oxígeno y al vapor de agua. Sin embargo, las propiedades funcionales de cada laminado fueron moduladas por la migración de compuestos entre las láminas durante la termocompresión. Todos los laminados con compuestos activos incorporados a la capa de PVA fueron eficaces para controlar el crecimiento microbiano en filetes de carne de vacuno envasada. Por tanto, el laminado de films de PVA y PLA parcialmente hidrolizado representa una estrategia adecuada para obtener materiales de envasado con propiedades funcionales más próximas a las de algunos plásticos sintéticos comúnmente utilizados en el envasado de alimentos. Asimismo, estos materiales con carvacrol o ácido ferúlico incorporados podrían utilizarse para el envasado de alimentos altamente susceptibles a procesos oxidativos o de degradación microbiológica para alargar su vida útil.

RESUM

L'ús massiu de plàstics sintètics i el seu impacte mediambiental, així com la necessitat de millorar la conservació dels aliments, fa necessari el desenvolupament d'alternatives biodegradables per a l'envasament actiu dels aliments, amb la finalitat d'allargar la seua vida útil. L'ús de compostos actius d'origen natural per a l'obtenció d'aquesta mena de materials és una nova tendència basada en la demanda del consumidor de productes més segurs i saludables. La present Tesi Doctoral, s'ha centrat en el desenvolupament de materials multicapa actius per a l'envasament, basats en polímers biodegradables amb propietats complementàries, amb la finalitat d'obtindre materials amb propietats mecàniques i de barrera adequades per a complir amb els requisits de l'envasament alimentari. Per a això, s'han considerat el poli (vinil alcohol) (PVA) (amb alta capacitat de barrera a l'oxigen), de diferent pes molecular i grau d'acetilació, i el poli àcid làctic (PLA) (amb alta capacitat de barrera al vapor d'aigua). Així mateix, es van triar el carvacrol (encapsulat o no en lecitina) i els àcids ferúlico i cinàmic, com a compostos antimicrobians/antioxidants, que es van incorporar en els films de PVA.

Es va estudiar l'encapsulació del carvacrol en liposomes de diferents tipus de lecitina per a millorar la retenció del carvacrol en la matriu polimèrica durant l'etapa de formació del film. La lecitina de soja enriquida en fosfatidilcolina va ser la més eficaç per a mantindre l'estabilitat de l'emulsió de carvacrol durant la formació del film, donant lloc a la seua major retenció en aquest. La incorporació de carvacrol (encapsulat o no en lecitina) al 5 o al 10%, respecte al polímer, va modificar lleugerament la microestructura i les propietats físiques de la pel·lícula, així com la cristalinidad i el comportament tèrmic del polímer. El PVA totalment hidrolitzat va proporcionar pel·lícules amb un millor rendiment mecànic i capacitat de barrera a l'oxigen, però amb menor retenció de carvacrol. Per contra, el PVA parcialment hidrolitzat va donar lloc a pel·lícules més homogènies, amb un major contingut de carvacrol, mentre que els grups acetilos van protegir el polímer de la termodegradación, desplaçant la temperatura de degradació a temperatures per sobre del punt de fusió. Això permet l'obtenció de films mitjançant les tècniques habituals del processament termoplàstic industrial. Per tant, el PVA parcialment acetilat té un gran potencial per a la producció de films actius amb carvacrol, amb una més àmplia gamma de possibilitats (*casting* o termoprocesat) que el PVA totalment hidrolitzat.

Es van incorporar també àcid cinàmic i ferúlic, amb un potencial impacte sensorial menor que el del carvacrol, al PVA parcial i totalment hidrolitzat, a l'1 i al 2% respecte al polímer, mitjançant *casting* de les solucions aquoses amb glicerol (10%) per a millorar la solubilitat dels àcids. Els films de PVA plastificats amb glicerol van mostrar una menor capacitat de barrera que els no plastificats.

La incorporació de l'àcid ferúlico va promoure majors canvis en les propietats dels films que l'àcid cinàmic, a causa d'un efecte d'entrecreuat, que va promoure la cristalinidad, la rigidesa i la capacitat de barrera del material. També es van obtindre pel·lícules plastificades amb glicerol a base de PVA parcialment hidrolitzat amb àcids fenòlics, mitjançant barrejat en fos i emotlament per compressió. Els films termoprocessats van ser menys extensibles i resistents al trencament i més permeables a l'oxigen i al vapor d'aigua. Estudis *in vitro* van demostrar que els films amb àcids fenòlics obtinguts per *casting* o termoprocessament van presentar activitat antioxidant i antimicrobiana, especialment amb l'àcid ferúlic.

Amb l'objectiu de complir els requisits d'envasament d'aliments, es van obtindre films tri-capa per termocompressió, compostos per una capa central de PVA, carregada amb compostos actius (carvacrol encapsulat o no en lecitina y àcid ferúlic), i dues capes externes de PLA termoprocessat, plastificat amb PEG. La termocompressió va ser eficaç per a l'adhesió interlaminar, proporcionant capes de grossària regular als laminatges. Les multicapes van mostrar un rendiment mecànic pròxim al dels films de PLA i una elevada capacitat de barrera a l'oxigen i al vapor d'aigua. No obstant això, les propietats funcionals de cada laminatge van ser modulades per la migració de compostos entre les làmines durant la termocompressió. Tots els laminatges amb compostos actius incorporats a la capa de PVA van ser eficaces per a controlar el creixement microbià en filets de carn de boví envasada. Per tant, el laminatge de films de PVA i PLA parcialment hidrolitzat representa una estratègia adequada per a obtindre materials d'envasament amb propietats funcionals més pròximes a les d'alguns plàstics sintètics comunament utilitzats en l'envasament d'aliments. Així mateix, aquests materials amb carvacrol o àcid ferúlic incorporats podrien utilitzar-se per a l'envasament d'aliments altament susceptibles a processos oxidatius o de degradació microbiològica per a allargar la seua vida útil.

PREFACE

DISSERTATION OUTLINE

This Doctoral thesis is organized in five sections: **Introduction**, **Objectives**, **Chapters**, **General Discussion** and **Conclusions**.

The **Introduction** section focuses on the use of bioplastics in the food packaging field, highlighting their application as carriers for natural active compounds in order to develop of active films with antimicrobial and antioxidant capacity. Likewise, the combination of biodegradable polymers with complementary functional properties in high-efficiency multilayer assemblies and their application for food preservation was emphasised. Next section, presents the general and specific **Objectives** of this Doctoral thesis.

The obtained results are organised into five Chapters. Each chapter is presented in article-style. **Chapter I, II, III** and **V** correspond to one scientific publication, and **IV** was divided into two scientific articles. Each scientific article was structured into: abstract, introduction, materials and methods, results and discussion, conclusions and references.

Chapter I entitled *"Liposomal encapsulation of carvacrol to obtain active poly (vinyl alcohol) films"*, compares the effectiveness of lecithin with different origin and composition in the liposomal encapsulation of carvacrol. The study of the liposome stability in aqueous medium (ζ-potential and particle size distribution over time) and the capacity of the liposomes to retain carvacrol in matrices of PVA with different molecular characteristics (molecular weight and acetylation degree) is described. Liposomes obtained with soy lecithin enriched with phosphatidylcholine led to the greatest carvacrol retention in the PVA films.

Chapter II entitled *"Effect of carvacrol in the properties of films based on poly (vinyl alcohol) with different molecular characteristics"*, analyses the effect of the incorporation of the emulsified carvacrol on the microstructural, thermal, tensile and barrier properties of active films based on PVA with different molecular characteristics. The results indicated the relevance of the molecular weight and the degree of hydrolysis of PVA in the degree of carvacrol retention and in the characteristics of the final films.

Chapter III entitled *"The Incorporation of carvacrol into poly (vinyl alcohol) films encapsulated in lecithin liposomes"* studies the incorporation of lecithin encapsulated carvacrol, using liposomes enriched with phosphatidylcholine, in two PVA matrices with different molecular characteristics, as a strategy to improve the carvacrol retention in the final active films. The results showed that the encapsulation of carvacrol enhances its retention in the PVA films, especially in partially hydrolysed PVA.

Chapter IV focus on the incorporation of phenolic acids (ferulic and cinnamic acids) into PVA matrices in order to obtain active films with less sensory impact applicable to food packaging, and is divided in two parts: **Chapter IV.I** entitled *"Effect of phenolic acids on the properties of films from poly (vinyl alcohol) of different molecular characteristics*" and **Chapter IV.II** entitle

"Effect of the processing method on the physicochemical and active properties of poly (vinyl alcohol) films incorporating phenolic acids". Both, the effect of the type of phenolic acid and the type of processing (casting and thermoprocessing) on the microstructure, thermal behaviour and tensile and barrier properties of PVA films are analysed. Thermoprocessing is only applied to partially hydrolysed PVA since acetate groups protect polymer for thermodegradation, opening the temperature window for thermoprocessing. The results showed that ferulic acid has greater potential for developing active PVA materials, due to its higher bioactivity and its ability to interact with PVA chains, improving the tensile and barrier performance of the films.

Chapter V entitled *"Antimicrobial PLA-PVA multilayer films containing phenolic compounds"* studies the combination of selected active PVA sheets (internal layers) and PEG plasticised PLA external layers in three-layer assemblies, to meet food packaging requirements in terms of barrier capacity and mechanical performance. Low molecular weight, partially acetylated PVA is chosen to this end due to its better capacity to load the active compounds (carvacrol and ferulic and cinnamic acids). The functional properties as packaging material of the three-layers are analysed, while their antimicrobial capacity is tested in beef meat fillets throughout cold storage time.

The **General Discussion** section analyses from a global perspective the most relevant results obtained from the different chapters and draws a comparison between the studied PVA films, containing different active compounds, and the three-layer films (laminates) obtained by their combination with PLA sheets, highlighting the advantages of the laminates for food preservation, particularly for beef meat product where are applied.

Finally, the **Conclusions** section presents the most relevant findings of this Doctoral Thesis.

DISSEMINATION OF RESULTS

INTERNATIONAL JOURNALS JCR

Published

- *Research articles:*

Liposomal Encapsulation of Carvacrol to Obtain Active Poly (Vinyl Alcohol) Films. Andrade, J., González-Martínez, C., & Chiralt, A. *Molecules* (2021), 26(6), 1589.

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The Incorporation of Carvacrol into Poly (vinyl alcohol) Films Encapsulated in Lecithin Liposomes. Andrade, J., González-Martínez, C., & Chiralt, A. (2020). *Polymers* (2020), 12(2), 497.

Effect of phenolic acids on the properties of films from Poly (vinyl alcohol) of different molecular characteristics. Andrade, J., González-Martínez, C., & Chiralt, A. *Food Packaging and Shelf Life* (2021) 29, 100711.

Submitted

- Research articles:

Effect of processing method on the physicochemical and active properties of Poly (vinyl alcohol) films incorporating phenolic acids. Andrade, J., González-Martínez, C., & Chiralt, A. *Food Packaging and Shelf Life*.

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CO-SUPERVISION OF Final degree project or Final master's project

- Final degree project

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- Final master's project

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Incorporación de ácidos fenólicos en películas de poli (vinil) alcohol elaboradas por el método de casting. Prieto Mota, Paula, (2020). M*áster universitario en gestión de Calidad y Seguridad Alimentaria.*

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TABLE OF CONTENTS

LIST OF ACRONYMS

AC	Active compound	PA	Phenolic acid
ASTM	American Society for Testing and Materials	PBC	Psychrotrophic bacteria count
C	Crystallinity	PDI	Polydispersity index
CA	Carvacrol	PEG	Polyethylene glycol
C_{ab}*	Chroma	PLA	Poly (lactic acid)
CNA	Cinnamic acid	PVA	Poly (vinyl alcohol)
DH	Hydrodynamic diameter	PVAc	Poly (vinyl acetate)
DH	Degree of hydrolysis	RH	Relative humidity
DSC	Differential Scanning Calorimetry	SFL	Sunflower lecithin
DTGA	Thermal weight loss derivate	SL	Soy lecithin
EM	Elastic modulus	SL-PC	Soy lecithin enriched in phosphatidylcholine
EO	Essential oil	TCC	Total coliforms count
E	Elongation at break	Tg	Glass transition temperature
F	Fully hydrolysed	TGA	Thermo Gravimetric Analysis
FA	Ferulic acid	Ti	Internal transmittance
FESEM	Field Emission Scanning Electron	Tm	Melting temperature
Gly	Glycerol	To	Onset temperature from TGA
h_{ab}*	Hue	Tp	Peak temperature from TGA
L	Lecithin/liposome	TS	Tensile Strength
L*	Luminosity	TVC	total viable count
LSD	Least significant difference	UV-VIS	Ultra Violet-Visible Spectroscopy
Mw	Molecular weight	WVP	Water vapour permeability
OP	Oxygen permeability	WVTR	Water vapour transmission rate
P	Partially hydrolysed	ΔHm	Melting enthalpy

I. INTRODUCTION

BIODEGRADABLE ACTIVE FILMS FOR FOOD PACKAGING APPLICATIONS

The Sustainable Development Goals (SDGs), were raised in the United Nations Conference on Sustainable Development, took place in Rio de Janeiro, Brazil in 2012. The SDGs are a universal call to face the environmental, political and economic challenges of the world, promoting a shared plan to seek peace and prosperity for the planet by 2030. The 17 SDGs are interrelated, meaning that the success of one affect that of others. Among the great challenges of current society are ensuring the food availability, necessary for a growing population, and reducing the production and consumption of plastics, which cause a serious environmental problem. Both aspects converge in at least six of the SDGs: Zero hunger (SDG2), Good health and well-being (SDG3), Responsible consumption and production (SDG12), Climate action (SDG13), Life below water (SDG14), Life on land (SDG15).

Every year 1.3 billion tons of food are lost or wasted across the food supply chain, nearly one-third of all production, which, added to trends in economic and population growth, puts enormous pressure on our natural resources (Selvam et al., 2021). One of the important factors to avoid food waste and ensure its availability is to promote the food preservation, increasing the shelf life. Packaging is one of the main preservation methods. In fact, one of the causes of food loss in developing countries is the lack of adequate packaging and transport solutions to maintain food quality and freshness from production to consumption (PlascticsEurope, 2020).

The use of plastics as packaging material has been increasing exponentially since its appearance, about 100 years ago. Plastic is lightweight and offers high performance, which can have economic benefits by reducing transport costs, extending the shelf life of the products (Matthews et al., 2021) and minimizing the negative impact of food waste (Yun et al., 2018). But plastic also has different drawbacks, specifically at the environmental level. The high volume of plastic production has reached 368 million tons per year, with a short time of use, mostly single-use, and waste management problems (Geueke et al., 2018), which, added to the non-biodegradable nature of plastic, has generated a growing concern about the plastic accumulation in our oceans and natural environment (Cordier & Uehara, 2019).

The demand for plastics in Europe reached 50.7 million tons between 2018 and 2019, of which 29.1 million tons of waste were collected in order to be treated, 32.5% were recycled, 42.6% were incinerated and 24.9% went to landfills. Of the total of plastics, 39.6% correspond to packaging materials (PE-LD / PE-LLD, PE-HO / PE-MD, PP, PS, PVC and PET etc) (EuropeanBioplastics, 2020). However, in the case of food packaging, recycling is difficult because they are loaded with residual organic matter that hinders the functionality of the material for later uses. In addition, food packaging often consists of several layers made of different types of polymers. These materials pose great challenges for recycling, either with

high costs, technological difficulties to separate the different plastic polymers or the impossibility of recycling mixed polymers (Faraca & Astrup, 2019). Furthermore, recycled plastics cannot be used for food packaging due to the risk of toxic substances migration.

Given the difficulties associated with the complex recycling of food packaging, materials with functional properties similar to conventional plastics, but that do not accumulate on the planet due to their long degradation period and that do not generate persistent microplastics are needed. In this sense, in order to create a sustainable environment and prevent the possible disposal of recalcitrant plastic waste in the environment, the production of bioplastics for food packaging application, especially biodegradables ones, have gained a lot of attention.

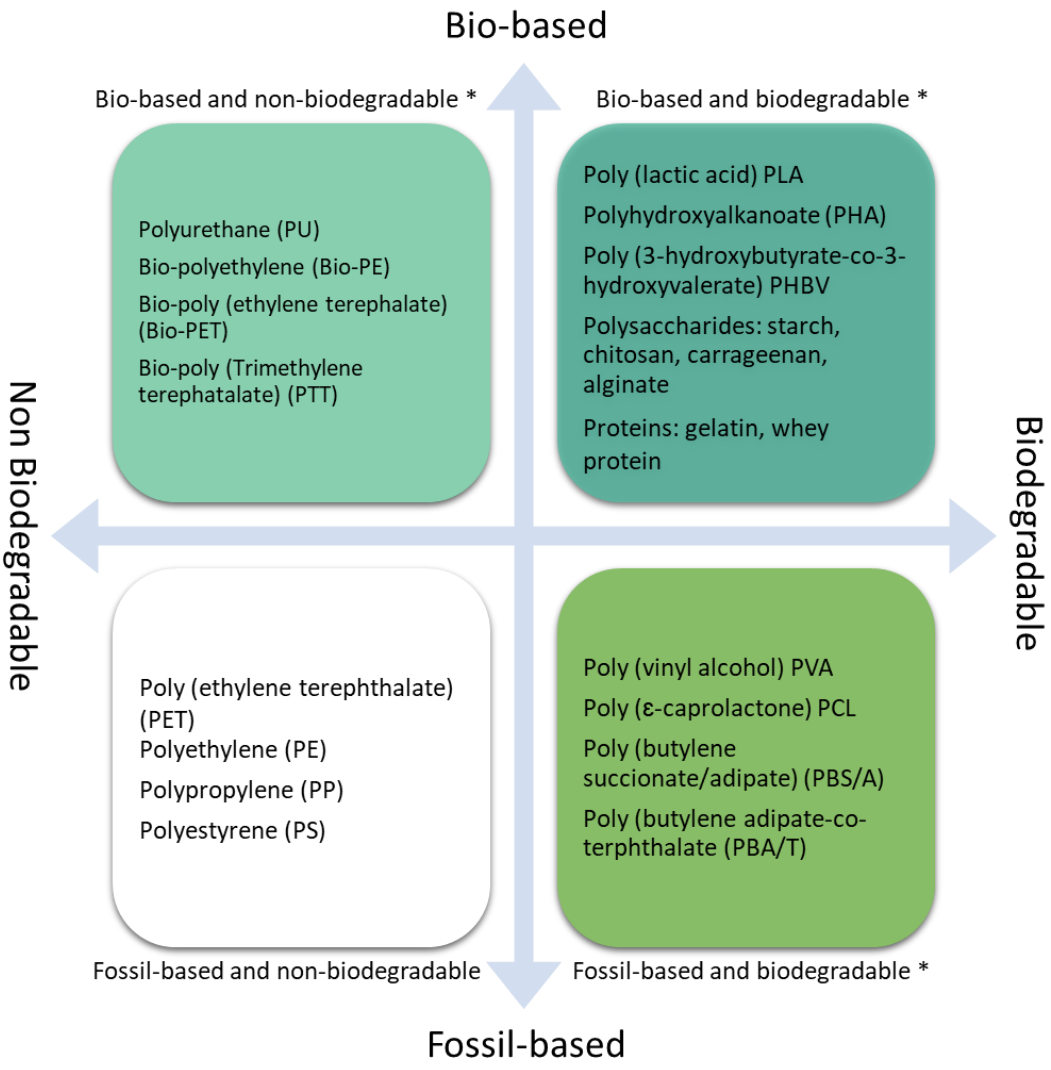
This chapter gives an overview of the state of the art of food packaging with active biodegradable materials, as a potential solution for improving food preservation, alleviating the environmental problem caused by the abuse of conventional plastics.

1. Bioplastics for food packaging

In **Figure 1**, the classification of the different plastics is presented depending on their origin, fossil or bio renewable sources, and their biodegradable or non-biodegradable nature. A plastic material is defined as bioplastic if is either bio-based, biodegradable, or has both properties (EuropeanBioplastics, 2020). According to the origin or the production method the biopolymers can be divided into three groups: those directly extracted from biomass of vegetable or animal origin; those produced by classical chemical synthesis from bio-based or fossil-based monomers; and those produced by wild or genetically modified microorganisms.

Biopolymers obtained from biomass are polysaccharides or proteins. Of polysaccharides, starch is abundant in many food products (corn, potato, cassava, etc.). Native starch consists of two types of glucose polymers, amylose and amylopectin. This is the highly used because it can be manufactured in large quantities, at relatively low cost, and easily handled to form films. The major challenge with native starch is its brittleness and hydrophilic nature (Zhong et al., 2020). Chitosan is another abundant polysaccharide, obtained from the shell of crustaceans, which also has antimicrobial properties (Chiralt et al., 2020). Others are obtained from algae such as carrageenan or alginate. Proteins, such as gelatine, obtained from by-products of meat or fish industry, as well as dairy and soy proteins have also been extensively studied (Chiralt et al., 2020). However, the constitutive monomers of these biopolymers are very polar, due to their abundant hydroxyl or amino) groups and, therefore, they have high water affinity and are quite water soluble (Collazo-Bigliardi et al., 2019; Ortega-Toro et al., 2017; Sapper et al., 2018, 2019). This provide very low water vapour barrier capacity to the materials, which is a negative factor for food preservation, where water exchanges must be controlled. In addition, this fact causes the moisture adsorption and modifications in the

material properties depending on the humidity of the environment or of the food in contact. This limits their use for many foods with high water activity. In contrast, they are a very effective barriers to the transport of gases, specifically to oxygen, thus preventing oxidative reactions causing food spoilage.



***Bioplastics**

Figure 1. Classification of plastics according to their origin and biodegradable/non biodegradable nature.

The **synthesis** of biopolymers can be carried out from bio-based monomers, such as those becoming from starch (in the case of polylactic acid: PLA), or from those fossil-based such as

occurred in polycaprolactone (PCL). These usually are hydrophobic polyesters, less water sensitive and with better water vapour barrier capacity than those of the previous group. However, these present higher oxygen permeability (Tampau et al., 2020a; Yi et al., 2009).Poly (vinyl alcohol) (PVA) is another biodegradable polymer obtained by synthesis, with high oxygen barrier capacity and good tensile properties, also being an interesting alternative for the development of food packaging materials (Cano et al., 2015; Tampau et al., 2020b).

The third group corresponds to those polymers obtained **biotechnologically** from microorganisms. Some polysaccharides, such as xanthan gum, gellan or pullulan are also obtained by fermentation (Sapper et al., 2019). But some polyesters can also be produced by this processing method; within this family are the so-called polyhydroxyalkanoates, with different properties, depending on the length of the hydrocarbon chain. One of the most promising is the poly (3-hydroxybutyrate-co-3-hydroxyvalerate) PHBV, whose biosynthesis process involves bacteria that can feed on food waste, which contributes to the re-use of waste, producing the polymers when a carbon source is in excess and at least one other nutrient essential for growth has been depleted (Hernández-García, Vargas, González-Martínez, et al., 2021; Pinto et al., 2021).

Some specific characteristics of the bioplastics used in the present study are described below.

1.1. Poly (vinyl alcohol)

Polyvinyl alcohol (PVA) is a semi-crystalline polymer, usually produced by alkaline hydrolysis of its precursor polyvinyl acetate (PVAc) (**Figure 1**). PVA is a highly versatile biopolymer, nontoxic, water soluble, with excellent properties of chemical resistance, mechanical, gas barrier, adhesive and film forming, with tolerance towards organic solvents (Liu et al., 2014; Loryuenyong et al., 2015). The controlled process allows for obtaining polymers with different extent of polymerization and degree of hydrolysis/re-esterification (Fukae et al., 1994). These characteristics have encouraged the use of PVA to develop biodegradable films. The specific functional properties of the materials based on PVA depend on various factors: the size of the molecular chains, the number of OH groups that remain free in the macromolecule, how many acetate groups and aldehyde radicals are bound to it and the extent to which ester bonds are created in the molecule (Julinová et al., 2018). These molecular characteristics also affect the polymer affinity and compatibility with other compounds that can be added as active compounds. Materials of highly hydrolysed PVA are the most suited to act as an oxygen barrier. However, a higher content in hydroxyl group in the matrix leads to a greater water affinity, which makes PVA films sensitive to the surrounding relative humidity. Then, their application as food packaging is restricted and requires blending or lamination with other non-polar polymers. In general, mechanical properties of synthetic biopolymers can be controlled,

which helps in acquiring desirable properties based on the application necessity (Liu et al., 2014; Loryuenyong et al., 2015).

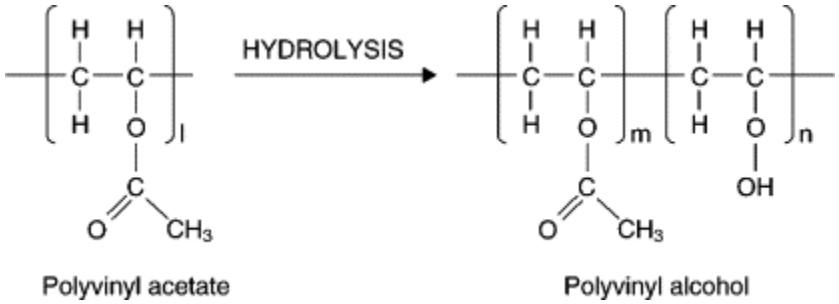


Figure 1. Poly (vinyl alcohol) development process Source: (Julinová et al., 2018).

Currently, PVA is used in a wide range of industries especially in fabric and paper sizing, fiber coating, adhesives, emulsion polymerization, films for packing and farming. Over the last decade, much attention has been recorded in PVA as biocompatible, low cytotoxic and degradable polymer for biomedical and biomaterial research fields. PVA hydrogels for biomedical applications have been used in drug delivery, cell encapsulation, wound dressings, artificial meniscus, vascular grafts and biosensors. PVA hydrogels have also been used as disposable contact lenses thanks to its high moisture retention, high oxygen permeability, optical clarity, compliance and softness (ben Halima, 2016).

1.2. Poly (lactic acid)

Poly (lactic acid) PLA is one of the most widely used biodegradable bioplastics, which constitutes a promising alternative to conventional plastics in food packaging. PLA is a linear aliphatic polyester, obtained industrially through lactide open ring polymerization or by the condensation-polymerization of the lactic acid monomer (Ahmed et al., 2009). The monomer of PLA is lactic acid, which can be produced by fermentation of renewable sources such as corn, cassava, potato and sugarcane (Altaf et al., 2006). Lactic acid has two active forms called L-lactic acid and D-lactic acid due to an asymmetric carbon atom. Thus, three stereochemical forms of PLA are possible: poly (L-lactide) (PLLA), poly (DL-lactide) (PDLLA) and poly (D-lactide) (PDLA).

PLA has numerous attractive properties compared to other aliphatic polyesters: high mechanical strength, high elastic modulus, low flammability and toxicity, biodegradability, biocompatibility, transparency, energy savings and easy processability (Ljungberg et al., 2005). However, it has significant problems such as a low oxygen barrier capacity, brittleness, and

can be unacceptably noisy due to the stiffness, when it is highly crystalline (Qi et al., 2017a; Saeidlou et al., 2012). These reasons often make it necessary to include plasticizers (PEG) to modulate tensile properties or lamination with high gas barrier materials. So, PLA and PVA exhibit complementary properties that could be combined to obtain laminated materials useful for food packaging purposes.

Safety evaluations have reported limited migration levels of polymeric components into the food this material is in contact with. The studies have shown there is no threat to human health as these migrants are expected to be metabolized to lactic acid, reaching levels far below to those acceptable for lactic acid as a food additive (Conn et al., 1995). These types of reports have granted approval of PLA for contact with food by the Food and Drug Administration (FDA). Food corporations such as Danone (Germany), Del Monte (USA) and Noble Juice (USA), as well as leading food retailers such as Wal-Mart (USA), SPAR (Austria) and Auchan (France) are already using PLA based packaging in some of their products (Castro-Aguirre et al., 2016). Several types of foods are currently packed and commercialized with PLA based materials. These foods are characterized by different physical states, a wide range of water activity (aw), pH and composition, with different shelf life. Some examples are potato crisps (amorphous solid, aw = 0.1–0.3), fresh salads and vegetables (biological gel, aw > 0.99), yogurts (gel, aw > 0.99) and citrus juices (acidic liquid, aw = 0.97–0.99) (Gerometta et al., 2019).

2. Biodegradation

The interest in biopolymers is mostly because of their biodegradability. Biodegradability of biopolymers means that they can be decomposed into their constituents by living organisms. Biological degradation involves cleavage of chemical bonds under specific ecological conditions by microorganisms or their hidden products, such as surfactants and enzymes, and it might be aerobic or anaerobic (Nasrollahzadeh et al., 2021). In this process, biopolymers are converted into energy, which microorganisms need to survive. Bacteria degrade biopolymers in marine sediment, soil, or compost (Hernández-García, Vargas, González-Martínez, et al., 2021). Biodegradation occasionally starts by non-biological degradation, for example, oxidation by atmospheric oxygen, hydrolysis or photodegradation (Nasrollahzadeh et al., 2021).

Generally, biodegradation is a four-step process: 1) biodeterioration, which comprises the breakdown of biodegradable materials into small pieces by the microorganisms action; 2) depolymerization, that involves catalytic agents generated by microorganism (enzymes and free radicals) to break down the polymeric chains and convert them into lower-molecular-weight polymers, giving rise to monomers, dimers and oligomers, which are able to cross the semi-penetrable bacterial membranes; 3) assimilation, whereby polymeric molecules are used

by microorganisms as energy and carbon sources and can produce new biomass, storage vesicles, and many kinds of metabolites, which help to maintain the structure, cellular action, and reproduction; 4) finally in the mineralization step, the metabolites may be excreted and reach the outside of the cellular medium (Chandra & Rustgi, 1998; Hernández-García, Vargas, González-Martínez, et al., 2021; Nasrollahzadeh et al., 2021). The products of this process are biomass, water, salts and gases.

Biodegradability of biopolymers depends on both the polymer molecular characteristics and environmental conditions, which influence the degree and rate of biodegradation. As concerns the polymer, the chemical structure, the kind of chemical bonds, the molecular weight distribution, the molecular complexity and the degree of crystallinity are critical factors that affect the biodegradation process. In general, polymers with a shorter chain, more amorphous and with less molecular complexity are more sensitive to the action of microbial enzymes. In contrast, wetness, oxygen accessibility, pH, the specific type of microorganisms presents, and the enzymes secreted by microorganisms, are the most important conditions of the environment to define degradation process (Nasrollahzadeh et al., 2021).

2.1. Polyvinyl alcohol degradation

The promising potential of synthetic PVA is its biodegradability which is considered as an unusual trait among the other synthetic carbon-chain polymers. The history of PVA biodegradation extends back over 1936 since the first report of degradation by *Fusarium lini*, phytopathogenic fungus, producing carbon dioxide and water as a result of the extracellular attack by a dehydratase (Nord, 1936). (Suzuki et al., 1973; Watanabe et al., 1975) and carried out extensive studies of PVA biodegradation. A variety of microorganisms able to assimilate PVA have already been reported. Most of PVA-degrading microorganisms were identified as aerobic bacteria belonging to Pseudomonas. Furthermore, some Gram-negative (*Alcaligenes faecalis* and *γ-proteobacteria*) and Gram-positive (*Bacillus megaterium, Paenibacillus amylolyticus, Microbacterium barkeri* and *Streptomyces venezuelae*) bacteria were included as PVA degraders. Some species degrade and assimilate PVA axenically, even though symbiotic is a rather common feature of PVA biodegradation.

PVA is biodegradable under a two-step metabolism consisting of oxidation and hydrolysis: oxidation of two side hydroxyl groups either by oxidase or dehydrogenase and subsequent hydrolysis (cleavage of the carbon-carbon chain between two carbonyl groups or a carbonyl group and an adjacent hydroxymethine group) either by hydrolase or aldolase. Enzymatic degradation of PVA takes place randomly and the resultant molecules distribute over smaller molecular weights. This enzymatic random endocleavage of PVA chains does not appear to be appreciably affected by polymer structural features, such as degree of polymerization and acetylation degree, at least for samples containing less than 20% residual acetyl groups.

However, a positive influence of the hydrophobic character (residual acetyl group content in PVA chain) of the polymeric substrates on the activity of specific PVA-dehydrogenase was demonstrated. The activity of other PVA chains cleaving enzymes, such as PVA-oxidase, also showed dependence on the macromolecular structure (Halima, 2016).

The biodegradability of PVA seems to be highly dependent on the environmental conditions. In general, PVA ideally biodegrades in an aerobic aquatic environment with diverse active organisms, whereas in some soils or composting conditions, the degradation rate was slower (Chiellini et al., 2003). To address the biodegradability limitations of PVA, some microorganisms that degrade PVA have been studied and isolated. The microbial communities were subjected to an acclimatization phase in environments rich in PVA to achieve the specificity of the bacteria for the degradation of this polymer. Of the bacterial PVA degraders, *Sphingomonads* were widely studied microorganisms, isolated from wastewater containing PVA, characterized by their PVA oxidase and alcohol dehydrogenase activity. Other specialized microorganisms are *Sphingopyxis sp. PVA3* and *Sphingopyxis sp. 113P3*, the former initially oxidizes PVA through PVA oxidase, while the latter depolymerizes PVA with PVA dehydrogenase. Glucose acts as a catabolite, this finding being particularly interesting considering that several PVA-based blends contain natural polysaccharides, particularly starch.

2.2. PLA degradation

PLA not only has complete biodegradability, but also not pollute environment after biodegradation. The mechanism of biodegradation had been more studied than in PVA. PLA degradation occurs mainly through scission of ester bonds. In addition, polymer degradation is induced by a range of factors in nature, such as oxidation, photodegradation, thermolysis or hydrolysis (Qi et al., 2017b).

During microbial degradation, PLA-degrading microorganisms first excrete extracellular depolymerase of PLA. The rapid production of extracellular depolymerase need usually to be stimulated by some inducers such as silk fibroin, elastin, gelatin, and some peptides and amino acids (Jarerat et al., 2004; Qi et al., 2017b; Tokiwa & Calabia, 2006; Yutaka & Hideo Tanaka, 2006). Most of the inducers have L-alanine units, which is similar to L-lactic acid units of PLA in the stereochemical position of chiral carbon. Following, the depolymerase attack intramolecular ester links of PLA, which result in production of oligomers, dimers and monomers. Afterwards the low molecular weight compounds enter in microbial membranes and decompose into carbon dioxide, water or methane by intercellular enzymes (Qi et al., 2017b; Tokiwa & Jarerat, 2004).

Two components, the appropriate microbes and a favourable environment, are required for the successful biodegradation of PVA. Analysis of PLA-degrading microorganisms is crucial for accelerating biochemical processes of PLA degradation. Therefore, the research on isolation of PLA-degrading microorganisms have been raised in recent years. Currently, multiple types of microbes that are able to degrade PLA have been isolated from soil or water. They mostly are actinomycetes, a fraction of them belong to bacteria and fungus. The degradation rate of PLA also depends on environmental factors, such as PH, humidity, temperature, oxygen and so on (Park & Xanthos, 2009). Nevertheless, the biodegradation of PLA under aquatic environment has been found to be very limited.

3. Active food packaging using bioplastics

Biopolymers can be the source for obtaining materials applied in advanced packaging methods, such as the active packaging. Active packaging interacts with food or its environment to extend its shelf life, improve its safety or its sensory properties, maintaining a high quality. To this end, they can absorb or release substances that allow this function to be carried out, sometimes without the need for direct contact with the product (Chen et al., 2018). Some materials can absorb free radicals or oxygen preventing their deteriorating action or release antimicrobials or antioxidants to favour the food preservation, making it safer and with an extended shelf life (Vilela et al., 2018; Yildirim et al., 2018).

For obtaining these materials, one possibility is the incorporation of the active compounds to the polymer master batch, either by their direct addition or previously encapsulated or anchored in different supports (micro or nanoparticles) by different techniques, in order to control their sustained release over time to the food. This release will depend on the food substrate (solid or liquid, aqueous or fatty, wet or dry, etc.) and on the molecular interactions that occur between the active compound and the packaging material (Muller et al., 2017; Requena et al., 2017). Likewise, interactions and potential migration of food components into the packaging material could also affect the polymer release properties, depending on the relaxation phenomena that migrated compounds could provoke in the polymer matrix (Requena et al., 2017).

As concerns active compounds, the food industry has traditionally used quite effective antimicrobials and antioxidants, incorporated directly into food, but currently some are questioned for their possible negative impact on consumer health. The current trends point to the use of compounds from natural resources (some of which have been traditionally used as spices or flavourings: oregano, cinnamon, cloves...) which have a proven preservative effect (Sharma et al., 2021). Plant essential oils or plant extracts are rich in phenolic compounds that have dual antimicrobial and antioxidant functions, at a more moderate level than synthetics, but with fewer negative health effects.

In general, in the plant world there are many natural compounds of this type that participate in the metabolic processes of plants, with different protective effects. Therefore, plants or food by-products are a source of safer functional compounds for food preservation. These can be used as ingredients, but instead of adding them to food, where they can cause changes in the sensory characteristics, they can be incorporated into the packaging material and from there exercise their active function. **Table 1** shows recent studies on active materials and their application for the preservation of different food systems, focusing on the use of plant-derived active compounds.

Essential oils (EOs) and their isolated compounds have been widely studied and incorporated into different polymeric matrices to obtain coatings or films applicable in active food packaging development. However, due to their chemical instability, they usually present short degradation periods due to external factors, such as light, oxidation or heating (Lima et al., 2019). Furthermore, their high volatility generates considerable losses of the active fraction in the packaging material (Sharma et al., 2020), thus reducing the active capacity of the material (Sánchez-González et al., 2011). The use of nano/micro-encapsulation techniques to protect and entrap these volatile compounds can mitigate this problem (Sánchez-González et al., 2011; Sapper et al., 2018).

Table 1. Recent studies on the active films based on biodegradable polymers.

Polymer matrix	Active compound	Processing method	Antimicrobial or antioxidant assessment	Main result	Reference
Chitosan (CH) or Corn starch (S)	Polyphenols rich thyme extract (TE)	Casting method	Active CH films presented more antioxidant capacity than active S films	CH-TE films presented better tensile response and greater antioxidant activity.	(Talón et al., 2017)
Oxidized (OS) and non-oxidized (S) corn starch blended with gelatin (G)	N-α-lauroyl-l-arginine ethyl ester monohydrochloride (LAE)	Thermoprocessing (C) or Casting method (T)	Thermoprocessed blend films with non-oxidised starch better preserved meat for lipid oxidation. Cast blend films with oxidized starch shows the best antibacterial action, but enhance lipid oxidation.	Thermoprocessed active OS:G film with LAE extended the shelf life of chicken breast fillets.	(Moreno et al., 2018)
Starch-gellan blend	Thyme essential oil (TEO) (free or lecithin encapsulated)	Casting method	In vitro antifungal activity against *Alternaria alternata* (AA) and *Botryotinia fuckeliana* (BF).	Lecithin encapsulation of TEO allowed for greater oil retention (45-55%), which enhanced the antifungical activity of the films.	(Sapper et al., 2018)
Corn starch	Eugenol (EU)	Casting method	Active films were highly effective at preventing sunflower oil oxidation in accelerated storage conditions.	Films with EU encapsulated preventing sunflower oil oxidation even throughout 53 days.	(Talón et al., 2019)

Polymer matrix	Active compound	Processing method	Antimicrobial or antioxidant assessment	Main result	Reference
Potato Starch	Extract of phenolic compounds from rice straw (EPC).	Thermoprocessing method	The obtained starch films exhibited good in-vitro antiradical scavenging activity against DPPH.	The addition of EPC improved the oxygen permeability without negative effect on the thermal stability and water vapor permeability.	(Menzel et al., 2020)
Cassava Starch	Ferulic acid (FA) and cinnamic acid (CA)	Thermoprocessing method	Both phenolic acids exhibited antibacterial effect. Films with CA and FA were more active against *L. Innocua* than against *E. coli*.	The highest effectiveness was obtained for films with cinnamic acid, against *E-coli and L. innocua*, in chicken breast and fresh-cut melon.	(Ordoñez et al., 2021)
PLA/PBSA	Thymol	Extrusion	The antifungal films delayed microbial growth and extended the shelf life of bread.	The antifungal packaging containing 6 wt% of thymol could extend the shelf life of bread to 9 days.	(Suwanamornlert et al., 2020)
PVA	Clove oil	Casting method	The films showed antioxidant and antimicrobial activity in vapour phase.	Shelf-life of *Trichiurus haumela* was extended for 2 days.	(Chen et al., 2018)
PVA	Tea polyphenol (TP)	Casting method	Bacteriostatic effect for *E. coli, S. aureus, B. cinerea* and *Rhizopus.*	PVA/TP films exhibited effective extension of strawberry shelf life.	(Lan et al., 2019)
PVA	Laurus nobilis–LEO- and Rosmarinus officinalis-REO essential oils	Electrospun mesh	Active containing LEO and REO reduced the lipid oxidation processes and *Listeria* counts during cold storage of chicken breast fillets.	The coatings enhanced the shelf-life of chicken breast fillets.	(Göksen et al., 2021)

Incorporation of EOs into hydrophilic biopolymers have been mainly carried out by emulsifying them in the polymer aqueous film forming solutions and active films were obtained by casting (Atarés & Chiralt, 2016). However, during the film drying step, emulsified essential oils are susceptible to destabilization processes (flocculation, coalescence, and creaming), which lead the drops to the film surface where the lipid compounds are lost by steam drag effect along with evaporated water (Cofelice et al., 2019; Requena et al., 2017). (Sapper et al., 2018)used the casting method to develop films based on starch-gellan blends containing emulsified or lecithin encapsulated thyme (*Thymus zygis*) essential oil. Liposomal encapsulation of the EO in lecithin allowed for greater oil retention (45–55%), which enhanced the antifungal activity of the films, against *Botryotinia fuckeliana* and *Alternaria alternata*. Liposomes improved the emulsion stability and limited of EO evaporation during the film drying step; thus, in films with non-encapsulated EO, the EO retention ranged between 4 and 26%. (Valencia-Sullca et al., 2016) reported a similar behaviour for chitosan-based films based with lecithin-encapsulated eugenol or cinnamon leaf essential oil. (Talón et al., 2019) applied spray-drying to encapsulate eugenol using whey protein isolate-maltodextrin and lecithin-maltodextrin wall-systems. This encapsulation process protected eugenol against evaporation and enhanced its thermal stability. The incorporation of eugenol microcapsules containing oleic acid (as a stabiliser component of the microcapsule) in the starch matrices promoted the eugenol retention during film formation and thus, these films exhibited the greatest antioxidant activity. Films prepared with encapsulated eugenol powder, containing lecithin and oleic acid, were highly effective at preventing sunflower oil oxidation even throughout 53 days of storage at 30 °C, maintaining lower and almost constant the values of peroxide index, conjugated dienes and trienes, in comparison with the control samples. Incorporation of active compounds into the polymer matrix can provoke microstructural changes that may affect its functional properties as packaging material. These changes usually occur even when essential oils (free or encapsulated) are incorporated into hydrophilic polymer matrices due to the lack of chemical affinity, that promote phase separation and a dispersed phase of lipid phase into the polymer continuous phase. Thus, the incorporation of lecithin encapsulated thyme essential oil reduced the stiffness, resistance at break and extensibility of the starch films (Sapper et al., 2018). Likewise, the addition of spray-dried eugenol, with lecithin or whey protein, generated starch films with lower mechanical resistant and less transparent (Talón et al., 2019).

Other interesting active compounds with great potential, although less studied, are phenolic acids and phenolic extracts. These are being explored in the development of active packaging, since most of them present thermal stability, low volatility and sensory impact, with a high antioxidant and antimicrobial potential (Lima et al., 2019; Mathew, 2015; Olszewska et al., 2020). Some studies have revealed that the incorporation of phenolic compounds into polymeric matrices also promotes the improvement of some functional properties, due to their affinity and great capacity of interaction with some polymeric matrices of proteinic or polysaccharidic nature (Benbettaïeb et al., 2020; Cao et al., 2007; Ou et al., 2005). (Talón et

al., 2017) reported that the aqueous thyme extract (TE) was efficiently incorporated into chitosan matrices, but not in starch matrices. Therefore, chitosan-based matrices carrying TE polyphenols exhibited better tensile response and greater antioxidant activity, which could be associated with the development of polyphenol-chitosan interactions that contributed to a better protection of the phenol functionality during film formation step. Whereas, in starch films, some losses of antioxidant activity occurred. This could be due to some degradation of TE during the drying of the films caused by the poor protective effect of starch. (Menzel et al., 2020) successfully incorporated the phenolic extract obtained of rice straw waste (major components: ferulic, p-coumaric and protocatechuic acid) into starch-based films. Homogeneous films with antioxidant properties were produced by melt blending and compression moulding. The obtained antioxidant starch films were slightly reddish-coloured and exhibited good in-vitro antiradical scavenging activity against DPPH. The addition of the antioxidant extract improved the oxygen barrier properties without negatively affecting the thermal stability and the water vapor barrier properties of the starch films. (Ordoñez et al., 2021) found that the incorporation of cinnamic or ferulic accid (1 and 2%) in starch matrices led to antimicrobial films, which were effective at inhibiting the growth of *E. coli* and *L. innocua*. Furthermore, interactions between the carboxyl groups of phenolic acids and hydroxyls of starch chains reduced the water affinity of the matrix and the water solubility of the material decreased between 3 and 25%.

Active films have been applied in the packaging of several food matrices to evaluate their preservation capacity. N-α-lauroyl-l-arginine ethyl (LAE) (antimicrobial substance derived from lauric acid and arginine) was incorporated into oxidised and non-oxidised starch blended gelatin obtained by melt melting (non-oxidised) and thermo compression or solution casting (oxidised). Antimicrobial blend films were applied in the packaging of chicken breast fillets and cold stored to analyse the shelf-life extension. Both thermo-processed and cast films significantly extended the shelf life of chicken, however, the cast films with oxidised starch promoted oxidative processes in meat, which affected the pH and colour parameters of packaged samples. Therefore, termo-processed, non-oxidised starch blend films containing LAE were recommended since they effectively extended the shelf life of chicken breast fillets (approximately 4 days) without affecting the meat oxidation (Moreno et al., 2018). (Ordoñez et al., 2021) also reported positive effects on the preservation of the microbial quality of chicken breast in contact with starch films containing ferulic or cinnamic acid. Films with 2% ferulic or cinnamic acid were effective at inhibiting the growth of *E.-coli* and *L. innocua* (Log CFU reduction> 2). Similar effect was also observed in fresh-cut melon in contact with this material. Active coating materials produced through electrospinning of polyvinyl alcohol (PVA) were also applied to chicken breast fillets to prolong their shelf-life. Essential oils from two broadly used spices (*Laurus nobilis* –LEO- and *Rosmarinus officinalis* -REO-) were loaded in the PVA mats. These active coatings containing LEO and REO extended the shelf-life of the

samples, reducing the lipid oxidation (43-65%) process and Listeria counts during cold storage (Göksen et al., 2021).

Trichiurus haumela preservation also was enhanced by packaging with active PVA films containing clove oil (Chen et al., 2018). The PVA films incorporated with clove oil showed effective antimicrobial and antioxidant activities in vapour phase which increased when the content of oil raised. The PVA film containing 9% clove oil showed the best quality protective effectiveness by inhibiting the bacterial growth and lipid oxidation of the *trichiurus haumela*. Its microbiological shelf-life could be extended for 2 days and a 28 % reduction of malonaldehyde was obtained with respect the control sample on 7 storage days. On the other hand, PVA films incorporated with tea phenolic extract exhibited great potential as a packaging material to extend the shelf life of strawberries (Lan et al., 2019). The active PVA film effectively limited microbial proliferation of *E. coli, S. aureus, B. cinerea* and *Rhizopus*. In addition, it retarded the loss of fruit weight and firmness. Antifungal films based on poly (lactic acid)/poly(butylene-succinate-co-adipate) (PLA/PBSA) blends, containing thymol were prepared using blown film extrusion technique (Suwanamornlert et al., 2020). The antifungal films were tested in bread packaging, compared with biaxially oriented polypropylene (BOPP) and neat PLA. Mould growth was observed on the bread after storage for 7 and 9 days, when packaged in active PLA/PBSA with 3 and 6% of thymol respectively. In contrast, the bread packaged in BOPP and PLA showed visible mould growth after 3- and 6-days storage, respectively. So, active PLA/PBSA packaging was effective at preserving the microbial quality of the bread, especially with the highest concentration of thymol.

4. Biodegradable multilayer films for food active packaging

The packaging properties can be significantly enhanced by combining or joining different plastics together by laminated constructions, using different technologies, such as co-extrusion process. Films containing several polymer layers of complementary properties can provide the packaging materials with adequate barrier and mechanical strength (Gherardi et al., 2016). In the food industry, multi-layer combination of synthetic plastic with other materials (laminates) are often used to achieve higher levels of food protection. In this sense, the search for more environmentally friendly that meet the food packaging requirements led to the study of multilayer films based on biodegradable polymers. Additionally, active compounds can be loaded one of the layers the multilayer assembly to accomplish its controlled release on the food surface, generating added value to the package for a better food protection and shelf-life extension. **Table 2** summarizes some studies and applications of multilayer films based on biopolymers.

Table 2. Recent studies on active multilayer films based on biodegradable polymers.

Multilayer polymers	Layer assembly method	Active compound	Main result	Antimicrobial or antioxidant assessment	Reference
Chitosan (CH) / Gelatin (G)	Cast G based films coated with CH film forming solutions.	Chitosan	CH coated films exhibited lower water barrier capacity than net CH films	In vitro/Disk diffusion tests with *L. monocytogenes* and *E-coli*. CH layer in bilayer exhibited antimicrobial activity against *E. coli*.	(Pereda et al., 2011)
Chitosan (CH)/Gelatin (G)	Cast G based films coated with CH film forming solutions.	Lauroyl arginate ethyl (LAE), incorporated in CH layer	CH coated films exhibited lower water barrier capacity and UV light barrier than net CH films.	In vitro/Disk diffusion tests with *Listeria monocytogenes, Escherichia coli, Salmonella typhimurium and Campylobacter jejuni.* CH layer in bilayer exhibited antimicrobial activity against against all tested microorganisms.	(Haghighi et al., 2019)
Chitosan (CH) /cassava starch (CS)	Thermocompression of cast CH films with thermoprocessed CS	Oregano (OEO) and cinnamon leaf (CLEO) essential oils, incorporated into CH layer	CH layer improve the mechanical resistance with respect to starch monolayers.	Aerobial, coliforms and total CFU counts in pork meat Lipid oxidation by TBARS Bi-layer film showed antimicrobial and antioxidant activity due to the action of CH layer. The addition of OEO or CLEO in CH layer did not promote antimicrobial action of in bilayers in pork meat.	(Valencia-Sullca et al., 2018)

Multilayer polymers	Layer assembly method	Active compound	Main result	Antimicrobial or antioxidant assessment	Reference
Chitosan (CH/Polycaprolactone (PCL)	Cast CH films were coated with PCL film forming solutions (C) or thermocompressed with PCL cast films (T).	Grape seed extract (GSE) incorporated into CH layers	Bilayers exhibited improved water barrier capacity, higher elastic modulus and tensile strength.	In vitro tests in liquid medium against *E. coli* and *L. monocytogenes.* DPPH scavenging capacity. Bi-layers showed antimicrobial and antioxidant activity (C>T). Possible oxidation of GSE during the thermocompression step.	(Sogut & Seydim, 2018)
Chitosan (CH)/polycaprolactone (PCL)	Cast CH films were coated with PCL film forming solutions.	Grape seed extract (GSE) incorporated into CH layers	Bilayers showed improve water vapour and oxygen barrier capacity.	Coliforms and total CFU counts in chicken breast. Lipid oxidation by TBARS. Bi-layer film showed antimicrobial antioxidant activity and extended shelf life of packed chicken breasts at least 3–6 days.	(Sogut & Seydim, 2019)
Sugar palm starch (SPS)/ poly (lactic acid) (PLA)	Coating.	Cast PLA films were coated with SPS film forming solutions.	PLA reduced water vapour permeability, water absorption capacity and solubility of multilayers.		(Sanyang, et al. 2016).
Cassava Starch (S) /PLA	Thermocompression of thermoprocessed S sheets with cast PLA films.	Cinnamaldehyde incorporated into PLA sheets	Bilayers exhibited better barrier capacity against water vapor and oxygen than isolate monolayers due to the complementary properties of both polymers.	In vitro test against E. *coli* and *L. innocua*. Films exhibited antibacterial effect against E. *coli* and *L. innocua*, when the culture medium was in contact with both starch and PLA layers, the starch contact being more effective.	Muller et al. (2017)

Multilayer polymers	Layer assembly method	Active compound	Main result	Antimicrobial or antioxidant assessment	Reference
PLA/MaterBi (starch-based thermoplastic material)	Co-extrussion		Bilayers exhibited higher stiffness than that of MaterBi and greater extensibility than of PLA.		Scaffaro et al. 2018
Pea starch (S) / PLA	Cast S films were coated with PLA film forming solutions		Improved water resistance (lower water uptake, solubility, and water vapor permeability) and tensile strength compared with those of S monolayer film.		Zhou et al., 2019
Potato starch (S) with crosslinking agents / polylactic acid (PLA).	Cast PLA sheets were coated with S film forming solutions		Improved mechanical moisture dependent properties (water permeability, solubility and absorption capacity) with respect S monolayer.		Gürler et al., 2020
PLA-PHBV-PEG blend (P)/ cassava starch (S)	Thermocompression of P cast films and compression moulded S films.	Carvacrol (CA) incorporated into P layer.	Improved tensile and moisture dependent properties (water permeability and absorption capacity) with respect S monolayer.	In vitro test against E. *coli* and *L. innocua*. Bi-layers inhibited the growth of L. *innocua and E. coli* from both P or S contact sides.	Requena et al., 2018
PLA-PHBV-PEG blend (P)/ cassava starch (S)- gellan blend.	Thermocompression of compression moulded films.		Improved tensile and moisture dependent properties (water permeability and absorption capacity) with respect S monolayer and lower oxygen permeability than P films.		Hernández-García et al., 2021

Multilayer polymers	Layer assembly method	Active compound	Main result	Antimicrobial or antioxidant assessment	Reference
PLA /Zein-chitosan- maize germ oil)	Cast PLA sheets were coated with Pickering emulsions of Zein-chitosan- maize germ oil	Thymol incorporated into the emulsion.	Improved mechanical resistance, ductility and barrier capacity against water vapor and oxygen.	In vitro/Disk diffusion tests with *E. coli* and *S. aureus* DPPH scavenging capacity. Bi-layer presented antimicrobial activity against all tested microorganisms and presented DPPH scavenging activity of up to 75%.	Zhu et al., 2018
Isolated soy protein (SPI) / poly (lactic acid) (PLA).	Cast SPI films were coated with PLA film forming solutions.	Natamycin or thymol incorporated into SPI layer.	Improved mechanical properties and water vapor barrier capacity, with lower water swelling index.	In vitro/Disk diffusion tests with mold, yeast, *E. coli* and *S. aureus*. Films containing natamycin inhibited the growth of mould and yeast. Films with thymol inhibited the growth of the two bacterial strains.	Gonzáles et al., 2013
PLA/Gelatin (G)	Thermocompression of compression moulded PLA sheets and cast G films	Epigallocatechin gallate incorporated into G layers	Improved mechanical properties, water vapor barrier capacity and UV–visible light barrier properties.	Psychrophilic and total CFU counts in packed striped catfish slices. Lipid oxidation by TBARS Lower psychrophilic bacteria count, peroxide value, and thiobarbituric acid reactive substances (TBARS) than samples packaged in control bags or in LLDPE bags.	Nilsuwan et al., 2019

Multilayer polymers	Layer assembly method	Active compound	Main result	Antimicrobial or antioxidant assessment	Reference
Thermoplastic corn starch (S) and PLA	compression moulded S films were deep coated with PLA film forming solutions		Improved water vapor and oxygen barrier capacity		Trinh et al., 2021
Thermoplastic wheat gluten (TG) /whey protein (WPI) or soy protein (SPI) isolates.	Compression moulded TG were Electrospun with WPI or SPI solutions	Alpha-tocopherol incorporated into WPI or SPI solutions	Improved water vapor barrier capacity.	ABTS scavenging capacity. The antioxidant activity of the active compound was preserved during the encapsulation process (up to 95%).	Fabra et al., 2016
Corn starch (S)/PCL/Starch (S)	PCL electrospun coating of compression moulded S sheet, plus thermocompression with the second S layer	Carvacrol incorporated into the PCL layer.	Improved (65-80%) water vapor barrier capacity.	Electrospun PCL fibre mats encapsulating CA were effective at controlling the growth of *E. coli*, but were not effective at controlling the growth of *L. innocua*. Similar results were obtained in three-layers.	Tampau et al., 2018

The studies shown in **Table 2** developed multilayer materials considering the combined effect of two or more polymers assembled mainly in two-layer or in three-layer systems. The different types of multilayer films were obtained by different methods: 1. Coating: the polymer solution of the second layer is poured directly on top of the previously dried layer. The first layer can be obtained by casting or thermo-processing, 2. Thermocompression: the preformed monolayers were assembled into a laminated structure by exposing the materials to high temperature and pressure; 3. Nanostructured coating: one of the layers was produced by means of the electro-hydrodynamic technique and polymeric mats were directly electrospun/electrosprayed onto one face of other film or the obtained mats were assembled with the other layers by thermocompression, and 4. Coextrusion.

The good adhesion between the layers is an important factor and interferes with the functional properties of the final material. Chitosan (CH) and gelatin (G) bilayer films have been developed, taking advantage of the high affinity between both polymer layers, confirmed by F-TIR analysis (Haghighi et al., 2019; Pereda et al., 2011). Good adhesion is due to electrostatic interactions and the formation of hydrogen bonds between CH and G at interfacial level (Haghighi et al., 2019; Pereda et al., 2011). This bi-layer system exhibited reduced water solubility and vapour permeability (WVP), as well as higher elongation capacity, without modifying the strength of the chitosan control film. All these improvements were attributed to the electrostatic interactions between cationic chitosan and anionic gelatin and hydrogen bond formation. An adequate interaction at the interface was also reported between chitosan (CH) and starch (S), resulting in an improvement in mechanical resistance and oxygen permeability in the CH-S bilayers, while the WVP values were between those found for the S and CH monolayers (Valencia-Sullca et al., 2018). Chitosan (CH) also presented high affinity and good adhesion with PVA films, allowing for the generation of matrices less susceptible to water adsorption. So, the PVA-CH bilayers exhibited reduced moisture content the swelling capacity with respect to the individual layers. The permeability of the bilayer system was controlled by the PVA layer, which presented the lowest WVP and OP values.

A critical feature in the development of packaging materials is the barrier capacity, which must be adequate for the preservation of the target foods. Polar polymers such as chitosan (CH), starch (S) or PVA have high oxygen barrier. However, their barrier capacity to water vapour is poor due to their hydrophilic nature. Therefore, multilayer materials usually combine polar and non-polar materials, with complementary barrier characteristics that allow for promoting the synergy of both polymers. **Table 2** shows several polymer combinations, the most effective being multilayer films with polar polysaccharides or proteins with less polar polyesters. To improve the barrier properties of the chitosan films, their lamination with non-polar polycaprolactone (PCL) was considered (Sogut & Seydim, 2018). The PCL layer was adhered to a CH layer incorporating nanocellulose (NC) and grape seed extract (GSE). Two assembly methods were tested, CH coating with PCL film forming solution or compression moulding of preformed films. In general, all the combinations presented better barrier

capacity than the chitosan monolayers. However, compression-moulding partially compromised the barrier capacity of the bilayers. The barrier efficiency reduction was attributed to the less oriented chain arrangement and possible GSE oxidation during thermocompression (Sogut & Seydim, 2018).

Bi-layer films based on starch layers from different sources and PLA layers have been studied by several authors (Gürler et al., 2020; Requena et al., 2018). The combination of these bioplastics with polar and non-polar nature, respectively, makes obtaining materials with appropriate barrier properties possible. The incorporation of PLA layer significantly reduced the WVP as well as the water absorption and solubility of bilayer films, which was attributed to the hydrophobic characteristic of the PLA layer (Scaffaro et al., 2018). As concerns the tensile properties, the presence of PLA layer usually makes the obtained laminate stiffer, and more brittle that net starch films.

In order to further improve the barrier and tensile properties of this bilayer material, thermoplastic starch was modified using maleic anhydride (MAH) (Trinh et al., 2021). Overall, the maleated TPS (MTPS) demonstrated lower crystallinity and polarity that led to improved interfacial interaction with the hydrophobic PLA layers. Due to the enhanced chemical interaction and mechanical interlocking between PLA and MTPS, the adhesive strength between each layer in the multilayer films was improved, resulting in superior mechanical and barrier properties (Trinh et al., 2021). In addition, the incorporation of nanoclays in the MTPS layer further enhanced the mechanical and barrier properties of the multilayer film assembly. This multilayer film showed 1300% improvement in moisture barrier, compared to TPS films, and 3300% improvement in oxygen barrier properties, compared to PLA films. For the same purpose, the use of maleic acid MA for starch crosslinking was evaluated. The results indicated the success of this technique on the functional properties of the bilayer, although the opacity of the material increased (Gürler et al., 2020). PLA layers have also been used in multilayer systems by coating with pickering emulsions (maize germ/chitosan colloid emulsion) (Zhu et al., 2018), soy protein (González & Alvarez Igarzabal, 2013)and gelatine (Nilsuwan et al., 2020). In all cases, the PLA layer improved the bilayer material's efficiency as a water vapor barrier, allowing an overall decrease of the swelling index and water solubility. Similarly, polar layers, with higher oxygen barrier ability than neat PLA films, generated a gas barrier effect suitable for the multilayer.

The use of polyester blends was also explored for the development of high moisture barrier multilayer films. Films of polyester blends (P), composed for PLA and PHBV, were obtained for their subsequent assembly with starch films (S), carrying essential compounds in the P layer. These active bilayers exhibited highly improved tensile properties and water vapour barrier capacity with respect to the starch monolayer (87% reduction in WVP, 840% increase in elastic modulus) (Requena et al., 2018). This polyester blend was also laminated with a starch based films containing gellan or xanthan gums to improve the starch mechanical performance. Both

gums also promoted the decrease of water vapour and oxygen permeability of starch films. This effect was also reflected in the P-S bilayers, especially when gellan gum was incorporated into the starch matrix (Hernández-García, Vargas, & Chiralt, 2021).

The electro-hydrodynamic process is an efficient and straightforward method that allows for obtaining micro and nanoscale polymer structures, able to encapsulate different compounds (Tampau et al., 2020b). This technic was used to obtain active PCL mats for coating starch films. CA-loaded electrospun PCL fibres were assembled between two starch sheets to form multilayer films. These multilayer films presented a great reduction in the water vapour permeability (65-80%) with respect to that of starch monolayers, without notable changes in the other packaging functions. This agrees with the effectiveness of the parallel assembly of the PCL sheet at limiting the transport of water molecules, as previously observed in starch-PCL bilayer films by (Ortega-Toro et al., 2015).

Multilayer films loaded with different active compounds, can be used to extend the food shelf life. **Table 2** indicates that a great variety of natural active compounds (essential oils, phenolic extracts, phenolic acids…) were incorporated into multilayer systems, which were mostly loaded into the polar layer. As is known, chitosan (CH) has an antimicrobial effect by itself. Therefore, bilayer films of chitosan and gelatin presented an inhibitory effect against *E-coli*, without any additional active compound (Pereda et al., 2011). The incorporation of ethyl lauroyl arginate (LAE) (0.1%, v / v) into the chitosan layers broadened the spectrum of antimicrobial action of the CH-G bilayer, inhibiting the growth of *Listeria monocytogenes*, *Escherichia coli, Salmonella typhimurium,* and *Campylobacter jejuni* (Haghighi et al., 2019). The high antimicrobial activity of LAE incorporated into films has been widely supported by different studies (Moreno et al., 2017).

Chitosan (CH) and starch (S) bilayers containing essential oils of oregano or cinnamon were applied to pork meat packaging. These materials were effective at controlling the growth of aerobic mesophylls and coliforms. However, the essential oils did not exhibit additional antimicrobial action to that of chitosan. This was attributed to the low final concentration of active compounds in the films due to the losses occurred during the casting and thermoprocessing methods of the films (Valencia-Sullca et al., 2018). In contrast, the incorporation of grape seed extract into the chitosan layer was a successful strategy to confer antioxidant and antimicrobial capacity to CH-PCL bilayers (Sogut & Seydim, 2018). The active CH-PCL bilayers applied to the packaging of fresh chicken breasts extended the shelf life at least 3 or 6 days. This packaging system reduced lipid oxidation, which was mainly attributed to their phenolic constituents, and gave rise to lower counts of total mesophilic aerobic and total coliforms compared to chitosan monolayers (Sogut & Seydim, 2019).

Some active compounds have also loaded in polyester matrices. Multilayer films of CA-loaded electrospun PCL fibres between two starch sheets were effective at controlling the growth of *E. coli*, but were not effective at controlling the growth of *L. innocua* (Tampau et al., 2018).

Likewise, cinnamaldehyde and carvacrol were incorporated into PLA (Muller et al., 2017)and PLA-PHBV blend (Requena et al., 2018) films, respectively, which were laminated with starch sheets by thermocompression. The antimicrobial activity against *E. coli* and *L. innocua* of the bilayer materials was tested in vitro for the two faces of the material. Both materials were effective against the growth of both bacteria while more marked antimicrobial effects were observed when the S layer was in contact with the culture media. This was attributed to the greater water affinity of the S layer, which promoted the swelling of the polymer matrix in contact with the culture medium, which, in turn, accelerated the release of the active compound. Nevertheless, the active compound diffused into both polyester (PLA or PLA-PHBV) layer and starch layers, giving rise to antimicrobial activity through both sides of the bilayers. The amount of active released and its release rate through each layer will determine how effective each side of the bilayer is at controlling the microbial growth inhibition (Muller et al., 2017; Requena et al., 2017).

Multilayer films with antioxidant capacity have also been developed and evaluated for the food packaging application. The electrohydrodynamic process was used to develop bioactive bilayer films for food packaging applications. Cast films of thermoplastic wheat gluten (WG) were coated with nanostructured polymer mats with alpha-tocopherol, produced by electro-hydrodynamic technique (Fabra et al., 2016). Alpha-tocopherol was encapsulated to form active electrospun coatings into hydrocolloid matrices (whey protein isolate, zein or soy protein isolate). The developed bilayer systems exhibited similar antioxidant activity to the neat alpha-tocopherol, which confirmed that the antioxidant encapsulation through electrospinning did not result in compound degradation (oxidation). (Nilsuwan et al., 2020) found that bilayer films of PLA and gelatin containing epigallocatechin gallate (EGCG) showed a high DPPH radical scavenging activity. These bilayer films were used to produce bags, in which striped catfish slices were packaged. The results showed that after 7 days of storage at 4 °C, the samples presented lower values of weight loss, peroxide value, and thiobarbituric acid reactive substances (TBARS) than those packaged in control bags or in LLDPE bags. The same trend was observed in bilayer films based on fish gelatin with EGCG, used for packaging of chicken skin oil (Nilsuwan et al., 2019). These samples showed lower peroxide value, TBARS and volatile compounds after 30 days of storage, in comparison with that packaged in LDPE pouch. Therefore, films incorporated with EGCG could be used as packaging materials for food products with high lipid content prone to lipid oxidation (Nilsuwan et al., 2019, 2020). (Lamarra et al., 2020) reported that CH-PVA bilayer films with gallic acid were effective at delaying the lipid autoxidation processes of walnut flour, reducing the formation of hydroperoxides and secondary oxidation compounds, compared to a polyethylene container. Similar results were obtained by (Ferreira et al., 2018) in accelerated deterioration conditions (24 h of light, 33% RH, 38 ° C for 14 days) for walnut oil samples packaged with FucoPol / chitosan bilayers.

Final remarks

Developing of active biodegradable packaging materials for food preservation is necessary to improve food quality and safety, minimizing food losses and reducing the negative impact of conventional plastics. Availability of biodegradable bioplastics is still scarce, compared to the potential industrial demand, and the research in this field is highly required. Many biopolymers becoming from biomass (polysaccharides and proteins) have been studied as to their potential use as packaging materials. Nevertheless, many of them are not thermoprocessable with the industrial equipment and possess limited properties for developing packaging. Synthetic biodegradable polymers offer new opportunities with more tailored properties. Likewise, the use of biotechnological process allows us the use of agricultural waste to obtain polymers with biodegradable nature, which contribute to circular economy and sustainability. The incorporation of active compounds from renewable sources also lead to promote the safe food preservation without environmental and health risks. Lamination of different polymer films, with complementary properties, is an adequate strategy to obtain high performance packaging materials for food packaging. These materials gain in performance when they incorporate active compounds that extend the shelf life of the food. Therefore, more research must be conducted to obtain active packaging materials from a sustainable point of view.

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II. OBJECTIVES

The **general objective** of this Doctoral thesis was the development of biodegradable active packaging materials based on polymers with complementary properties in order to obtain effective mechanical and barrier properties that meet food packaging requirements, allowing to extend the product shelf-life. To this end, PVA with different molecular characteristics and PLA were considered, as well as carvacrol (lecithin encapsulated or not) and ferulic and cinnamic acid, as potential active (antimicrobial/antioxidant) compounds loaded into the PVA matrices.

For this purpose, the **specific objectives** were:

1. To study the liposomal encapsulation of carvacrol for its effective incorporation into PVA matrices, reducing the losses of the active compound during the film processing.
2. To study the effect of different molecular characteristics (molecular weight and degree of hydrolysis) of PVA matrices in the final properties of the cast films incorporating emulsified carvacrol.
3. To obtain and characterize cast PVA films with different molecular characteristics (molecular weight and degree of acetylation) incorporating encapsulated carvacrol in lecithin liposomes.
4. To obtain and characterize cast PVA films with different molecular characteristics (molecular weight and degree of acetylation) incorporating phenolic acids (ferulic and cinnamic).
5. To study the influence of the processing method, casting and thermo-processing, on the characteristics of partially acetylated PVA films incorporating phenolic acids.
6. To design and characterization multilayer films, combining active PVA layers obtained by casting or thermo-processing, and amorphous PLA for direct contact with wet food.
7. To study the effectiveness of PLA-PVA-PLA active three-layers at the preserving fresh beef meat and its extending self-life.

III. CHAPTERS

CHAPTER I. Liposomal encapsulation of carvacrol to obtain active poly (vinyl alcohol) films

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ABSTRACT

Lecithins of different origins and compositions were used for the liposomal encapsulation of carvacrol within the framework of the development of active films for food packaging. Liposomes were incorporated into aqueous polymeric solutions from fully (F) and partially (P) hydrolysed Poly (vinyl alcohol) (PVA) to obtain the films by casting. Particle size distribution and ζ-potential of the liposomal suspensions, as well as their stability over the time, were evaluated. Liposomal stability during film formation was analysed through the carvacrol retention in the dried film and the film microstructure. Subtle variations in the size distributions of liposomes from different lecithins were observed. However, the absolute values of ζ-potential were higher (-52, -57 mV) for soy lecithin (SL) liposomes, followed by those of soy lecithin enriched with phosphatidylcholine (SL-PC) (-43, -50 mV) and sunflower lecithin (SFL) (-33, -38 mV). No significant changes in the liposomal properties were observed during the study period. Lyotropic mesomorphism of lipid associations and carvacrol leakage occurred to differing extents during the film drying step, depending on the membrane lipid composition and surface charge. Liposomes obtained with SL-PC were the most effective at maintaining the stability of carvacrol emulsion during film formation, which led to the greatest carvacrol retention in the films, whereas SFL gave rise to the least stable system and the highest carvacrol losses. PVA_P was less sensitive to the emulsion destabilisation due to its greater bonding capacity with carvacrol. Therefore, PVA_P with carvacrol-loaded SL-PC liposomes has great potential to produce active films for food packaging applications.

Keyword

Food packaging; lyotropic mesomorphism; phosphatidylcholine; partially hydrolysed PVA; fully hydrolysed PVA.

1. INTRODUCTION

The incorporation of active compounds of natural origin in biodegradable polymeric matrices has been extensively studied as a strategy for developing active food packaging with the ability to prolong the shelf life of foods, through the control of microbial or oxidative degradative phenomena (Altan et al., 2018; Andrade et al., 2020a; Gómez-Estaca et al., 2014; Gursul et al., 2019; Sharma et al., 2020). The extension of the shelf-life of meat or fish products, as well as that of fresh cut fruits and vegetables, requires the incorporation of antimicrobials or antioxidant compounds that can be carried out in the packaging material. A monoterpenoid phenol found in the essential oil of oregano (Origanum vulgare), thyme (Thymus vulgaris L.), marjoram (Origanum majorana), and similar aromatic plants (De Vincenzi et al., 2004), carvacrol has been widely studied due to its excellent activity as an antimicrobial (Veldhuizen et al., 2006) and antioxidant (Gursul et al., 2019) agent. However, low solubility, oxidation and loss through volatilization are the main limitations to the development of active materials that incorporate this compound (Requena et al., 2017; Sapper et al., 2018a).

Liposomes represent an efficient approach to the encapsulation of essential oils and their main constituents, thus improving their ability for water dispersion, chemical stability (Coimbra et al., 2011; Sebaaly, Charcosset, et al., 2016; Sebaaly, Greige-Gerges, et al., 2016) and controlled release (Carvalho et al., 2016; Hammoud et al., 2019). Phospholipids are the main constituents of liposomes; their amphiphilic character allows for their self-assembly in an aqueous medium, in which polar and non-polar regions are driven to align with neighbouring molecules to form favourable interactions, permitting an arrangement in bilayer lipid membranes that generally give rise to spherical vesicles (Cullis et al., 1986; Gruner et al., 1985). These vesicles can encapsulate the active compounds according to the chemical affinity (Gillet et al., 2009). Each phospholipid has specific characteristics, such as geometric isomerism, the degree of saturation of the fatty acids in the hydrophobic tails and the structure of the head group, which influence the lipid membrane packing (Gruner et al., 1985; Sebaaly, Greige-Gerges, et al., 2016; Taladrid et al., 2017a). Likewise, the polar head of the phospholipids is of great importance, as it determines the negative or positive surface charge of the vesicles, thus defining their kinetic stability (Müller et al., 2001). Electrostatic repulsion between the particles depending on the charge level and ionic strength of the medium, and their balance with the Van der Waals attractive forces determine the particle aggregation kinetics, which affects the protection and release of the encapsulated compounds (Seelig et al., 1987).

The efficiency of encapsulation, the control of leaks during storage, the kinetic stability over time and the release dynamics of the encapsulated compound in liposomal systems mainly depend on the membrane packing characteristics, such as the thickness, fluidity, permeability and rotational mobility of phospholipids and the packing density. The molecular interactions between the encapsulated compound and the vesicle components are highly relevant when

explaining the mechanisms involved in both the compound load and release (Ramazani et al., 2016).

Liposomal encapsulation has been used to incorporate essential oils, or their constituents, into hydrophilic systems, such as hydrocolloid aqueous solutions for film formation, in order to limit the emulsion destabilisation phenomena (droplet flocculation, coalescence or creaming); this leads to an accelerated loss of the active compound during the film drying step, with the subsequent reduction in the bioactivity of the material (Talón et al., 2019; Valencia-Sullca et al., 2016). Previous studies into starch-gellan films containing liposome encapsulated thyme essential oil (Sapper et al., 2018a) and chitosan films with liposome encapsulated eugenol or cinnamon leaf essential oil (Valencia-Sullca et al., 2016) reported an increase in the retention of the active compound in the dried polymer films, with a notable increase in the bioactivity of the material. However, a partial release of the active compound during the film drying step and the formation of new phospholipid associations were also reported. The structural rearrangement of lipid membranes is associated with the changes in the molecular interactions in the system associated with water loss, which leads to a new thermodynamic state with minimal free energy (lyotropic mesomorphism) (Cullis et al., 1986; Gruner et al., 1985).

The characteristics of liposomes depending on their composition as well as their kinetic stability, have been widely studied in aqueous media (Asbahani et al., 2015; Gibis et al., 2014; Sebaaly et al., 2015; Silva et al., 2010). However, the stability of liposomal structures and their encapsulation capacity when incorporated into polymer solutions and matrices are still issues to be addressed, given how interesting they are for the purposes of developing active polymeric materials. In this sense, the objective of this study was to characterise liposomal systems obtained with different lipid compositions (different lecithins), encapsulating carvacrol, by using different processing methods (rotor-stator homogenisation and sonication). Likewise, the different liposomal systems were incorporated into two types of PVA polymeric matrices (fully (F) and partially (P) hydrolysed) to obtain active films by casting. The degree of carvacrol retention in the films after the film drying step was analysed as well as their microstructure. The efficiency of the different lecithin at promoting carvacrol retention in the film was analysed in each polymeric matrix in order to identify the best lecithin-PVA combination for the obtaining of carvacrol-rich films.

Liposomes obtained with phosphatidylcholine-rich soy lecithin (SL-PC) were the most effective at maintaining the stability of carvacrol emulsion during film formation, which led to the maximum level of carvacrol retention in the films. Likewise, P-PVA systems were less sensitive to the emulsion destabilisation effects and to the kind of lecithin used in the liposome formation. These materials are of great potential for the production of active films for food packaging applications.

2. MATERIALS AND METHODS

2.1. Materials

The liposomal vesicles were obtained using different types of lecithin (LEC); soybean lecithin enriched (74%) in phosphatidylcoline PC (SL-PC) (Lipoid S75, Lipoid GmbH, Ludwigshafen, Germany), soy lecithin (SL) (Guinama, Prague, Czech Republic) and sunflower lecithin (SFL) (Cargil S.L.U., Barcelona, Spain), whose phospholipid compositions are shown in **Table 1**. Carvacrol was purchased from Sigma-Aldrich (Steinheim, Germany). Two types of poly (vinyl alcohol) (PVA): fully hydrolysed (F: Mw 89,000-98,000; 99-99.8% hydrolysed) and partially hydrolysed (P: Mw 13,000-23,000; 87-89% hydrolysed) (Sigma-Aldrich, Steinheim, Germany), were used as polymeric matrices to develop the films. Phosphorus pentoxide salt (P2O5) and UV-grade methanol were supplied by Panreac Química S.A. (Barcelona, Spain).

Table 1. Phospholipid composition of soybean lecithin enriched in phosphatidylcoline (SL-PC), soy lecithin (SL) and sunflower lecithin. SFL).

Phospholipid	% (w/w)		
	SL-PC	SL	SFL
PC-phosphatidylcholine	74	14	12
PI-Phosphatidylinositol		12	4
PE-Phosphatidylethanolamine	11	10	11
LPC-Lysophosphatidylcholine	3	0	3
PA-Phosphatidic acid	1	4	2

2.2. Liposome production

Lecithin (LEC) (5% w / w) was initially dispersed in distilled water using magnetic stirring for 30 minutes at 800 rpm. The CA was incorporated into the mixture using CA: LEC ratios of 0: 1, 0.5: 1 and 1: 1. To obtain nanoliposomes in the initial dispersion, two methods were applied: homogenisation using a rotor-stator mixer (IKA digital T25 UltraTurrax, Staufen, Germany), at 1100 rpm for 5 min and sonication (35 kHz) for 10 minutes with 1 s pulses, using an ultrasonic device (Vibra Cell, Sonics & Material, Inc. USA), maintaining the sample in an ice bath to avoid heating. All samples were kept in the dark at 4 °C till their use.

2.3. Liposome properties

The particle size distributions, the hydrodynamic diameter (D_H) and the polydispersity index (PDI) were obtained in triplicate using the dynamic light scattering-DLS technique by means of Zetasizer NanoZS, using DTS 1070 cell (Malvern Instruments Zen 3600, United Kingdom). The ζ-Potential was calculated by means of Henry's equation from the electrophoretic mobility

of the vesicles. Measurements were taken at 25 °C, after 100-fold dilution of the liposomal suspension in distilled water. The measurements were taken in triplicate at different times (after 1, 4 and 8 days) in order to evaluate the physical stability of the sample throughout time.

2.4. Preparation of films

The PVA films were obtained by casting. Polymer solutions (PVA_F 5 wt.% and PVA_P 10 wt.%) were prepared in distilled water using magnetic stirring (1200 rpm) at 100 °C for 3 h. Liposomes were added to the polymer solutions to achieve a lecithin:polymer ratio of 10%. Later, different concentrations of CA were added (0, 5 or 10 wt. % with respect to the polymer) (Andrade et al., 2020b). All formulations were degassed by using a vacuum pump before being evenly spread onto Teflon plates of 150 mm in diameter, using a constant equivalent mass of polymer per plate of 2 g. The films were dried under controlled temperature (25 ± 2 °C) and relative humidity (54 ± 2%) for 48 h. The analyses of final CA content in the films and microstructural analyses were carried out in films conditioned at 0% RH by using P2O5.

2.5. Final carvacrol content in the films

The analysis of CA retained in the different film formulations was carried out by spectrophotometry after the extraction of CA from the dry films. Thus, film samples (4 cm^2) were immersed in 50 mL of a 50% aqueous solution of UV-grade methanol and kept under stirring (300 rpm) for 48 h at 23 °C. The absorbance (A) of the aliquots was measured at 274 nm by using a spectrophotometer (Evolution 201 UV-Vis, Thermo-Fisher Scientific Inc., Waltham, MA, USA). The CA-concentration (C) in the films was determined by means of a standard curve, which was obtained from solutions with different concentrations of carvacrol (10 - 50 µg/mL) in the same solvent ($C = 63.61A$, $R^2 = 0.998$). As backgrounds, the corresponding extracts obtained under the same conditions from the CA-free films were used. The final content of CA (%) in the films was calculated as the ratio between the amount of CA extracted from the film with respect to the corresponding amount of initially incorporated CA.

2.6. Microstructure of films

The cross-section microstructure of the films was evaluated by using a Field Emission Scanning Electron Microscope (FESEM) (ZEISS®, model ULTRA 55, Germany) and an acceleration voltage of 2 kV. To obtain the cross-section micrographs of the films, the samples were immersed in liquid nitrogen, cryofractured and coated with platinum before obtaining the images.

2.7. Statistical analysis

The statistical analysis of the data was carried out using Statgraphics Centurion XVI.II. The results were submitted to a Multi-factor ANOVA to estimate the significance of the factors that influence the response variables. Fisher's least significant difference (LSD) was used with a confidence level of 95%.

3. RESULTS AND DISCUSSION

3.1. Liposomal characteristics

Figure 1 shows the different size distributions of newly prepared liposomal systems. All treatments presented a main peak, between 179 nm and 294 nm, with a very low intensity peak at around 4000 nm. Thus, nanosized particles were obtained in all cases, with a small ratio of micro-droplets probably attributed to the aggregation of other lipid molecules present in the raw lecithin. The distribution curves of the carvacrol-free liposomes generally presented a small shoulder at the onset of the main curve, whereas the incorporation of CA promoted a narrower distribution, without the initial shoulder. This suggests structural changes in the liposomal membranes, promoted by the carvacrol-lipid interactions. These changes implied a decrease in both the hydrodynamic diameter (D_H) and polydispersity index (PDI) (Table 1).

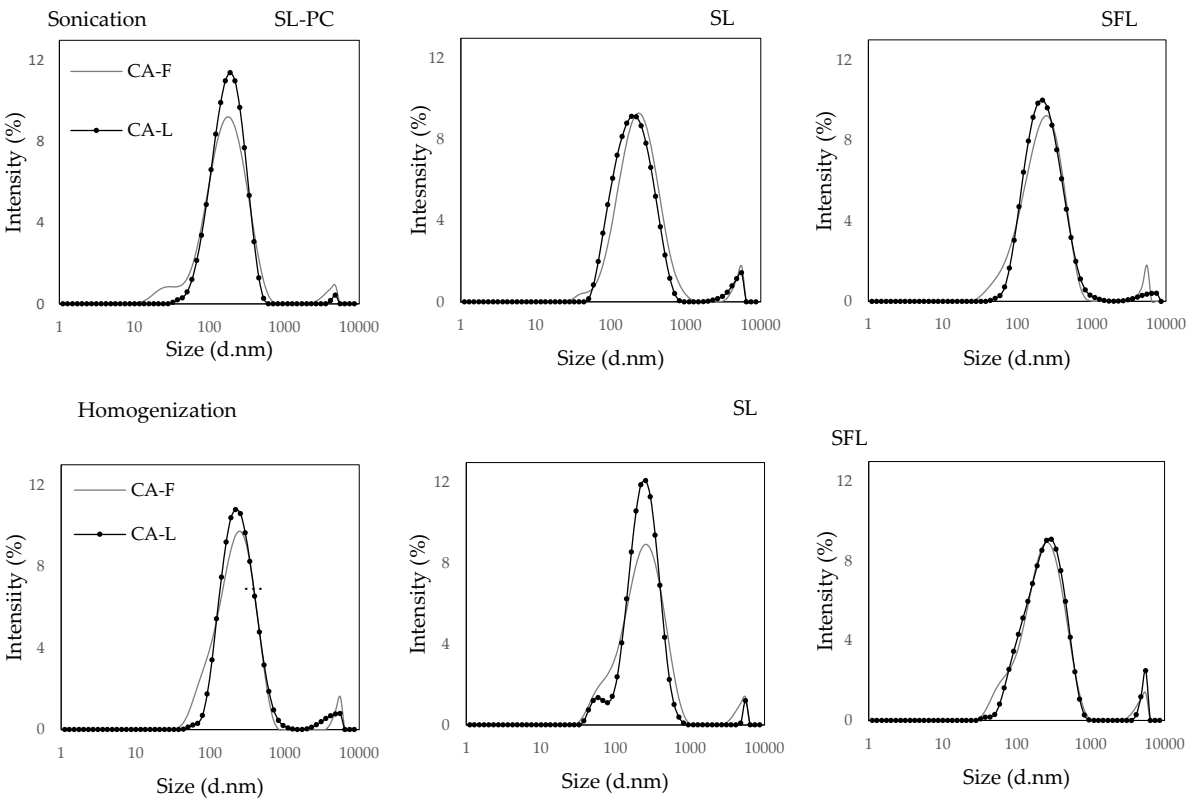


Figure 1. Particle size distribution of carvacrol-free (CA-F) and carvacrol-loaded liposomes (CA-L) (CA:LEC 1:1) using different lecithins and preparation methods.

Table 2 shows the values of the hydrodynamic diameter (D_H), the polydispersity index (PDI) and the ζ-potential of the different liposomal systems, with different lecithin and carvacrol ratios, obtained by rotor-stator homogenisation and sonication. The influence of the factors, type of lecithin, carvacrol-loaded ratio (CA:LEC) and preparation method, on the different properties of liposomes (response variables) was statistically analysed and the significance level for each one is also shown in Table 1.

Table 2. Properties (hydrodynamic diameter (D_H), polydispersity index (PDI) and the ζ-potential (ζ)) of liposomal systems obtained with the different types of lecithin (Soy lecithin enriched in phosphatidylcoline (SL-PC), soy lecithin (SL) and sunflower lecithin (SFL)), using different carvacrol-lecithin ratios (CA:LEC 0:1, 0.5:1, 1:1) and preparation methods (sonication and rotor-stator homogenisation).

LEC	CA:LEC	Method					
		Sonication			Homogenisation		
		D_H (nm)*	PDI	ζ (mV)	D_H (nm)*	PDI	ζ (mV)
SL-PC	0:01	195 (10)	0.28 (0.01)	-46 (2)	250 (14)	0.29 (0.03)	-43 (1)
	0.5:1	211 (1)	0.24 (0.02)	-47 (2)	249 (12)	0.32 (0.01)	-44 (1)
	1:01	207 (2)	0.17 (0.01)	-50 (3)	242 (5)	0.19 (0.01)	-45 (2)
SL	0:01	271 (20)	0.31 (0.03)	-52(1)	293 (42)	0.32 (0.04)	-52 (2)
	0.5:1	219 (8)	0.32 (0.01)	-53 (1)	232 (30)	0.33 (0.01)	-56 (2)
	1:01	235 (9)	0.27 (0.05)	-55 (1)	257 (13)	0.31 (0.01)	-57 (2)
SFL	0:01	236 (22)	0.30(0.02)	-34 (1)	294 (40)	0.35 (0.03)	-33 (1)
	0.5:1	192 (5)	0.35 (0.02)	-38 (1)	179 (7)	0.36 (0.03)	-37 (1)
	1:01	218 (20)	0.25 (0.03)	-37 (1)	233 (11)	0.36 (0.01)	-37 (1)

*Value corresponds to the main peak of distribution size (containing ≥ 94% of particles). Factors with a statistically significant effect with 95% confidence level. D_H: Method (P-value: 0.0003), lecithin (P-Value: 0.0048), CA:LEC (P-value: 0.0001); PDI: Method (P-value: 0.0001), lecithin (P-Value: 0.0002), CA:LEC (P-value: 0.0001); ζ: lecithin (P-value: 0.0001), CA:LEC (P-value: 0.0001).

The hydrodynamic diameter of liposomes ranged between 179 and 294 nm and the amplitude of the diameter distribution (PDI) between 0.17 and 0.36. The values of these parameters were significantly affected (95% confidence level) by all the factors considered. In general, sonication promoted the formation of smaller liposomes with lower PDI, in agreement with that previously reported by other authors for this method, which produces homogeneous dispersions of nanometric liposomes (Pereira-Lachataignerais et al., 2006; Silva et al., 2010; Taladrid et al., 2017b). The sonication effects are based on the cavitation of the bubbles, which provokes a localised, short-lived pressure increase, as well as microstreaming effects and

shock waves that rupture lipid vesicles and promote the formation of more homogeneous systems (Greenly & Tester, 2015). A linear decrease in the particle size and polydispersity of liposomes as a result of increasing the time and intensity of sonication has previously been reported (Silva et al., 2010; Taladrid et al., 2017b).

The type of lecithin also had a significant effect on the particle size and distribution. The liposomes from SL-PC were the smallest with the narrowest distributions (D_H: 195-250 nm; PDI: 0.17-0.32), followed by those of SL (D_H: 219-293 nm; PDI: 0.27-0.33) and SFL (D_H: 179-294 nm; PDI: 0.25-0.36). The phospholipid composition of each lecithin gave rise to distinct liposomal topologies (McMahon & Gallop, 2005). The high concentration of phosphatidylcholine (PC: 74%) of SL-PC, could lead to liposomes with more homogeneous, cohesive and stable lipid membranes. PC have relatively large head groups, which promotes the spontaneous formation of lipid bilayers, defining the specific membrane curvature and the shape of the liposomes (Yang, 2016). In contrast, the SL and the SFL, with a lower concentration of PC, present other phospholipid and non-phospholipid impurities (aminoacids, free fatty acids), giving rise to liposomal membranes of mixed composition, probably less cohesive and more fluid, which is generally reflected in a higher D_H and a greater variation in the size distribution (higher PDI). As is well known, the physicochemical properties of liposomes are dictated by their lipid composition, since each phospholipid has a different molecular structure and geometry that mark the structural characteristics of the liposomes. The specific membrane packing parameter is mainly determined by the size of the head groups and hydrophobic tails (Yang, 2016). Thus, longer-chain phospholipids generate liposomes with good cohesion due to the greater attraction force of Van der Waals between the chains.

Regardless of the CA:LEC ratio in the systems, the incorporation of carvacrol led to a reduction in the liposomal size, which was especially notable in the SL and SFL liposomes, whereas the size of SL-PC liposomes was less sensitive to the carvacrol load. The size reduction provoked by the carvacrol load is coherent with changes in molecular interactions within the membrane introduced by the phenolic compound. These promoted membrane reorganisations and subsequent changes in D_H and PDI. Similar results were reported for liposomes from sunflower seed lecithin, with 20% of PC- encapsulating carvacrol (Valencia-Sullca et al., 2016). In contrast, the liposome encapsulation of eugenol led to an increase in liposomal size due to different changes in molecular interactions associated with its molecular structure (Sebaaly, Charcosset, et al., 2016; Sebaaly, Greige-Gerges, et al., 2016). The changes in both size and ζ-potential depended on the molecular structure (size and shape), polarity, and chemical affinity of the loaded phenolic compounds (Maherani et al., 2013; Rafiee et al., 2017). Thus, phenolic compounds with several phenolic hydroxyl groups can interact cooperatively with lipid polar heads, inducing conformational changes in the membranes (Wink, 2010). In contrast, more hydrophobic molecules can interact with the acyl chains of the bilayer inside the lipid membrane, which could provoke a decrease in the liposome size. Furthermore, changes in the

fluidity of the membrane can explain the liposome size change (Olbrich et al., 2000; Yi et al., 2009).

The values of ζ-potential were highly negative in the obtained liposomal dispersions, which is associated with a high degree of stability, since repulsive electrostatic forces are promoted against attractive Van der Waal forces (Müller et al., 2001). The ζ-potential was significantly affected by the type of lecithin and CA load ($P < 0.05$). In this sense, different values were obtained for each type of lecithin; SL liposomes (-52, -57 mV) had the highest absolute value, followed by SL-PC liposomes (-43, -50 mV) and SFL liposomes (-33, -38 mV). CA incorporation enhanced the negative charge of the liposomes, probably due to the membrane changes associated with the carvacrol load, which can promote the greater exposure of negative charges.

Differences in the ζ-potential values of each lecithin liposome must be attributed to its specific composition, since it is mainly associated with the negative charge of the PO_4^{-3} head group of the phospholipids, and the presence of impurities, such as free fatty acids, which are associated in the lipid membrane (Taladrid et al., 2017b). In this sense, the presence of anionic lipids, such as phosphatidic acid (PA) and phosphatidylinositol (PI), can induce an increase in the electronegativity of liposomes (Wang & Wang, 2008), which agrees with the highest ζ-potential of SL that contains the highest ratio of PA and PI (**Table 1**).

3.2. Liposomal stability

The stability of liposomal systems was evaluated through the changes in their size and charge throughout time. **Figure 2** shows the values of the hydrodynamic diameter and the ζ-potential of the different liposomes after 1, 4 and 8 days of storage at 4 °C in darkness. Every liposomal system exhibited very slight changes in both D_H and ζ-potential, regardless of the presence or absence of carvacrol. Therefore, no destabilisation of the colloidal systems during the evaluated period could be deduced. The high surface charge (ζ-potential ≥ $|-30\mathrm{mV}|$) of liposomes favours the stability of the colloidal system due to the electrostatic repulsion of individual particles (Joseph & Singhvi, 2019; Lu et al., 2014).

The slight changes in the D_H and in the ζ-potential over time (**Figure 2**) indicated the liposomal dynamic character, promoted by the small fluctuations in the particle environment and the dynamic equilibrium between associated molecules in the colloidal system. This dynamic equilibrium is responsible for the typical characteristics of the liposomal system, such as the fluidity and flexibility of the lipid bilayer, and the continuous membrane reorganisation in response to the mobility and molecular rotation rate of lipids and phospholipids (Gibis et al., 2014; Li et al., 2021). Although no significant differences between the stability of CA-free and CA-loaded liposomes were detected, other authors (Reiner, Fraceto, et al., 2013; Reiner,

Perillo, et al., 2013) reported that the spatial rearrangement of the liposomal membrane, caused by carvacrol, increased the system stability due to the reduction of the repulsive forces among the head groups of phospholipids; this decreased the degree of mobility of the hydrophobic tails and led to closer molecular packing.

Stability studies of soybean and salmon lecithin liposomal systems, encapsulating polyphenols and curcumin, respectively, indicated that the significant changes in liposomal characteristics (which occurred after around 5 weeks) could be associated with the oxidation and hydrolytic degradation of the unsaturated fatty acids of phospholipids, causing a decrease in the absolute value of ζ-potential and an increase in the D_H of the particles (Gibis et al., 2014; Hasan et al., 2014).

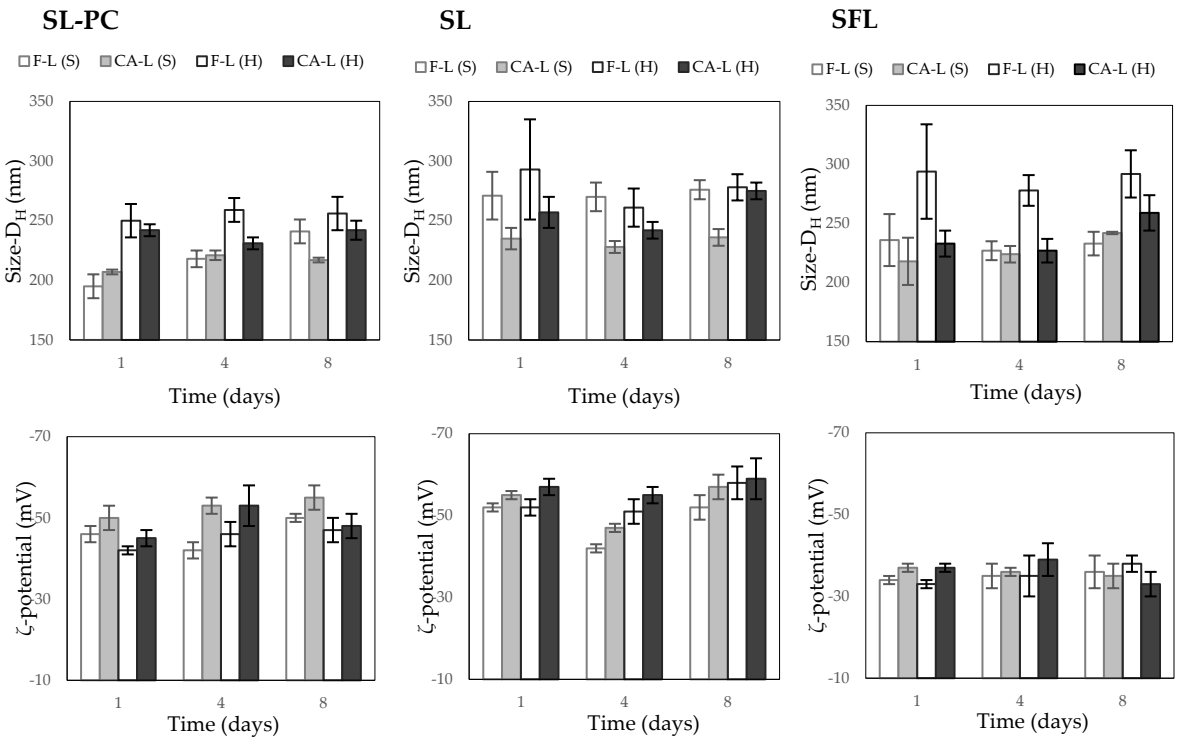


Figure 2. Development over time of the hydrodynamic diameter (D_H) and the ζ-potential of liposomes from soy lecithin enriched in phosphatidylcoline (SL-PC), soy lecithin (SL) and sunflower lecithin (SFL), carvacrol-free (F-L) or CA-loaded (CA-L) (CA:LEC 1:1) liposomes, obtained by sonication (S) or rotor-stator homogenisation (H).

3.3. Carvacrol content and film microstructure of PVA matrices with liposomes

The different CA-loaded liposomes were incorporated into aqueous solutions of fully (F-PVA) and partially (P-PVA) hydrolysed PVA to obtain carvacrol-loaded films by casting. The different efficiency of each liposomal system and polymer matrix to retain the volatile carvacrol was evaluated through the analysis of the CA content in the dry films (**Table 2**) and the film microstructure (**Figure 3**). Very big losses of volatile compounds, such as carvacrol, have been observed during the film-forming step (drying) from the casting of aqueous polymer solutions with emulsified organic compounds. Throughout the drying step, emulsion destabilisation mechanisms (flocculation, coalescence and creaming) take place in line with water evaporation. As a consequence, large droplets of the organic compounds migrate to the film surface and evaporate, together with water molecules, by steam drag effect (Perdones et al., 2016), which constitutes the main reason for the losses of these compounds in the cast films.

The liposome encapsulation of volatile compounds, such as carvacrol, can reduce these losses due to the stabilising effect of lipid membranes. Nevertheless, lipid associations, such as liposomes, are also very sensitive to water availability, exhibiting lyotropic mesomorphism and phase transitions as a function of the water content (Gruner et al., 1985). This implies that when liposomes are incorporated into aqueous film-forming solutions, e.g. to obtain films loaded with carvacrol, they will also suffer changes in their association. Likewise, the molecular interactions of lecithin lipids and carvacrol with the film polymer would play an important role in the properties and in the stability of the aqueous emulsion and, therefore, in the final content of the volatile compound in the film. In fact, in the presence of a polymer, different structural changes in liposomes could also be expected during the drying of the film, as well as the release of carvacrol from the liposomes into the polymer-rich phase. The changes will progress in line with the process of water evaporation during film formation. These dynamic changes will give rise to a final carvacrol content in the films, resulting from both the different destabilisation phenomena that have occurred and the final molecular rearrangement according to the chemical affinity and interactions.

Table 2 shows the amount of carvacrol retained in the film with respect to the initially incorporated amount (% retention) for the two types of PVA polymers and the different liposomal systems (with different lecithin, carvacrol load and preparation method). The CA retention was significantly ($P < 0.05$) affected by the type of lecithin and by the liposome preparation method, as well as by the type of PVA. Films with SL-PC liposomes had the highest final CA content, probably due to the lipid composition of lecithin, rich in phosphatidylcholine. In fact, a more homogeneous, more cohesive and less fluid liposomal membrane could be obtained for SL-PC (Vélez et al., 2017), with a greater control of the CA leakage. Furthermore, the greater packing density of lipid bilayer could be less susceptible to both the phenomenon of destabilisation and to the structural modifications associated with water loss during film drying. In general, the liposomes obtained by sonication also showed slightly greater CA-

retention in the films, probably due to their smaller size and lower PDI in comparison with those obtained by rotor-stator homogenisation. This could favour their kinetic stability, reducing the rate of particle aggregation and coalescence (Dos reis coimbra & Teizeira, 2016).

On the other hand, partially hydrolysed PVA presented the highest CA retention values (between 54% and 74%), compared to fully hydrolysed PVA, with percentage retention values between 22% and 57%. It is worth highlighting that during the film formation process, liposomal systems present carvacrol leakage, due to the lyotropic mesomorphism of liposomes (Andrade et al., 2020b; Sapper et al., 2018b). Thus, the degree of carvacrol retention would depend not only on liposomal encapsulation systems, but also on the interaction of the active compound released with the polymer matrix. PVA_P has residual acetyl groups (12%) in the polymer chain, which acquire a state of resonance and provide the matrix with a negative charge (Wiśniewska et al., 2016). This donor electron pair provides the P-PVA chains with Lewis base character, which promotes the formation of Lewis adducts with carvacrol (Lewis acid). This mechanism enhances the chemical affinity between carvacrol and the polymer chains, increasing their retention in the film (Andrade et al., 2020b, 2020a; Tampau et al., 2020). In this sense, it was possible to observe that the type of lecithin had less influence on the CA retention for P-PVA matrices, since the carvacrol released from liposomes could be better entrapped through its bonding to the PVA_P chains. In contrast, the type of lecithin greatly affected the carvacrol retention in the PVA_F matrix with a lower chemical affinity for carvacrol. The carvacrol retention in this polymer matrix markedly reflects the different stabilising capacity of each lecithin, SL-PC being the most effective and SFL the least at stabilising the carvacrol-loaded liposomes during film formation.

Table 2. Carvacrol retention (ratio of determined carvacrol with respect to the incorporated amount, in percentage) in dry PVA films for fully (F) and partially (P) hydrolysed polymer and different carvacrol-loaded liposomal systems.

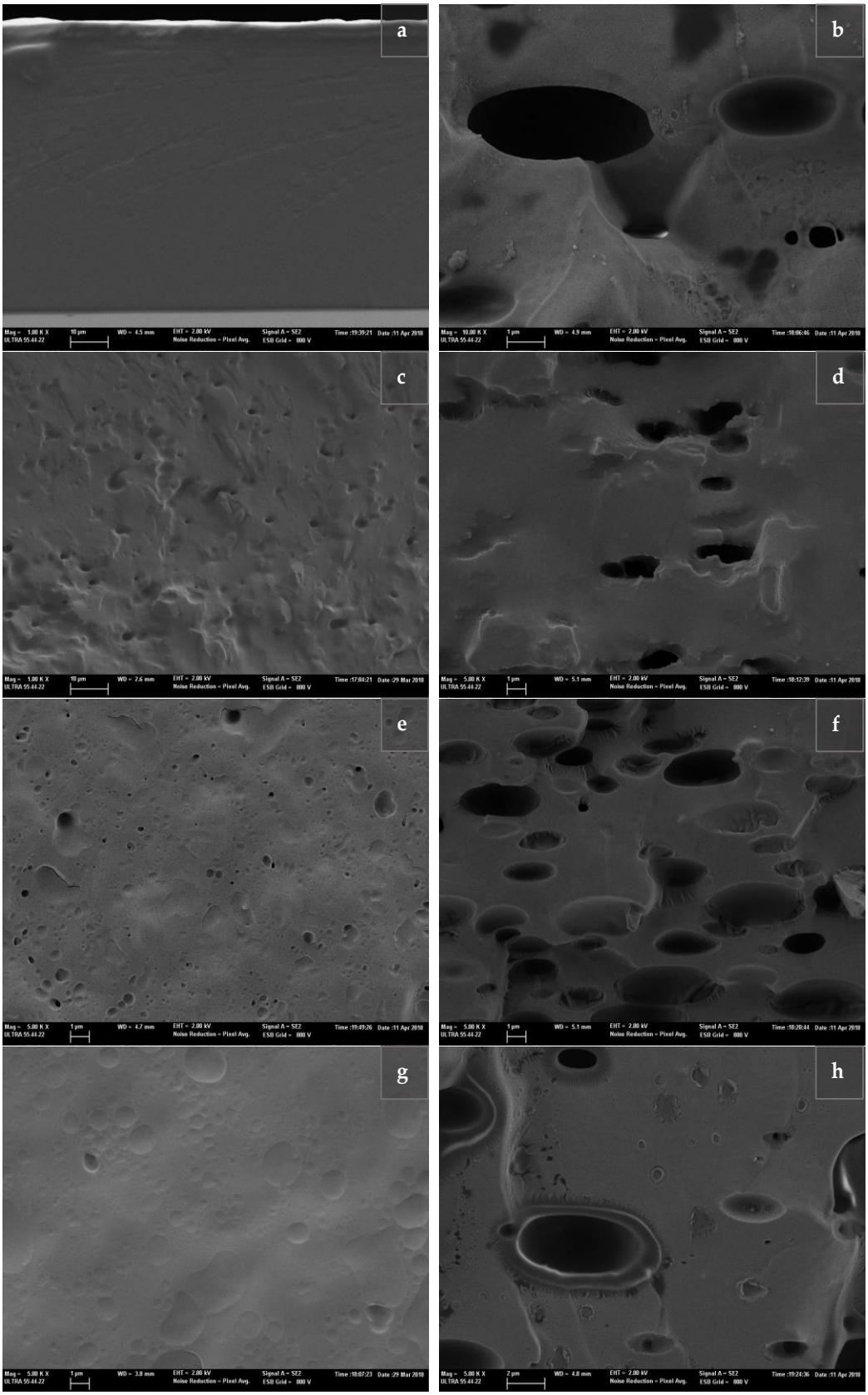
LEC	CA:LEC	CA retention (%)			
		PVA_F		PVA_P	
		Sonication	Homogenisation	Sonication	Homogenisation
SL-PC	0.5:1	54 (2)	53 (4)	74 (2)	71 (5)
	1:01	57 (3)	54 (3)	67 (3)	65 (3)
SL	0.5:1	47 (1)	41 (1)	66 (1)	64 (1)
	1:01	45 (4)	46 (4)	66 (7)	62 (5)
SFL	0.5:1	28 (1)	22 (2)	61 (7)	54 (8)
	1:01	27 (2)	24 (4)	63 (3)	60 (3)

Factors with a statistically significant effect with a 95% confidence level. CA-retention: Method (P-value: 0.0036), lecithin (P-Value: 0.0001), PVA type (P-value: 0.0001), and the interaction between Lecithin and PVA (P-value: 0.0001)

Figure 3 shows FESEM micrographs of the cross-section of PVA films (F and P) with liposomes from different kinds of lecithin, loaded or not with carvacrol. These micrographs reveal qualitative differences related to the arrangement of the components in the samples. A first remarkable feature was the smooth appearance of PVA_P films with free carvacrol (non-liposome encapsulated) (**Figure 4a**), in contrast to the carvacrol droplets observed in the PVA_F matrix (**Figure 4b**). This fact demonstrates the good integration of this phenolic compound in PVA_P matrix, without visible dispersed droplets, while the PVA_F films presented a clear phase separation. This was attributed to the formation of Lewis adducts between the phenolic compound and the negatively-charged chains of PVA_P, previously described (Greenly & Tester, 2015; Pereira-Lachataignerais et al., 2006). Nevertheless, the incorporation of carvacrol-free liposomes into PVA_P and PVA_F matrices led to heterogeneous matrices that exhibited a dispersed phase of altered liposomes (**Figures 4c** and **4d**, respectively).

Different microstructural features were also observed for films with carvacrol-loaded liposomes as a function of the kind of lecithin in agreement with the different stabilising effects of the lipid membranes during film formation. The more stable membranes exhibit a lower level of disruption during the water evaporation step, remaining less altered in the films and entrapping carvacrol more efficiently. In the PVA_P matrix with carvacrol- loaded SL-PC liposomes (**Figure 4e**), a relatively fine dispersion of lipid particles (about 1 µm or smaller), partially altered in shape, could be observed as a result of mesomorphic transitions in the lipid membranes. According to the high chemical affinity between carvacrol and PVA_P chains, the leakage of carvacrol from the liposomes would not imply the presence of free carvacrol droplets. The SL-CA and SFL-CA liposomes also appeared dispersed in the PVA_P matrix (**Figures 4g** and **4i**), similar in appearance to those of the SL-PC liposomes, but exhibiting larger particle traces and more coalescing droplets.

In contrast, much larger particles could be observed in PVA_F films with carvacrol- loaded liposomes (**Figures 4f, 4h** and **4j**), indicating a greater progress of coalescence during film drying. For SFL liposomes, a particularly high level of lipid separation was observed in the matrix, thus showing the poor stabilising capacity of this type of lecithin. The microstructural observations agree with the different carvacrol retention in the films associated with the stabilising capacity of the lipid membranes (**Table 2**). This ability was affected by polymer-lipid interactions, linked to the degree of hydrolysis of the PVA chains and the lipid composition of the liposome membrane. Despite the slightly higher surface charge of SL liposomes, the high ratio of PC in the SL-PC membrane seems to produce more stable structures in aqueous PVA solutions that provide emulsified systems with greater stability during the water evaporation step, helping to keep the carvacrol contained in the dry films. SFL Liposomes, with a more heterogenous lipid composition and lower ζ-potential values, were less effective at stabilising emulsified carvacrol in aqueous PVA solutions.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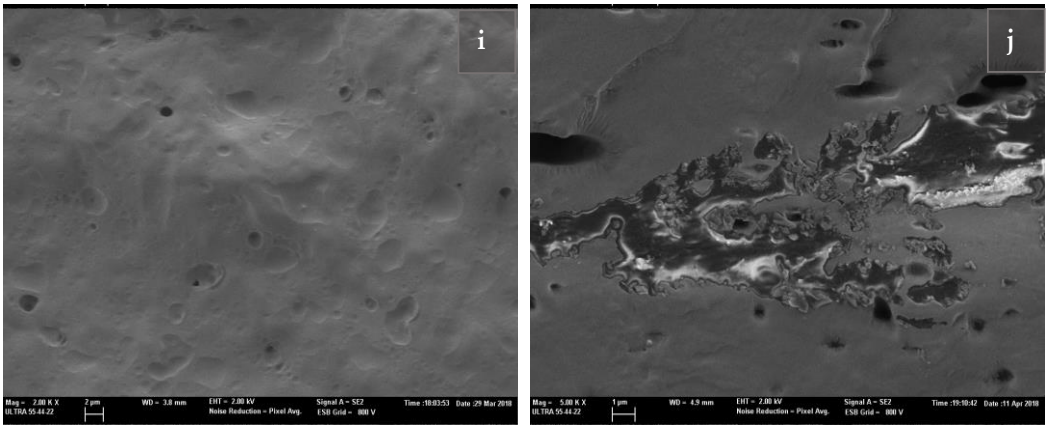

Figure 3. FESEM micrographs of the cross-section of PVA_F and PVA_P films with CA-loaded liposomes (CA:LEC 1:1) using different types of lecithin (soy lecithin enriched in phosphatidylcoline PC (SL-PC), soy lecithin (SL) and sunflower lecithin (SFL)) and the sonication method. **a**: PVA_P with free CA, **b**: PVA_F with free CA, **c**: PVA_P with CA-free SL-PC, **d**: PVA_F with CA-free SL-PC, **e**: PVA_P with CA-loaded SL-PC, **f**: PVA_F with CA-loaded SL-PC, **g**: PVA_P with CA-loaded SL, **h**: PVA_F with CA-loaded SL, **i**: PVA_P with CA-loaded SFL **j**: F-PVA_F with CA-loaded SLF.

4. CONCLUSION

The composition of lecithin of differing origins affected the stability of the carvacrol-loaded liposomes incorporated into the PVA films obtained by casting the aqueous polymer solutions. The lyotropic mesomorphism of lipid associations and the carvacrol leakage occurred to different degrees during the film drying step, depending on the lipid composition of the membrane and its resulting surface charge. Lipid interactions with the polymer also play an important role in the liposomal stability and the final carvacrol retention in the films. Liposomes obtained with phosphatidylcholine-rich soy lecithin (SL-PC) were the most effective at maintaining the stability of the carvacrol emulsion during film formation, which led to the highest carvacrol retention, whereas SFL gave rise to less stable systems and greater carvacrol losses. Likewise, systems with partially hydrolysed PVA were less sensitive to the emulsion destabilisation effects and to the kind of lecithin used in the liposome membranes due to the greater chemical affinity and bonding capacity between carvacrol and polymer chains. Therefore, PVA_P with carvacrol-loaded SL-PC liposomes and with a carvacrol retention capacity of about 70% in films, has great potential to produce active films for food packaging applications. In this sense, the antioxidant and antimicrobial properties of the potentially active materials with carvacrol should be evaluated in further studies, both through in vitro tests and using different food matrices.

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CHAPTER II. Effect of carvacrol in the properties of films based on poly (vinyl alcohol) with different molecular characteristics

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ABSTRACT

Polyvinyl alcohol (PVA) is a hydrophilic linear polymer obtained from the controlled hydrolysis of polyvinyl acetate (PVAc). The molecular weight (Mw) and degree of hydrolysis (DH) of PVA are considered relevant in both the functionality of the polymer and its capacity for film formation. This study analysed the influence of the Mw and DH of PVA on both the film's ability to incorporate carvacrol (CA), for the purposes of obtaining active films for food packaging application, as well as on the film microstructure and thermal behaviour and its functional properties as packaging material. CA was incorporated at 5 and 10 g/100 g polymer by emulsification in the polymer-water solutions, while the films were obtained by casting. The higher Mw polymer provided films with a better mechanical performance but less CA retention and a more heterogeneous structure. In contrast, low Mw, partially acetylated PVA gave rise to homogenous films with a higher CA content that increased the mechanical resistance and stretchability of the films. The melting temperature of this polymer with acetyl groups was lower than the degradation temperature, which makes thermoprocessing feasible.

Keywords

Molecular weight; degree of hydrolysis; thermal behaviour; cross-linking; food packaging.

1. INTRODUCTION

The development of biodegradable packaging materials has become a matter of great importance, especially within the food sector. This industry currently reports one of the highest rates of non-biodegradable plastic consumption destined for food packaging, generating a negative impact on the environmental balance, problematic that must be counteracted through the progressive change in the packaging system (European Bioplastics, 2017). Food packaging materials must meet not only determined requirements to ensure food preservation and safety: adequate barrier properties to water vapour and gases, proper mechanical performance and optical properties, along with thermal characteristics to ensure their processability, but also the requirements of environmental sustainability (Bhagabati et al., 2019). To this end, different biodegradable polymers (biobased or not), as well as their blends, have been evaluated and characterized. Some non-biobased, biodegradable polymers, such as poly (Ɛ-caprolactone) (PCL), poly (butylene succinate/adipate) (PBS/A), poly (butylene adipate-co-terphthalate) (PBA/T) and poly (vinyl alcohol) (PVA), have been extensively studied because of their high versatility. In these polymers, it is possible to adjust some of their molecular parameters by controlling their processing factors, thereby modifying the characteristics and functionality of the material.

Some molecular characteristics of these biodegradable polymers, such as chain length, molecular weight, number and types of functional groups or tacticity (which is related to the stereoregularity of the chain), as well as the processing conditions, are factors which determine the physical and functional properties of the resulting materials. (Bhagabati et al., 2019) report that the polymer crystallinity and oxygen barrier capacity and transparency of PCL films have been shown to be highly dependent on the molecular weight. The molecular and chemical structure of the PBA and the PBS were relevant factors in the thermal behaviour and in the biodegradation by enzymatic hydrolysis of these polyester films (Bai et al., 2018). (Perilla, 2007) showed that the degree of hydrolysis of the PVA molecular chains generated significant differences in the glass transition temperature (Tg), the melting temperature (Tm) and the thermal degradation mechanisms of the PVA films. (Aruldass et al., 2019) reported an increment in the crystallinity and water solubility of the PVA films when increasing the hydrolysis degree of PVA. The greater PVA crystallinity promoted, in turn, the development of smoother film surfaces.

PVA is produced by partial or complete hydrolysis of the polyvinyl acetate, eliminating the acetate groups; this means that both the molecular weight and the degree of hydrolysis of the PVA can be controlled to obtain materials with different properties and functionality (Juan David, 2019). The high number of hydroxyl groups in the molecular chain of PVA confers it with a highly hydrophilic nature, enhancing biocompatibility, and promotes the formation of hydrogen bonds, affecting the physical properties of the material (Cano et al., 2015). These characteristics have encouraged the use of PVA to develop biodegradable films, using pure

PVA or blended with other biopolymers, such as starch (Aydin & Ilberg, 2016; Cano et al., 2015; Domene-López et al., 2018; Hilmi et al., 2019; Kahvand & Fasihi, 2019; Tian et al., 2017), proteins (Ghaderi et al., 2019; Lara et al., 2019), chitosan (Ghaderi et al., 2019; Tang et al., 2007; Thanyacharoen et al., 2018) or cellulose and derivatives (Cazón et al., 2019; Ghorpade et al., 2019) in order to obtain biodegradable materials with adequate properties for different uses.

One relevant aspect related with food preservation is the availability of active packaging. The incorporation of active compounds, with antioxidant or antimicrobial activity, in different polymeric matrices has been studied to obtain antimicrobial or antioxidant packaging materials (Atarés & Chiralt, 2016; Gómez-Estaca et al., 2014; Mousavi Khaneghah et al., 2018). In this sense, the compatibility of the active compounds with the polymer matrix and the controlled release to the food mainly depend on the chemical nature of the compounds involved and their molecular interactions. These interactions are of great importance since they determine the applicability of the active films.

Several studies have been carried out with the aim of developing active films based on PVA using fully hydrolysed, high molecular weight polymer. Thus, (Kavoosi et al., 2014) and (Chen et al., 2018) obtained active films of fully hydrolysed (1–2% acetate groups) PVA, incorporating essential oils of *Zataria multiflora* and clove, respectively. These films exhibited effective antimicrobial and antioxidant activities in the vapour phase. Nevertheless, the low affinity between the essential oil compounds and the PVA chains negatively influenced the mechanical resistance, oxygen barrier properties and thermal stability of the films. In contrast, the incorporation of tea polyphenols in nanocomposites of PVA with Montmorillonite significantly improved the tensile strength and the water vapour and oxygen barrier capacity, due to the formation of hydrogen bonds between the polymer matrix and phenols (Chenwei et al., 2018). Other studies analysed the properties of PVA-based blend films incorporating different natural active compounds, such as curcumin (Ma et al., 2017), gallic acid (Yoon et al., 2017), mint and pomegranate peel extract (Kanatt et al., 2012). None of these studies have evaluated the effect of the PVA molecular characteristics on the film properties and functionality, which is relevant to determine the more appropriate polymer for a given food packaging use.

The aim of this study was to develop and characterise active PVA films containing carvacrol (CA), by using two types of PVA with different hydrolysis degree and molecular weight. The effect of the molecular characteristics of the polymer on the CA retention in the film, the film microstructure, polymer crystallization degree, thermal behaviour and barrier and tensile properties of the films has been analysed.

2. MATERIALS AND METHODS

2.1. Materials

Two types of poly (vinyl alcohol) (PVA) with different molecular weight and degree of hydrolysis (PVA_F: Mw 89,000-98,000; 99-99.8% hydrolysed and PVA_P: Mw 13,000-23,000; 87-89% hydrolysed) and carvacrol were purchased from Sigma-Aldrich (Steinheim, Germany) and magnesium nitrate ($Mg(NO_3)_2$), phosphorus pentoxide (P_2O_5) salts and UV-grade methanol were supplied by Panreac Química S.A. (Barcelona, Spain).

2.2. Film preparation

The films were prepared by casting of the aqueous solution of the polymer. Polymer (PVA_F 5% wt. and PVA_P 10% wt.) solutions in distilled water were prepared using magnetic stirring (1200 rpm) at 100 °C for 3 h. CA was incorporated into the PVA solution at 5% wt. and 10% wt. (with respect to the polymer) by applying sonication (20 kHz for 5 min, with pulses of 1 s), using a ultrasonic processor (Avantor, 505-Vibra Cell, USA). All formulations were degassed by using a vacuum pump and spread evenly onto Teflon plates of 150 mm in diameter, using a constant equivalent mass of polymer per plate of 2 g. The films were dried for approximately 48 h under controlled relative humidity (RH) and temperature (T) conditions (RH: 50 ± 2% and T: 25 ± 2 °C). Subsequently, the films were conditioned for one week at 53% RH, using $Mg(NO_3)_2$ over-saturated solution, before their characterisation. Therefore, the final CA content in the film (CA retention), microstructure and thermal analyses were carried with films conditioned at 0% RH using P_2O_5. **Table 1** shows the different film formulations with the respective mass fractions of the components.

2.3. Characterisation of the active PVA films

2.3.1. *CA retention in the films*

CA retained in the different formulations was determined through the extraction of CA contained in the dried films and its spectrophotometric quantification. The extraction was carried out from film samples of 4 cm^2 in area in 50 mL of a 50% (v/v) aqueous solution of UV-grade absolute methanol and kept under stirring at 300 rpm for 48 h at 25 °C. Absorbance ($\boldsymbol{x}$) of the extracts was measured at 274 nm, wavelength of maximum absorption of carvacrol, using a spectrophotometer (Evolution 201 UV-Vis, Thermo Fisher Scientific, USA). The CA concentration ($\boldsymbol{y}$) in the films was determined by means of a standard curve obtained with CA solutions containing between 10 and 50 µg/mL in the same solvent ($y = 63.61x, R^2 = 0.998$). The backgrounds used for the measurements were the corresponding extracts obtained under the same conditions from the CA-free films. CA content in the film was expressed as mg/g dry film and mg/g polymer, while CA retention in the films was expressed,

in percentage, as the ratio between the mass of CA extracted from the film with respect to the corresponding mass of CA initially incorporated.

2.3.2. *Microstructure of the films*

The microstructure of the films was observed using a Field Emission Scanning Electron Microscope (FESEM) (ZEISS®, model ULTRA 55, Germany), at an acceleration voltage of 2 kV. The film samples were cryofractured by immersion in liquid nitrogen, platinum coated and the cross-section images obtained.

The film thickness was measured with a digital electronic micrometer (Palmer, COMECTA, Barcelona, Spain) to the nearest 0.001 mm at six random positions.

2.3.3. *Fourier transformed infrared spectroscopy (FT-IR)*

The vibration mode of the chemical groups of the polymer was assessed in films equilibrated at 53% RH at 25 ℃ through attenuated total reflectance ATR-FTIR analysis using a Nicolet 5700 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The average spectra were collected from 64 scans with a resolution of 4 cm^{-1} in the 4000–400 cm^{-1} range. The analyses were performed in triplicate and at three different locations in each sample.

2.3.4. *Thermal behaviour*

The thermal behaviour of the films was characterised using thermogravimetric analysis (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland) and differential scanning calorimetry (DSC 1 StareSystem, Mettler-Toledo, Inc., Switzerland). TGA analysis was performed by heating from 25 ℃ to 700 ℃ at 10 ℃/min under a 10 mL/min nitrogen stream. DSC curves were obtained by multiple scan. A first heating from -25 ℃ to 250 ℃ at 10 ℃/min, holding for 2 min at 250 ℃. Samples were then cooled to -25 ℃ and held for 2 min before the second heating from -25 to 250 ℃ at 10 ℃/min. Analyses were carried in triplicate for each sample.

2.3.5. *X-ray diffraction*

The X-ray diffraction spectra of the films were recorded with a D8 Advance X-ray diffractometer (Bruker AXS, Karlsruhe, Germany) between 2θ: 10° and 50°, with a step size of 0.05, using Kα Cu radiation (λ: 1.542 Å), 40 kV and 40 mA. The degree of crystallinity (Xc) of the samples was estimated from the ratio of crystalline peak areas and the integrated area of X-R diffractograms and expressed as a percentage. The diffraction scattering curve was deconvoluted with Lorentz model using the OriginPro 8.5 software, for crystalline and amorphous peaks.

2.3.6. *Tensile properties*

Tensile properties were determined using a universal testing machine (Stable Micro Systems, TA.XT plus, Haslemere, England), following the standard method ASTM D882-02 (ASTM, 2002). For each formulation, eight test specimens (25 mm x 100 mm) were cut and conditioned for one week (RH: 53% and T: 25 ℃) and subjected to the extension test. The initial separation of the clamps was 50 mm and elongation speed 50 $mm.min^{-1}$. From the stress (σ)-Henky deformation (εH) curves, the elastic modulus (EM) and tensile strength (TS) and elongation at break point (ε) were obtained. Measurements were carried out 8 times for each sample.

2.3.7. *Barrier properties*

Water vapour permeability (WVP) was analysed following a modification of the E96/E95M-05 gravimetric method (E. ASTM, 2003). The film samples of each formulation were placed on Payne permeability cups (3.5 cm in diameter, Elcometer SPRL, Hermelle/s Argentau, Belgium) at 25 ℃ and 53-100% RH gradient, which was created with an oversaturated $Mg(NO_3)_2$ solution (inside the desiccator where cups were placed) and distilled water (5 mL inside the cup). In order to reduce the resistance to transport of water vapour, a fan was positioned above each cup. The cups were weighed periodically every 1.5 h for 24 h using an analytical balance (±0.00001 g). To calculate WVTR, the slopes in the steady state period of the weight loss vs. time curves were determined by linear regression. WVP was calculated according to (Cano et al., 2014). For each type of film, WVP measurements were carried out in triplicate.

The oxygen permeability (OP) was determined following a modification of the standard method F1927-07 (F.-07 ASTM, 2004). For this analysis, the Ox-Tran system (Mocon, Minneapolis, US) at 23 ℃ and 53% RH was used. Sample films (50 cm^2) were exposed to pure nitrogen flow on one side and pure oxygen flow on the other side. OP was calculated by dividing the oxygen transmission rate by the partial pressure of oxygen and multiplying it by the average film thickness. Each film formulation was analysed in triplicate.

2.3.8. *Optical properties*

The optical properties (transparency and colour coordinates) were determined by measuring the reflectance spectra of the samples from 400 to 700 nm, on white (R) and black (R_0) backgrounds, as well as the reflectance of the white backing (R_g), using a spectrocolorimeter (CM-3600d Minolta CO., Tokyo, Japan). Three measurements were taken from each film and three films were considered per formulation. The transparency was measured through the internal transmittance (Ti), applying the Kubelka-Munk theory for multiple scattering (Eq. 1). The CIE L*a*b* colour coordinates were obtained from the reflectance of an infinitely thick film (R∞) (Eq. 2) spectra, by using D65 illuminant and observer 10°, observer according to (Hutchings, 1999). Psychometric coordinates, Chroma (Cab*) and hue (hab*) were also determined using Eqs. (5) and (6).

$$T_i = \sqrt{(a + R_0)^2 - b^2} \quad (1)$$

$$R_\infty = a - b \quad (2)$$

$$a = \frac{1}{2}\left[R + \frac{R_0 - R + R_g}{R_0 \times R_g}\right] \quad (3)$$

$$b = (a^2 - 1)^{1/2} \quad (4)$$

$$C^*_{ab} = \sqrt{(a^{*2} + b^{*2})} \quad (5)$$

$$h^*_{ab} = arctg\ \frac{b^*}{a^*} \quad (6)$$

2.3.9. *Statistical analysis*

The statistical analysis of the data was carried out using Statgraphics Centurion XVI.II. The results were submitted to an analysis of variance (ANOVA). Fisher`s least significant difference (LSD) was used at the 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Carvacrol retention

The percentages of CA retention and final CA contents in the two types of PVA films are shown in **Table 1**. The type of PVA significantly affected the final content of CA in the films, the compound retention being higher in the matrix with lower degree of hydrolysis (PVA_P). This may be attributed to the greater compound affinity with the polymer chains due to the presence of residual acetyl groups, which confer a more hydrophobic nature on the polymer. Films obtained by the casting of aqueous solutions of hydrophilic polymers, containing essential oils, lose a great part of the emulsified oil during the drying step because of the emulsion destabilization (droplet flocculation, coalescence and creaming) and steam drag of the surface oil in line with water evaporation (Perdones et al., 2016). Factors improving the emulsion stability, such as the presence of amphiphilic compounds or high viscosity, mitigate these losses (Perdones et al., 2016). Likewise, a greater affinity between the essential oil compounds and the polymer chain can favour the bonding of active compounds in the polymer matrix, increasing their retention in the film.

Table 1. Nominal fraction (x) of the components in the different film formulations, CA content (extracted) in the final films and retention percentage (ratio between the mass of CA extracted from the film with respect to that incorporated). Mean values and standard deviation.

Sample	x_{PVA}	x_{CA}	CA content in the films		CA-Retention (%)
			(mg CA/ g dry film)	(mg CA/ g polymer)	
PVA_F	1	-	-	-	-
PVA_F-CA_5	0.95	0.05	23 (1)^a	24 (1)^a	48 (2)^a
PVA_F-CA_{10}	0.91	0.09	46 (2)^c	51 (2)^c	51 (2)^a
PVA_P	1	-	-	-	-
PVA_P-CA_5	0.95	0.05	28 (2)^b	29 (1)^b	58 (3)^b
PVA_P-CA_{10}	0.91	0.09	57 (2)^d	63 (2)^d	63 (2)^c

Different superscript letters within the same column indicate significant differences among films (P<0.05).

*(single column)

3.2. Film microstructure

The microstructural analysis permits the identification of the arrangement of the different components in the film, which can be correlated with the film's functional properties, such as barrier, mechanical or optical. During the film drying step, the solvent evaporation leads to an increase in both the viscosity of the continuous phase and in the concentration of the dispersed phase that affect the kinetics of the destabilization process of emulsified film

forming systems. This leads to changes in the particle size distribution of the dispersed lipid fraction, affecting the internal structure of the film and the final properties of the film matrix (Atarés & Chiralt, 2016; Song et al., 2018). The cross section of the obtained films (**Figure 1**) showed marked differences in the internal structure for PVA_F and PVA_P polymers, which may be related with their different chemical affinity with CA and the viscosity of the film-forming systems.

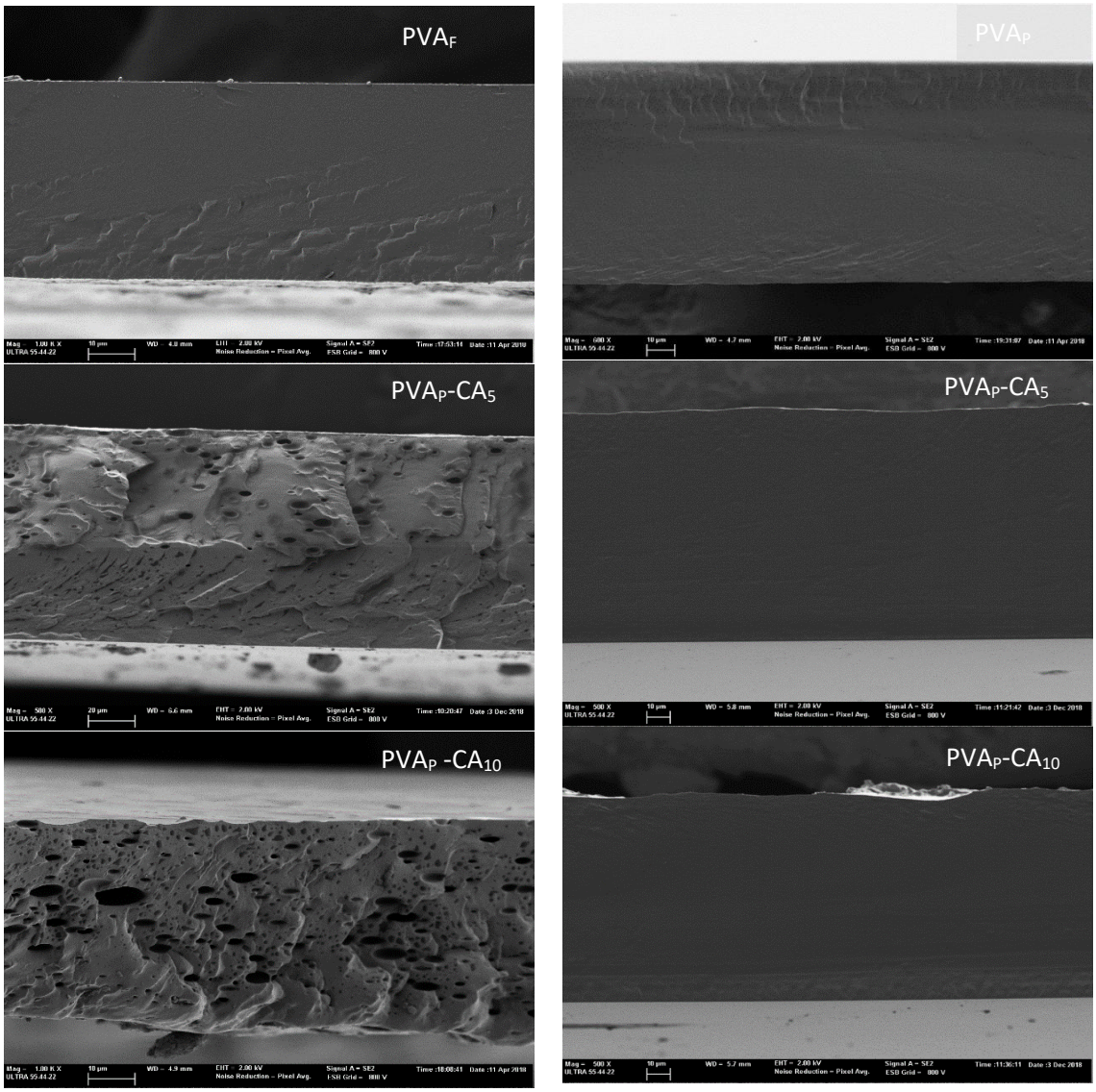

Figure 1. FESEM micrographs of the cross-section of the PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA).

In films from fully hydrolysed PVA (PVA_F), CA was distributed stochastically in droplets of highly variable sizes, whereas no lipid droplets were observed in partially acetylated PVA

(PVA_P) despite the higher CA content determined in the latter case. The high degree of hydrolysis and the longer size of the chains favour the chain intermolecular forces in polymer A, leading to greater cohesion forces in the matrix, whereas the chemical affinity with CA is lower than in PVA_P. This low chemical affinity provokes the CA phase separation (visible droplets), as observed in **Figure 1**, and limits its retention in the matrix. This effect was also reported by other authors (Chen et al., 2018) in PVA films (with the same M_W and degree of hydrolysis) for clove oil at concentrations higher than 3%. The lack of visible CA droplets in films from polymer PVA_P, with higher final CA content, suggests that the remaining CA is bonded to the polymer chains, generating a homogeneous structure able to link a determined amount of the compound without phase separation in the matrix. The smaller molecular weight of PVA_P imparts lower viscosity to the film-forming systems which should limit the system's ability to stabilise the emulsified CA against creaming and its subsequent losses by steam drag effect during the film drying step. However, the acetylated groups in the chains could favour the bonding of CA molecules by chemical interactions in a non-emulsified form. As reported by Wiśniewska et al. (2016), acetyl groups in the chains ionize (**Figure 2**), generating negative charges (lone electron pairs) that can act as electron donors (Lewis base). On the other hand, the hydroxyl group of CA, acting as Lewis acid, could form Lewis adducts with the lone electron pairs of negatively charged PVA chains. A previous study (Tampau et al., 2020) reported a decrease of zeta potential in acetylated PVA aqueous solutions when carvacrol was incorporated, which supports this hypothesis. This reaction could contribute to the higher CA retention in matrix PVA_P, without phase separation, as observed in **Figure 1**. In fact, taking into account the molar ratio between CA and acetylated groups of PVA_P, deduced from the degree of hydrolysis and the mass ratio of CA and polymer, an excess of acetylated groups could be deduced for both 5 and 10 g CA/ 100 g polymer (7 and 3.6 moles of acetylated groups/CA mole, respectively). Therefore, the evident differences in the film microstructure and CA retention between the two types of PVA must be attributed to the different degree of hydrolysis and molecular weight of the polymer chains.

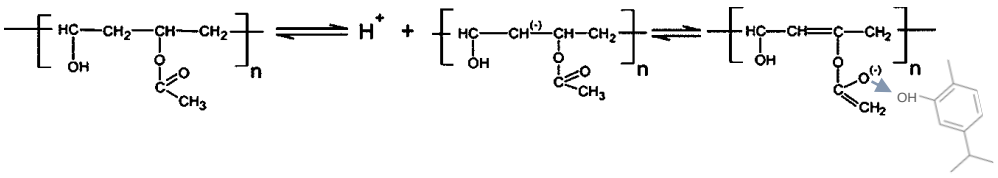

Figure 2. Ionization mechanism of acetylated PVA chains (Wiśniewska et al., 2016) and proposed Lewis adduct formation with carvacrol.

FTIR spectra (**Figure 3**) of the films have been obtained to analyse possible changes in the molecular vibration modes associated with the interactions with CA. All spectra of PVA_F films

show a similar pattern. The spectra show the typical broad peak at 3260 cm^{-1} associated with the intermolecular hydrogen bonding and the hydroxyl (O-H) stretching vibration. The C-H asymmetrical and symmetrical stretching vibration occurs at 2930 cm^{-1} and 2910 cm^{-1}, respectively. Other peaks appear at 1416 cm^{-1}, 1326 cm^{-1}, 1085 cm^{-1} and 832 cm^{-1}, which are related to CH_2 bending, motion of the carbon skeleton (C-H), C-O stretching and C-C stretching (Abral et al., 2019; Cano et al., 2015; Chenwei et al., 2018). FTIR spectra of films from partially acetylated PVA_P presented the same peaks, slightly displaced, with three additional peaks associated with the stretching vibrations bands of the carbonyl (C=O) and acetyl groups that were observed at 1732 cm^{-1}, 1372 cm^{-1} and 1240 cm^{-1}.

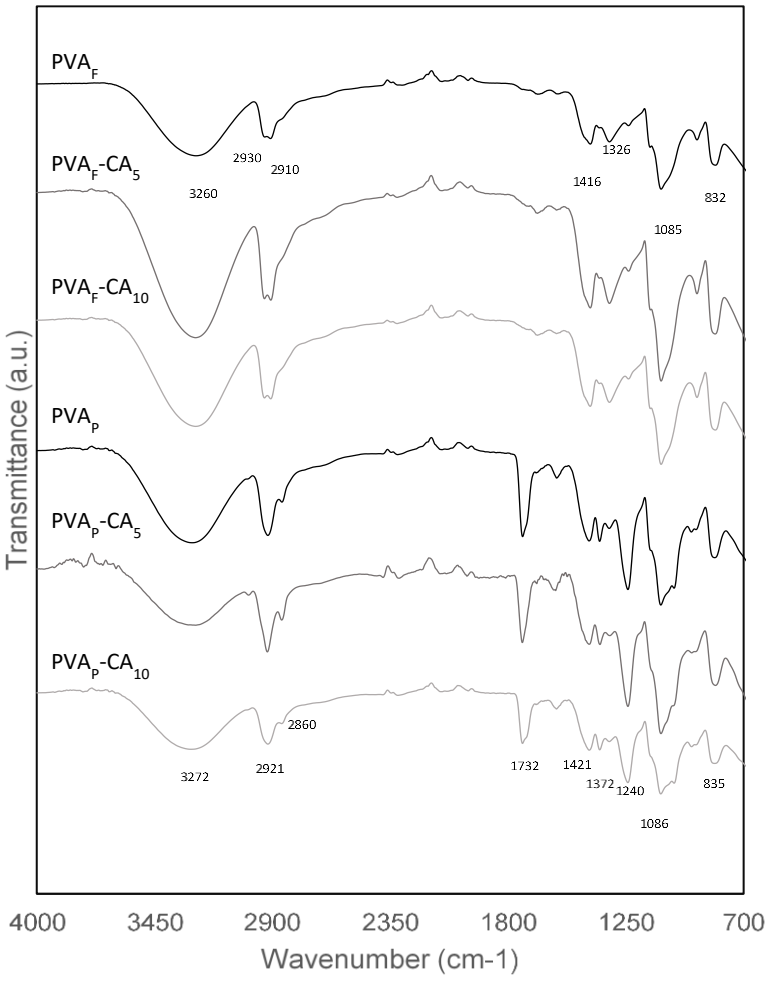

Figure 3. FTIR spectra of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA).

In neither case did the presence of CA cause changes in the vibration band of PVA. Not even the described interaction between CA and acetyl groups in PVA_P chains caused changes in the vibration mode of carbonyls. This can be attributed to the very low molar ratio of CA in the films, which prevents the quantitative observation of its characteristic vibration bands, and

the lack of covalent interactions between CA and PVA groups affecting the vibration mode of the chain bonds. The characteristic FTIR peaks of carvacrol have been observed by other authors (Altan et al., 2018; Buendía−Moreno et al., 2020) at 3500-3300 cm^{-1} (O-H stretch), 2868-2958 cm^{-1} (C-H stretch), 1620-1485 cm^{-1} (C-C stretch), 1240 cm^{-1} (C-O stretching vibration in aromatic ring) and 800 cm^{-1} (aromatic C-H bending).

3.3. Thermal behaviour and crystallinity of the films

The DSC analysis was carried out in two heating steps, whose thermograms are shown in **Figure 4**. The presence of first and second order transitions in all samples corroborates the semi-crystalline character of the PVA in the film samples. The first heating scan reflects the state of the polymer after the casting process, while the second heating scan shows the thermal behaviour of the material once its previous thermal history has been erased by the polymer melting in the first heating step. **Table 2** shows the temperature and enthalpy values for the different thermal events shown in **Figure 4**.

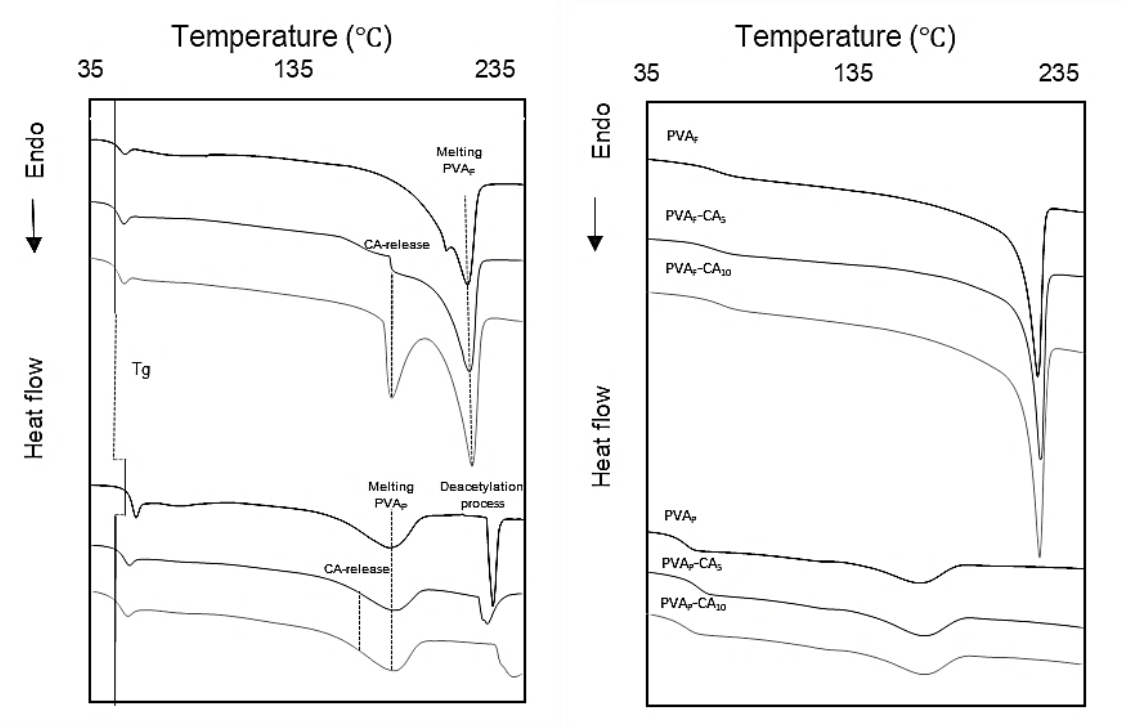

Figure 4. DSC curves of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA). On the left, the first heating scan and on the right the second heating scan.

Glass transition temperature (Tg) between 48-53 ℃ appeared in both polymers (PVA_F and PVA_P), where a relaxation enthalpy can be observed in the first heating step associated with the polymer ageing during storage (Wang et al., 2009). In the first heating step, the Tg values of PVA_F were lower than those of PVA_P despite the latter's lower molecular weight. This could be attributed to different factors, such as the greater restrictions in the molecular mobility imposed by the acetyl groups in the amorphous phase generated during casting or to the differences in the bonded water that can plasticise the matrix to a different extent. Due to the slow process of film formation by casting (in line with water evaporation), macromolecules of different molecular weight in each polymer solution could be preferably located in the amorphous phase due to the different crystallization restrictions associated with the steric hindrance in the molecular arrangement. The lowest molecular weight chains seem to preferably constitute the amorphous phase of matrix PVA_F, whereas the opposite behaviour seems to occur for the partially acetylated polymer (PVA_P). The high content of bonded water in PVA_F, commented on below, can also have a plasticising effect on matrix PVA_F, thus decreasing the Tg value. In this sense, it is remarkable that Tg values of PVA_F were higher (73 ℃) once the bonded water was eliminated during the first heating and the polymer melted and recrystallized in the melt (second heating step). This notable variance in Tg values suggests a different average molecular weight of the macromolecules constituting the amorphous phase both in cast films and after melting and heating till 250 ℃. For PVA_P, the Tg values were only slightly higher in the second heating step, which could be mainly attributed to the loss of bonded water during the first heating, with the formation of an amorphous phase of similar composition to that formed in cast films.

No effect of the CA incorporation on the Tg values of PVA_F was observed, although CA plasticised matrix PVA_P, decreasing the Tg values of the polymer in cast films (first heating), in agreement with the previously described interactions between CA and acetylated PVA_P. These interactions can enhance the free volume of the chains, promoting molecular mobility. In the second heating, no significant differences in Tg values were observed for the different samples obtained with PVA_P, which confirms the CA delivery during the first heating.

Both polymers exhibited several endothermic events between 180 ℃ and 240 ℃ that can be attributed to the polymer melting, CA evaporation or endothermic degradation events as the temperature increases. To better understand the origin of these events, the TGA and DGTA curves (**Figure 5**), showing the different weight loss steps, were taken into account. This is because both the CA losses detected in TGA, as well as some incipient thermodegradation steps of the polymer, could imply endothermic peaks. TGA and DTGA curves exhibit notable differences between the thermal behaviour of samples from PVA_F and PVA_P. In both cases, DTGA curves showed a first weight loss step corresponding to the loss of bonded water in the polymer matrix (conditioned at 0% RH): 3% of bonded water was lost by the non-acetylated PVA_F, while only 1.5% of bonded water was determined for matrices PVA_P, in agreement with their more hydrophobic nature provided by acetyl groups. The second weight loss step of TGA

curves, which only appears in samples containing CA, must be attributed to the CA thermo-release at about 196 ℃ and 150 ℃ for polymers PVA_F and PVA_P, respectively. The amount of CA released in this step (determined from the integration of the corresponding peak in DTGA curves) ranged between 40-60% of the final CA content determined in the films (**Table 1**), the percentage being higher when the carvacrol content increased. This suggests that a part of carvacrol was strongly bonded to the polymer matrix and it was not thermo-released, previously to the polymer degradation step, as has been also observed by other authors (Tampau et al., 2020). In this sense, it is remarkable that no quantitative mass of CA was released at 196 ℃ in sample PVA_F with 5% nominal CA content, probably due to that this low content was strongly bonded to the polymer matrix. However, a very small endothermic event was detected in the first heating step of the DSC before the melting endotherm.

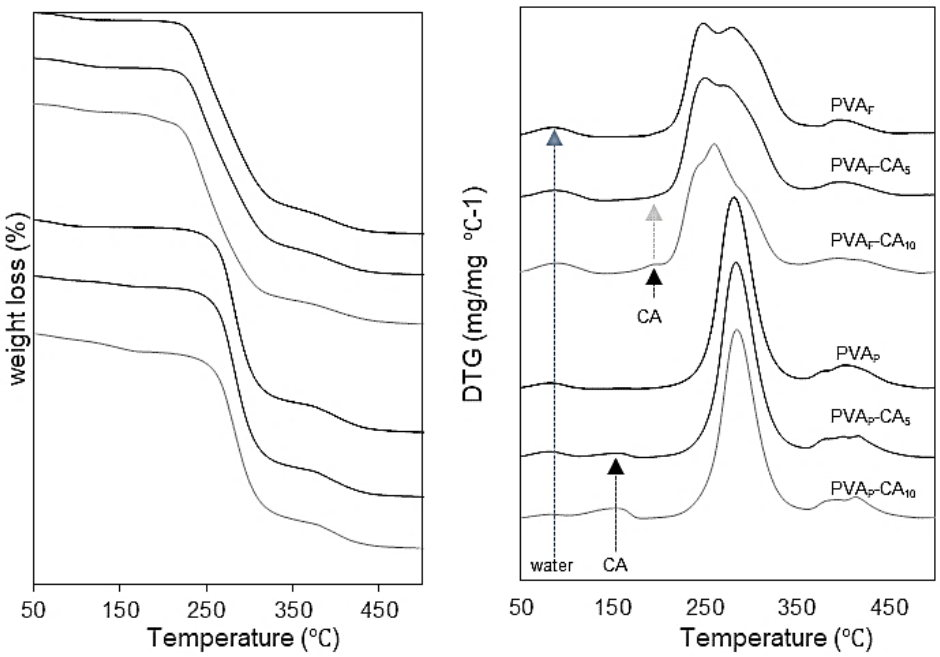

Figure 5. TGA (left) and DTGA (right) curves of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA).

The earlier partial release of CA in PVA_P at about 150 ℃ could be due to the lower melting temperature of this polymer (Tm: 180 ℃, first heating step in Table 2) than that of PVA_F (Tm: 225 ℃). The carvacrol release that occurred was associated with the polymer melting and this event could affect the melting enthalpy of PVA_P in the first DSC heating step. Likewise, the first endotherm, at about 190 ℃ in samples PVA_F containing CA, must be attributed to the CA evaporation, as deduced from the TGA detection of the CA thermo-release at this temperature.

A second endotherm, at between 230-245 ℃, appeared in the first heating step for samples PVA_P that could be attributed to endothermic events associated with polymer thermodegradation, such as the deacetylation process, as reported by other authors (Rimez, Rahier, van Assche, Artoos, & van Mele, 2008; Rimez, Rahier, van Assche, Artoos, Biesemans, et al., 2008). Deacetylation is autocatalytic and corresponds to the first degradation mechanism of acetylated PVA and, in inert conditions (N_2 flow), the deacetylation step as well as the chain scission reaction show endothermic effects. In DGTA curves, the PVA_P degradation process starts at about 220 ℃ under N_2 flow. Then, endothermic degradation events are reflected in the first heating step of DSC curves (**Figure 4**). The changes occurred in the polymer chains during this first degradation step significantly affected the melting temperature of the polymer (Tm: 168 ℃) in the second heating scan. This suggests that the chain crystalline packing and crystal sizes were notably influenced by the acetylation degree and partial degradation of the polymer.

Table 2. Glass transition and melting temperature and enthalpy of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA).

Sample	First heating scan			Second heating scan		
	Tg (℃)	Tm (℃)	ΔHm (J.g-1)	Tg (℃)	Tm (℃)	ΔHm (J.g-1)
PVA_F	48.0 (0.2)^{a}	225 (5)^{b}	78.6 (0.4)^{c}	73 (3)^{b}	225 (1)^{b}	74 (3)^{b}
PVA_F-CA_5	48.1 (0.2)^{a}	224 (2)^{a}	72 (2)^{c}	74 (3)^{b}	226 (1)^{b}	69 (3)^{b}
PVA_F-CA_{10}	48.1 (0.1)^{a}	223 (1)^{a}	63 (3)^{b}	71 (3)^{b}	225 (1)^{b}	71 (7)^{b}
PVA_P	53.8 (0.4)^{c}	183 (1)^{a}	40 (4)^{a}	56 (3)^{a}	168 (1)^{a}	25 (2)^{a}
PVA_P-CA_5	49.9 (0.8)^{b}	184 (2)^{a}	37 (6)^{a}	58 (6)^{a}	168 (1)^{a}	22 (1)^{a}
PVA_P-CA_{10}	49 (2)^{ab}	184 (1)^{a}	39 (2)^{a}	53 (2)^{a}	169 (1)^{a}	23 (2)^{a}

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

The degradation of the polymeric material occurred in two stages; the first started at about 200 ℃ (PVA_F) or 220 ℃ (PVA_P) and represented around 70% of the weight loss of the samples and the second stage started at 360 ℃ with about 12% of weight loss. The temperature peak of the first main step was 248 ℃ for PVA_F and 281 ℃ for PVA_P. For the acetylated chains, such as polymer PVA_P, the detachment of acetyl groups from the chains, forming water, acetaldehyde and acetic acid, has been described as the first degradation mechanism (Perilla, 2007). For polymer PVA_F, the degradation peak in DGTA curves showed an overlapping of different weight loss events, suggesting the action of different simultaneous degradation events. This could be related with the fact that both the melting of the crystalline fraction (Tm: 225 ℃) and degradation occur in the same temperature range and, melt and crystalline phases should degrade differently. In contrast, the degradation of PVA_P occurred once melted above 180 ℃ (**Table 2**) in a more continuous degradation process. The second stage of polymer

degradation above 360 ℃ was related to the degradation of low molecular weight products from the decomposition of the main chain, or of heavier structures formed in the previous degradation stage (Perilla, 2007).

Thus, molecular properties of PVA affected the thermal behaviour of the material. The degree of hydrolysis of the polymer was highlighted, since the acetate groups in the PVA_P-chains appear to have a thermo-protective effect, as described by (Cristancho et al., 2013), who report that the presence of the carbonyl group (C=O) in the PVA-chains increases the absolute value of the energy required for the material degradation. In this sense, Perilla (2007) applied both the Friedman and the Freeman-Carroll methods to determine the activation energy (E_a) of PVA degradation, and found that E_a was inversely proportional to the degree of hydrolysis of the polymer. This is of particular importance for the feasibility of the thermal processing of polymers. Thermoprocessing requires the melting temperature to be rather lower than the degradation temperature. This occurs in acetylated low molecular weight PVA_P but not in fully hydrolysed high molecular weight PVA, which could not be submitted to the thermoplastic industrial processes because it has no adequate thermal processing window. In the second heating step of the DSC analysis, a part of PVA_F would be degraded, since the temperature of the first heating reached 250 ℃. So, both the obtained values of Tg and Tm would be affected by this partial degradation. In fact, the much higher values of Tg obtained in the second heating could be due to the higher mean molecular weight of the remaining molecules. The melting temperature coincides with the value of the first heating step, which suggests that no significant changes in the crystalline fraction occurred as a result of the partial degradation at 250 ℃.

Other authors (Cano et al., 2015; Juan David, 2019) only reported Tg and Tm values from the second heating step of the DSC analysis under similar conditions, which agree with those found in this study. Nevertheless, during the first heating, the state of the polymer was modified by both melting above Tm and partial thermogradation. So, the second heating DSC analysis did not reflect the real polymer state in cast films. The melting enthalpy values and melting temperature of PVA_P in particular were higher for the cast samples (first heating) than for the melt samples (second heating), which could point to the formation of bigger, more stable crystals in cast films due to the greater mobility of molecules in the water solution than in the melt.

The crystallinity of PVA in the cast films was analysed by DRX analysis, since the different overlapped endothermic events occurring during DSC analyses did not permit its evaluation from the melting enthalpy data. The DRX patterns of pure PVA-films and PVA-films with different CA ratios are shown in **Figure 6**. PVA films showed a crystalline peak at around 2θ = 19.7° with a shoulder at 22.6° and a small broad peak at 41.5°. The PVA_P-films showed a slight decrease in the crystalline peak's intensity and an increase in the width of the main crystalline peak with respect to the PVA_F-films, which suggests a lower percentage of crystallinity and

smaller crystals in PVA_P (Safna Hussan et al., 2019). The crystallinity quantified through the peak area is indicated in Figure 6 for each sample and was higher for polymer PVA_F than for PVA_P, both slightly increasing when CA was present. The greater crystallinity of PVA_F can be explained by the more homogenous chain structure (without acetyl groups) that enhances both the capacity to form inter-chain hydrogen bonds and the crystalline arrangement. PVA_P, with partially acetylated chains, has steric hindrance that limits a more ordered, crystalline molecular arrangement.

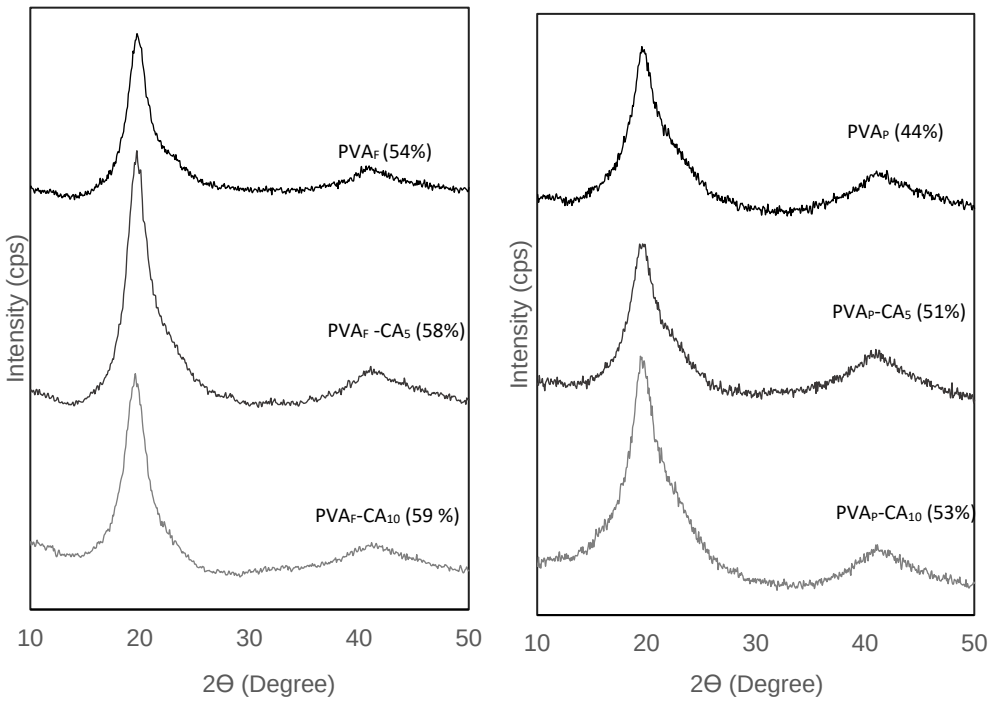

Figure 6. X-Ray diffraction spectra of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA). Percentages of crystallinity are shown for each sample.

3.4. Tensile, barrier and optical properties of the films.

Table 3 shows the values of tensile parameters (elastic modulus: EM, tensile strength: TS and elongation: E, at break) of the different films. Films obtained with PVA_F exhibited better mechanical performance than those with PVA_P, which can be attributed to the formation of more inter-chain hydrogen bonds due to the longer molecular chains with a greater proportion of hydroxyl groups. In contrast, the acetyl groups in PVA_P interrupted the hydrogen bond formation in the shorter chains. This limits the cohesion forces in the matrix and reduces the mechanical performance of the material. Restrepo et al. (2018) also found that the

increase in molecular weight and the degree of hydrolysis improved the mechanical properties of the PVA materials.

Table 3. Thickness, tensile parameters (Tensile strength (TS), elongation (E), at break and elastic modulus (EM)) and barrier properties (water vapour permeability: WVP and oxygen permeability: OP) of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA). Mean values and standard deviation.

Sample	Thickness (μm)	TS (MPa)	E (%)	EM (MPa)	WVP x 10^3 (g/m. h. kpa)	OP x 10^8 (cm^3/m. h. kpa)
PVA_F	101 (2)^{bc}	153 (8)^{d}	135 (6)^{c}	80 (4)^{b}	2.47 (0.06)^{a}	0.38 (0.01)^{a}
PVA_F-CA_5	106 (2)^{c}	138 (13)^{c}	133 (5)^{c}	79 (5)^{b}	2.60 (0.30)^{a}	1.07 (0.10)^{c}
PVA_F-CA_{10}	108 (2)^{a}	134 (5)^{c}	132 (5)^{c}	82 (6)^{b}	3.10 (0.60)^{b}	1.55 (0.04)^{e}
PVA_P	95 (2)^{b}	44 (6)^{a}	97 (6)^{a}	54 (5)^{a}	2.90 (0.02)^{ab}	0.53 (0.05)^{b}
PVA_P-CA_5	101 (2)^{bc}	68 (4)^{b}	121 (7)^{b}	53 (4)^{a}	3.12 (0.03)^{b}	1.40 (0.06)^{d}
PVA_P-CA_{10}	99 (2)^{b}	66 (3)^{b}	118 (4)^{b}	50 (3)^{a}	2.45 (0.05)^{a}	6.11 (0.06)^{f}

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

The incorporation of CA did not affect the elastic modulus (EM) or extensibility (E) of films from PVA_F, but reduced their resistance to break (TS), as previously reported by (Chen et al., 2018) for PVA (99% hydrolysed) matrices with incorporated clove oil (CO). This must be attributed to the presence of a lipid dispersed phase (**Figure 1**) that interrupted the polymer network and weakened its mechanical resistance. In contrast, the incorporation of CA into the PVA_P matrices increased the film's resistance (TS) and elongation (E), without modifying EM. The CA bonded to PVA_P enhanced the chain slippage during stretching, making the films more extensible without break. Then, the greater chemical affinity of CA with PVA_P gave rise to monophasic films, where CA acted by enhancing their mechanical performance. Tongnuanchan et al. (2012) reported that some compounds in essential oils might be able to improve the polymer tensile properties due to the rearrangement of the polymer network in line with the developed molecular interactions. In this sense, some studies reported an increase in the TS of soy protein isolate films (Atarés et al., 2010) and chitosan films (Ojagh et al., 2010) when cinnamon essential oil was incorporated into the matrices.

Water vapour permeability (WVP) and oxygen permeability (OP) are relevant properties for the applicability of the films as food packaging materials. The optimal values of permeability to water vapour and oxygen are marked by the kind of packaged food, depending on the required conditions to maintain the product quality (Valencia-Sullca et al., 2016). **Table 3** shows the values of water vapour and oxygen permeability of the different films. Films from PVA_F exhibited a better barrier capacity against both water vapour and oxygen than PVA_p film, due to the formation of a more cohesive matrix, with higher crystallinity degree, as

commented on above. Likewise, as occurred in the mechanical behaviour, a different effect was provoked in each matrix by the incorporation of CA. This effect was only significant ($p<0.05$) at the highest ratio of CA and implied a slight increase in the WVP for the PVA_F, and a decrease in the matrix PVA_P. The different structural arrangement of CA, previously commented on, affected the different response of the matrices to the transfer of water molecules.

As concerns the oxygen barrier capacity, an increase in OP was observed in both PVA_F and PVA_P films when CA was present in the film. In both cases, the higher the CA content, the higher the OP. Given that PVA exhibited a good oxygen barrier capacity, in line with its hydrophilic nature, the incorporation of non-polar compounds, with an increased oxygen solubility, represented a negative effect in the material barrier capacity. This effect was more marked in the PVA_P films with the highest content of CA (10%), probably due to the observed plasticizing action that promoted molecular mobility and mass transfer processes.

PVA_F, with higher molecular weight and greater crystallinity degree, exhibited better barrier properties than PVA_P. Nevertheless, carvacrol incorporation promoted crystallization in both matrices PVA_F and PVA_P, but this was not reflected in a generalized enhancement of the film barrier capacity. This can be attributed to the fact that other structural changes, related with the different molecular interactions of carvacrol with the polymer chains, also play a relevant role in the feasibility of mass transfer phenomena through the matrix. These interactions were greatly affected by the presence of acetyl groups in the PVA chains.

The colour parameters of Lightness (L *), Chrome (C_{ab} *) and hue (h_{ab} *) of the different samples and the internal transmittance values at 460 nm (T_i), used as a transparency indicator, are shown in **Table 4**. Films from PVA_P were lighter with lower hue values than those of PVA_F; this is probably due to the different refractive indexes of the matrices, of differing compactness, which affect the light interactions. The presence of CA in the PVA_F films slightly reduced the lightness, chrome values and transparency (Ti) in line with the CA concentration. However, this did not significantly affect these values in the PVA_P films. This agrees with the formation of a dispersed CA phase in the PVA_F matrices (**Figure 1**) which provoked light scattering, reducing the film transparency and affecting the colour parameters. In the homogenous PVA_P matrices containing carvacrol, no significant effect of the compound was observed on the film optical properties. Despite the commented differences in the optical properties of the films, the visual appearance was very similar for all cases and, globally, all films were highly transparent and colourless.

Table 4. Lightness (L *), chrome (Cab *), hue (hab *) and internal transmittance values at 460 nm (Ti) of the of PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA). Mean values and standard deviation.

Sample	L*	C_{ab}*	h_{ab}*	T_i (460 nm)
PVA_F	88 (2)^{b}	3 (1)^{b}	114 (11)^{c}	0.86 (0.01)^{bc}
PVA_F-CA_5	87 (2)^{b}	3.5 (0.7)^{b}	112 (3)^{bc}	0.85 (0.01)^{b}
PVA_F-CA_{10}	82 (2)^{a}	2.7 (0.4)^{a}	120 (5)^{d}	0.83 (0.01)^{a}
PVA_P	92 (1)^{c}	3.4 (0.5)^{b}	104 (2)^{a}	0.86 (0.01)^{bc}
PVA_P-CA_5	92 (2)^{c}	2.7 (0.5)^{a}	108 (5)^{ab}	0.85 (0.01)^{c}
PVA_P-CA_{10}	92 (1)^{c}	2.9 (0.4)^{ab}	106 (2)^{a}	0.85 (0.01)^{bc}

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

4. CONCLUSION

The molecular weight and degree of hydrolysis of the PVA significantly affected both the microstructure of the films containing carvacrol and the thermal behaviour of the polymer matrices. Semicrystalline structures were obtained in cast films from both polymers, the crystallinity being slightly higher in de-acetylated high Mw polymer (PVA_F). Low Mw, partially acetylated PVA_P melts at a lower temperature than high Mw PVA_F while its thermodegradation occurs at a higher temperature due to the protective effect of acetyl groups. This makes it possible for the polymer thermoprocessing to obtain films by means of the usual thermoplastic industrial process. Likewise, the presence of acetyl groups in the chain promoted chemical affinity with active compounds, such as carvacrol, allowing a greater retention in the matrix and, thus a more effective way of obtaining active films for food packaging. This better chemical affinity between the active compound and polymer chains gave rise to homogenous films (without phase separation) that positively affected the tensile properties of the films. No relevant differences between the water vapour barrier capacity of either kind of PVA films were observed and, although the oxygen barrier and mechanical performance of the high Mw PVA_F films was better than that of the low Mw, partially acetylated PVA_P films, the incorporation of carvacrol enhanced the resistance to break and stretchability in the latter, but negatively affected the cohesion forces (tensile strength) of the matrix in the former. Therefore, PVA_P is of great potential for the production of active films with CA, by casting or thermoprocessing, with a broader range of possible uses than high Mw, de-acetylated PVA_F.

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CHAPTER III. The incorporation of carvacrol into poly (vinyl alcohol) films encapsulated in lecithin liposomes

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ABSTRACT

Lecithin-encapsulated carvacrol has been incorporated into poly (vinyl alcohol) (PVA) for the purpose of obtaining active films for food packaging application. The influence of molecular weight (Mw) and degree of hydrolysis (DH) of the polymer on its ability to retain carvacrol has been analysed, as well as the changes in the film microstructure, thermal behaviour, and functional properties as packaging material provoked by liposome incorporation into PVA matrices. The films were obtained by casting the PVA aqueous solutions where liposomes were incorporated until reaching 0 (non-loaded liposomes), 5 or 10 g carvacrol per 100 g polymer. The non-acetylated, high Mw polymer provided films with a better mechanical performance, but less CA retention and a more heterogeneous structure. In contrast, partially acetylated, low Mw PVA gave rise to more homogenous films with a higher carvacrol content. Lecithin enhanced the thermal stability of both kinds of PVA, but reduced the crystallinity degree of non-acetylated PVA films, although it did not affect this parameter in acetylated PVA when liposomes contained carvacrol. The mechanical and barrier properties of the films were modified by liposome incorporation in line with the induced changes in crystallinity and microstructure of the films.

Keywords

Food packaging; encapsulation; PVA; degree of hydrolysis

1. INTRODUCTION

Packaging is necessary for preserving food quality and extending its shelf life (Cruz et al., 2018), but the accelerated generation of non-biodegradable plastic wastes, many of them from the food packaging sector, has generated a serious environmental problem that impacts consumer awareness. Many efforts have been directed towards the search for materials based on biodegradable polymers and the development of new packaging models to address this issue. Of these, the use of biodegradable materials with antimicrobial and antioxidant properties represents innovation in the concept of food packaging (Fang et al., 2017). These materials seek to reduce the environmental impact, while also ensuring the food quality and safety.

Poly (vinyl alcohol) PVA is a biodegradable synthetic polymer that was obtained from the controlled hydrolysis of polyvinyl acetate (PVAc), with high (≈99%), medium (≈88%), or low (≈78%) hydrolysis degree (Thong et al., 2016). The use of this polymer for food packaging purposes could be of great interest, as it is transparent, non-toxic, odourless and water-soluble, while exhibiting good mechanical properties (Li et al., 2019). The degree of hydrolysis and the molecular weight are relevant molecular parameters in the polymer functionality, since these affect the physical properties, such as viscosity and film-forming capacity, as well as the elasticity and tensile strength of their films (Muppalaneni, 2013). These molecular characteristics also affect the polymer affinity and compatibility with other compounds that can be added as active components (Cano et al., 2015), which makes the polymer more or less appropriate for the development of active films.

Several compounds of natural origin, such as essential oils and plant extracts, which exhibit antimicrobial and antioxidant properties, can be used in the development of active materials (Bakkali et al., 2008). Carvacrol is a monoterpenoid phenol that is found in the essential oil of oregano (Origanum vulgare), thyme (Thymus vulgaris L.), marjoram (Origanum majorana), and similar aromatic plants (de Vincenzi et al., 2004), which is widely studied for its outstanding antimicrobial (Veldhuizen et al., 2006) and antioxidant action (Gursul et al., 2019).

Essential oils or their components have been emulsified in the polymer aqueous solutions to obtain active films from water soluble polymers by casting (Atarés & Chiralt, 2016). However, this process usually involved significant losses of the volatile compounds, mainly during the film drying step. In this step, emulsified essential oils are susceptible to destabilization processes, such as droplet flocculation, coalescence, and creaming, which lead the drops to the surface of the film where the lipid compounds are lost by steam drag effect along with evaporated water (Cofelice et al., 2019; Requena et al., 2017). Therefore, the active capacity of the films is significantly reduced in line with the decrease in the retention capacity of the active components (Sánchez-González et al., 2011). The use of nano/micro-encapsulation

techniques to entrap these volatile compounds could mitigate this problem (Asbahani et al., 2015; Sapper et al., 2018).

Liposomes are phospholipid vesicles that are organized in one or several concentric bilayers, with an aqueous inner core, which is able to self-assemble in aqueous solutions by hydrophobic effects (Callegarin et al., 1997). They represent an efficient approach for encapsulating essential oils, thus improving their solubility and chemical stability in water systems (Coimbra et al., 2011; Sebaaly, Charcosset, et al., 2016; Sebaaly, Greige-Gerges, et al., 2016), also allowing their more controlled release (Carvalho et al., 2016; Hammoud et al., 2019). Liposomal encapsulation has been proved to enhance the retention of different essential oils during film formation by casting (Sapper et al., 2018; Valencia-Sullca et al., 2016). However, the inclusion of liposomes in the different polymeric matrices also generated changes in the microstructure of the films. The changes in the solvent properties during the film drying step alter the vesicular structure due to the liotrophic mesomorphism of polar lipids. Previous studies reported the formation of lamellar lipid associations in starch-gellan (Sapper et al., 2018) and chitosan films (Valencia-Sullca et al., 2016) with incorporated lecithin liposomes that were loaded with essential oil compounds. These changes at the microstructural level promoted modifications in the film functional properties. Nevertheless, liposomes notably improved the retention capacity of the active compounds in the film matrix.

In this study, carvacrol encapsulated in lecithin liposomes was incorporated into two types of poly (vinyl alcohol) (PVA), in order to analyse the influence of the molecular weight (Mw) and the degree of hydrolysis of PVA on the ability to form active films. To this end, the microstructural, thermal, mechanical, and barrier properties of the films with encapsulated carvacrol were evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Two kinds of poly (vinyl alcohol) (PVA) with different molecular weight and degree of hydrolysis (PVA_F: Mw 89,000–98,000; 99-99.8% hydrolysed and PVA_P: Mw 13,000–23,000; 87–89% hydrolysed), carvacrol (CA) (Sigma-Aldrich, Steinheim, Germany), and soy lecithin (Lipoid S75, Lipoid GmbH, Ludwigshafen, Germany), containing 72% phosphatidylcholine (SL-PC), 10% phosphatidulethanolamine and 2% lysophosphatidylcholine, were used in the preparation of the films. Magnesium nitrate ($Mg(NO_3)_2$), phosphorus pentoxide (P_2O_5) salts, and UV-grade methanol were supplied by Panreac Química S.A. (Barcelona, Spain).

2.2. Preparation of liposome dispersions

Lecithin was initially dispersed in distilled water (5% w/w) while using magnetic stirring for 15 min at 800 rpm. Afterwards, the dispersion was subjected to sonication (35 kHz) for 10 min with pulses of 1 s, by using an ultrasonic device (Vibra Cell, Sonics & Material, Inc., Newtown, CT, USA). In this step, carvacrol (2.5 or 5% w/w) was incorporated into the lecithin dispersions. Thus, three liposome dispersions were obtained: one containing non-loaded liposomes (L) and two carvacrol loaded (LCA5, LCA10). The dispersions were placed in an ice bath during the ultrasound application to prevent sample heating.

2.3. Preparation of films

The PVA films were obtained by casting the PVA aqueous solutions. Polymer solutions (PVA_F 5% wt. and PVA_P 10% wt.) were prepared in distilled water while using magnetic stirring (1,200 rpm) at 100 °C for 3 h. Liposome dispersions (L, LCA5, LCA10), were added to the polymer solutions to reach a lecithin:polymer ratio of 10% with variable ratios of CA (0%, 5% or 10% wt. with respect to the polymer). The control films were obtained with the pure polymer solutions. All of the formulations were degassed by using a vacuum pump and spread evenly onto Teflon plates of 150 mm in diameter, while using a constant equivalent mass of polymer per plate of 2 g. The films were dried under controlled temperature (25 ± 2 °C) and relative humidity (54 ± 2%) for 48 h. **Table 1** shows the different film formulations with the respective mass fractions of the components.

The films were conditioned for one week at 53% relative humidity (RH), while using $Mg(NO_3)_2$ over-saturated solution, before their characterisation. Meanwhile, the final CA content in the film (CA retention), microstructure, and thermal analyses were carried out with films conditioned at 0% RH using P_2O_5.

2.4. Characterization of the active poly (vinyl alcohol) films

2.4.1. *CA retention and structural arrangement*

The determination of CA that was retained in the different formulations after film drying was carried out by the total extraction of CA from the films and its spectrophotometric quantification. The film samples (4 cm^2) were immersed in 50 mL of a 50% aqueous solution of UV-grade methanol, and then kept under stirring (300 rpm) for 48 h at 25 °C. Aliquots of the samples were extracted and the absorbance (A) was measured at 274 nm, by using a spectrophotometer (Evolution 201 UV-Vis, Thermo Fisher Scientific Inc., Waltham, MA, USA). The CA concentration (C) in the films was determined by means of a standard curve, which was obtained while using solutions containing between 10 and 50 µg/mL of carvacrol in the same solvent ($C = 63.61A$, $R^2 = 0.998$). The backgrounds used for the measurements were the corresponding extracts that were obtained under the same conditions from the CA-free films. CA retention (%) in the films was calculated as the ratio between the mass of CA extracted from the film with respect to the corresponding mass of CA initially incorporated.

The structural arrangement was evaluated in the cross-sections of the films, by using a Field Emission Scanning Electron Microscope (FESEM) (ZEISS®, model ULTRA 55, Oberkochen, Germany), at an acceleration voltage of 2 kV. The film samples were cryofractured by immersion in liquid nitrogen and then coated with platinum before obtaining the images.

A Nicolet 5700 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to obtain the FTIR spectra of the film samples. The average spectra were collected from 64 scans with a resolution of 4 cm^{-1} in the 4000–400 cm^{-1} range. These were performed in triplicate and at three different locations in each sample.

The X-ray diffraction spectra of the films were recorded with a D8 Advance X-ray diffractometer (Bruker AXS, Karlsruhe, Germany) that was in the range 2θ: 10° and 50°, with a step size of 0.05, while using Kα Cu radiation (λ: 1.542 Å), 40 kV and 40.mA. The diffraction curves were deconvoluted by using the Lorentz model and the OriginPro 8.5 software to obtain crystalline and amorphous regions. The degree of crystallinity of the samples (Xc, expressed as a percentage) was estimated from the ratio of crystalline peak areas and the total area of the original diffractograms.

2.4.2. *Thermal behaviour*

The thermal behaviour of the films was assessed by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). TGA analysis was performed by heating the samples in alumina crucibles from 25 °C to 700 °C at 10 °C/min. under a 10 mL/min. nitrogen stream, by using (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland). The DSC measurements of samples in aluminium crucibles were carried out under nitrogen flow (10 mL/min.) while applying heating (first and second) and cooling scans in a differential

scanning calorimeter (DSC 1 stareSystem, Mettler Toledo, Schwarzenbach, Switzerland). The temperature scanning profile was: a first heating from −25 °C to 250 °C at 10 °C/min., holding for 2 min. at 250 °C, sample cooling to −25 °C, holding for 2 min. at −25 °C; and, a second heating from −25 to 250 °C at 10 °C/min. All of the measurements were taken in triplicate.

2.4.3. *Functional and optical properties*

The tensile properties of the films (elastic modulus (EM), tensile strength (TS), and elongation at break point (E)) were determined from the tensile stress (σ) vs. Hencky strain (εH) curves, following the standard method ASTM D882-02 (ASTM, 2002). The test specimens (25 mm x 100 mm) were mounted in the film extension grips of the universal testing machine (Stable Micro Systems, TA.XT plus, Haslemere, England); the initial separation of the clamps was 50 mm and stretched at 50 mm.min.$^{-1}$ until breaking. The measurements were carried out in eight samples for each treatment.

Water vapour permeability (WVP) was analysed following a modification of the E96/E95M-05 gravimetric method (E. ASTM, 2003). The film samples of each formulation were placed on Payne permeability cups 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argentau, Belgium) at 25 °C and 53–100% RH gradient, which was created with an oversaturated $Mg(NO_3)_2$ solution (inside the desiccator where the cups were placed) and distilled water (5 mL, inside the cup). A fan was positioned above each cup in order to reduce the resistance to transport of water vapour. The cups were weighed periodically every 1.5 h for 24 h while using an analytical balance (±0.00001 g). The slopes of the weight loss *vs.* time at the steady state period were determined by linear regression to calculate the water vapour transmission rate (WVTR). WVP was calculated as described by Cano et al. (2014). For each type of film, WVP measurements were carried out in triplicate.

The oxygen permeability (OP) was analysed in film samples (50 cm^2) by using an Ox-Tran system (Mocon, Minneapolis, US) at 23 °C and 53% RH, following a standard method F1927-07 (F.-07 ASTM, 2004). The films were exposed to pure nitrogen flow on one side and pure oxygen flow on the other side. The oxygen transmission rate was multiplied by the average film thickness and divided by the partial pressure of oxygen to calculate the OP. Each film formulation was analysed in triplicate. The film thickness was measured with a digital electronic micrometer (Palmer, COMECTA, Barcelona, Spain) to the nearest 0.001 mm at six random positions. Ten measurements were taken for each sample to determine the average.

The optical properties (transparency and colour coordinates) were determined by using a spectrocolorimeter (CM-3600d Minolta CO., Tokyo, Japan) to obtain the reflectance spectra of the samples from 400 to 700 nm, on white (R) and black (R_0) backgrounds, as well as the reflectance of the white backing (R_g). The transparency was measured through the internal transmittance (Ti), while applying the Kubelka-Munk theory for multiple scattering (Equation (1)). The CIE L*a*b* colour coordinates were obtained from the reflectance of an infinitely

thick film (R_∞) (Equation (2)) spectra, using the 10° observer and the D65 illuminant as reference, according to Hutchings (1999). Psychometric coordinates, Chroma (C_{ab}*) and hue (h_{ab}*) were also determined while using Equations (5) and (6). Three measurements were taken from each film and three films were considered per formulation.

$$T_i = \sqrt{(a + R_0)^2 - b^2} \quad (1)$$

$$R_\infty = a - b \quad (2)$$

$$a = \frac{1}{2}\left[R + \frac{R_0 - R + R_g}{R_0 \times R_g}\right] \quad (3)$$

$$b = (a^2 - 1)^{1/2} \quad (4)$$

$$C^*_{ab} = \sqrt{(a^{*2} + b^{*2})} \quad (5)$$

$$h^*_{ab} = arctg\ \frac{b^*}{a^*} \quad (6)$$

2.4.4. *Statistical analysis*

The statistical analysis of the data was carried out while using Statgraphics Centurion XVI.II. The results were submitted to an analysis of variance (ANOVA). Fisher`s least significant difference (LSD) was used at the 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Microstructure

Figure 1 shows the FESEM micrographs of the cross-section of the different PVA films (PVA_F and PVA_P). Homogeneous PVA films were obtained with both polymers, PVA_F and PVA_P, but a dispersed phase in the polymer matrix can be observed for formulations containing lecithin liposomes loaded, or not, with carvacrol. This dispersed phase corresponds to the different incorporated lipid materials (lecithin liposomes with and without carvacrol), which are not miscible with the polymer. The lipid particles are larger than the liposomes initially incorporated (180 nm) into the aqueous phase, which indicates that liposomes, either loaded or not with carvacrol, changed during the film formation. Throughout the film drying step, the evaporation of the solvent, where the initial liposomes were dispersed, provoked changes in the liposomal structure due to the modification of the lipid-solvent interactions, thus giving rise to different mesomorphic species (lyotropic mesomorphism) containing lecithin components and carvacrol that remain dispersed in the polymer matrix, as previously observed by other authors (Andreuccetti et al., 2011; Sapper et al., 2018). The carvacrol that was released from liposomes could partially evaporate during the film drying step in line with the coalescence and creaming of the formed droplets and steam drag effect on the film surface (Perdones et al., 2016).

The volume fraction of the particles and the particle size distribution were different for a determined lipid composition in PVA_F and PVA_P, as shown in **Figure 1**. A higher proportion of dispersed lipid particles was observed in the PVA_F matrix, where the dispersed particles were smaller for the highest ratio of carvacrol. In contrast, PVA_P matrix exhibited a lower ratio of dispersed phase, with particle size also being affected by the carvacrol ratio (0, 5 or 10 g/100 g polymer) with a constant amount of lecithin. For non-loaded liposomes, the particle size was quite homogenous and bigger than that of carvacrol-loaded liposomes, for which a higher proportion of smaller particles could be observed. These microstructural differences between PVA_F and PVA_P films could be attributed to the molecular differences in the polymer chains. While PVA_F is highly hydrophilic with a high number of hydroxyl groups, the presence of residual acetyl groups in the PVA_P chains provides them with an amphipathic nature (Limpan et al., 2012) that promotes the chemical affinity between the polymer and the lipid compounds. This greater affinity enhances the molecular integration of the lipids in the matrix, thus giving rise to a smoother and more homogeneous microstructure than that obtained with PVA_F.

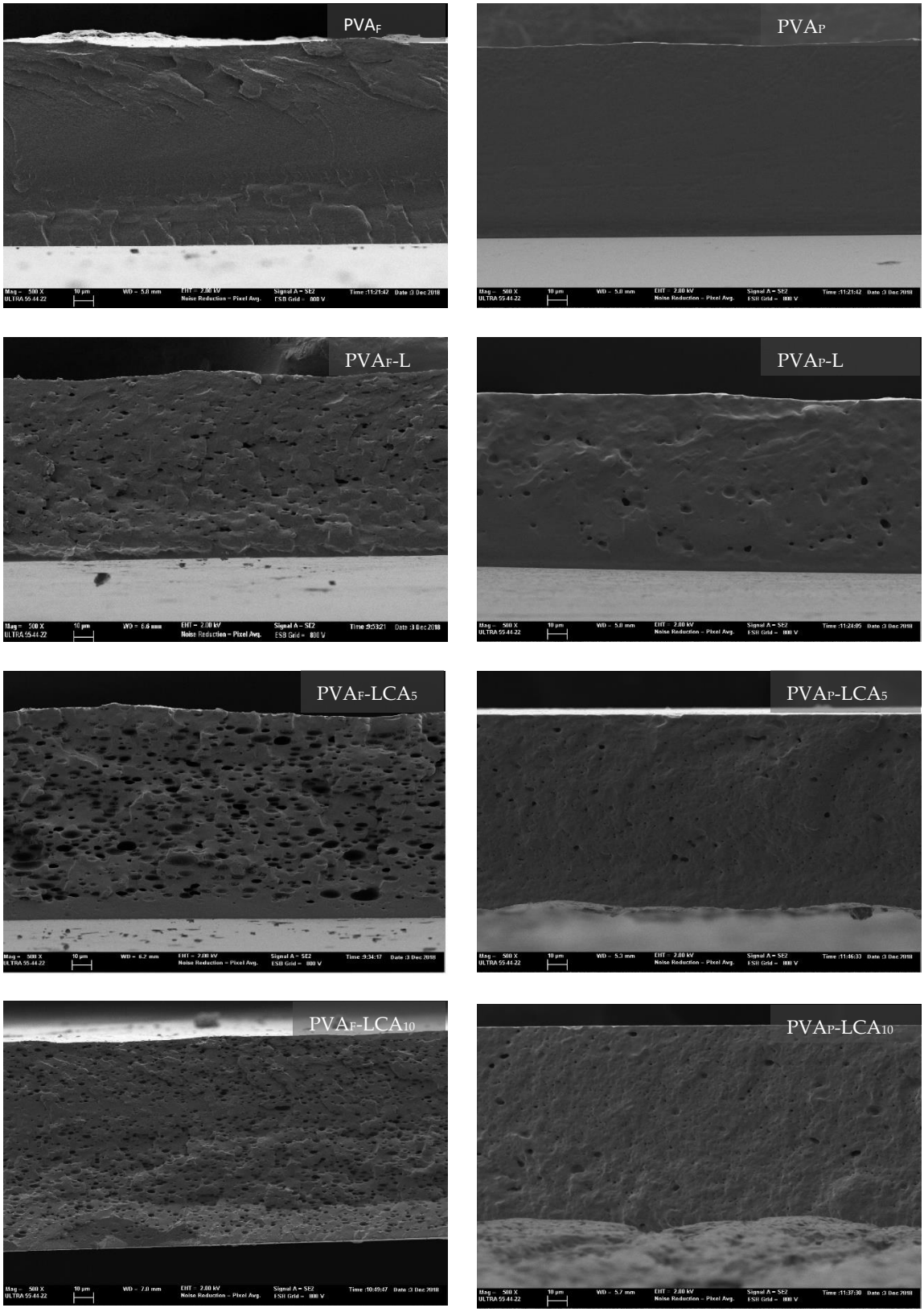

Figure 1. Field Emission Scanning Electron Microscope (FESEM) micrographs of the cross-section of the poly (vinyl alcohol) PVA_F and PVA_P films with lecithin liposomes (L) and carvacrol loaded liposomes (LCA) (5 or 10 g/100 g PVA) (magnification: 500X; bar: 10 µm).

In both polymers PVA_F and PVA_P, the highly-loaded carvacrol liposomes seem to have greater stability, remaining smaller in the dry film, which was probably due to the better structural cohesion of the liposomal membrane promoted by carvacrol. Other authors (Reiner, Fraceto, et al., 2013; Reiner, Perillo, et al., 2013) also observed greater stability of the lipid associations with carvacrol by analysing liposomal systems by means of epifluorescence imaging and H-NMR spectroscopy techniques. These authors reported that the spatial rearrangement of carvacrol in the liposomes resulted in the reduction of repulsive forces among the head groups of phospholipids, which decreased the mobility degree of hydrocarbon chains, thus allowing for closer molecular packing. However, liposomes, carvacrol-loaded or not, generated subtle structural changes in the PVA_P films, regardless of the carvacrol ratio. This could be explained by the greater chemical affinity of carvacrol with the polymer that can enhance its bonding to the polymer chains, limiting its interactions with the lecithin structures, thus giving rise to a partition of the compound between the polymer chains (bonded fraction) and lecithin liposomes (dispersed fraction). In fact, PVA_P matrices containing different ratios of free carvacrol did not exhibit a dispersed phase, showing a homogeneous structure (Andrade et al., 2020). Subsequently, the lipid particles dispersed in PVA_P matrices must be mainly attributed to the lecithin lipid associations coming from the destructuring of incorporated liposomes during the film drying step, which can also entrap a part of the carvacrol. Afterwards, a partition of carvacrol between the polymer chains and lecithin lipid associations could be assumed in the PVA_P matrices, which would explain the microstructural differences between samples PVA_F and PVA_P.

Table 1 shows the mass fraction of the different components of the films and the carvacrol concentration in the matrix as well as the percentage of CA retention (with respect to what was initially incorporated) in the final film. The specific molecular characteristics of each type of PVA generated different CA-retention levels. PVA_P Matrix retained a significantly ($p<0.05$) higher amount of CA (67% and 74%) than matrix A (55% and 57%). This has also been observed for emulsified carvacrol in PVA_F and PVA_P films and it was attributed to the greater chemical affinity of CA with PVA_P (Andrade et al., 2020). The formation of Lewis adducts between CA and the residual acetate groups of PVA_P chains has been reported to describe the specific molecular interactions. When comparing the amount of carvacrol retained in the films by liposome encapsulation with that obtained by direct emulsification (Andrade et al., 2020), an increase of about 15% in carvacrol retention was observed when using liposomes as carvacrol carriers. This fact indicates that liposomal encapsulation enhances the retention of non-polar active compounds, as carvacrol, in hydrophilic polymer matrices that were obtained by casting the aqueous polymer dispersions, as has also been observed by other authors (Sapper et al., 2018; Talón et al., 2019; Valencia-Sullca et al., 2016). Subsequently, although the liposomes lost the initial structure, the modified structures also contributed to stabilizing the non-polar compounds (such as carvacrol) against flocculation, coalescence, and creaming, which are mainly responsible for their losses at the film surface in line with water evaporation.

Table 1. Nominal mass fraction (x) of the different components of the films, extracted carvacrol in the final film, and its retention percentage. Mean values and standard deviation.

Sample	X_{PVA}	X_{CA}	X_L	Extracted CA		CA-Retention (%)
				(mg CA/ g PVA)	(mg CA/ g dry film)	
PVA_F	1	-	-	-	-	-
PVA_F-L	0.91	-	0.09	-	-	-
PVA_F-LCA_5	0.87	0.04	0.09	28 (1)	24 (1)	55 (3)[a]
PVA_F-LCA_{10}	0.84	0.08	0.08	57 (2)	48 (2)	57 (30)[a]
PVA_P	1	-	-	-	-	-
PVA_P-L	0.91	-	0.09	-	-	-
PVA_P-LCA_5	0.87	0.04	0.09	37 (2)	32 (2)	74 (2)[c]
PVA_P-LCA_{10}	0.84	0.08	0.08	67 (2)	61 (2)	67 (3)[b]

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

The crystallization pattern and degree of crystallinity of the films were analysed through the DRX spectra (**Figure 2**), all of which showed the typical PVA crystalline peaks at around 2Θ of 20° and 41°. The crystallinity of PVA_F films (51%) was higher than that of PVA_P films (45%), as previously described by Andrade et al. (2020), which was attributed to the presence of acetyl groups in the PVA_P chains that provoke a steric hindrance for the crystalline molecular arrangement. The incorporation of liposomes (loaded or not with CA) led to a significant decrease in the crystalline fraction of matrix PVA_F. This could be due to the presence of the dispersed lipid phase that might disrupt the normal organization of polymeric chains. The PVA_F-LCA_5 samples had the lowest crystallinity value (25%), in line with the more heterogeneous distribution of lipid fraction in the matrix (**Figure 1**), as previously commented on. On the other hand, even though the non-loaded liposomes promoted a drop in crystallinity of PVA_P films (35%), the liposomes containing CA gave rise to films with crystallinity that was similar to that of pure polymer films. Andrade et al. (2020) observed an increase in the crystallinity of PVA_P films when these contained carvacrol, which was attributed to the specific interactions of the carvacrol molecule and PVA chains that could favour the molecular arrangement in the crystalline domains. Subsequently, the decrease in crystallinity provoked by the lecithin incorporation was less significant when liposomes carried carvacrol, whose delivery and interaction with the polymer chains enhance crystallinity. The different crystallinity degree of the films affected their functional properties, as described below.

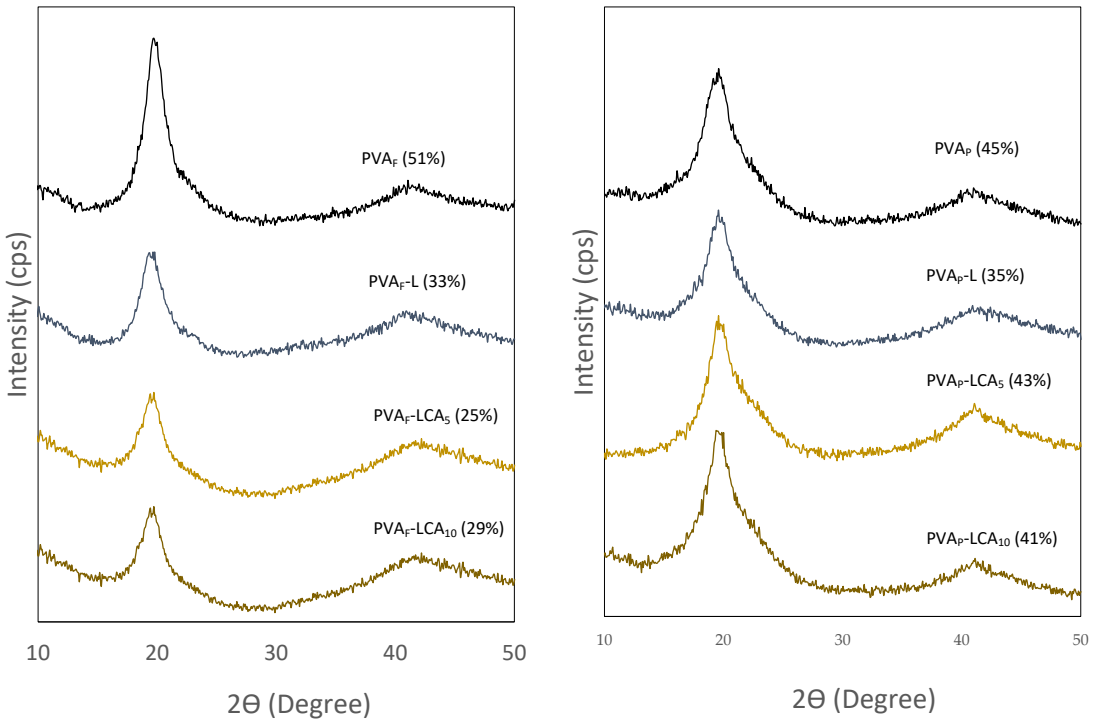

Figure 2. X-Ray diffraction spectra of the PVA films (PVA$_F$: left and PVA$_P$: right) without and with carvacrol (5 or 10 g/100 g PVA) previously encapsulated in lecithin liposomes. Percentages of crystallinity are shown for each sample.

The molecular interactions between loaded compounds and polymer chains could lead to molecular vibration changes that are reflected in the FTIR spectra. The FTIR spectra of all PVA films (**Figure 3**) showed the characteristic bands of PVA; the strong broadband between 3600 cm^{-1} and 3000 cm^{-1} corresponding to the stretching vibration of hydroxyl (O-H) and the intermolecular hydrogen bond. Two peaks that appear between 3000 cm^{-1} and 2800 cm^{-1} are related to the -CH_2 asymmetrical and symmetrical stretching vibration of the alkyl groups. Other peaks that were related to the -CH_2 bending (1416 cm^{-1}), the deformation of C-H vibrations (1326 cm^{-1}), C-O stretching (1085 cm^{-1}), and C-O-C ring vibration (832 cm^{-1}) were also observed due to the motion of the carbon skeleton of PVA (Abral et al., 2019; Cano et al., 2015). In matrix PVA$_P$, two additional peaks that were associated with stretching vibrations of the ester group and the carbonyl (C=O) group stretching (corresponding to the residual acetyl groups) were observed at 1240 cm^{-1} and 1732 cm^{-1} (Altan et al., 2018; Buendía−Moreno et al., 2020; Neira et al., 2019; Trindade et al., 2019).

The incorporation of liposomes in PVA$_F$ films led to the appearance of characteristic peaks of phosphatidylcholine corresponding to the asymmetrical and symmetrical CH stretching at 2920 cm^{-1} and 2850 cm^{-1}, C=O stretching at 1730 cm^{-1}, and C=C stretching vibration at around

1240 cm^{-1} (Pinilla et al., 2019; Taladrid et al., 2017). In films of PVA_P, the characteristic peaks of phosphatidylcholine are overlapped with those of the polymer. The typical FTIR peaks of carvacrol have been observed by other authors (Altan et al., 2018; Buendía−Moreno et al., 2020; Neira et al., 2019; Trindade et al., 2019) at 3500–3300 cm^{-1} (-OH stretch), 2868–2958 cm^{-1} (C-H stretch), 1620⁻1485 cm^{-1} (C-C stretch), 1240 cm^{-1} (C-O stretching vibration in aromatic ring), and 800 cm^{-1} (aromatic C-H bending). In no case did the presence of CA cause changes in the vibration band of PVA. The described interaction between CA and acetyl groups in PVA_P chains did not cause changes in the vibration mode of carbonyls. This can be attributed to both the very low molar ratio of CA in the films, which prevents the quantitative observation of its characteristic vibration bands, and the lack of covalent bonds between CA and PVA groups.

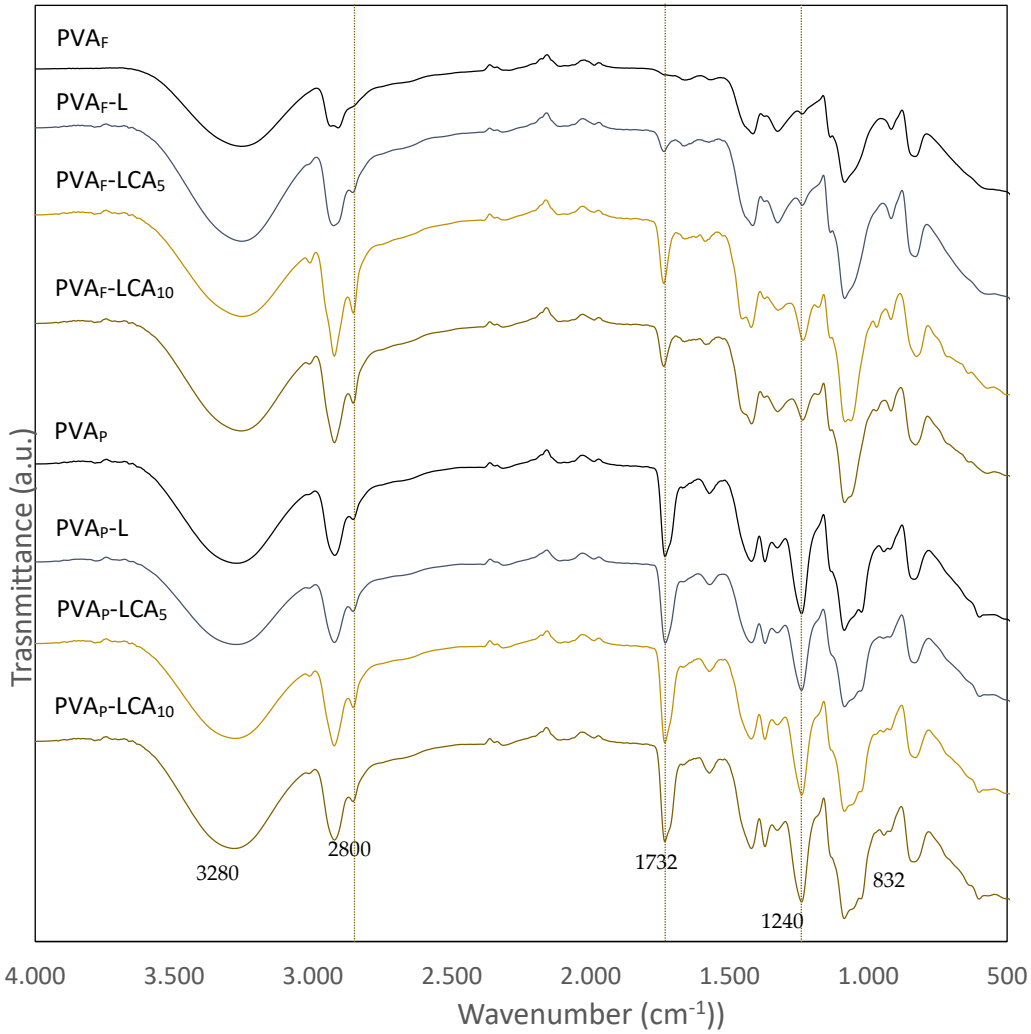

Figure 3. FTIR spectra of the PVA_F and PVA_P films without and with carvacrol (5 or 10 g/100 g PVA) previously encapsulated in lecithin liposomes (L).

3.2. Thermal behaviour

The alterations that the heating can cause in the materials, such as dehydration, oxidation, combustion, and decomposition, were studied through thermogravimetric analysis (TGA). **Figure 4** shows the TGA and DTGA (first derivate) curves, which showed the different weight loss steps of material as a function of temperature. The first step, between 50 °C and 120 °C, corresponds to the sample water loss due to the vaporization of the bonded water of the polymer matrix. Every sample of PVA_F, including those with liposomes, had 3% of bonded water. In contrast, only 1.6% bonded water was determined in PVA_P films, which was in agreement with its more hydrophobic nature, associated with the acetylated hydroxyls. The incorporation of carvacrol into PVA_P films, and the developed interactions with the polymer, also reduced the polymer bonded water to 0.6%. A second weight loss step that only appeared in samples containing carvacrol is attributable to this compound thermo-release. In this step, the peak temperatures were roughly at 196 °C and 150 °C for PVA_F and PVA_P films, respectively. The different temperature in each polymer could be attributed to their different melting behaviour, as described below, since carvacrol will be effectively delivered from the polymer melt. The integration of the DTGA peak of carvacrol evaporation permits the estimation of the amount of carvacrol that was thermo-released from the PVA_F and PVA_P matrices. The estimated amounts were 60–70% from the compound that was retained in the case of PVA_F matrix (poorer in carvacrol) and 33–46% in PVA_P matrix (richer in carvacrol). Therefore, PVA_P matrix, with a greater carvacrol affinity, allowed for a better carvacrol retention in the films with more thermal stability.

At higher temperatures, the polymer degradation occurred in two different steps; the first was associated with the detachment of side groups from the chains, forming water in the case of PVA_F and acetaldehyde and acetic acid, as additional by-products, in PVA_P with acetylated hydroxyls. The second stage of polymer degradation has been related to the degradation of low molecular weight products from the decomposition of the main chain, or of heavier structures that formed in the previous degradation steps (Perilla, 2007). In polymer PVA_F, the first degradation step occurred between 219 °C and 333 °C, exhibiting two peaks that indicates the overlapping of different weight loss events with the respective degradation mechanisms. This could be related with the fact that the melting of the crystalline fraction (Tm: 225 °C) and degradation both occurred in the same temperature range, generating fractions in different physical states that should degrade differently (Andrade et al., 2020). The incorporation of liposomes into the PVA matrix provoked a shift in the degradation temperature to higher values (onset at 255 °C), thus increasing the thermal stability of the polymer matrix, while only one degradation peak temperature was observed between 297 °C and 314 °C. The thermo-protection effect of lecithin in PVA_F films could be due to the presence of carbonyl from lecithin. Some authors (Cristancho et al., 2013; Perilla, 2007) reported the thermo-protective effect of carbonyls in PVA degradation, due to the increase in the value of the activation

energy that was associated with the material degradation. In fact, for the partially acetylated PVA_P, the polymer degradation occurred at higher temperatures in a single degradation peak at 281 °C. Moreover, degradation occurred after the polymer melting at 185 °C.

The incorporation of liposomes into PVA_P matrices also implied an increase in the degradation peak temperature of the polymer (302 °C), although the mass loss step started at 210 °C (as compared to 230 °C in lecithin-free matrices). **Figure 4** also shows the wide thermo-degradation curve of lecithin with overlapped multiple peaks that were associated with the degradation of its different components (phosphatidylcholine (75%), phosphatidylethanolamine (10%), lysophosphatidylcholine (2%), and free fatty acids, triglycerides). At 210 °C, the quantitative degradation of the lecithin present in the films, which overlapped with the polymer degradation, can cause the apparent onset at 210 °C for polymer PVA_P degradation. Accordingly, lecithin provides thermo-protection to both PVA_F and PVA_P polymers in the obtained films probably due to the presence of carbonyls in the blend. The temperature peak for the second stage of polymer degradation was above 400 °C for PVA_F and PVA_P matrices and also slightly shifted towards higher temperatures when liposomes were incorporated. Moreover, there was a significant change in the percentage of mass loss that was associated with the two different degradation stages of polymer PVA_F. The presence of lecithin provoked an increase in the mass loss of the second step with respect to the first, which could be due to the interactions that developed between lipids and polymer during heating that alter the degradation pattern of the PVA. Accordingly, lecithin provided greater thermal stability to PVA, which is particularly relevant for non-acetylated PVA_F with overlapped melting and degradation events.

The PVA is a semi-crystalline polymer with amorphous and crystalline fractions, which is reflected in first and second order transitions in the DSC thermograms (**Figure 5**). **Table 2** shows glass transition and melting temperatures determined from the thermograms corresponding to the first and second heating scans. The former reflects the thermal behaviour of the cast films, while, in the second heating scan, the polymer melting in the previous heating step has erased the thermal history. In the first heating, a relaxation enthalpy that is associated with the glass transition can be observed as a result of molecular relaxation after aging (Cai et al., 1996). The glass transition temperature (Tg) in PVA_F (46 ℃) was lower than in PVA_P (54 ℃). This could be due to different factors, including the degree of hydrolysis; PVA_F matrix, with a high degree of hydrolysis (99%), has a greater number of hydroxyl groups available to bind water, whose plasticizing effect decreases the Tg. In the same sense, the presence of residual acetyl groups in partially hydrolysed chains of PVA_P, allows less water binding, as commented on above. Other factors that are associated with the film preparation process could also affect the Tg value. The distribution of polymer chains of different lengths in the amorphous or crystalline phases due to the crystallization restrictions that are associated with the steric hindrance could occur. Shorter molecular chains could be located in the amorphous phase of PVA_F matrix.

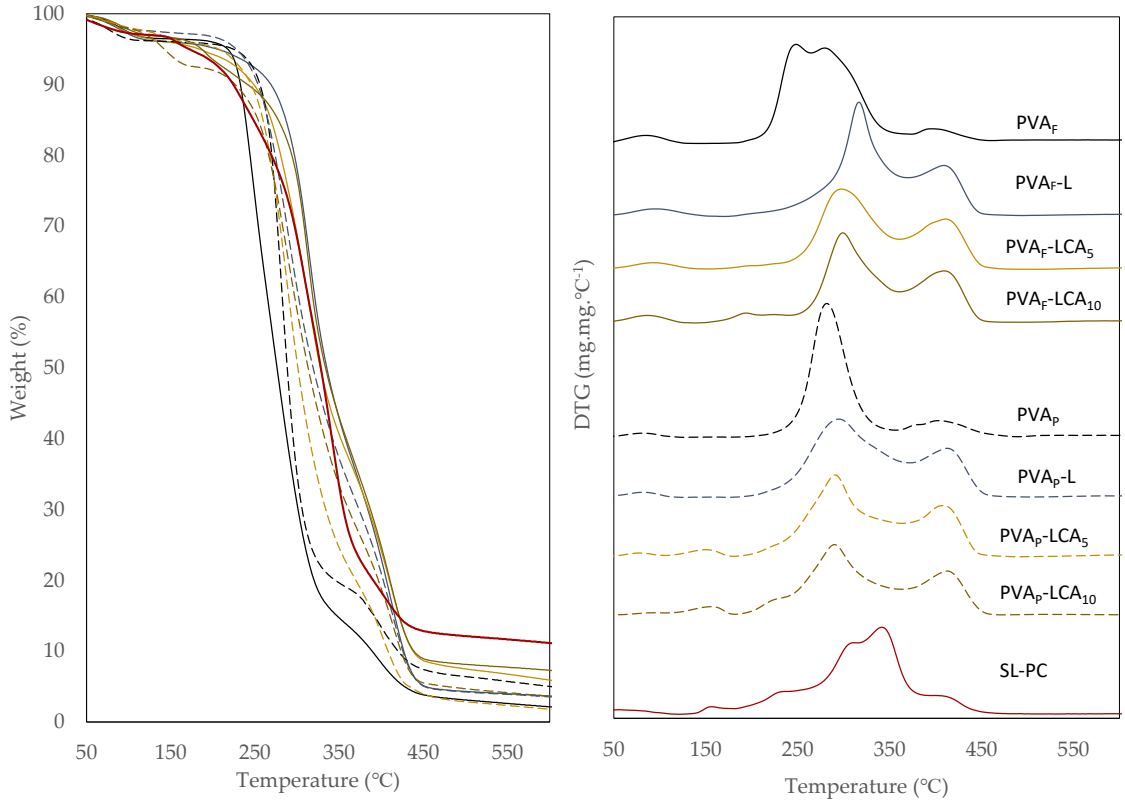

Figure 4. Thermogravimetric analysis (TGA) (left) and DTGA (right) curves of the PVA films (PVA_F and PVA_P) without and with carvacrol (5 or 10 g/100 g PVA) previously encapsulated in lecithin liposomes (L), and of the soy lecithin enriched with phosphatidylcholine (SL-PC).

The incorporation of liposomes with and without carvacrol slightly increased the Tg of PVA_F, whereas the opposite effect was observed for carvacrol-loaded liposomes in PVA_P. This suggests that the lecithin components had an anti-plasticizing effect in fully hydrolysed PVA_F, whereas the carvacrol, as released from liposomes, plasticized PVA_P, as previously observed by Andrade et al. (2020). In the second heating scan, the loss of bonded water in the matrix and the different chain rearrangement after melting increased the Tg values in PVA_F polymer, especially in the samples with lecithin, which confirm its anti-plasticizing effect in PVA_F. The Tg values of PVA_p sample in the second heating were only slightly higher than in the first, which could be mainly attributed to the loss of bonded water during the first heating, with the formation of an amorphous phase with a similar composition to that present in the cast films. The incorporation of lecithin into PVA_p also had an anti-plasticizing effect after the first heating, but this effect seemed to be mitigated by the carvacrol plasticization when carvacrol was present. The apparent presence of carvacrol in the samples that were previously heated up to 250 °C suggests the thermo-protection of CA by liposomal encapsulation. A previous study (Andrade et al., 2020) showed no evidence of CA after the sample heating up to 250 °C, when CA was incorporated in matrices PVA_F and PVA_p by direct emulsification.

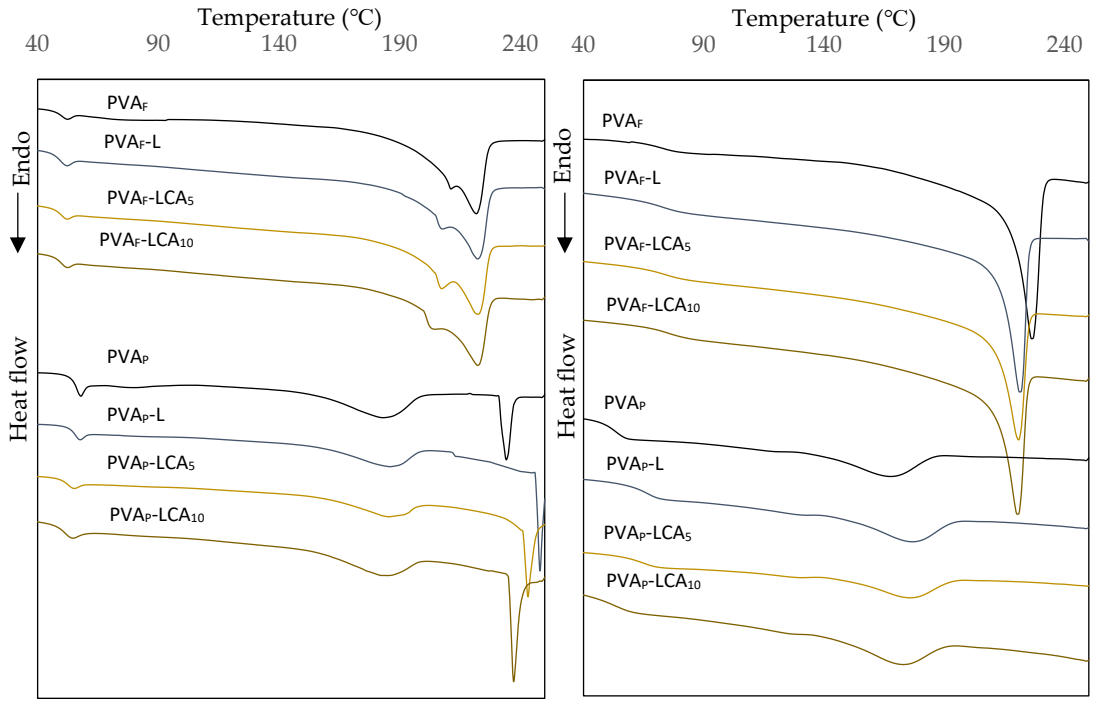

Figure 5. Differential scanning calorimetry (DSC) curves of the PVA films (PVA_F and PVA_P) with and without carvacrol (5 or 10 g/100 g PVA) previously encapsulated in lecithin (L). On the left, the first heating scan and on the right the second heating scan.

DSC thermograms of the first heating scan (**Figure 5** show several endothermic events between 140 °C and 250 °C for both PVA_F and PVA_P polymers. Polymer PVA_F presented peaks at 215 °C and 225 °C that suggest two successive melting events, which is in line with the existence of crystalline forms of different sizes leading to different melting temperatures. When liposomes were incorporated, the melting enthalpy increased, which suggests that lipid compounds could enhance the PVA crystallization in the smallest crystals, since it was the peak at the lower temperature that was promoted. The partial vaporization of CA thermo-released in this temperature range could also contribute to the enthalpy increase. PVA_P matrix exhibited a melting endotherm at 183 ℃ with enthalpy values that were lower than PVA_F matrix. In this case, the incorporation of lipid compounds (lecithin and CA) did not modify the Tm value or the enthalpy. Nevertheless, a second endothermic peak was present in all of samples PVA_P above 230 °C, which must be attributed to the early endothermic degradation reactions (deacetylation) of the polymer that start at this temperature (Canevarolo, 2004; Perilla, 2007).

In the second heating scan, PVA_F only presented one endothermic peak, at 225 °C, as previously reported by Cano et al. (2015) and Salazar (2019), whereas PVA_P exhibited the

melting peak at 168 °C. The Tm value was significantly higher in PVA_F matrix, coherently with the differences between the polymers' molecular weights and the steric effect of the side acetyl groups of PVA_P on the crystalline arrangement of the chains. PVA_F did not modify the Tm with respect to the previous step, which indicated a similar composition in its crystalline fraction even after heating the sample up to 250 °C. However, the presence of lipid compounds slightly reduced the peak temperature and enthalpy, which indicates that the dispersed lipids affected the crystalline arrangement, thus reducing the degree of crystallinity and promoting the formation of smaller crystals, as also deduced from RDX analyses. In contrast, PVA_P behaved in the opposite way; Tm decreased from 183 °C in the first heating to 168 °C, which could be due to polymer changes occurring above 230 °C (degradation event). However, the presence of lipids in the matrix increased the Tm to 175 °C. The different effect of lipids on the material may lie in the different degree of lipid-polymer affinity. In PVA_P, the interactions with lecithin lipids seemed to enhance the crystal size, while carvacrol promoted crystallinity, as observed in DRX analyses.

Table 2. Glass transition and melting temperature and enthalpy of the PVA films (PVA_F and PVA_P).

Sample	First heating scan			Second heating scan		
	Tg	Tm1	ΔHm (J/g PVA)	Tg	Tm	ΔHm (J/gPVA)
PVA_F	46.1 (0.2)^{a}	225 (5)^{a}	79 (1)^{b}	72 (2)^{cd}	225 (1)^{d}	73 (2)^{c}
PVA_F-L	47.4 (0.5)^{b}	222 (1)^{a}	97 (6)^{c}	82 (1)^{e}	221 (1)^{c}	71 (8)^{bc}
PVA_F-L CA_5	48.9 (0.1)^{c}	223 (2)^{a}	116 (7)^{d}	78 (2)^{de}	221 (1)^{c}	77 (2)^{c}
PVA_F-L CA_{10}	49.0 (0.6)^{cd}	223 (2)^{a}	115 (13)^{d}	79 (2)^{de}	220 (1)^{c}	65 (10)^{b}
PVA_P	53.8 (0.4)^{e}	183 (1)^{a}	55 (2)^{a}	56 (3)^{a}	168 (1)^{a}	25 (2)^{a}
PVA_P-L	54.3 (0.7)^{e}	186 (1)^{a}	54 (13)^{a}	64 (4)^{bc}	176 (1)^{b}	34 (4)^{a}
PVA_P-L CA_5	50.0 (0.8)^{d}	185 (1)^{a}	53 (7)^{a}	58 (9)^{ab}	174 (2)^{b}	25 (1)^{a}
PVA_P-L CA_{10}	48.9 (0.4)^{c}	186 (1)^{a}	54 (5)^{a}	58 (4)^{ab}	174 (2)^{b}	28 (2)^{a}

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

3.3. Functional properties

The functional properties of the films are closely related to the molecular arrangement. Consequently, the chemical nature of the polymer and other included compounds, as well as their molecular interactions, play a crucial role in developing materials with specific requirements (McHugh & Krochta, 1994). The molecular weight and the degree of hydrolysis of PVA had a marked influence on the tensile parameters (elastic modulus: EM, tensile strength: TS, and elongation: E, at break) and on the barrier properties (water vapour permeability: WVP; oxygen permeability: OP) of the films, as shown in **Table 3**.

The films that were obtained with PVA_F exhibited better mechanical properties (higher values of elastic modulus, resistance to break, and stretchability) than those that were obtained from PVA_P. This has been attributed to the greater ability of the longer, deacetylated chains to form inter-chain hydrogen bonds. In contrast, the acetyl groups in PVA_P interrupted the hydrogen bond formation in the shorter chains, which resulted in less cohesive matrices with lower crystallinity and, subsequently, reduced tensile performance. Restrepo et al. (2018) also found improved mechanical properties of the PVA materials when the molecular weight and degree of hydrolysis increased. The barrier properties of the films also reflected these effects. Thus, the films of PVA_F, with greater structural cohesion and crystallinity, exhibited better barrier capacity to both water vapour and oxygen than those of PVA_P.

Table 3. Tensile parameters (Tensile strength (TS), percentage elongation (E) and elastic modulus (EM)) and barrier properties (water vapour permeability, WVP; oxygen permeability, OP of the films. Mean values and standard deviation.

Sample	Thickeness (µm)	TS (MPa)	E (%)	EM (MPa)	WVP x 10^3 (g/m. h. kPa)	OP x 10^8 (cm^3/m. h. kPa)
PVA_F	101 (2)^b	153 (8)^f	135 (6)^d	80 (4)^d	2.47 (0.06)^a	0.38 (0.01)^a
PVA_F-L	134 (2)^d	131 (7)^e	138 (5)^d	65 (8)^C	2.90 (0.30)^b	2.52 (0.24)^b
PVA_F-L CA_5	131 (2)^d	111 (10)^d	137 (6)^d	67 (4)^c	3.60 (0.40)^c	5.47 (0.04)^c
PVA_F-L CA_{10}	132 (2)^d	132 (12)^e	142 (8)^d	63 (5)^c	3.30 (0.02)^{bc}	1.72 (0.03)^b
PVA_P	95 (2)^a	44 (6)^{ab}	97 (6)^b	54 (5)^b	2.90 (0.02)^b	0.53 (0.05)^a
PVA_P-L	122 (2)^c	40 (4)^a	86 (5)^a	43 (2)^a	3.50 (0.20)^c	16.10 (0.90)^f
PVA_P-L CA_5	121 (2)^c	53 (5)^b	119 (4)^c	42 (2)^a	3.00 (0.30)^b	7.45 (0.08)^d
PVA_P-L CA_{10}	124 (2)^c	71 (3)^c	140 (2)^d	40 (2)^a	3.10 (0.10)^b	10.3 (0.75)^e

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

The inclusion of liposomes, loaded or not with carvacrol, reduced the elastic modulus and resistance to break (TS) of PVA_F films. The presence of a dispersed phase in the matrix, due to the low lipid-polymer affinity, interferes in the polymer chain association, thus weakening the matrix cohesion forces (Talón et al., 2019). In sample PVA_F-LCA_5, with a more heterogeneous microstructural arrangement and lower crystalline fraction (25%), this weakening effect was more marked. In contrast, PVA_P films exhibited different behaviour, depending on the carvacrol load of liposomes. In all cases, liposomes reduced the elastic modulus of the films due to the weakening effect of the dispersed phase in the matrix cohesion forces, thus resulting in less rigid materials. Sapper et al. (2018) also reported this effect for starch-gellan films with liposomes loaded or not with thyme essential oil. However, resistance and elongation at break increased in line with the carvacrol ratio, with the films becoming more stretchable. This agrees with the specific interactions of the acetyl group with CA that facilitate the slippage of the chains during the film stretching, delaying the break. Tongnuanchan et al.

(2012) reported that some compounds in essential oils might be able to cross-link chains, thereby improving the tensile properties. In this sense, some authors reported the increase in TS when cinnamon essential oil was incorporated into the soy protein isolate films (Atarés et al., 2010) or chitosan films (Ojagh et al., 2010).

Barrier properties were also modified by including liposomes in the matrices. In both polymers, WVP slightly increased when liposomes were incorporated, whereas more notable changes were observed for OP. Different factors affected the molecular permeability in the matrix: the molecular diffusion of permeant that is affected by the microstructural features, such as the degree of crystallinity and the tortuosity factor that was introduced by the dispersed phase, and the molecular solubility in the matrix, affected by the chemical affinity of the permeant with the matrix components. In this sense, water molecules are highly soluble in the polymer continuous phase and less soluble in the lipid dispersed phase, whereas oxygen molecules behave in the opposite way. Thus, the balance of the different factors can explain the changes that were induced by liposomes in the barrier capacity of the films. The structural differences introduced by the dispersed phase only caused a small increase in the WVP values, given the high water solubility in the PVA continuous phase. In contrast, lipid incorporation enhanced the oxygen solubility in the matrix, promoting the increase in the OP values, additionally to that provoked by the structural changes. Samples PVA_F-LCA_5 and PVA_P-L, in particular, exhibited the lowest barrier capacity against both water vapour and oxygen in line with their lower crystalline fraction and greater microstructural heterogeneity, when compared with the other samples of the respective polymer. The incorporation of carvacrol-loaded liposomes gave rise to lower OP values in PVA_P films than non-loaded liposomes, which could be attributed to the greater crystallinity induced by carvacrol in these films (**Figure 2**). In polymer PVA_F, this effect of carvacrol was only observed for sample PVA_F-LCA_{10}, which exhibited a greater dispersed phase with lower particle size, all of which implies a higher tortuosity factor for mass transfer.

Table 4 shows the colour parameters (lightness (L*), Chroma (C_{ab}*), and hue (h_{ab}*)), and internal transmittance values at 460 nm (Ti), used as a transparency indicator, of the different samples. In general, the lightness values were lower in PVA_F films than in PVA_P films, while hue was higher in PVA_F, which was probably due to the different refractive indices of the matrices, associated with the corresponding microstructural arrangement affecting the light interactions. The incorporation of liposomes, loaded or not with CA, significantly reduced the lightness and hue and increased chrome (more saturated colour) of films PVA_F and PVA_P, in line with the colour imparted by lecithin (Sapper et al., 2018; Talón et al., 2019). In addition, the Ti values of the films with liposomes decreased, especially in films A. Similar effects were observed by other authors (Jiménez et al., 2014; Talón et al., 2019), when lecithin liposomes were incorporated into corn starch-sodium caseinate films.

Table 4. Lightness (L *), chrome (Cab *), hue (hab *), and internal transmittance values at 460 nm (Ti) of the of the PVA films. Mean values and standard deviation.

Sample	L*	Cab*	hab*	T_i (460 nm)
PVA_F	88 (2)^{C}	3 (1)^{a}	114 (11)^{e}	0.86 (0.01)^{e}
PVA_F-L	78 (1)^{a}	10.8 (0.6)^{cd}	99 (2)^{ab}	0.82 (0.01)^{ab}
PVA_F-L CA_5	78 (2)^{a}	8.7 (0.9)^{b}	105 (2)^{d}	0.82 (0.01)^{bc}
PVA_F-L CA_{10}	78 (2)^{a}	10.6 (0.9)^{c}	100 (1)^{bc}	0.81 (0.01)^{a}
PVA_P	92 (1)^{d}	3.4 (0.5)^{a}	104 (2)^{cd}	0.86 (0.01)^{e}
PVA_P-L	81 (1)^{b}	11.1 (0.6)^{cd}	96 (1)^{a}	0.84 (0.01)^{d}
PVA_P-L CA_5	81.8 (0.3)^{b}	11 (1)^{d}	98 (1)^{ab}	0.84 (0.01)^{d}
PVA_P-L CA_{10}	81.0 (0.9)^{b}	13 (1)^{e}	96 (1)^{ab}	0.83 (0.01)^{c}

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

4. CONCLUSION

The incorporation of liposome-encapsulated carvacrol into PVA films notably affected the microstructure of the films, their thermal behaviour, and their functional properties as packaging material, depending on the molecular weight and degree of hydrolysis of the polymer. Liposome-encapsulated carvacrol was better integrated in the polymer matrices of low M_W, partially acetylated PVA, generating a more homogenous structure, where carvacrol enhanced the degree of crystallinity, whereas liposomes reduced the higher crystallinity in high-M_w, non-acetylated PVA. A thermal protective effect of acetyl groups was observed in PVA, which was also observed when lecithin was incorporated as liposomes into the films. This effect shifted the thermodegradation temperature of PVA towards higher values, which were above its melting temperature, thus making it possible for polymer thermoprocessing to obtain films by means of the usual thermoplastic industrial process. Likewise, the presence of acetyl groups in the chain promoted chemical affinity with less polar compounds, such as carvacrol or lecithin components, permitting a greater carvacrol retention in the matrix and, thus, a more effective way of obtaining active films for food packaging. Liposome encapsulation also promoted the retention of carvacrol in the films, as compared with that incorporated as a non-encapsulated compound. Carvacrol-loaded liposomes reduced the elastic modulus and tensile strength of the PVA films, more markedly in the non-acetylated polymer, in line with the different changes in crystallinity and microstructure. Likewise, liposomes increased the oxygen permeability of the films according to the introduced structural modifications, but they maintained adequate values for food packaging applications. In general, the acetylated PVA exhibited better capacity as a carrier of carvacrol, encapsulated in liposomes, with great potential for the production of active films for food packaging applications.

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CHAPTER IV. Incorporation of phenolic acids in poly (vinyl alcohol) matrices for the active films development

IV.I. Effect of phenolic acids on the properties of films from poly (vinyl alcohol) of different molecular characteristics

IV.II. Effect of the processing method on the physicochemical and active properties of poly (vinyl alcohol films incorporating phenolic acids

IV.I. Effect of phenolic acids on the properties of films from poly (vinyl alcohol) of different molecular characteristics

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ABSTRACT

Cinnamic and ferulic acid were incorporated into highly hydrolysed and partially acetylated poly (vinyl alcohol) (PVA) for the purpose of obtaining active films. The microstructure, crystallinity, tensile and barrier properties and thermal behaviour were analysed in order to ascertain the changes provoked by phenolic acids in the film properties, depending on the kind of PVA. Phenolic acids were more compatible with the partially hydrolysed PVA. Ferulic acid led to greater changes in PVA films than cinnamic acid, promoting the crystallinity, stiffness and barrier capacity to water vapour and oxygen, although in no case the thermal stability of the polymers was affected. Films of partially hydrolysed PVA, with lower melting (170 °C) and higher thermodegradation (onset: 220 °C) temperature, could be thermoprocessed by means of the technology commonly used in the plastics industry. Therefore, the incorporation of ferulic or cinnamic acid into this matrix could be of great potential in the development of active packaging for food preservation, on the basis of the antioxidant and antimicrobial activity of phenolic acids.

Keywords

Cinnamic acid; ferulic acid; thermal stability; crystallinity; active films; food packaging.

1. INTRODUCTION

Physicochemical, enzymatic and microbial alterations occurring during food transport and storage lead to significant losses that could be reduced through adequate packaging systems. These losses are reported to be around a third of all the food produced for human consumption (1.3 billion tonnes) every year around the globe, representing a waste of resources used in production, such as land, water, energy and inputs, which increases the greenhouse gas emissions in vain. Thus, food loss and waste has become an issue of great public concern, as is reflected in the 2030 Agenda for Sustainable Development (Ishangulyyev, Kim, & Lee, 2019). Likewise, the use of biodegradable materials for the development of packaging systems is necessary to mitigate the great environmental impact of the massive use of plastics.

The development of active packaging that guarantees the preservation, safety and quality of food until its consumption constitutes a good strategy by which to minimise the food losses. For this purpose, a series of active compounds can be incorporated into the packaging systems with different functions, such as oxygen, moisture or ethylene absorbing properties, gas release properties or antimicrobial and antioxidant capacity (Vilela et al., 2018; Yildirim et al., 2018). Within the great variability of naturally occurring active compounds, phenolic compounds such as terpenes, flavonoids and phenolic acids stand out, as well as essential oils, mainly constituted by volatile phenolic compounds. Essential oils and their isolated components have been incorporated into different polymeric matrices to obtain coatings or films applicable in active food packaging development. However, due to their chemical instability, they usually present short degradation periods due to external factors, such as light, oxidation or heating (Fernández-López & Viuda-Martos, 2018). Furthermore, its great volatility generates considerable losses of the active fraction in the packaging material (Sharma, Barkauskaite, Jaiswal, & Jaiswal, 2020), while the low retained fractions can negatively affect the organoleptic properties of the packaged foods, resulting in odour and flavour changes (Ribeiro-Santos, de Melo, Andrade, & Sanches-Silva, 2017), reducing consumer acceptance.

In order to avoid some of the problems commented on above, a wide variety of other potentially active phenolic compounds, such as phenolic acids or phenolic extracts, is being explored in the development of active packaging, since most of them present low volatility and sensory impact with a high antioxidant and antimicrobial potential (Lima et al., 2019; Mathew, 2015; Olszewska, Gędas, & Simões, 2020). Some studies have revealed that the incorporation of phenolic acids into polymeric matrices promotes the improvement of some functional properties, due to their great capacity of interaction with some polymeric matrices of proteinic or polysaccharidic nature. Thus, Benbettaïeb, Tanner, Cayot, & Karbowiak (2018) studied the incorporation of ferulic acid and caffeic acid into gelatin/chitosan matrices to obtain active films by casting. The incorporation of these active compounds improved the

water vapour permeability by 30% and increased the mechanical resistance of the composite films by 50%. Likewise, the elongation of the films increased from 23% to 50% with the incorporation of the mixture of the two acids. These effects were attributed to a crosslinking effect, since caffeic and ferulic acids can interact with various active sites of gelatin and chitosan by means of hydrogen bonds and charge-charge interactions between the primary ammonium groups of the protein and the carboxylate group of the phenolic acids. Cao, Fu, & He (2007) also reported the formation of hydrogen bonds and hydrophobic interactions between the galloyl group of tannic acid and the hydrophobic fraction of the gelatin in film formulations. Ou, Wang, Tang, Huang, & Jackson (2005) showed the ability of ferulic acid to for adducts with the amino acids of soy protein, which generates an increase in the tensile resistance and elongation, as well as a reduction in oxygen permeability. Other authors have also observed an improvement in the thermal stability and mechanical behaviour of the films when gallic acid (G) or ferulic acid (F) were incorporated into gelatin/chitosan, gum tragacanth/polyvinyl alcohol and chitosan/polyvinyl alcohol blend films (Benbettaïeb et al., 2020; Goudar et al., 2020). Furthermore, these films showed good antioxidant and antimicrobial activities against common pathogenic bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*.

Poly (vinyl alcohol) or PVA is a biodegradable, water soluble, non-toxic polymer with a high oxygen barrier capacity, obtained from the controlled hydrolysis of poly (vinyl acetate). PVA can be obtained with different molecular weights and degrees of hydrolysis, both features defining the microstructural arrangement of the polymeric matrices, their functional properties and the ability to incorporate active compounds. This particularity generates a wide margin in developing PVA-based materials for food packaging purposes (Andrade, Johana; González-Martínez, Chelo; Chiralt, 2020; Andrade, González-Martínez, & Chiralt, 2020). The incorporation of active components into the PVA matrices is of great potential for the development of new active, biodegradable materials for food packaging applications, but the effect of this incorporation on the functional properties of materials must be studied.

The aim of this study is to analyse the impact of the incorporation of cinnamic and ferulic acids on the microstructural, barrier, mechanical and thermal properties of films based on two types of poly (vinyl alcohol) of different molecular weights and degrees of hydrolysis.

2. MATERIALS AND METHODS

2.1. Materials

Two kinds of poly (vinyl alcohol) (PVA) of different molecular weights (Mw) and degrees of hydrolysis (PVA_F: Mw 89,000–98,000; 99-99.8% hydrolysed and PVA_p: Mw 13,000–23,000; 87–89% hydrolysed), glycerol, cinnamic acid (CNA) and ferulic acid (FA) as phenolic acids (PA) (Sigma-Aldrich, Steinheim, Germany) were used in the preparation of the films. Magnesium nitrate ($Mg(NO_3)_2$), phosphorus pentoxide (P_2O_5) salts, and UV-grade methanol were supplied by Panreac Química S.A. (Barcelona, Spain).

2.2. Preparation of films

The PVA films were obtained by casting the polymeric aqueous solutions. For the preparation of the polymer solutions, glycerol (10% wt. with respect to the polymer) and phenolic acids (1% or 2% wt. with respect to the polymer) were added to distilled water previously heated to 100 °C. After the dissolution of the acids, the polymer (PVA_F 5% wt. or PVA_P 10% wt.) was incorporated by stirring (1,200 rpm) and heating (100 °C) for 3 h, until polymer solutions were obtained. All the formulations were degassed by using a vacuum pump and they were subsequently spread evenly onto Teflon plates (150 mm in diameter), using a constant equivalent mass of polymer per plate of 2 g. The solutions were dried at controlled temperature (25 ± 2 °C) and relative humidity (54 ± 2%) for 48 h to obtain the cast films. Plasticised films without PA were used as controls. **Table 1** shows the different film formulations with the respective mass fractions of the different components.

The films were conditioned for one week at 53% relative humidity (RH) by using $Mg(NO_3)_2$ over-saturated solution, before the characterisation of their mechanical and barrier properties. The final phenolic acid content, microstructure and thermal analyses were carried out in films conditioned at 0% RH by using P_2O_5.

2.3. Characterisation of the active PVA films

Final content of phenolic acids

The final content of the phenolic acids (PA) from the dried PVA samples was determined by their extraction with solvent and spectrophotometric quantification. PA were extracted by immersing 25 mg film samples in 10 mL of a 50% aqueous solution of UV-grade methanol, placed in amber vials that were shaken at 300 rpm for 48 h. The absorbance (*A*) of the solutions was measured at wavelengths of 320 nm for ferulic acid (FA) and 270 nm for cinnamic acid (CNA), using a spectrophotometer (Evolution 201 UV–Vis, Thermo Fisher

Scientific, USA). The concentration of phenolic acids (C_{PA}) was obtained using the equation of the standard curve of each acid: ferulic acid ($C_{PA} = (A - 0.005)/0.0852, R^2 = 0.995$) and cinnamic acid($C_{PA} = (A - 0.02)/0.1372, R^2 = 0.992$). The backgrounds used for the measurements were the corresponding extracts from the PA-free films, obtained under the same conditions. The results were expressed, in percentage, as the ratio between the mass of PA (mg) extracted from the film with respect to the corresponding mass of initially incorporated PA. The measurements were taken in triplicate.

Table 1. Nominal mass fraction of the film components and final content of phenolic acids (PA) determined by extraction and UV quantification. The ratio between the determined final content and the incorporated amount of PA (% Retention) was also shown. Mean values and standard deviation in brackets.

Sample	X_{PVA}	X_{GLY}	X_{PA}	Final PA content in the films	
				mg PA/g PVA	%Retention
PVA_F	0.91	0.09	0	-	-
PVA_F-CNA_1	0.9	0.09	0.01	9.55 (0.07)	95.5 (0.7)^{e}
PVA_F-CNA_2	0.89	0.09	0.02	12.6 (0.6)	78 (3)^{ab}
PVA_F-FA_1	0.9	0.09	0.01	8.33 (0.02)	83.2 (0.2)^{bc}
PVA_F-FA_2	0.89	0.09	0.02	15.3 (0.1)	76.3 (0.5)^{a}
PVA_P	0.91	0.09	0	-	-
PVA_P-CNA_1	0.9	0.09	0.01	9.08 (0.06)	90.8 (0.6)^{de}
PVA_P-CNA_2	0.89	0.09	0.02	17.2 (0.8)	86 (3)^{cd}
PVA_P-FA_1	0.9	0.09	0.01	7.8 (0.3)	78(3)^{ab}
PVA_P-FA_2	0.89	0.09	0.02	15.4 (0.1)	77.0 (0.3)^{ab}

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

2.3.1. *Microstructure, X-ray diffraction analysis and FTIR characterisation*

The microstructure of the films was observed using a Field Emission Scanning Electron Microscope (FESEM) (ZEISS®, model ULTRA 55, Germany), at an acceleration voltage of 2 kV. The film samples were cryofractured by immersion in liquid nitrogen, platinum coated, and the cross-section images obtained.

The crystalline structure of the materials was analysed through the X-ray diffraction spectra of the films by means of a D8 Advance X-ray diffractometer (Bruker AXS, Karlsruhe, Germany). The range 2θ of evaluation was from 10° to 50°, with a step size of 0.05, using KαCu radiation (λ: 1.542 Å), 40 kV and 40.mA. The diffraction curves were deconvoluted by using the Lorentz model with the OriginPro 8.5 software to define the crystalline and amorphous regions in the

diffraction curves. The degree of crystallinity of the samples was estimated from the ratio of the crystalline peak areas and the total area of the diffractograms.

The chemical compositions of different samples were analysed by means of attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FTIR) (BRUKE, VERTEX 80, Germany) over the range 4000-600cm^{-1}. These analyses were carried out in triplicate and at three different locations in each sample.

2.3.2. *Tensile properties*

The tensile properties of the films, elastic modulus (EM), tensile strength (TS), and elongation at break point (E), were determined from the tensile stress (σ) vs. Hencky strain (εH) curves, following the standard method ASTM D882-02 (ASTM, 2002). The test specimens (25 mm x 100 mm) were mounted in the film extension grips of the universal testing machine (Stable Micro Systems, TA.XT plus, Haslemere, England). The initial separation of the clamps was 50 mm and the films were stretched at 50 mm.min^{-1} until break. The measurements were taken in eight samples of each treatment.

2.3.3. *Water solubility and barrier properties*

The water solubility test was carried out with 1 cm^2 films conditioned at 0% RH. The previously weighed samples were placed on a weighed mesh inside a crucible with 10 mL of distilled water and kept in a temperature control chamber (25 °C) for 24 h. Subsequently, the water was removed and the sample residue contained in the mesh was dried in a convection oven (J.P. Selecta, S.S., Barcelona, Spain) at 60 °C, for 48 h. The samples were then stored in a desiccator with P_2O_5 until reaching a constant weight. The solubility of the films was obtained as the percentage of mass loss of the dry film with respect to the initial dry mass. All samples were evaluated in triplicate.

Water vapour permeability (WVP) was analysed in triplicate following a modification of the method E96/E95M-05 (E. ASTM, 2003). Samples of each formulation were placed on Payne permeability cups (3.5 cm in diameter) (Elcometer SPRL, Hermelle/s Argentau, Belgium) at 25 °C. In order to obtain a RH gradient of 53 to 100%, the cups were placed inside a desiccator containing an oversaturated $Mg(NO_3)_2$ solution, while 5 mL of distilled water was placed inside the cup. A fan was positioned on top of each cup to reduce the resistance to water vapour transport. The cups were weighed periodically every 1.5 h for 24 h using an analytical balance (0.00001 g). The slopes of the weight loss *vs.* time during the steady state period were determined by linear regression to calculate the water vapour transmission rate (WVTR). WVP was calculated as described by Cano *et al.* (2014).

The oxygen permeability (OP) in film samples (50 cm^2) was analysed in triplicate using an Ox-Tran system (Mocon, Minneapolis, US) at 23 °C and 53% RH, following a standard method F1927-07 (ASTM, 2004). The films were exposed to pure nitrogen flow on one side and pure

oxygen flow on the other side. The oxygen transmission rate was multiplied by the average film thickness and divided by the partial pressure of oxygen to calculate the OP. The film thickness was measured with a digital electronic micrometer (Palmer, COMECTA, Barcelona, Spain) to the nearest 0.001 mm at six random positions.

2.3.4. *Thermal behaviour*

The thermal behaviour of the films was assessed by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The DSC measurements were taken under nitrogen flow (10 mL/min.), using aluminium crucibles by applying heating (first and second) and cooling scans in a differential scanning calorimeter (DSC 1 stareSystem, Mettler Toledo, Schwarzenbach, Switzerland), with a constant heating or cooling rate (10 °C/min). The temperature scanning profile was: a first heating from -25 °C to 250 °C, holding this temperature for 2 min, a subsequent cooling to -25 °C, holding this temperature for 2 min; and a second heating to 250 °C. TGA analysis was performed by heating the samples in alumina crucibles from 25 °C to 700 °C at 10 °C /min, under a constant nitrogen flow (10 mL/min), by using a thermogravimetric analyser (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland). All of the measurements were taken in triplicate.

3. RESULTS AND DISCUSSION

3.1. Content of phenolic acids in the films

Table 1 shows the final content of both ferulic acid (FA) and cinnamic acid (CNA) in the films determined by solvent extraction and UV quantification. In every case there was a reduction in the content with respect to the initially incorporated amount. The remaining percentage with respect to the incorporated amount ranged between 77-95%, depending on the sample. The higher the amount of phenolic acid incorporated, the lower the percentage retained, especially in the case of FA. The same tendency in PA retention was observed for both PVA matrices of different molecular weights and degrees of acetylation. Considering the non-volatile nature of the PA, it can be assumed that the lack of a complete extraction of the incorporated amount must be attributed to the compound degradation during the film preparation process (e.g. by compound oxidation) or to the strong PA-polymer interactions, which would lead to a tighter bonding of the PA to the PVA chains, thus reducing the amount of compound available to be effectively released into the solvent during the extraction process. These interactions can be mainly attributed to the hydrogen bonds established between the hydrophilic groups in PVA and the carboxyl or hydroxyl groups of PA. The presence of the hydroxyl group in the phenyl ring of FA would promote the establishment of hydrogen bonds between the polymeric chains, leading to its more effective anchorage in the matrices. However, the lack of significant differences in the amount of PA retained due to the different PVA matrices (with different molecular structures) and the fact that a lower percentage is retained when a greater amount is incorporated point to compound degradation as the main cause of the reduction in the retention percentage.

Retention percentages of FA were significantly ($p<0.05$) lower than those of CNA regardless of the PVA matrix. This is coherent with the greater sensitivity of FA to oxidation by thermal or oxygen effects. In fact, the antioxidant activity of CNA derivatives depends primarily on the number of hydroxyl and methoxy groups attached to the phenyl ring (Aguilar-Hernández et al., 2017; Bezerra et al., 2017). Ferulic acid is a free radical scavenger, but also an inhibitor of enzymes that catalyse free radical generation and an enhancer of scavenger enzyme activity. Nonetheless, its use is limited by its tendency to rapid oxidation (Aguilar-Hernández et al., 2017; Bezerra et al., 2017).

3.2. Microstructure, crystallinity and FTIR characterization of the films.

The microstructural analysis has been carried out using the FESEM micrographs of the cross section of the films (**Figure 1**) while polymer crystallinity was evaluated from the XRD spectra (**Figure 2**). The micrographs of samples for PVA_F matrix (with higher molecular weight and fully hydrolysed chains) and for PVA_P matrix (with the partially hydrolysed chains) showed marked

differences that must be related to the molecular characteristics of each polymer, since these define specific levels of interaction between components in the material (Andrade et al., 2020). Thus, the phenolic acids, regardless of the type or concentration, promoted some microstructural changes when incorporated into PVA_F matrix, whereas these were not observed in PVA_P matrix.

This suggests that the phenolic acids are better integrated in PVA_P matrix, where the PA molecules could be bonded to the chains in polymer PVA_P, giving rise to a homogeneous structure. Then, a good compatibility could be observed between the active compounds and the polymer in line with the established bonds. The bonding mechanisms could be described in terms of the ionisation pattern of acetylated PVA described by Wiśniewska et al. (2015), which provides the chain with a negative charge , located on the carbonyl oxygen of the acetyl groups (resonant structure). This donor electron pair enhances the Lewis base character of PVA, promoting the formation of Lewis adducts with phenolic acids, which enhances the chemical affinity between the active compounds and polymer. Similar findings were reported by Andrade, Johana; González-Martínez, Chelo; Chiralt (2020) and Tampau, González-Martínez, & Chiralt (2020) for partially acetylated PVA_P when carvacrol was incorporated. In contrast, in non-acetylate PVA_F matrices different domains showing a filamentous structure were observed in films with phenolic acids (images at higher magnification in **Figure 1**). This kind of structure could be attributed to the local aggregation of PA molecules (dispersed phase) giving rise to over concentrated regions of phenolic acids in the matrix. These locally concentrated regions of phenolic acids could be plasticised, giving rise to this kind of rubbery fracture.

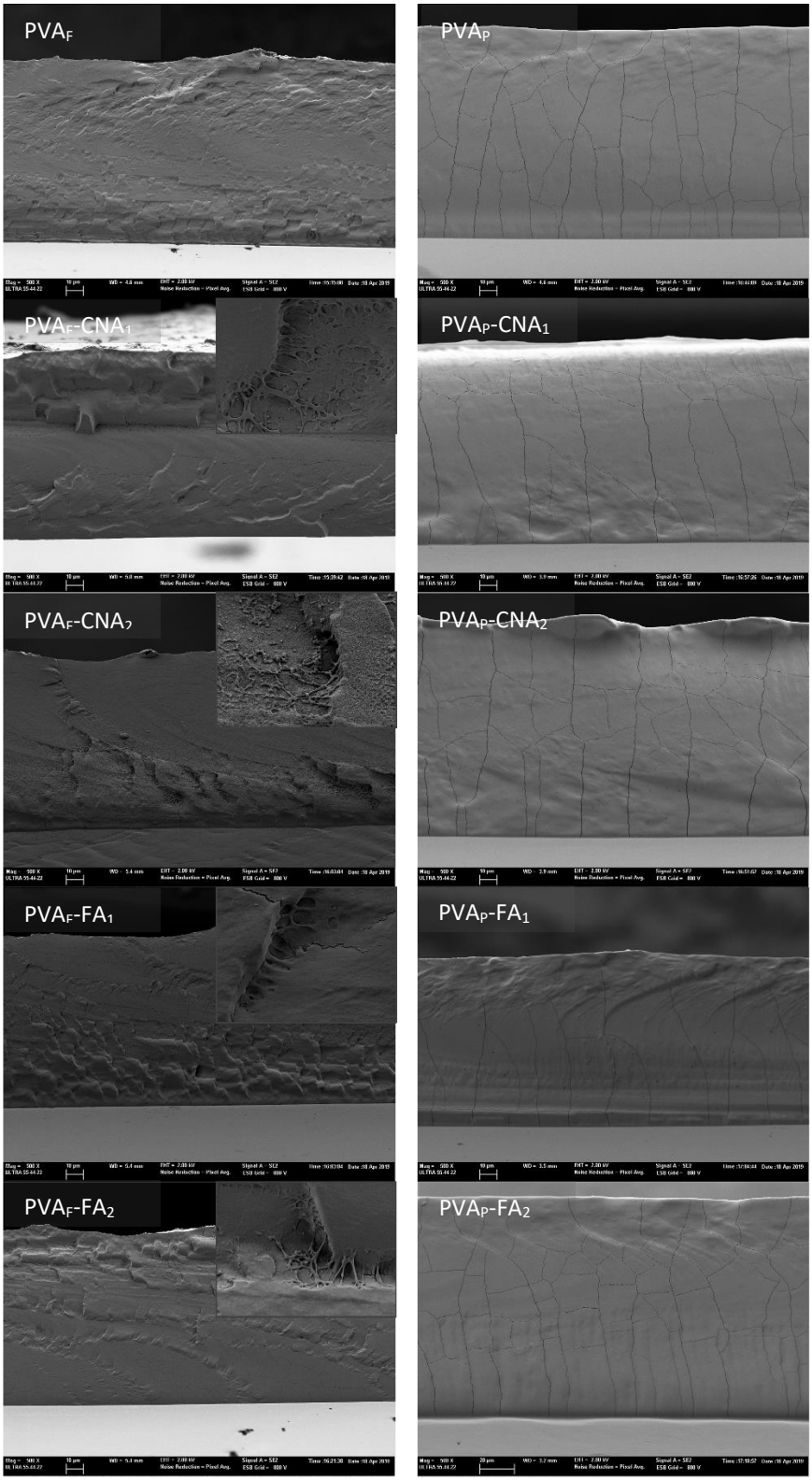

Figure 1. Field Emission Scanning Electron Microscope (FESEM) micrographs of the cross-section of the PVA_F and PVA_P films with cinnamic acid (CNA) or ferulic acid (FA) (1 or 2 g/100 g PVA) (500X). Embedded micrographs correspond to higher magnification images.

Figure 2 shows the XRD spectra of PVA films, with the quantified degree of crystallinity. The typical crystalline peaks of PVA were observed at 2θ = 19.5° (main peak) and at 41° (small broad peak). PVA_F films showed a greater intensity of the main crystalline peak, while PVA_P films presented a reduction in the crystalline peak intensity and slightly broader peaks. This was reflected in the marked difference between the degree of crystallinity of matrices PVA_F (58%) and PVA_P (26%) and also indicates the presence of smaller crystals in PVA_P films (Safna Hussan, Thayyil, Jinitha, & Kolte, 2019). The molecular characteristics of polymer plays an important role in the crystalline structural arrangement PVA_F, highly hydrolysed, has a more linear chain structure than PVA_P, which enhances the ability to form interchain hydrogen bonds creating a uniform structure with greater crystallinity (Choy et al., 2020). In contrast, PVA_P has steric hindrance caused by the side residual acetyl groups (13%) in their shorter molecular chains, which interferes in the crystal lattice formation (Andrade et al., 2020). Likewise, the greater number of chain ends in the low molecular weight polymer, PVA_P, also interferes with the crystal organisation due to its greater mobility, thus also helping to reduce the degree of crystallinity (Koltzenburg, Maskos, & Nuyken, 2017).

The effect of the incorporated phenolic acids on the film crystallinity depended on the acid and its concentration as well as on the type of polymer. Thus, the addition of CNA reduced the crystallinity of the PVA_F films, more markedly when it was incorporated at 2% (65% reduction), but did not notably affect the crystallinity in PVA_P films. The reduction in the crystallinity of PVA_F films could probably be due both to the aggregation of the acid molecules during the film drying, introducing discontinuities into the PVA matrix, and to local plasticisation effects, as deduced from the FESEM images (**Figure 1**). In contrast, when FA was added to the films, crystallinity was promoted by about 14% in PVA_F films, when using the highest concentration of acid, and by around 20% in PVA_P films at every concentration. These marked differences could be attributed to the greater ability of ferulic acid to form interchain hydrogen bonds, favouring the chain arrangement in crystalline domains. This effect was also reported for hydrogels of fully hydrolysed poly (vinyl alcohol) after its reaction with ferulic acid in the presence of laccase (Bersanetti et al., 2019).

The FTIR spectra (**Figure 2**) of the films have been obtained to analyse the molecular vibration modes associated with the components of polymeric matrices, as affected by their interactions. The spectra of the films show the typical PVA pattern. The typical broad peak between 3260 cm^{-1} 3280 cm^{-1} is associated with the intermolecular hydrogen bonding and the hydroxyl (O-H) stretching vibration. The C-H asymmetrical and symmetrical stretching vibrations occur at 2950 cm^{-1} and 2900 cm^{-1}, respectively. Other peaks appear at around 1410 cm^{-1}, 1320 cm^{-1}, 1080 cm^{-1} and 835 cm^{-1}, which are related to the CH_2 bending, the motion of the carbon skeleton (C-H), the C-O stretching and C-C.

The acetylated polymer, PVA_P, presented three additional peaks associated with the stretching vibration bands of the carbonyl (C=O) and acetyl groups that were observed at 1737

cm^{-1}, 1704 cm^{-1}, 1376 cm^{-1} and 1250 cm^{-1}. The typical peaks of cinnamic acid (Chandran, Nithya, Sankaran, Gopalan, & Ganesan, 2006; Nolasco, Amado, & Ribeiro-Claro, 2009) and ferulic acid (Aarabi, Honarvar, Mizani, Faghihian, & Gerami, 2016) were not detected in the FTIR spectra of films containing these compounds, probably due to their low concentration in the matrices and the overlapping with the PVA peaks. The lack of peak displacement in the films with phenolic acid, compared with the pure polymers, reflected the absence of covalent bonds between the chain groups and phenolic molecules.

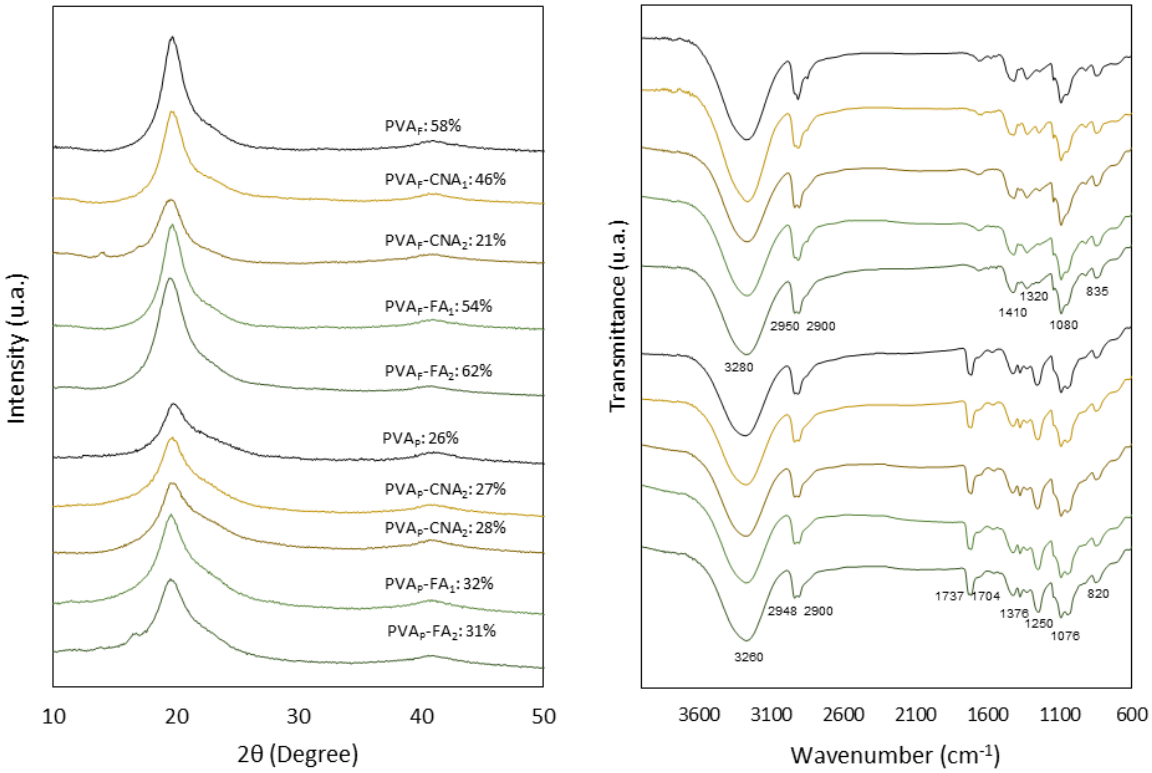

Figure 2. X-Ray diffraction spectra, percentage of crystallinity (left) and FTIR spectra (right) of the PVA_F and PVA_P films with and without cinnamic acid (CNA) or ferulic acid (FA) at different concentrations (1 or 2 g/100 g PVA).

3.3. Tensile properties

The tensile parameters (elastic modulus: EM, tensile strength: TS and elongation: E, at break), together with the thickness of the different films, are shown in **Table 2**. The samples were around 100 μm thick, with the exception of those containing cinnamic acid that were thicker. The higher the acid concentration, the thicker the film, which suggests that cinnamic acid tends to increase the intermolecular spacing in both matrices, PVA_F and PVA_P. Likewise, a high degree of variability in the thickness was observed for sample PVA_F-CNA_2, which may be due

to the previously mentioned phase separation that could occur with a stochastic distribution in the films.

The PVA_F films, with longer, linear molecular chains and a higher degree of crystallinity, exhibited greater resistance to break (TS), stiffness (EM) and stretchability (E) than PVA_P films, according to the direct relationship between the structural cohesion of the matrix and its tensile performance (Restrepo et al., 2018). This trend was also observed by Andrade et al. (2020), comparing fully and partially hydroxylated non-plasticised PVA films. When comparing the tensile parameters of the glycerol plasticized PVA films **(Table 2)** with the values found for non-plasticised peers (Andrade, Johana; González-Martínez, Chelo; Chiralt., 2020), the expected effect was observed and both PVA matrices became more stretchable. Nevertheless, the fully hydroxylated PVA matrix became more resistant to break (24% increase) and stiffer (14% increase) than non-plasticised films, while the elastic modulus of the PVA_P films decreased by around 9% with respect to the non-plasticized film. These results point to the different role of the glycerol in the different PVA matrices. More favourable glycerol interactions were observed in matrix A, probably due to the greater ability to establish hydrogen bonds between glycerol and the highly hydroxylated molecular chains, thus enhancing the cohesion forces in the matrix, while favouring the chain slippage capacity and film stretchability.

Table 2. Thickness, tensile parameters (Tensile strength (TS), elongation (E) and elastic modulus (EM)), barrier properties (water vapour permeability (WVP) and oxygen permeability (OP)) and water solubility of the PVA_F and PVA_P films without and with phenolic acids, cinnamic CNA and ferulic FA (1 or 2 g/100 g PVA). Mean values and standard deviation.

Sample	Thickness (µm)	TS (MPa)	E (%)	EM (MPa)	Water solubility (%)	WVP x 10^3 (g/m.h. kPa)	OP x 10^8 (cm3/m.h. kPa)
PVA_F	101 (4)^{a}	200 (10)^{c}	146 (4)^{c}	93 (3)^{d}	43 (6)^{b}	6.0 (1.0)^{ab}	2.7 (0.6)^{b}
PVA_F-CNA_1	111 (4)^{abc}	210 (20)^{c}	148 (6)^{c}	93 (7)^{d}	38 (1)^{ab}	6.4 (0.9)^{ab}	1.4 (0.3)^{a}
PVA_F-CNA_2	127 (33)^{d}	110 (20)^{b}	125 (3)^{b}	77 (7)^{c}	36 (5)^{ab}	8.0 (3.0)^{c}	7.0 (2.0)^{c}
PVA_F-FA_1	104 (7)^{ab}	212 (15)^{c}	152 (6)^{c}	96 (13)^{de}	34 (1)^{a}	5.4 (0.7)^{a}	1.8 (0.9)^{ab}
PVA_F-FA_2	103 (6)^{ab}	202 (18)^{c}	146 (4)^{c}	101 (11)^{e}	31 (3)^{a}	5.7 (0.5)^{ab}	1.1 (0.1)^{a}
PVA_P	106 (17) ^{ab}	47 (9)^{a}	111 (9)^{a}	49 (3)^{a}	80 (6)^{c}	8.2 (0.7)^{c}	13.0 (2.0)^{e}
PVA_P-CNA_1	118 (16)^{bcd}	47 (12)^{a}	107 (8)^{a}	52 (3)^{ab}	75 (3)^{c}	7.0 (2.0)^{abc}	12.0 (1.0)^{e}
PVA_P-CNA_2	123 (17)^{cd}	44 (10)^{a}	107 (8)^{a}	53 (4)^{ab}	82 (5)^{c}	8.0 (0.5)^{c}	11.8 (0.4)^{de}
PVA_P-FA_1	103 (16)^{ab}	53 (6)^{a}	111 (4)^{a}	56 (5)^{b}	74 (1)^{c}	6.5 (0.5)^{ab}	10.0 (0.9)^{d}
PVA_P-FA_2	108 (17)^{abc}	58 (6)^{a}	109 (6)^{a}	57 (4)^{b}	90 (1)^{d}	6.5 (1.7)^{abc}	11.3 (1.1)^{de}

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

The incorporation of phenolic acids into both PVA films slightly modified their mechanical properties, depending on the compound and its concentration. Thus, CNA only affected the mechanical properties of the films when incorporated at 2% in PVA_F matrices, with a

significant ($p<0.05$) reduction in the elastic modulus, resistance to break and extensibility of the films. This coincides with the observed marked reduction in crystallinity that negatively affected the strength, stiffness, and elongation capacity of the films. No significant effect of CNA on the tensile properties of PVA_P was observed at any concentration, in agreement with the lack of changes in crystallinity.

In contrast, the incorporation of FA gave rise to significantly stiffer PVA films (greater EM values), when incorporated into matrices PVA_F and PVA_P, in agreement with the promoted crystallinity of the films (**Figure 2**); this effect was more marked in PVA_F matrix. Moreover, the presence of both hydroxyl and carboxyl groups in F enhances the establishment of hydrogen bond interactions with the PVA chains, which promoted the polymer inter-chain forces. Similar results were obtained by Luzi et al. (2018) for fully hydrolysed PVA films incorporating gallic acid or quercetin.

3.4. Water solubility and barrier properties

Table 2 shows the values of water solubility and the barrier properties of the different samples. The water solubility of biodegradable films is an important factor in developing potential substitutes for synthetic plastics. This depends on the molecular chain packing (amorphous or crystalline), the ratio of acetylated OH groups in the molecule and the molecular weight of the polymer (Koltzenburg et al., 2017). Thus, the solubility of PVA_F films (43%) was significantly lower ($p<0.05$) than that of PVA_P films (80%), coherent with its higher molecular weight, low degree of acetylation and higher degree of crystallinity (**Figure 2**). For the polymer dissolution, the binding forces that maintain the cohesion of the matrix must be overcome; these are especially strong in the crystalline zones and in materials with long and homogeneous molecular chains (Ravve, 2012).

The solubility of the films was significantly affected ($p<0.05$) by the incorporation of ferulic acid, depending on the PVA matrix. Thus, FA reduced the water solubility of PVA_F films in line with the increase in the polymer crystallinity and the molecular structure of FA (trans-cinnamic acid-bearing methoxy and hydroxy substituents at positions 3 and 4 respectively on the phenyl ring) that can promote interchain hydrogen bonds. These bonds promote the binding between PVA chains and also reduce the polarity of the matrix by limiting the availability of the chain OH groups to interact with water molecules. Similar results were reported by other authors working on PVA films with phenolic compounds (Bersanetti et al., 2019; Liu, Wang, Lan, & Qin, 2019). In contrast, the addition of the highest proportion of ferulic acid (2%) to the PVA_P films significantly increased (by around 11%) the solubility, in comparison to the control film (PVA_P), which could be attributed to the lower degree of crystallinity of these films and the partial potential deacetylation of the PVA_P chains due to acid hydrolysis that would enhance the water affinity of the chains.

As concerns the barrier properties, the matrix PVA_F films presented better barrier properties than those of matrix PVA_P, in agreement with the greater degree of crystallinity and cohesion forces associated with their molecular characteristics (**Figure 2**). In addition, the presence of residual acetyl groups in the PVA_P chains, which changes the global polarity of the matrix, also contributes to the much higher OP values of PVA_P films, since the oxygen solubility in the matrix increases due to its higher chemical affinity.

The addition of ferulic acid slightly enhanced the barrier properties of PVA_F and PVA_P films, according to the increase in the polymer crystallinity and the binding capacity of ferulic molecules, as previously described. The greater compactness of the matrix reduces the free volume of the polymer and limits the mobility of the polymer chains; consequently, the number of permeation paths through the polymer network is also limited, which also contributes to the improvement of the barrier capacity. In contrast, the barrier properties of films were barely affected by the incorporation of cinnamic acid, except in sample PVA_F-CNA_2, where both the WVP and OP values increased, probably due to the great reduction in the crystallinity of the films.

3.5. Thermal behaviour

The PVA is a semi-crystalline polymer in which both crystalline and amorphous domains coexist, as is reflected in first and second order phase transitions shown in the DSC thermograms (**Figure 3)**. The glass transition melting temperatures (Tg and Tm) and melting enthalpy (ΔH) from the second heating scan, when the thermal history of the films was erased, are shown in **Table 3**.

Table 3. Glass transition (Tg), melting temperature (Tm) and enthalpy (ΔH) of the PVA_F and PVA_P films without and with phenolic acids, cinnamic CNA and ferulic FA, (1 or 2 g/100 g PVA). Mean values and standard deviation in brackets.

Sample	Second heating scan		
	Tg (°C)	Tm (°C)	ΔH (J/g PVA)
PVA_F	45 (2) ^{de}	217 (1) ^{d}	60 (1) ^{bc}
PVA_F-CNA_1	46 (1) ^{e}	217 (1) ^{de}	66 (4) ^{c}
PVA_F-CNA_2	42 (2) ^{cde}	214 (1) ^{c}	66 (2) ^{c}
PVA_F-FA_1	47 (1) ^{e}	218 (1) ^{de}	60 (5) ^{b}
PVA_F-FA_2	44 (4) ^{de}	218 (1) ^{e}	61 (8) ^{bc}
PVA_P	37 (3) ^{abc}	154 (3) ^{a}	24 (1) ^{a}
PVA_P-CNA_1	39 (7) ^{bcd}	157 (1) ^{b}	21 (3) ^{a}
PVA_P-CNA_2	36 (3) ^{ab}	157 (1) ^{b}	24 (4) ^{a}
PVA_P-FA_1	38 (1) ^{abc}	156 (1) ^{b}	23 (4) ^{a}
PVA_P-FA_2	33 (3) ^{a}	156 (1) ^{ab}	21 (1) ^{a}

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

The Tg of phenolic-free polymers PVA_F (39 °C) and PVA_P (34 °C) were notably lower than those obtained for non-plasticised PVA films reported in previous studies (Tg PVA_F: 72 °C; Tg PVA_P: 56 °C) (Andrade et al., 2020), in agreement with the plasticising effect of glycerol. The incorporation of phenolic acids did not provoke significant changes in the Tg values of either polymer, except for the PVA_P-FA_2 sample where a small decrease was observed, probably due to some effects of a partial acid hydrolysis, as previously deduced from the water solubility increase in this sample. The melting behaviour (Tm and ΔH values) of polymers PVA_F and PVA_P, after the melting-crystallisation process occurred in the first heating and cooling steps, was barely affected by the presence of phenolic acids. The Tm values were slightly lower than in non-plasticised polymers (225 and 168 °C, respectively for PVA_F and PVA_P), which suggests that glycerol interfered with the crystallisation pattern, giving rise to smaller crystals. Likewise, the melting enthalpy was also reduced in polymer PVA_F after the incorporation of glycerol. The ΔH values of the PVA_F samples (60 J/g) were higher than those of the PVA_P samples (24 J/g), coinciding with the greater crystallinity of this matrix. However, the ΔH values of neither matrix reflected any differences in crystallinity associated with the incorporation of phenolic acids deduced from the XRD analysis. This must be attributed to the melting of cast films in the first heating scan, and the formation of a new crystalline arrangement during the cooling step. This second crystallisation did not reproduce the differences in crystallinity provoked by the incorporation of phenolic acids in the casting process, which were well correlated with the observed differences in the mechanical and barrier properties of the cast films.

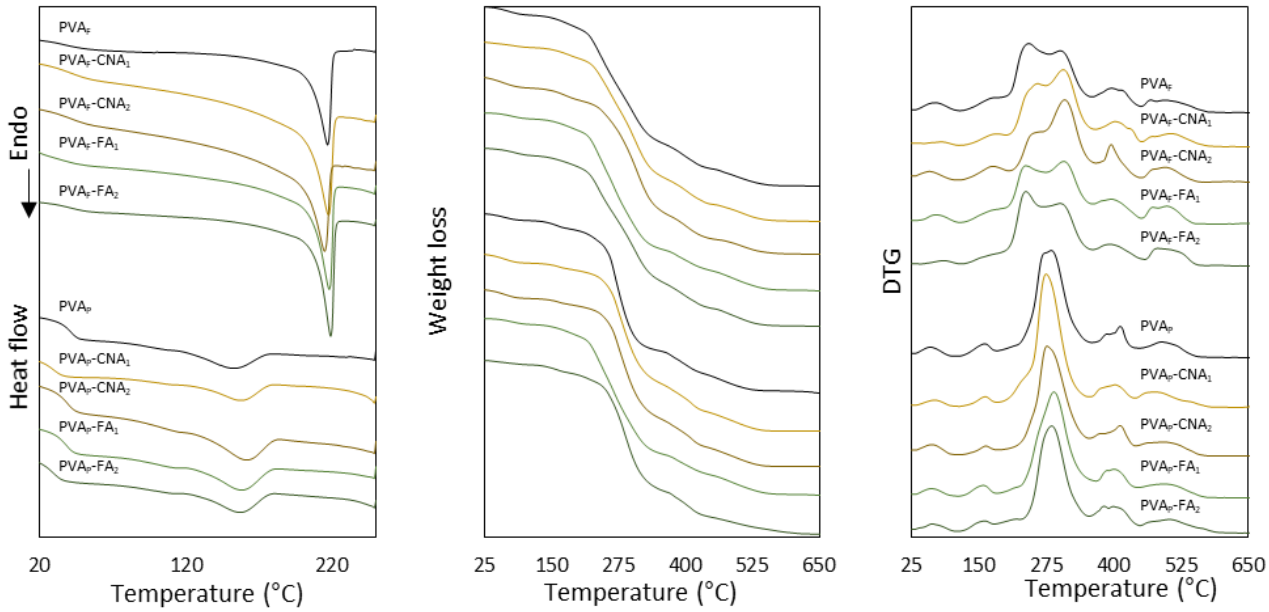

Figure 3. DSC curves (second heating scan) (left) and Thermogravimetric analysis (TGA) and DTGA (right) of the PVA_F and PVA_P films without and with cinnamic acid (CNA) or ferulic acid (FA) (1 or 2 g/100 g PVA).

3.6. Thermal degradation

The thermal degradation of the samples was analysed through the curves of the thermogravimetric analysis (TGA) and its derivative (DTG), shown in **Figure 3**. The DTG profiles indicated significant differences between the degradative behaviour of matrices PVA_F and PVA_P, in line with their different molecular properties (Andrade, Johana; González-Martínez, Chelo; Chiralt, 2020). The first peak in the DGTA curves (40 ℃ - 110 ℃) corresponds to the vaporisation of the bonded water in the polymer matrices (conditioned at 0% RH). Polymer PVA_F had 2.5% bonded water, while polymer PVA_P contained only 1.6%, which is coherent with its more hydrophobic nature, due to its partially acetylated chains. The second step, with temperature peaks at 185 ℃ and 160 ℃ in matrices PVA_F and PVA_P, respectively, could be associated with the partial release of glycerol, since this was not observed in the glycerol-free PVA films (Andrade, Johana; González-Martínez, Chelo; Chiralt, 2020). This difference in the glycerol release temperature could be due to the different attractive forces between glycerol and PVA chains with different ratios of hydroxyl groups. The thermo-released glycerol (5.2% from matrix PVA_F and 4% from matrix PVA_P) did not correspond to the total glycerol incorporated into the matrices, which suggests that a fraction of the plasticizer were more tightly bonded to the polymer chains.

The degradation of the polymeric material occurred in several stages; the first stage was associated with the detachment of side groups from the chains, giving rise to by-products, such as water in the case of polymer PVA_F, and acetaldehyde, acetic acid and water in polymer PVA_P, due to the thermal cleavages of esters in their acetylated polymer chains (Perilla, 2007). In polymer PVA_F, this stage showed an overlapping of different degradative events that occurred simultaneously between 200 ℃ and 360 ℃ (peaks: 250 ℃ and 307 ℃), as reported by different authors (Andrade et al., 2020; A. Cano et al., 2015). Since the fusion of the crystalline fraction (Tm: 213 ℃) occurred within this temperature range, crystalline and amorphous fractions would coexist and degrade differently (Andrade, Johana; González-Martínez, Chelo; Chiralt, 2020). In polymer PVA_P, degradation occurred between 220 ℃ and 350 ℃ (peak: 276 ℃) after the melting of the crystalline fraction (Tm: 170 ℃), resulting in a unique degradation pattern. The degradation of polymer PVA_P shifted to higher temperatures due to the thermo-protective action of the acetyl group in the PVA chains, as has been described by Cristancho et al. (2013) and Perilla (2007). Subsequently, the second and third stages of polymer degradation occurred at temperatures above 350 ℃. These stages were possibly related to the degradation of low molecular weight products from the breakdown of the polymer backbone, or to heavier structures formed in previous stages of degradation (Holland & Hay, 2001; Perilla, 2007).

The thermal degradation patterns of the individual phenolic acids, occurring between 190 ℃ and 230 ℃ for cinnamic acid and between 180 ℃ and 235 ℃ for ferulic acid, were not observed in the thermograms of the films, probably due to their low concentrations in the

films or the overlapping with the polymer degradation events. However, in the degradation patterns of samples PVA_F, especially with cinnamic acid, slight changes were observed in the main degradation curve of the polymer (200 ℃ - 360 ℃), thus suggesting that the presence of phenolic acids interferes with the degradation mechanisms of polymer PVA_F. Nevertheless, the thermal stability of matrices PVA_F and PVA_P remained unaffected ($p>0.05$) by the inclusion of phenolic acids, without changes in the temperature range of degradation. In contrast, some authors found that interactions between –OH groups of PVA and –OH moieties of phenol compounds conferred a thermal shift towards higher temperatures on the PVA films when using tannic acid and quercetin (Dhand et al., 2016).

4. CONCLUSION

The incorporation of cinnamic and ferulic acids at 1 or 2 wt. % gave rises to small changes in the physical properties of the glycerol plasticised films of highly hydrolysed and partially acetylated PVA. The changes were associated with the establishment of hydrogen bonds between the hydroxyl groups in PVA and the carboxyl and hydroxyl groups of phenolic acids. Phenolic acids were more compatible with the partially acetylated PVA, as revealed by the more homogeneous film microstructure. Ferulic acid promoted greater changes in PVA films than cinnamic acid, which can be attributed to the presence of a phenolic hydroxyl group, which may promote interchain hydrogen bonds and a cross linking effect. Thus, films containing ferulic acid exhibited a higher degree of crystallinity, were mechanically stiffer and less water-soluble, and had enhanced barrier properties (lower WVP and OP values). Phenolic acids did not reduce the thermal stability of the PVA polymers, whose temperature range of degradation was not altered. Considering the better mechanical and barrier properties of fully hydrolysed, high molecular weight PVA films, the incorporation of ferulic or cinnamic acid in this matrix could be of great potential for the purposes of developing active packaging materials for food preservation, but these could not be obtained by the usual thermal processes employed in the plastics industry due to the fact that the melting temperature exceeded the thermodegradation temperature. In contrast, partially acetylated PVA exhibited a lower melting temperature and greater thermostability and could be used to obtain active thermoprocessed materials for food packaging applications on the basis of the antioxidant and antimicrobial activity of phenolic acids. The effectiveness of these films as antioxidant and antimicrobial materials must be analysed in further studies.

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IV.II. Effect of the processing method on the physicochemical and active properties of poly (vinyl alcohol) films incorporating phenolic acids

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ABSTRACT

Poly (vinyl alcohol) films containing cinnamic acid (CNA) and ferulic acid (FA) at 1 wt % and 2 wt % were successfully produced by solvent-casting the polymeric solution and melt blending and compression moulding. The effect of the processing method and the phenolic acid content on the microstructure, crystallinity and the optical, thermal, barrier and mechanical properties of partially hydrolysed poly (vinyl alcohol) based films have been analysed, as well as the antioxidant and antimicrobial properties of the films. The processing method influenced the microstructural arrangement of the matrix, leading to differences in the degree of crystallinity and in the tensile and barrier properties of the films. The incorporation of phenolic acids, especially ferulic acid, enhanced the barrier properties of the materials without affecting their thermal stability. The presence of phenolic acids in the films clearly induced the inhibition of *Listeria innocua* growth and had a positive antioxidant response, thus suggesting the great potential of these active materials for food packaging applications.

Keywords

Cinnamic acid; ferulic acid; active films; cast-films; thermoprocessed-films; food packaging.

1. INTRODUCTION

Plastic packaging has become an essential part of the food supply chain due to its significant role in the preservation of the quality of the food product until its final consumption (Trabold & Babbitt, 2018). In fact, during the initial stages of the COVID-19 pandemic, consumer preference for packaged food has skyrocketed, probably due to the increase in online ordering and food delivery, as packaging is a way of offering consumers reassurance (Kakadellis et al., 2021; Vanapalli et al., 2021). However, the increasing pressure on the generation of waste has brought plastic food packaging back into the spotlight amidst growing public concern about the environmental impact of plastic pollution (Kakadellis et al., 2021).

A great deal of research has successfully reported the development of biodegradable polymer-based materials that constitute an alternative for the progressive replacement of synthetic plastics (Brockhaus et al., 2016; RameshKumar et al., 2020). Pol (vinyl alcohol) (PVA) is a semi-crystalline, water-soluble and biodegradable polymer that has a high degree of biocompatibility, while being capable of self-crosslinking due to the high density of hydroxyl groups located on its side chains (Havstad, 2020). PVA is easily produced by the saponification of poly (vinyl acetate), a process that allows the molecular weight and the degree of hydrolysis to be controlled and, consequently, permits the development of materials with differing degrees of crystallinity, solubility in water and tensile, barrier and thermal properties (Andrade et al., 2020b). The molecular characteristics provide PVA with a marked affinity for several active compounds (Andrade et al., 2020b; Cano et al., 2015), which, when effectively incorporated into the polymeric matrix, could enable the development of active packaging materials. Active packaging systems are characterised by interacting dynamically with the target intrinsic and/or extrinsic factors of the packaged product, whose action enhances the protective function of the package (Lim, 2015). Additionally, the partially hydrolysed PVA could be thermo-processed due to the thermo-protective action of the residual acetate groups in the polymer chain, providing flexibility in the type of processing used for the development of PVA-based materials (Andrade et al., 2020b).

The processing conditions may result in modifications of the structural arrangement, especially of the crystalline fraction of semi-crystalline polymers, which are relevant in view of their effect on the density and the mechanical and optical performance of the final product, as well as, to a lesser extent, on its physical aging and long-term stability (Mileva et al., 2018). The processing factors that are the major influencers on the characteristics of the material are the thermal history, pressure and flow phenomena. Depending on the polymer structure (molecular weight and polydispersity), the effect of the processing conditions, such as the increase in cooling rate, can range from simple reductions in crystallinity to the formation of a completely amorphous structure, nearly always involving changes in tensile and optical properties. The effects of flow and pressure, especially important in the injection moulding process, generally modify nucleation, crystal growth and orientation, with a clear correlation

with stiffness and elongation at break, the effect of which is dominated by the stress applied to the material (Mileva et al., 2013). Thus, the different processing methods and their inherent conditions lead to the development of materials with specific final characteristics, which could determine their application. These changes that films may undergo could also affect their functionality when incorporating active agents, especially when using phenolic compounds, such as phenolic acids (PA), capable of interacting with the hydroxyl and acetate groups of the PVA matrix (Lan et al., 2019). Of the phenolic acids, cinnamic and ferulic acids are of increasing potential interest because of their proven antioxidant and antimicrobial properties (Lima et al., 2019; Mathew & Abraham, 2008; Olszewska et al., 2020).

This study aims to evaluate the impact of two types of film processing, casting and thermo-processing (melt blending and compression moulding), on the crystallinity and the mechanical and barrier characteristics of partially hydrolysed PVA films, containing or not cinnamic and ferulic acids as active agents. To our knowledge, these analyses have not yet been addressed and are of great interest due to the importance of developing new biodegradable active materials for safe and sustainable food packaging.

2. MATERIALS AND METHODS

2.1. Materials

Partially hydrolysed poly (vinyl alcohol) (PVA_P) (Mw 13,000–23,000; 87–89% hydrolysed), glycerol as plasticizer and phenolic acids (PA), cinnamic (CNA) and ferulic acid (FA), were purchased from Sigma-Aldrich (Steinheim, Germany). Magnesium nitrate ($Mg(NO_3)_2$), phosphorus pentoxide (P_2O_5) salts, UV-grade methanol and UV-grade ethanol were supplied by Panreac Química S.A. (Barcelona, Spain).

2.2. Preparation of films

Two processing methods, casting (C) of the polymeric aqueous solutions and melt blending and compression moulding (T), were used to obtain the poly (vinyl alcohol) films.

To cast films, glycerol (10% wt. with respect to the polymer) and phenolic acids (1% or 2% wt. with respect to the polymer) were added to distilled water previously heated to 100 °C. After the dissolution of the acids, the polymer (10% wt.) was incorporated by stirring (1,200 rpm) and heating (100 °C) for 3 h, until polymer solutions were obtained. Subsequently, all the formulations were degassed by using a vacuum pump and the solution (equivalent amount to 2 g of polymer per plate) was subsequently spread evenly onto Teflon plates (150 mm in diameter). The films were then obtained by drying at controlled temperature (25 ± 2 °C) and relative humidity (55 ± 2%) for 48 h.

Thermoprocessed-films were obtained by the compression-moulding of pellets using a hot plate-press (Model LP20, Labtech Engineering, Thailand). Pellets were obtained by melt blending the different components by using an internal mixer (HAAKE™ PolyLab™ QC, Thermo Fisher Scientific, Germany) at 160 °C and 50 rpm, for 10 min. In every case, 10 wt% of glycerol was used with respect to PVA and the PA was added to the mixture at 1 or 2 g/100 g polymer. The pellets were cold-milled in a Universal Mill (IKA, model M20, Germany) and the preconditioned particles (at 53% RH and 25ºC) were compression moulded with a preheating step for 3 min at 160 °C, followed by 3 min thermocompression at 100 bars. Thereafter, a 3 min cooling cycle was applied. Plasticised films without PA were used as controls. The different film formulations and the mass ratio of the respective components are shown in **Table 1**.

Cast and thermoprocessed films were conditioned for one week at 25ºC and 53% relative humidity (RH) by using $Mg(NO_3)_2$ over-saturated solution before the characterisation of their functional properties. Films were conditioned at 0% RH, using P_2O_5, for the analyses of the final PA content in the film, water solubility, microstructure and thermal behaviour.

2.3. Characterisation of the active PVA films

2.3.1. *Final content of phenolic acids, moisture content and water solubility of the films*

The final content of the phenolic acids (PA) was determined by solvent extraction and spectrophotometric quantification. The PA extraction was carried out by immersing dry samples (25 mg) in 10 mL of a 50% methanol aqueous solution under stirring at 300 rpm for 48 h. The absorbance (*A*) of the solutions was measured at wavelengths of 320 nm for ferulic acid (FA) and 270 nm for cinnamic acid (CNA), using a spectrophotometer (Evolution 201 UV–Vis, Thermo Fisher Scientific, USA). The PA concentration (C_{PA}) was obtained using the calibration curve of each acid: ferulic acid ($C_{PA} = (A - 0.005)/0.0852, R^2 = 0.995$) and cinnamic acid ($C_{PA} = (A - 0.02)/0.1372, R^2 = 0.992$). The corresponding extracts from the PA-free films were used as backgrounds. The mass of extracted PA (mg) was compared to the corresponding mass of initially incorporated PA and the percentage of retention was determined. The measurements were taken in triplicate.

The moisture content of the previously conditioned films (53% RH; 25 °C) was analysed gravimetrically. Four weighed samples per treatment were dried in a convection oven (JP Selecta S.A., Barcelona, Spain) at 60 °C for 24 h; subsequently, these were equilibrated (0% RH;25 °C) until constant weight.

The water solubility was evaluated by a modification of the method described by Talón, Vargas, Chiralt, & González-Martínez (2019). Dry film samples (2 cm x 2 cm) inside a mesh, were weighed and placed in a crucible with 10 mL of distilled water for 24 h at 25 °C. The meshes with the remaining film sample were dried in an oven (J.P. Selecta, S.A., Barcelona, Spain) at 60 °C for 72 h and, subsequently, were transferred to a desiccator with P_2O_5 until reaching constant weight. The assay was performed in triplicate and the results were expressed as g of solubilised film/100 g initial film.

2.3.2. *Microstructure and X-ray diffraction analysis*

The microstructure of the cross-section films was observed using a Field Emission Scanning Electron Microscope (FESEM) (ZEISS®, model ULTRA 55, Germany), at an acceleration voltage of 2 kV. Previously, the film samples were cryofractured by immersion in liquid nitrogen and platinum coated.

The degree of crystallinity was analysed through the X-ray diffraction spectra of the films by means of a D8 Advance X-ray diffractometer (Bruker AXS, Karlsruhe, Germany). The range 2θ of evaluation was from 10° to 50°, with a step size of 0.05, using KαCu radiation (λ: 1.542 Å), 40 kV and 40.mA. The diffraction curves were deconvoluted by using the Lorentz model with the OriginPro 2021 software to define the crystalline and amorphous regions. The ratio of the crystalline peak area and the total area of the diffractograms defined the degree of crystallinity of the samples.

2.3.3. *Optical properties*

The optical properties were determined by measuring the reflectance spectra of the samples from 400 to 700 nm of wavelength using a spectrocolorimeter (CM-3600d Minolta CO., Tokyo, Japan), using both black and white backgrounds, following the method reported by (Sapper et al., 2018). The transparency was measured through the internal transmittance (Ti), applying the Kubelka-Munk theory for multiple scattering. CIE L*a*b* colour coordinates and chromatic parameters (chroma and hue) were obtained from the reflectance of an infinitely thick layer of the material by considering D65 illuminant and 10º observer, according to Hutchings (1999). Three measurements were taken from each film and three films were considered per formulation.

2.3.4. *FTIR characterisation*

The attenuated total reflection Fourier transformed infrared spectra (ATR-FTIR) (BRUKE, VERTEX 80, Germany), over the range 4000-600cm^{-1}, were analysed for the different samples. These analyses were carried out in triplicate and at three different locations in each sample.

2.3.5. *Tensile properties and barrier properties*

The tensile properties of the films were measured using a Universal Machine (Stable Micro Systems, TA.XT plus, Haslemere, England). Equilibrated test specimens (25 mm x 100 mm) were mounted in the film extension grips with an initial separation of 50 mm and stretched at 50 $mm.min^{-1}$ until break, following the standard method ASTM D882-02 (ASTM, 2002). Elastic modulus (EM), tensile strength (TS), and elongation at break point (E) were determined from the tensile stress (σ) vs. Hencky strain (εH) curves. The measurements were taken in eight samples of each treatment.

The water vapour permeability (WVP) was analysed in triplicate following a standard method, E96/E95M-05 (E. ASTM, 2003). In order to obtain a RH gradient of 53 to 100%, the samples were placed on Payne permeability cups (3.5 cm in diameter) (Elcometer SPRL, Hermelle/s Argentau, Belgium) containing 5 mL of distilled water, which were placed inside a desiccator at 25 °C with an oversaturated $Mg(NO_3)_2$ solution. The cups were weighed periodically every 1.5 h for 24 h using an analytical balance (0.00001 g). The slopes of the weight loss *vs.* time during the steady state period were determined by linear regression to calculate the water vapour transmission rate (WVTR). WVP was calculated as described by Cano, Jiménez, Cháfer, Gónzalez, & Chiralt (2014).

The oxygen permeability (OP) in film samples (50 cm^2) was analysed in triplicate using an Ox-Tran system (Mocon, Minneapolis, US) at 23 °C and 53% RH, following a standard method, F1927-07 (F.-07 ASTM, 2004). The OP was calculated from the oxygen transmission rate multiplied by the average film thickness and divided by the partial pressure of oxygen. The

film thickness was measured with a digital electronic micrometer (Palmer, COMECTA, Barcelona, Spain) to the nearest 0.001 mm at six random positions.

2.3.6. *Thermal behaviour*

The thermal behaviour of the films was assessed by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). For DSC measurements, the sample was inserted into a hermetically-closed aluminium pan and placed in a differential scanning calorimeter (DSC 1 stareSystem, Mettler Toledo, Schwarzenbach, Switzerland), with an empty aluminium pan used as a reference. The temperature scanning profile was a first heating step from -25 °C to 250 °C, holding this temperature for 2 min, followed by a cooling to -25 °C, holding this temperature for 2 min and a second heating step to 250 °C; all of the scans were run at 10 C/min. For TGA analysis purposes, the samples were heated in alumina crucibles from 25 °C to 700 °C at 10 °C /min, by using a thermogravimetric analyser (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland). All of the measurements were taken in triplicate using nitrogen (10 mL/min) as purge gas.

2.3.7. *Antioxidant activity*

The antioxidant capacity of the PVA films was evaluated in terms of the radical scavenging capacity of the ferulic and cinnamic acids released from the films into different food simulants. The simulants used were ethanol at 50% (v / v) in water, as a food simulant of an alcoholic beverage or o/w emulsions, and ethanol at 95% (v / v), as a simulant of more fatty foods. The DPPH method was used, as proposed by Aragón-Gutiérrez et al. (2021), with some modifications. A film sample of each formulation (50 mg) was immersed in 10 mL of the simulant and kept under constant stirring (300 rpm) at 20 ° C, for 48 h. Afterwards, filtered aliquots of 500 µL of each extract were mixed with 2 mL of a 0.06 mM DPPH solution in methanol (Abs 515nm = 0.7 ± 0.2) in a closed cuvette, which was kept in the dark at room temperature for 60 min. The absorbance was measured at 515 nm by using a spectrophotometer (Evolution 201 UV-Vis, Thermo Fisher Scientific, USA). The ethanolic extracts of the films without phenolic acids were used as a control. All of the tests were carried out in triplicate and the DPPH radical scavenging activity was expressed as % according to Equation (1)

$$Scavenging\ of\ DPPH\ (\%) = \frac{A_C - A_S}{A_C} x100 \quad (1)$$

Where A_C and A_S are the absorbance of the black control and the tested sample, respectively.

2.3.8. *Antimicrobial activity*

The antimicrobial activity of the active PVA films was evaluated by means of the agar diffusion method with slight modifications (Brito et al., 2021) against *Escherichia coli* (CECT 515), Gram (+), and *Listeria innocua* (CECT 910), Gram (−), obtained from Spanish Type Culture Collection (CECT, Burjassot, Spain). The bacterial strains, stored under protective conditions (glycerol 30%) at -25 °C, were regenerated as described by Valencia-Sullca et al. (2016), by incubating them at 37 °C for 24 h in tryptic soy broth (TSB) (Scharlab, S.L., Barcelona, Spain) and harvested in their exponential growth phase. The active cultures were properly diluted in tryptone phosphate water (Sharlab S.A., Barcelona, Spain) to obtain a target inoculum of 10^5 colony-forming units (CFU)/ml.

The films were cut into discs (10 mm in diameter) and sterilised under UV light before the antimicrobial tests. Circular samples were carefully placed on the inoculated plates with 1 mL inoculum. Culture media in the plates were 10 mL of violet red bile agar (VRBA) (Scharlab, S.L., Barcelona, Spain) for *E. coli* and palcam agar base (PAB) (Scharlab, S.L., Barcelona, Spain), enriched with palcam selective supplement, for *L. innocua* (Scharlab, S.L., Barcelona, Spain). The petri dishes were then incubated at 10 °C for 7 days in the incubation chamber. The plates were examined to measure the halo of inhibition of the film discs. Five replicates were carried out for each film.

3. RESULTS AND DISCUSSION

3.1. Phenolic acid and moisture content in the films, and their water solubility

The final phenolic acid content (PA) in the films is shown in **Table 1**. In every case, the remaining amount of PA was lower than that initially incorporated. The retention percentage ranged between 90-77% for cast films and 72-20% for thermoprocessed samples. The losses were higher for films containing ferulic acid, especially in thermoprocessed films. Therefore, the losses of active compound depended on the type of phenolic acid and the type of film processing. The combined effect of high temperature, shearing stress and pressure conditions given in thermo-processing provoked significantly greater reductions in the final PA concentration of the thermoprocessed films than those obtained in cast-films. Cinnamic acid (CNA) has greater thermal stability than ferulic acid (FA), which could explain its greater retention in the films. However, the onset temperature of degradation (230 °C for cinnamic acid and 210 °C for ferulic acid) of both acids is higher than that applied during melt blending (160 °C) or compression moulding. Therefore, the losses could not be principally attributed to compound degradation brought about by thermal effect, but rather to the fact that their antioxidant nature causes oxidative degradation, under the process conditions. In fact, the UV spectra of the compounds extracted from films for their spectrophotometric analyses revealed changes in the UV spectral pattern with respect to the standard compounds in the case of ferulic acid (**Figure 1**) that had a greater antioxidant capacity (Li et al., 2021). These changes must be associated with the presence of modified molecular structures produced by the partial oxidation of the active. (Aragón-Gutiérrez et al., 2021) also reported 30% losses of ferulic acid in EVOH films obtained by melt extrusion at 190 °C, but with a shorter residence time (3 min). Oxidative processes will also be affected by the exposure time of the material to the processing conditions. A reduction of 40% in the case of ferulic acid and 5% in that of cinnamic acid were also observed for in starch films obtained by thermo-processing at 130 °C (Ordoñez et al., 2021).

The equilibrium moisture of PVA films was affected by the PA incorporation, which reduced the water sorption capacity in both cast and thermoprocessed films. This may be due to the formation of hydrogen bonds between the OH groups of the polymer chains and the carboxyl or phenolic groups of PA, which should reduce the number of active points of the PVA chains for bonding water molecules (Hernández-García et al., 2021). In general, thermoprocessed films exhibited slightly lower values of moisture content than cast films, which could be attributed to the different chain rearrangement obtained in each case and the different interchain bond established, which can modify the active points of the matrix to bond water molecules.

Table 1. Nominal mass fraction of the film components and final content of cinnamic (CNA) or ferulic acids (FA) at 1 or 2%. The ratio between the determined final content and the incorporated amount of phenolic acid (% retention), the equilibrium moisture content and the water solubility (% of solubilised solids) were also shown for films obtained by casting (C) and thermo-processing (T). Mean values and standard deviation.

Sample	X_{PVA-P}	X_{GLy}	X_{PA}	Final PA content in the films		Equilibrium Moisture Content	Water solubility
				mg PA/g PVA	Retention (%)	(%)	(%)
PVA_C	0.91	0.09	0	-	-	6.0 (0.1) ^{d,2}	80 (6) ^{a,2}
PVA_C-CNA_1	0.9	0.09	0.01	9.1 (0.16)	90.8 (0.6) ^{c,2}	5.7 (0.2) ^{bc,2}	75 (3) ^{a,1}
PVA_C-CNA2	0.89	0.09	0.02	17.2 (0.8)	86.0 (4.0) ^{b,2}	5.5 (0.2) ^{ab,2}	82 (5) ^{ab,1}
PVA_C-FA_1	0.9	0.09	0.01	7.8 (0.3)	78.0 (3.0) ^{a,2}	5.5 (0.1) ^{a,1}	74 (1) ^{a,1}
PVA_C-FA_2	0.89	0.09	0.02	15.4 (0.1)	77.0 (0.3) ^{a,2}	5.9 (0.1) ^{cd,2}	90 (1) ^{b,1}
PVA_T	0.91	0.09	0	-	-	5.8 (0.5) ^{b,1}	68 (3) ^{a,1}
PVA_T-CNA_1	0.9	0.09	0.01	8.32 (0.03)	83.0 (0,3) ^{d,1}	5.3 (0,0) ^{a,1}	72 (3) ^{ab,1}
PVA_T-CNA_2	0.89	0.09	0.02	14.4 (0.5)	72.0 (3.0) ^{c,1}	5.2 (0.1) ^{a,1}	85 (1) ^{c,1}
PVA_T-FA_1	0.9	0.09	0.01	1.99 (0.04)	19.9 (0.4) ^{a,1}	5.4 (0.0) ^{a,1}	83 (7) ^{c,1}
PVA_T-FA_2	0.89	0.09	0.02	5.5 (0.2)	27.0 (1.0) ^{b,1}	5.3 (0.0) ^{a,1}	81 (9) ^{bc,1}

Different superscript letters indicate significant differences between formulations within the same processing method, while different numbers indicate significant differences between formulations with equivalent mass fractions but processed by different methods ($p < 0.05$).

As regards the water solubility values, films without phenolic acids obtained by casting (PVAc) presented significantly higher solubility values (80%) than thermoprocessed films (PVA_T) (68%). However, the incorporation of phenolic acids promoted changes in the water solubility of the films, but these were only significant in thermoprocessed films. This could be due to the partial hydrolysis induced in the polymer chains by phenolic acids at the high processing temperature.

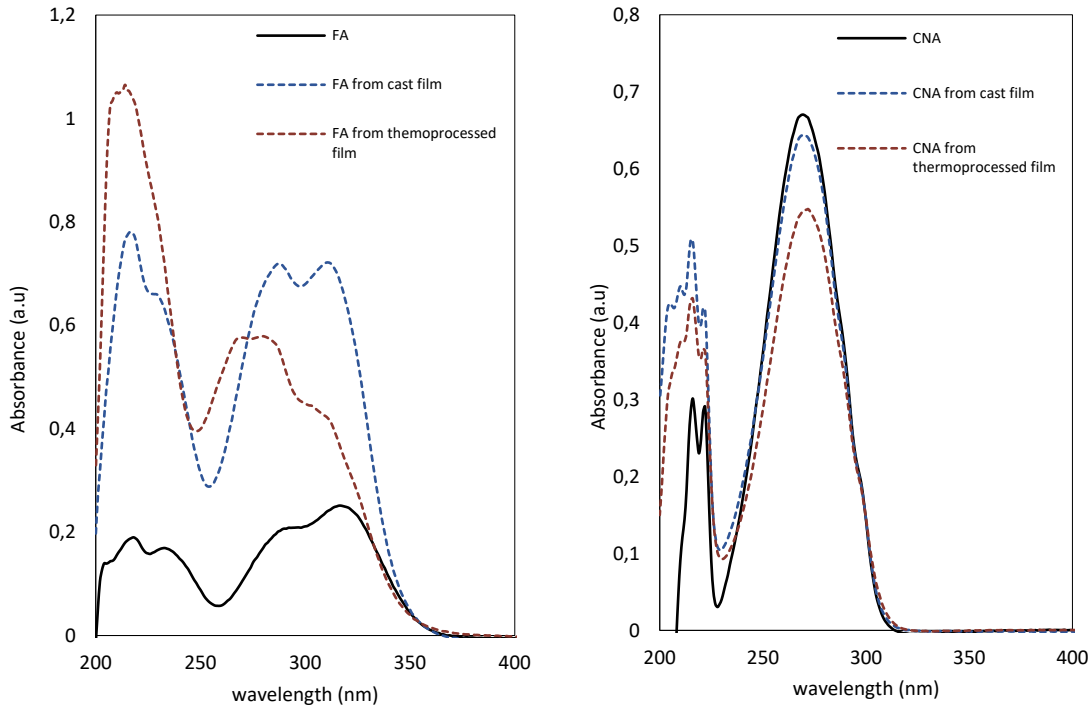
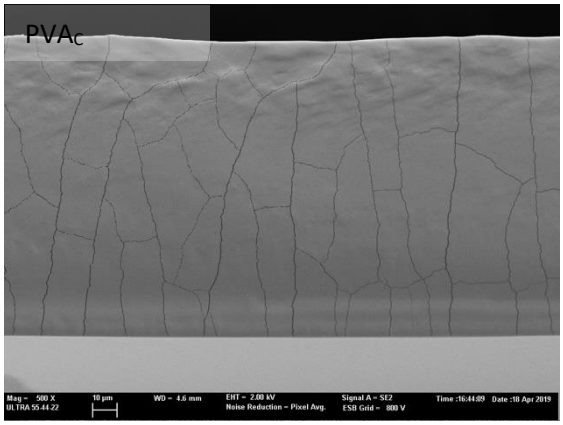
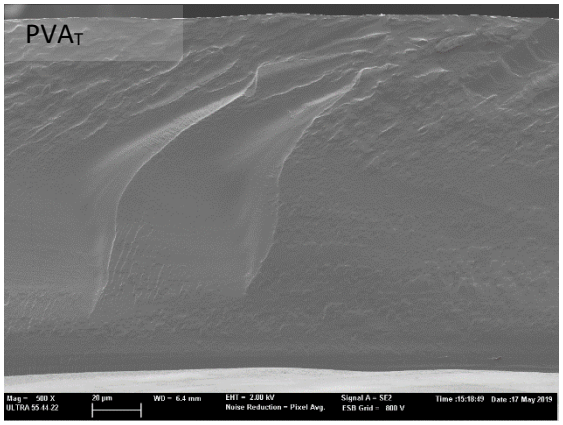


Figure 1. UV absorbance spectra for standard ferulic (FA)/cinnamic (CNA) acid and compounds extracted from cast and thermo-processed films using 50% ethanol in water.

3.2. Microstructure and crystallinity of the films.

The PVA films obtained both by casting and by thermo-processing presented a smooth and homogeneous appearance, as observed in **Figure 2**. The incorporation of PA did not generate marked modifications in the structure of the film matrix, which suggests that the acids presented a chemical affinity with the polymeric matrix. Nevertheless, at higher magnification, different structural features could be observed for films with phenolic acids, as shown in **Figure 2** for ferulic acid. Thermoprocessed films exhibited some domains with a rubbery cryofracture (image of higher magnification in Figure 2), which suggests the presence of highly plasticised regions in the film. Likewise, few dispersed acid particles of approximately 200 nm were observed in all the cast films (image of higher magnification in Figure 2). This could be attributed to the local aggregation/precipitation of PA molecules during film drying, caused by oversaturation in the polymeric solution in line with water loss. This local oversaturation of acids at high temperatures (thermoprocessing) could promote partial hydrolyses at some points of the PVA matrix, thus provoking local plasticised regions with the observed rubbery fracture.


PVA$_C$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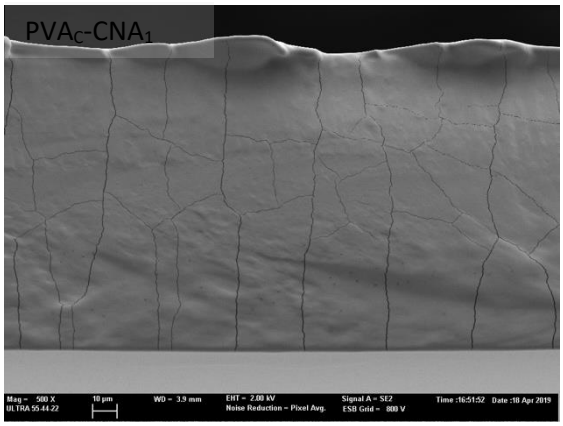
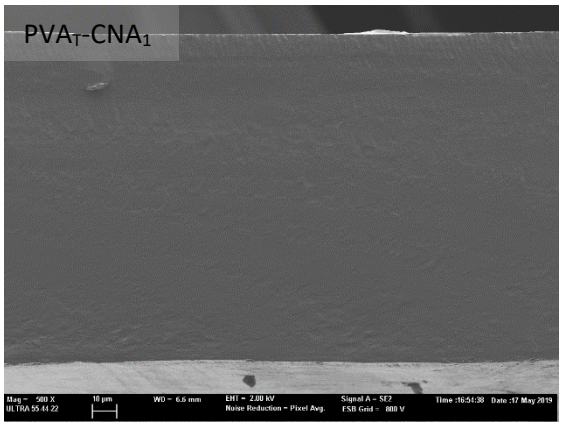

PVA$_C$-CNA$_1$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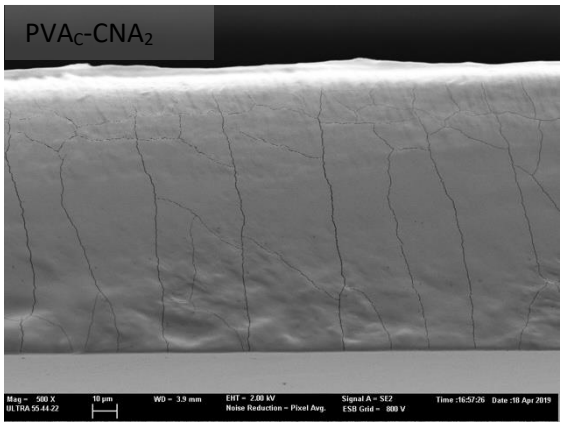
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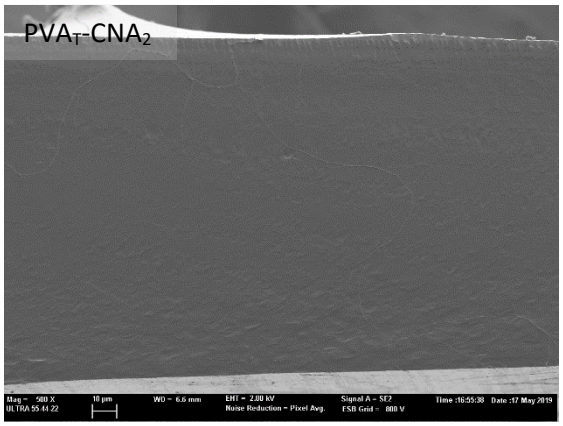
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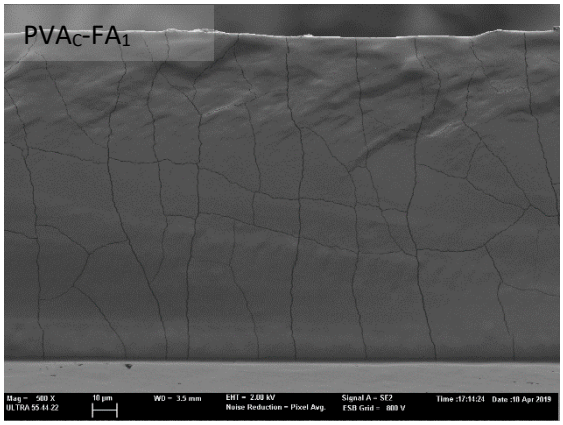
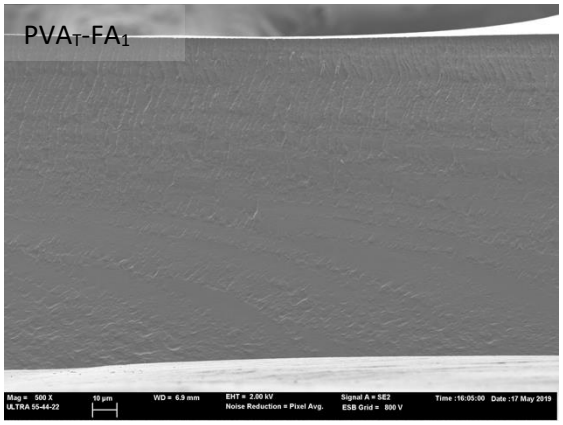

PVA$_C$-FA$_1$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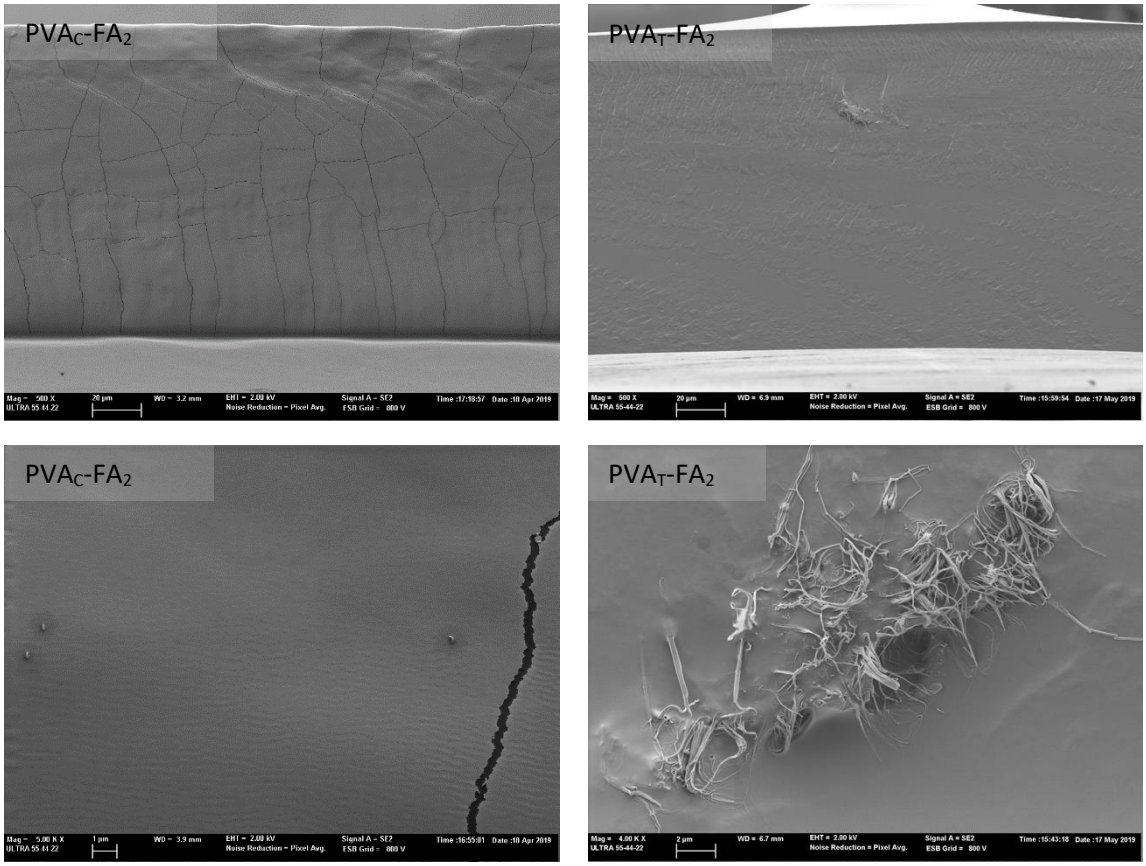

Figure 2. Field Emission Scanning Electron Microscope (FESEM) micrographs of the cross-section of the PVA films with cinnamic (CNA) or ferulic (FA) acid (1 or 2 g/100 g PVA) obtained by casting (C) or thermo-processing (T) (500X). Micrographs at higher magnification (4k-5k X) were included for films with 2% ferulic acid.

Figure 3 shows the XRD pattern and the degree of crystallinity of the PVA samples. As can be observed, the thermoprocessed and cast films presented differentiated patterns, both corresponding to the characteristic XRD pattern of the PVA monoclinic cell. The diffraction peaks of the thermoprocessed films were sharper, allowing for the identification of a greater number of peaks: the two main peaks (a doublet at 2θ = 19.5°/20° and the second at 40.4°) and other peaks with less intensity at 11.5 ° and 32.8 °, as well as a shoulder that protrudes from the main peak at 22.8 °. In contrast, only two main peaks were observed (at 2θ 20° and 40.4°) in the diffraction trace of the cast films. Therefore, the degree of crystallinity of the active-free PVA films obtained by thermoprocessing was greater (35%) than that obtained by casting (26%). These results are coherent with what was reported by Di Vona (2015), who stated that the processing conditions of semicrystalline polymers affect the crystal structure, the degree of crystallinity, the perfection of the crystals and the orientation of both the crystalline and amorphous phases in the films. Assender & Windle (1998) also claimed that

the intensity of the peaks depends on the orientation of the hydrogen bonds, both intermolecular and intramolecular, found within a crystalline lattice. Therefore, it can be concluded that the PVA film processing method influenced the orientation of the hydrogen bonds in the crystalline unit cell, giving rise to different diffraction traces of the films and crystallinity.

The incorporation of phenolic acids also promoted some changes in the XRD patterns and in the degree of crystallinity of the films. Whereas this incorporation, enhanced the film crystallinity in cast films, this did not occur in thermoprocessed films. For both kinds of films, the addition of ferulic acid implied greater crystallinity than that of cinnamic acid. In thermoprocessed films, the incorporation of ferulic acid enhanced the sharpness of the diffraction peaks, which is mainly visible in the 19.5°/ 20° doublet; in cast films, however, the highest concentration of ferulic acid gave rise to an additional peak at 17.2°, also reported as characteristic of PVA crystallisation. The p-hydroxyl group in ferulic acid could promote the interchain bonds, affecting the orientation of the hydrogen bonds inside the unit cell.

Therefore, thermoprocessing and the incorporation of ferulic acid promoted the crystallisation of PVA; thermal treatment in the presence of phenolic acids, however, generally implied a reduction in crystallinity, probably due to the occurrence of partial hydrolyses in the PVA chains that affect the overall chain interactions in the matrix and the plasticisation of local regions where acids could become oversaturated, as observed in FESEM images.

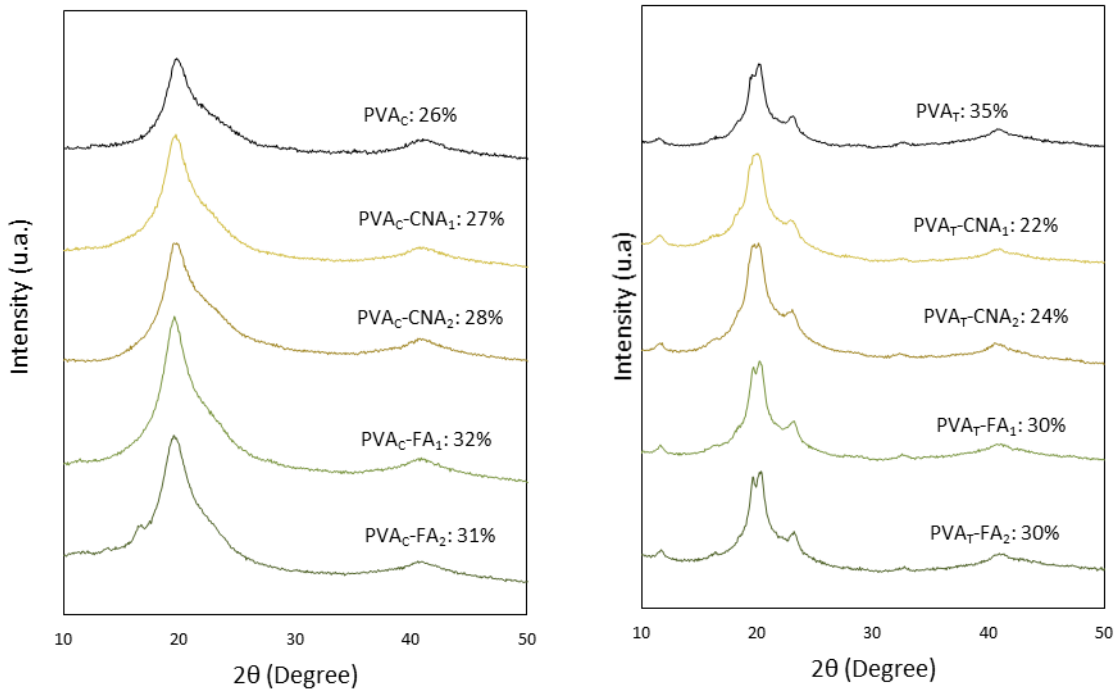

Figure 3. X-Ray diffraction spectra and percentage of crystallinity (%) of the cast (left) and thermo-processed (right) films with and without cinnamic (CNA) or ferulic acid (FA) at different concentrations (1 or 2 g/100 g PVA).

3.3. FTIR characterisation

The FTIR spectra of the active-free PVA films and those containing phenolic acids are shown in **Figure 4**.

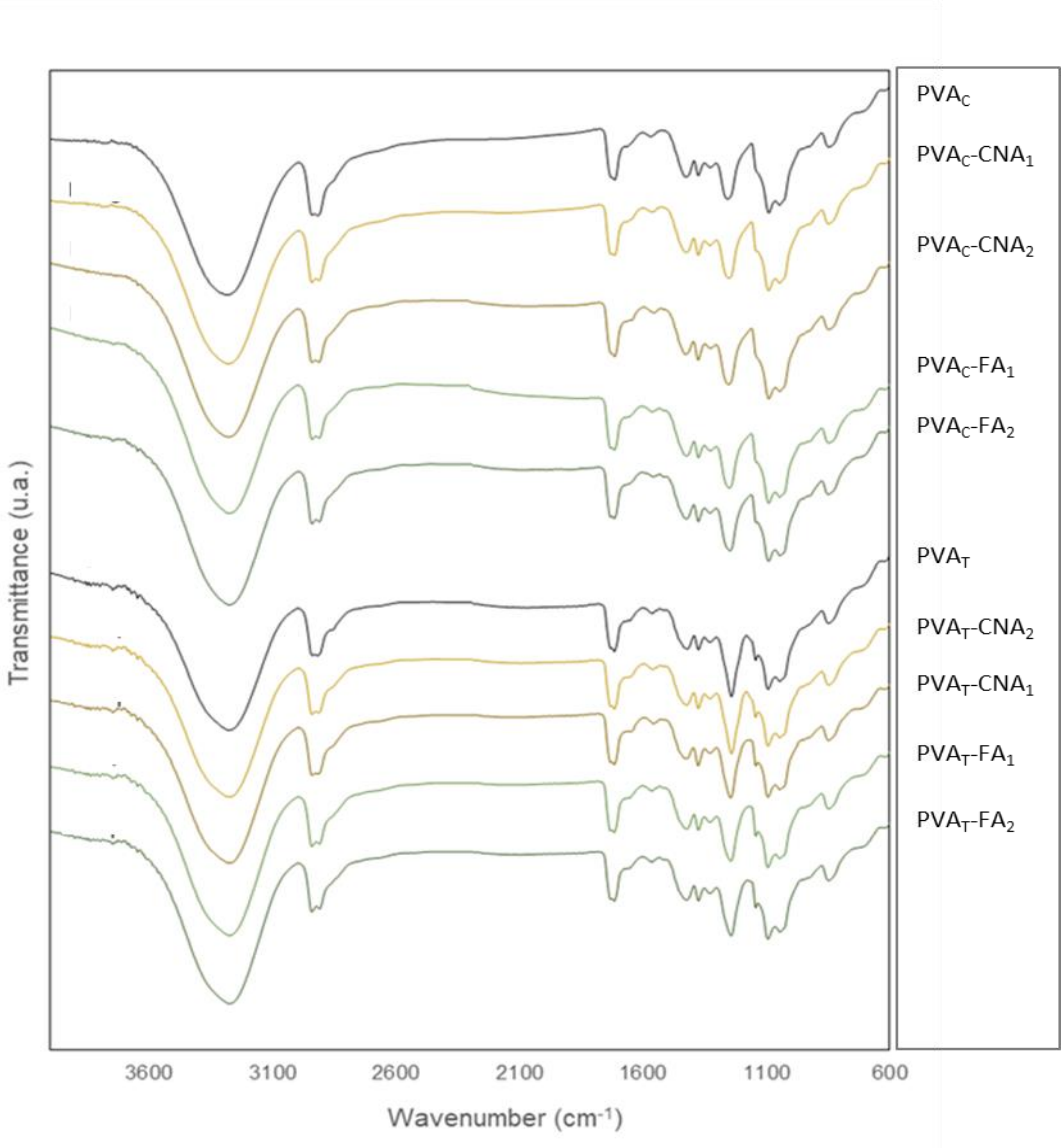

Figure 4. FTIR spectra of the PVA films without and with cinnamic (CNA) and ferulic (FA) acids at 1 or 2 g/100 g PVA obtained by casting (C) or thermo-processing (T).

The spectra of the films show the typical PVA bands, without being affected by the type of processing; a broad peak at 3270 cm^{-1} associated with intermolecular hydrogen bonding and hydroxyl stretching vibration (O-H) and consecutive peaks at 2930 cm^{-1} and 2900 cm^{-1} due to asymmetric and symmetric stretching vibrations. Peaks at 1410 cm^{-1}, 1310 cm^{-1}, 1080 cm^{-1} and 825 cm^{-1} are related to CH_2 bending, carbon skeleton movement (C-H), C-O and C-C stretching, respectively. The presence of residual acetate groups in the polymer chain is represented by peaks at 1737 cm^{-1}, 1706 cm^{-1}, 1365 cm^{-1} and 1250 cm^{-1} associated with the stretching vibration bands of the carbonyl and acetyl groups. The typical peaks of cinnamic acid (Chandran et al., 2006; Nolasco et al., 2009) and ferulic acid (Aarabi et al., 2016) were not detected in the FTIR spectra of the films containing these compounds, probably due to their low concentration in the matrices and the overlapping with the PVA peaks. Similar results were found by (Aragón-Gutiérrez et al., 2021) for EVOH films incorporating 0.25 and 1% (w/w) of ferulic acid.

3.4. Tensile properties

The thickness values and tensile and barrier properties of the films are shown in **Table 2**. The compression-moulded films were significantly thicker ($p<0.05$) than the cast films due to the higher surface solid density (2 and 4 g polymer per film, respectively, for cast and thermoprocessed films). The incorporation of PA reduced the film thickness in thermoprocessed samples, which may be attributed to the higher degree of flowability of the material during the thermocompression. This increase in the flowability might be due to the partial hydrolyses in PVA chains and the subsequent local plasticisation, as commented on in FESEM analyses. The tensile strength and elongation at break and stiffness of the PVA films were also influenced ($p<0.05$) by the method used to process the material. Thus, thermoprocessing generated 30% stiffer films, whereas the solvent-casting process promulgated the development of materials that were highly stretchable (E) and resistant to break (TS), and whose values almost doubled those obtained by their thermoprocessed peers. The greater stiffness of thermoprocessed films is coherent with their greater degree of crystallinity. In contrast, in the cast films, the polymer chains can unfold and orient themselves throughout the drying step, thus giving rise to a network with more oriented chains, which enhanced chain slippage during film stretching, favouring the film extensibility and, subsequently, the resistance to break. During melt blending and compression moulding, the greater viscosity of the melt limited the opportunities for the polymer chain orientation, thus promoting the development of less stretchable, more brittle matrices. So, the two film-processing methods generated materials with different molecular arrangements and degrees of crystallinity that would present different degrees of impediment in the sliding of the molecular chains, giving rise to materials of varying stiffness, elongation and resistance.

Moreover, differences in the thickness and moisture contents of the films will also affect their tensile behaviour.

Table 2. Thickness, tensile strength (TS), elongation at break (E%) and elastic modulus (EM), water vapour permeability (WVP) and oxygen permeability (OP) of the PVA films without and with cinnamic CNA and ferulic FA acids (1 or 2 g/100 g PVA) obtained by casting (C) or thermo-processing (T). Mean values and standard deviation.

Sample	Thickness (μm)	Tensile strength TS (MPa)	Elongation E (%)	Elastic modulus (MPa)	WVPx10^3 (g/m^2.h. kPa)	OPx10^8 (cm^3/m.h. kPa)
PVA_{P-C}	104 (7) ^{a,1}	47 (9) ^{a,2}	111 (9) ^{a,2}	49 (3) ^{a,1}	8.2 (0.7) ^{b,1}	13.0 (2.0) ^{b,1}
PVA_{P-C}-CNA_1	118 (6) ^{b,1}	47 (12) ^{a,2}	107 (8) ^{a,2}	52 (3) ^{ab,1}	7.0 (.02) ^{ab,1}	12.0 (1.0) ^{ab,1}
PVA_{P-C}-CNA_2	124 (4) ^{b,1}	44 (10) ^{a,2}	107 (8) ^{a,2}	53 (4) ^{bc,1}	8.0 (0.5) ^{b,1}	11.8 (0.4) ^{ab,1}
PVA_{P-C}-FA_1	105 (8) ^{a,1}	53 (6) ^{ab,2}	111 (4) ^{a,2}	56 (5) ^{c,1}	6.5 (0.5) ^{ab,1}	10.0 (0.9) ^{a,1}
PVA_{P-C}-FA_2	103 (11) ^{a,1}	58 (6) ^{b,2}	109 (6) ^{a,2}	57 (4) ^{c,1}	6.5 (0.2) ^{a,1}	11.3 (1.1) ^{ab,1}
PVA_{P-T}	204 (17) ^{b,2}	25 (2) ^{b,1}	58 (7) ^{ab,1}	68 (4) ^{c,2}	11.4 (0.8) ^{b,2}	30 (4) ^{c,2}
PVA_{P-T}-CNA_1	189 (14) ^{ab,2}	23 (3) ^{ab,1}	55 (11) ^{a,1}	63 (2) ^{b,2}	10.5 (0.3) ^{ab,2}	24 (1) ^{b,2}
PVA_{P-T}-CNA_2	186 (8) ^{a,2}	20 (2) ^{a,1}	57 (4) ^{ab,1}	55 (7) ^{a,1}	9.1 (0.5) ^{a,2}	20 (2) ^{ab,2}
PVA_{P-T}-FA_1	183 (12) ^{a,2}	22 (3) ^{a,1}	64 (7) ^{ab,1}	52 (2) ^{a,1}	10.2 (0.9) $^{ab.2}$	17 (2) ^{a,2}
PVA_{P-T}-FA_2	182 (13) ^{a,2}	23 (3) ^{a,1}	66 (5) ^{b,1}	53 (6) ^{a,1}	9.6 (1.4) ^{a,2}	17 (2) ^{a,2}

Different superscript letters indicate significant differences between formulations within the same processing method, while different numbers indicate significant differences between formulations with equivalent mass fractions but processed by another method ($p < 0.05$).

The incorporation of PA did not significantly affect ($p > 0.05$) the elongation at break of the films. However, changes in the stiffness and mechanical resistance were observed, depending on the processing method. In cast films, PA increased stiffness and strength of the films, while the opposite behaviour was observed in thermoprocessed films probably due to the above-mentioned effect of hydrolyses. The established polymer-PA interactions (interchain bonds through the chain hydroxyls and carboxyl and phenol groups) seem to enhance the load mechanical parameters (TA, EM) in cast films, although this effect overlapped with the potential effect of hydrolyses in thermoprocessed films, giving rise to weakened matrices. In every case, ferulic acid promoted the film elastic modulus and resistance to break to a greater extent, regardless of its concentration, while cinnamic acid only provoked a significant effect when incorporated at the highest concentration. This greater effect of ferulic could be attributed to its specific molecular structure, with a p-hydroxyl group, that better allows for interchain bonds and the crosslinking effect.

3.5. Barrier properties

PVA films present good barrier capacity against oxygen due to its polar nature and the low oxygen solubility in the matrix, which limits permeability. The highly cohesive nature of the polymer matrix due to the interchain hydrogen bonds also contributes to a reduction in the oxygen permeability through the limitation of molecular mobility and diffusion. In contrast, given the high degree of polarity of the polymer, the water molecules in the matrix are highly soluble, which favours their permeation through the matrix.

As expected, the values of OP (13.10^{-8} cm^3 / m.h.kPa) and WVP ($8.2.10^{-3}$ g / m^2.h.kPa) of the phenolic-free, glycerol-plasticised PVA cast films (Pc) were higher than those reported for non-plasticised cast films (Andrade et al., 2020b). This is coherent with the plasticising effect of glycerol in the polymer matrix which leads to weaker interactions between the chains and, therefore, to greater molecular mobility and free volume in the polymer system (Hedenqvist, 2012).

As can be observed in **Table 2**, thermoprocessed PVA films exhibited significantly worse barrier properties ($p<0.05$) than cast films despite their higher crystallinity values, which could be explained by the better packing of oriented chains in cast films, which inhibited molecular diffusion. Similar results were found by Moreno et al. (2017) for starch-gelatine films obtained by both casting and by compression moulding.

Although the barrier properties of the films were only slightly improved by the incorporation of PA, this effect was more marked in thermoprocessed films, where WPV was reduced by up to about 10% and OP by up to 43%, when ferulic acid was incorporated. The hydrogen bonds and Lewis adducts established between PA and the PVA chains may contribute to a reduction in molecular mobility, thus reducing the rate of molecule transport throughout the matrix. In addition, the antioxidant nature of phenolic acids could also contribute to the reduction in oxygen molecule transport, improving the barrier capacity of the material. Ferulic acid was more effective than cinnamic acid at improving the barrier properties of the PVA films, especially the oxygen barrier capacity. Its molecular structure (with a p-hydroxyl group), which allows for interchain bonds, the promotion of crystallinity and a higher antioxidant capacity than cinnamic acid (Li et al., 2021), better contributes to an enhancement in the oxygen barrier properties of the films than cinnamic acid. This trend has also been reported for EVOH films with 0.25% and 0.5% of ferulic acid, which caused a decrease (25% and 28%, respectively) in the values of the oxygen transmission rate of the films (Aragón-Gutiérrez et al., 2021).

3.6. Optical properties of the films

The colour parameters (lightness (L*), Chroma (Cab*) and hue (hab*)), and internal transmittance pattern, used as a transparency indicator, of the different samples are shown in **Table 3** and **Figure 5**, respectively.

Table 3. Lightness (L*), chrome (Cab*), hue (hab*) and internal transmittance values at 460 nm (Ti) of the cast and thermoprocessed films of partially hydrolysed PVA films.

Sample	L*	Cab*	hab*	Ti (460 nm)
$PVA_{P\text{-}C}$	90 (1) ^{a,1}	3.0 (0.1) ^{a,1}	102 (1) ^{c,1}	0.86 (0.01) ^{c,1}
$PVA_{P\text{-}C}$-CNA_1	90 (1) ^{a,1}	3.0 (0.1) ^{a,1}	102 (1) ^{c,1}	0.86 (0.01) ^{c,1}
$PVA_{P\text{-}C}$-CNA_2	90 (2) ^{a,1}	2.9 (0.1) ^{a,1}	102 (2) ^{c,1}	0.86 (0.01) ^{c,1}
$PVA_{P\text{-}C}$-FA_1	89 (2) ^{a,1}	5.9 (0.4) ^{b,1}	99 (1) ^{b,2}	0.84 (0.01) ^{b,2}
$PVA_{P\text{-}C}$-FA_2	89 (1) ^{a,2}	5.6 (0.6) ^{b,1}	97 (1) ^{a,1}	0.83 (0.01) ^{a,2}
$PVA_{P\text{-}T}$	90 (1) ^{c,1}	3.0 (0.1) ^{a,1}	102 (1) ^{d,1}	0.86 (0.01) ^{c,1}
$PVA_{P\text{-}T}$-CNA_1	90 (1) ^{c,1}	4.6 (0.3) ^{b,2}	102 (1) ^{cd,1}	0.86 (0.01) ^{c,1}
$PVA_{P\text{-}T}$-CNA_2	89 (1) ^{c,1}	4.5 (0.3) ^{b,2}	101 (1) ^{c,1}	0.86 (0.01) ^{c,1}
$PVA_{P\text{-}T}$-FA_1	88 (1) ^{b,1}	10 (1) ^{c,2}	97 (1) ^{b,1}	0.80 (0.01) ^{b,1}
$PVA_{P\text{-}T}$-FA_2	86 (1) ^{a,1}	19 (2) ^{d,2}	96 (1) ^{a,1}	0.74 (0.01) ^{a,1}

Different superscript letters indicate significant differences among formulations within the same processing method, while different numbers indicate significant differences between formulations with equivalent mass fractions but processed by another method ($p < 0.05$).

The incorporation of phenolic acids, especially ferulic, significantly reduced the hue values and increased the chrome, leading the film towards a more saturated yellowish appearance, regardless of the processing method. Nevertheless, thermoprocessed films incorporating PA were more saturated in colour and darker than cast films, which could be derived from the partial oxidation of acids under the thermal processing conditions. This also affected the loss of transparency (mainly at low wavelengths: 400 and 500 nm) of the films with ferulic acid, especially in the case of 2% ferulic, thermoprocessed films.

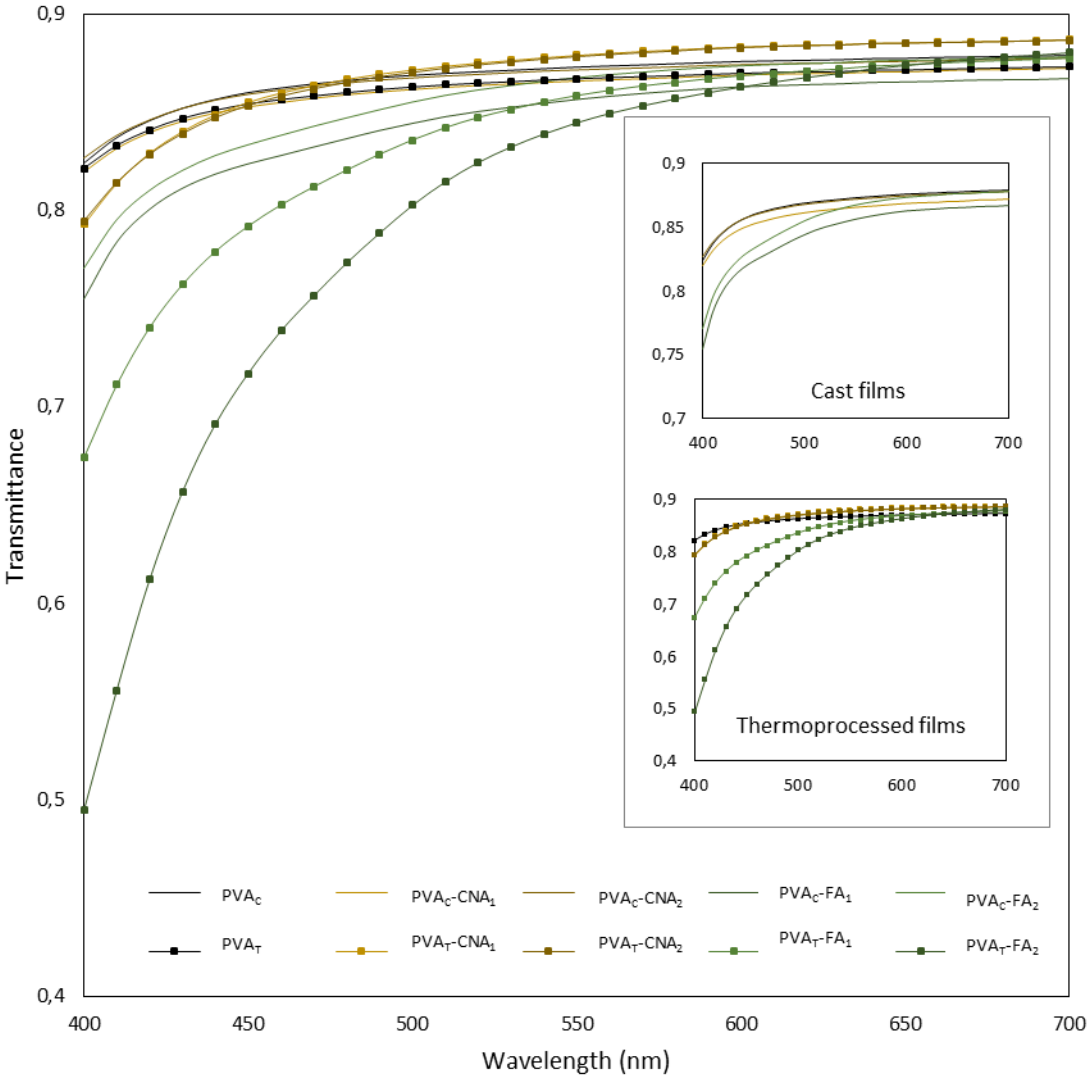

Figure 5. Internal transmittance pattern of cast (C) and thermoprocessed (T) films of partially hydrolysed PVA films with the incorporation of cinnamic (CNA) and ferulic (FA) acid (1 or 2 g/100 g PVA).

3.7. Thermal behaviour

The first and first and second order thermal transitions, associated with semi-crystalline materials, were analysed from the second heating scan of the DSC analyses, as shown in **Figure 6**. The values of glass transition temperature, melting temperature and melting enthalpy are shown in **Table 4**.

The thermal properties of the films were affected by the incorporation of glycerol in the polymeric matrix, which produces significantly lower Tg and Tm values than those reported for non-plasticised films, (Tg: 56ºC, Tm: 168ºC). This effect is coherent with the plasticising

effect of glycerol in the amorphous fraction and the formation of smaller crystals, due to the interference of glycerol in the crystallisation of the polymer (Andrade et al., 2020a). In general, the processing method did not generate significant effects on the melting behaviour of the materials. Nevertheless, the phenolic-free films obtained by thermo-processing (PVA_T) presented higher enthalpy values than films obtained by casting (PVA_C), in line with their higher crystallinity, as deduced from DRX spectra.

No remarkable changes were observed in the Tg, Tm or ΔH values of the films when incorporating PA. Nevertheless, the glass transition temperature (Tg) in both cast and thermoprocessed films decreased ($p<0.05$) when using the highest concentration of ferulic acid. This suggests a certain plasticising effect on the amorphous fraction of the material, as previously reported for EVOH films incorporating different concentrations of ferulic acid and in thermoprocessed starch films with PA (Aragón-Gutiérrez et al., 2021). The partial hydrolysis caused by acids under the process conditions could explain this mild plasticising effect.

Table 4. Glass transition (Tg), melting temperature (Tm) and enthalpy (ΔH) of the PVA films without and with phenolic acids (1 or 2 g/100 g PVA). Mean values and standard deviation.

Sample	Second heating scan		
	Tg (ºC)	Tm (ºC)	ΔH (J/g PVA)
$PVA_{P\text{-}C}$	37 (3) ^{b,1}	154 (3) ^{a,1}	24 (1) ^{a,1}
$PVA_{P\text{-}C}$-CNA_1	37 (2) ^{b,1}	157 (1) ^{b,1}	21 (3) ^{a,1}
$PVA_{P\text{-}C}$-CNA_2	35 (1) ^{ab,1}	157 (1) ^{ab,1}	24 (3) ^{a,1}
$PVA_{P\text{-}C}$-FA_1	38 (1) ^{b,1}	156 (1) ^{ab,1}	23 (3) ^{a,1}
$PVA_{P\text{-}C}$-FA_2	33 (2) ^{a,1}	156 (1) ^{ab,1}	21 (1) ^{a,1}
$PVA_{P\text{-}T}$	37 (1) ^{bc,1}	158 (1) ^{a,1}	27 (2) ^{b,2}
$PVA_{P\text{-}T}$-CNA_1	39 (1) ^{cd,1}	158 (1) ^{a,1}	22 (1) ^{a,1}
$PVA_{P\text{-}T}$-CNA_2	36 (1) ^{ab,1}	161 (2) ^{a,2}	25 (2) ^{ab,1}
$PVA_{P\text{-}T}$-FA_1	39 (1) ^{d,1}	159 (1) ^{a,2}	28 (1) ^{b,2}
$PVA_{P\text{-}T}$-FA_2	35 (1) ^{a,1}	158 (2) ^{a,1}	24 (4) ^{ab,1}

Different superscript letters indicate significant differences among formulations within the same processing method, while different numbers indicate significant differences between formulations with equivalent mass fractions but processed by another method ($p<0.05$).

3.7.1. *Thermal degradation*

The thermal degradation of the samples was analysed using the curves of the thermogravimetric analysis (TGA) and its derivative (DTG), shown in **Figure 6**. The thermal degradation pattern, observed in the DTG curves, comprises multiple consecutive peaks corresponding to the degradation of the components in the polymeric matrix. The first stage corresponds to the vaporisation of the bonded water, which appeared at 62 °C and 82 °C in the casting (1.8% mass loss) and thermoprocessed (0.8% mass loss) films, respectively. The partial release of glycerol represents the second peak that occurred in the cast films (4% mass

loss) at 160 °C and the thermoprocessed films (2.5% mass loss) at 167 °C. The thermo-released glycerol was less than 10% wt., concentration initially incorporated, which suggests that a fraction of the plasticiser remained closely bonded to the polymer chains due to its high affinity. The polymer degradation occurred in several stages; the first stage was associated with the detachment of side groups (hydroxyl and acetyl) from the chains, giving rise to by-products, such as acetaldehyde, acetic acid and water (Perilla, 2007). In the cast films, the main polymer degradation peak occurred at 274 °C, while in thermoprocessed films this peak moved towards higher temperatures, reaching 285 °C. This behaviour coincides with that previously reported by other authors for other matrices, such as starch-gelatine and PLA (Moreno et al., 2017; Rhim et al., 2006), indicating the early formation of degradation compounds during thermoprocessing, which may be responsible for the colour changes of these films, as previously commented on. This also points to the different chemical composition of the materials after being partially hydrolysed and/or degraded during the thermal process. Two subsequent peaks of polymer degradation were observed between 350 °C and 650 °C, which were related to the degradation of low molecular weight products from the polymer backbone break, or of heavier structures formed in previous stages of degradation (Holland & Hay, 2001; Perilla, 2007).

The thermal degradation of pure phenolic acids, between 230 °C and 280 °C (peak: 267 °C) for cinnamic acid and between 210 °C and 270 °C (peak: 250°C) for ferulic acid, was not appreciated in the thermograms of the films, due to their low mass fraction and the fact that they overlapped with the polymer degradation events.

The incorporation of PA only affected the thermal degradation pattern of films with ferulic acid obtained by casting, regardless of its concentration. In these films, a 10 °C increment in the temperature of the main degradation peak of PVA was observed. This indicates that this phenolic acid interfered with the thermal stability of the polymer, probably due to the interaction between its carboxyl and phenolic hydroxyl group with the hydroxyl/acetyl groups of PVA chains, giving rise to more thermally stable cross-linked films with a larger crystalline fraction, as deduced from the XRD analysis. This effect was also reported in EVOH films incorporating ferulic acid in different proportions (Aragón-Gutiérrez et al., 2021) and in PVA films with tannic acid and quercetin (Luzi et al., 2019). These acids acted as natural stabilisers against polymer degradation, in response to their characteristic chemical structure with several phenolic rings with hydroxyl groups (Luzi et al., 2019).

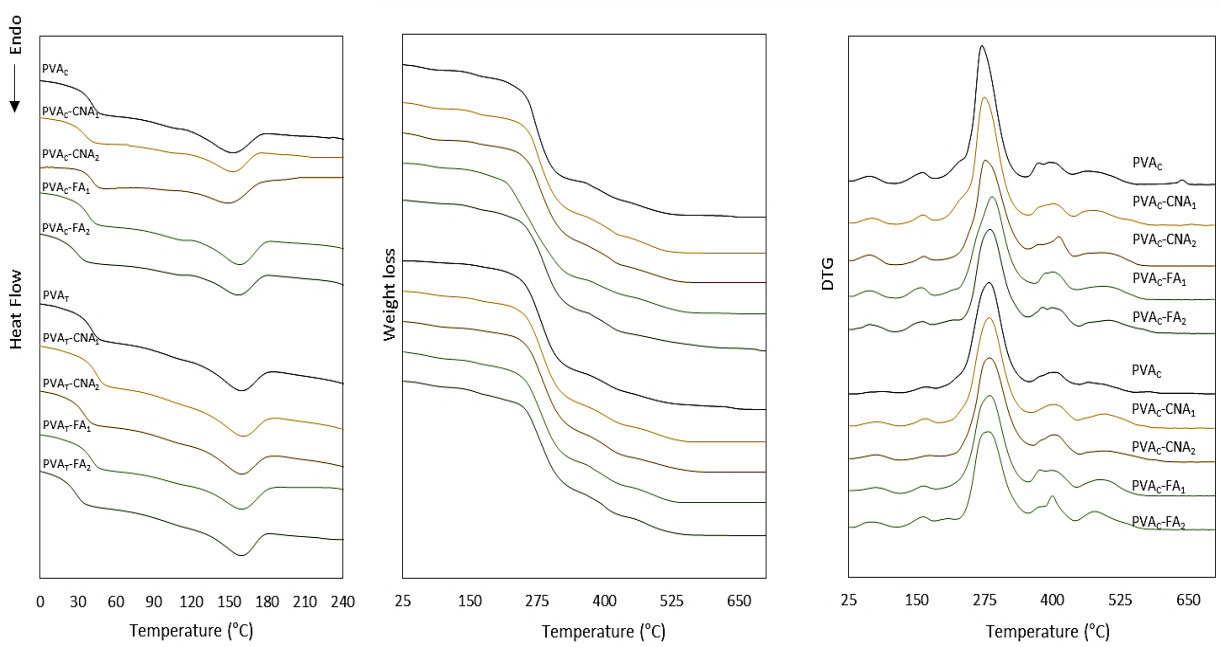

Figure 6. DSC (second heating scan) (left), thermogravimetric analysis (TGA) (middle) and DTGA (right) curves of the partially hydrolysed PVA films without and with cinnamic (CNA) or ferulic (FA) acid (1 or 2 g/100 g PVA).

3.8. Antioxidant and antimicrobial capacity of the films

The antioxidant capacity of the active films was evaluated through the inhibition power of the DPPH radical of the active compounds delivered from the films into food simulants: ethanol 50% (v/v) as a simulant of O/W emulsified foods and ethanol 95% (v/v), as a simulant of lipophilic foods. No significant radical scavenging activity was observed for the films with cinnamic acid; the inhibition values ranged between 0.5 and 2%. In contrast, the inhibition capacity of ferulic acid was very high, ranging between 32% and 81% as can be observed in **Figure 7**, depending on the film processing method and food simulant. This difference is directly related to the molecular structure, depending on the number and position of the hydroxyl groups on the benzene ring (Li et al., 2021). Thus, ferulic acid is an o-hydroxycinnamic acid with a methoxy group in the ortho position with respect to the hydroxyl in the benzene ring, a structural factor that greatly improves the hydrogen donation capacity of the phenol. The absence of a phenolic hydroxyl in the cinnamic acid structure reduces its antioxidant capacity (Li et al., 2021). Ferulic acid incorporated into EVOH films has also been proven to be highly effective at scavenging a DPPH radical (Aragón-Gutiérrez et al., 2021).

The inhibition values obtained for films containing ferulic acid are shown in **Figure 7**. These values will be related to the total amount of compound released from the films into the food simulant. This release depends on the solubility of the compound in the simulant and their

relative chemical affinity with both the polymer matrix and the solvent, according to the corresponding partition coefficient, as well as on the relaxation of the polymer in contact with the solvent, which promotes the release rate. As can be observed in **Figure 7**, the antioxidant capacity of the films was affected by the film processing method and the food simulant. Thus, the thermoprocessed films presented a lower radical scavenging capacity than the cast films, this being about 35% and 65% for films with 1% and 2% ferulic acid, respectively. This can be attributed to the partial oxidation of the compound during the thermal treatment, as commented on above, which reduced the radical inhibition capacity of the films. In cast films, the inhibition of DPPH was about 80 % in every case, except in the least polar simulant (ethanol 95%) when the films contain 1% of compound, where an inhibition of only about 56% was observed, probably due to the smaller amount of ferulic acid released.

Finally, the greatest antioxidant capacity was found in the most polar simulant (ethanol 50%) due to the greater affinity with the PVA matrix, which favoured its swelling, thus enhancing the release of phenolic acids available to inhibit the free radicals.

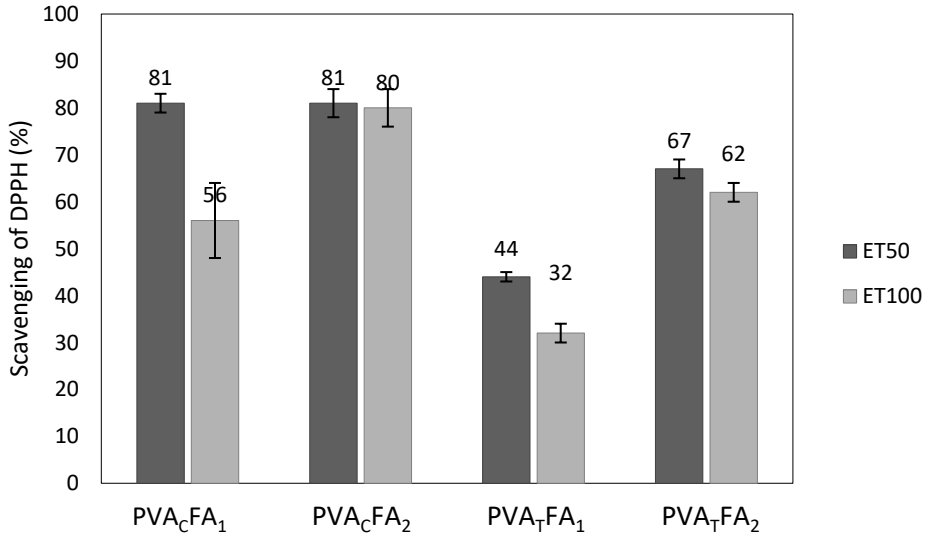

Figura 7. Antioxidant activity measured through the scavenging capacity of DPPH of two food simulants (50 and 95 % ethanol in water) in contact (48 h) with PVA films containing ferulic acid (FA) at 1 or 2 %, obtained by casting (C) or thermoprocessing (T).

The antimicrobial activity was only evaluated in the films with the greatest PA content (2%) by means of the disk difussion method. The diameter of the inhibition halo of each film disk are shown in **Table 5**, for *Escherichia coli* Gram (-) and *Listeria innocua* Gram (+), as the diameter of the growth inhibition zone around the film disk. This method reflects the diffusion capacity

of phenolic acids from the polymeric matrix and the sensitivity of each bacteria to compounds diffused into the agar.

The antibacterial activity of the films depended on the processing method, the type of PA incorporated and the bacteria. Thus, *L. innocua*, with inhibition halos of between 26 and 30 mm, was more sensitive to the antimicrobial effect of the active films than *E.coli,* which exhibited smaller inhibition diameters. Ordoñez et al., (2021) also reported this trend, when studying thermo-processed cassava starch films incorporating CNA and FA, coherent with the higher MICs of phenolic acids of *E. Coli*.

As regards the film processing method, the cast films seemed to be more effective at controlling the microbial growth than the thermoprocessed ($p<0.05$); this difference was only significant ($p<0.05$) when using CNA acid. So, in general, the greatest antibacterial activity was found for cast films, regardless of the PA incorporated. In thermoprocessed films, those with ferulic acid exhibited greater inhibition halos ($p<0.05$) than those with cinnamic acid, despite the higher degree of oxidation of ferulic acid in the films and the lower MIC of cinnamic acid for both bacteria (Ordoñez et al., 2021). This suggests that the oxidated compounds of ferulic acid could also exhibit an antimicrobial effect (Aljawish et al., 2014).

Table 5. Diameters of inhibition halos (mm) of bacterial growth obtained in the agar difussion test for film disks containing ferulic acid (FNA) or cinnamic acid (CA) of cast (C) and thermoprocessed (T) films.

Sample	Inhibition halo	
	***L. Innocua* (mm)**	***E. coli* (mm)**
$PVA_{P\text{-}C}$-CNA_2	29 (2) ^{a,2}	15 (3) ^{a,2}
$PVA_{P\text{-}C}$-FA_2	30 (2) ^{a,1}	13 (2) ^{a,1}
$PVA_{P\text{-}T}$-CNA_2	26 (1) ^{a,1}	6 (2) ^{a,1}
$PVA_{P\text{-}T}$-FA_2	29 (2) ^{b,1}	10 (2) ^{b,1}

Different superscript letters indicate significant differences among formulations within the same processing method, while different numbers indicate significant differences between formulations with equivalent mass fractions but processed by different methods ($p <0.05$).

4. CONCLUSION

The incorporation of cinnamic and ferulic acids into the PVA films significantly modified the physicochemical and functional properties of the films. These changes were affected by the processing method (casting or thermoprocessing) and the type and concentration of phenolic acid. Thus, the addition of these active compounds improved the barrier properties of the films, especially when incorporating ferulic acid, which favours a greater degree of crosslinking in the matrix and polymer crystallinity. As concerns the processing method, cast films were more extensible and resistant to break, although thermoprocessed films were stiffer with a lower barrier capacity against water vapour and oxygen. In none of the cases was the thermal stability of the material compromised, while ferulic acid enhanced the thermal stability of the polymer in cast films. The PVA films with ferulic acid exhibited a remarkable antioxidant capacity and films with both ferulic and cinnamic acids had antilisteria properties. These results indicate that PVA films with ferulic or cinnamic acid, with modulated mechanical, barrier and antioxidant and antibacterial properties can be obtained by using casting or thermoprocessing, depending on the target application. Therefore, these are of great potential in the development of different materials for active food packaging purposes, such as coatings or films.

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CHAPTER V. Antimicrobial PLA-PVA multilayer films containing phenolic compounds

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ABSTRACT

Multilayer materials with good interlayer-adhesion were obtained by thermocompression for laminating an internal poly (vinyl alcohol) (PVA) layer with two external poly (lactic acid) (PLA) layer. Carvacrol (CA) or ferulic acid (FA) were incorporated into the internal PVA sheet to obtain active materials. The multilayer films were characterised as to their microstructure, thermal behaviour, tensile and barrier properties. Furthermore, the antimicrobial capacity of the packaging materials was analysed in packaged beef meat samples for 17 days at 5 ^{o}C, through the total viable counts, psychrotrophic bacteria, total coliforms and *Escherichia coli*. Thermocompression was successfully used to obtain the three-layer films with good layer adhesion, while the process promoted the inter-sheet migration of plasticisers and active compounds to a different extent, which slightly affected their functional properties as packaging material. The laminates exhibited tensile properties close to those of the PLA films, but with enhanced stretchability. Compared to the monolayers, the barrier capacity of multilayers was much improved by combining polyester and PVA layers, which provide the laminate with water vapour and oxygen barrier capacity, respectively. Active containing multilayers were effective at controlling microbial growth in beef meat during cold storage. Therefore, the materials developed were functionally adequate for food packaging purposes and successfully promoted the meat preservation.

keywords

Carvacrol; ferulic acid; food packaging; barrier capacity; antimicrobial capacity.

1. INTRODUCTION

The food industry is constantly working on new methods of transformation and preservation, in order to guarantee products with high quality and safety standards throughout the production, storage and distribution chain. During food storage, some negative physicochemical and microbiological phenomena trigger changes that compromise the safety and cause odour, colour or texture defects, which finally lead to its degradation (Sousa Gallagher et al., 2011). Microbial deterioration is the result of various metabolic pathways, such as those involved in energy production or the synthesis of molecules essential for bacterial growth. This mechanism depends both on the microorganisms initially present and on their biotic and abiotic interactions (Zagorec & Champomier-Vergès, 2017). Kerry & Tyuftin (2017) stated that abiotic conditions, such as storage temperature or the packaging system, exert a selective pressure on microbial growth in food products. In this sense, packaging systems can extend the shelf life of food by slowing down the multiple, complex metabolic processes involved in its deterioration. To this end, several packaging strategies have been applied, including the use of modified atmosphere packaging (MAP), high barrier materials and active packaging (Kerry & Tyuftin, 2017).

The packaging properties can be significantly enhanced by combining or joining different plastics together by laminated constructions or through the co-extrusion process. Films containing layers of different materials provide desirable properties, such as high barrier, mechanical strength or heat sealing, that no single material possesses (Gherardi et al., 2016). The most commonly-used plastics in the food industry are polyethylene (various types based on molecular weight), polypropylene (PP), ethylene vinyl acetate, polyvinyl chloride (PVC), polyvinylidene chloride (PVDC), polyamide (PA), polyethylene terephthalate (PET) and polystyrene (PS) (Kerry & Tyuftin, 2017). Nevertheless, in the context of the environmental problems associated with the generation of huge amounts of plastic waste, the transition towards the use of biodegradable packaging is preponderant. The combination of biodegradable polymers, both polar and nonpolar in nature, have been used to obtain high barrier multilayer systems, in order to prevent the excessive exchange of gases between the packaged food and the external atmosphere. Some examples include starch films with electrospun PCL fibres (Tampau, González-Martínez, & Chiralt, 2020; Trinh et al., 2021), starch and PLA bilayers (Muller et al., 2017), starch and PLA-PHBV-PEG blend bilayers (Hernández-García et al., 2021; Requena et al., 2018), where the benefits of combining polar and non-polar polymer sheets has been proved.

The incorporation of active compounds into packaging systems has generated great interest in the food sector due to its ability to extend the shelf life of foods (Cruz-Romero et al., 2013). These compounds can either be incorporated into the packaging material directly or through sachets or labels; they may also directly coat the surface of the container in contact with the food. Furthermore, multilayer films of packaging materials have also been proposed as a

means of controlling the release of active compounds from the packaging towards the packaged product. Of the active compounds under analysis, phenolic compounds, such as some major constituents of essential oils, are some of the most widely studied and have remarkable broad-spectrum antimicrobial activity (Sharma et al., 2020). For the purposes of using these compounds in food systems, they are often previously encapsulated due to their volatility, heat sensitivity and organoleptic effect on food (Delshadi et al., 2020). With less sensory impact and remarkable antioxidant and antimicrobial activity, the use of phenolic and hydroxycinnamic acids in food applications has also been the subject of increasing interest (Aragón-Gutiérrez et al., 2021; Ordoñez et al., 2021). The antimicrobial effectiveness of phenolic compounds is related to the number and position of hydroxyl groups in the phenolic ring (Kachur & Suntres, 2020), since it has been shown that they can act as a proton exchanger, thus reducing the gradient across the cytoplasmic membrane and, consequently, resulting in the collapse of the proton motive force and the depletion of the ATP reserve, leading to cell death (Ben Arfa et al., 2006; Veldhuizen et al., 2006).

Poly (vinyl alcohol) PVA is a biodegradable polymer of great potential use in food packaging because it is colourless, non-toxic, transparent and has high oxygen barrier capacity. PVA films can be obtained by casting the polymer solution or by thermo-processing, depending on their molecular characteristics (molecular weight and degree of chain acetylation) (Andrade et al., 2020a). PVA has the ability to incorporate a high variety of active compounds, such as carvacrol, ferulic and cinnamic acid, into its matrix to develop active films (Andrade et al., 2020b, 2021, 2021b). However, because of the high degree of hydroxylation of its molecular chain, the PVA- based materials are highly permeable to water vapour and have a high degree of water sensitivity and solubility. These characteristics limit its use for direct contact with high moisture or water activity foods. However, PVA lamination with materials such as biodegradable polyesters is an effective alternative method to obtain materials with adequate barrier properties for food packaging. Poly (lactic acid) PLA is a biodegradable polyester suitable for food application due to its high moisture barrier capacity, mechanical resistance and biocompatibility (Muller et al., 2017). However, the high degree of rigidity of the material makes the use of plasticisers necessary to improve its stretchability.

The aim of this study was to develop and characterise high barrier capacity multilayer materials combining PLA and PVA films with active properties for food packaging purposes. To this end, an inner PVA sheet, incorporating phenolic compounds, such as carvacrol, lecithin-encapsulated carvacrol or ferulic acid, was laminated with two external sheets of PLA plasticised with PEG1000. The multilayer materials were characterised both as to their functional characteristics as packaging and to their antimicrobial performance in packaged beef meat samples cold stored at 5^{o}C.

2. MATERIALS AND METHODS

2.1. Materials

Amorphous PLA 4060D (density of 1.24 g/cm^3 and average molecular weight of 106,226 D with 40% of low molecular weight fraction (275 D) as reported by (Muller et al., 2017), was supplied by Natureworks (U.S.A). Partially hydrolysed poly (vinyl alcohol) (PVA) (Mw 13,000–23,000; 87–89% hydrolysed), glycerol (GLY), poly (ethylene glycol) with a molecular weight of 1,000 Da (PEG), as plasticisers and phenolic compounds, carvacrol (CA) and ferulic acid (FA), were purchased from Sigma-Aldrich (Steinheim, Germany). Soy lecithin with 72% phosphatidylcholine Lipoid S75 (SL-PC) was supplied by Lipoid GmbH (Ludwigshafen, Germany). Magnesium nitrate ($Mg(NO_3)_2$), phosphorus pentoxide (P_2O_5) salts, UV-grade methanol and UV-grade ethanol were supplied by Panreac Química S.A. (Barcelona, Spain). The microbiological media (Maximum Recovery Diluent, Plate Count Agar (PCA), M RS Agar, Brilliance™ *E-coli*/Coliform Selective Medium), were supplied by Oxoid (Oxoid Ltd., Basingstoke, England). Tryptic Soy Agar and Yeast extract granulate were supplied by Merck (Merck KGaA, 64271 Darmstadt, Germany).

2.2. Processing of multilayer films

Multilayer materials were composed of two outer sheets of PLA containing an internal layer of poly (vinyl alcohol) PVA with and without active compounds (carvacrol, lecithin encapsulated carvacrol or ferulic acid). The different monolayer films were previously obtained and afterwards assembled by thermocompression

2.2.1. *Carvacrol-loaded PVA films*

Carvacrol-loaded films were prepared by casting (C), considering the volatility and thermal sensitivity of carvacrol. Carvacrol was incorporated by direct emulsification (sample PVA_C-CA) or lecithin encapsulated (sample PVA_C-LCA). Liposomes were prepared with a SL-PC lecithin, following the method described by Andrade et al. (2020). The polymer solutions (PVA 10% wt.) were prepared in distilled water using magnetic stirring (1,200 rpm) at 100 ^oC for 3 h. Carvacrol, directly emulsified (CA) or encapsulated in liposomes (LCA), was incorporated into the polymer solutions to reach 10% wt. with respect to the polymer. Carvacrol-free films (PVA_c and PVA_cL) were also prepared as controls. All of the formulations were degassed by using a vacuum pump and spread evenly onto Teflon plates of 150 mm in diameter, using a constant equivalent mass of polymer per plate of 4 g. The films were dried under controlled temperature (25 ± 2 ^oC) and relative humidity (54 ± 2%) for 48 h.

2.2.2. *Ferulic acid loaded PVA films.*

Films containing ferulic acid (FA) were obtained by the melt blending and compression moulding of pellets using a hot plate-press (Model LP20, Labtech Engineering, Thailand) (Thermoprocessing (T)). The pellets were obtained by melt blending the different components (PVA:GLY:FA at weight ratio 100:10:2) by using an internal mixer (HAAKE™ PolyLab™ QC, Thermo Fisher Scientific, Germany) at 160 °C and 50 rpm, for 10 min and the subsequent cold grinding of the mixture in a refrigerated batch mill (Model M20, IKA, Germany). The conditioned pellets (25 °C and 53% RH) were placed on Teflon sheets, preheated for 3 min at 160 °C, afterwards compressed at 100 bar and 160 °C for 3 min; thereafter, a 3 min cooling cycle was applied until the temperature reached approximately 70 °C. Ferulic acid-free films were also obtained and used as control films.

The PVA films were conditioned at 53% relative humidity by using $Mg(NO_3)_2$ over-saturated solution until used. **Table 1** shows the different multilayer film composition with the respective mass fractions of the components.

2.2.3. *PLA films*

PLA films (P) were obtained by the melt blending and compression moulding of PLA and PEG (10% wt. with respect to polymer) as plasticiser. The melt blending of PLA:PEG process was carried out in an internal mixer (HAAKE™ PolyLab™ QC, Thermo Fisher Scientific, Germany) at 150 °C, rotor speed 50 rpm, for 8 min. After processing, the blends were cold ground in a refrigerated batch mill (Model M20, IKA, Germany) and conditioned at 25 °C and 0% RH. The conditioned pellets (3 g) were placed on Teflon sheets and preheated at 160 °C for 3 min in a hot plate press (Model LP20, Labtech Engineering, Thailand). The films were obtained by compression moulding at 160 °C and 150 bars for 3 min, with a final cooling cycle of 3 min. The films thus obtained were kept in a desiccator at 0%RH until used.

2.2.4. *Assembly of multilayer films*

The multilayer assembly PLA-PVA-PLA was carried out by compression moulding in a hydraulic press (Model LP20, Labtech Engineering, Thailand), preheated at 110 °C for 2 min, and subsequently heated at 110 for 3 min, followed by a cooling cycle of 3 min until 70 °C. All multilayer films were stored at 25 °C and 53% RH.

Table 1. Nominal mass fraction of different components in monolayers and theoretical and measured thickness values of multilayer films. Mean values and standard deviations.

Sample	PLA (P)		PVA					Thickness (μm)	
	x_{PLA}	x_{PEG}	x_{PVA}	x_{GLY}	x_{LEC}	x_{CA}	x_{FA}	Theoretical*	Measured
P-PVA_C	0,91	0,09	1	-	-	-	-	410 (17)	358 (14) ^{ab}
P-PVA_C-CA			0.91	-	-	0.09	-	427 (13)	361 (19) ^{ab}
P-PVA_C-L			0.91	-	0.09	-	-	438 (18)	374 (21) ^{bc}
P-PVA_C-LCA			0.84	-	0.08	0.08	-	436 (21)	362 (31) ^{ab}
P-PVA_T			0.91	0.09	-	-	-	427 (21)	338 (13) ^{a}
P-PVA_T-FA			0.89	0.09	-	-	0.02	436 (18)	346 (18) ^{ab}

Different superscript letters within the same column indicate significant differences between films (P<0,05).

*Sum of thickness values of individual monolayers

2.3. Characterisation of the multilayer films

2.3.1. *Microstructural analysis*

The microstructure of the samples was observed using a Field Emission Scanning Electron Microscope (FESEM) (ZEISS®, model ULTRA 55, Germany), at an acceleration voltage of 2 kV. The multilayers were previously cryo-fractured by immersion in liquid nitrogen and coated by platinum sputtering to observe the cross-section.

2.3.2. *Thermal behaviour*

The thermal behaviour of the materials was assessed by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). For DSC measurements, each sheet was carefully separated from its corresponding multilayer, the sample (about 7-8 mg) were placed in hermetically closed aluminium crucibles and analysed in a differential scanning calorimeter (DSC 1 stareSystem, Mettler Toledo, Schwarzenbach, Switzerland). An empty aluminium pan was used as reference. The temperature scanning profile for PVA sheets was a first heating from -25 °C to 250 °C, holding this temperature for 2 min, followed by a cooling to -25 °C, holding this temperature for 2 min and a second heating to 250 °C. For PLA sheets, the scanning profile was a first heating from -25 °C to 200 °C, holding this temperature for 5 min, followed by a cooling to -10 °C, holding this temperature for 5 min and a second heating to 200 °C. Every scan was carried out at 10 C/min. The thermal degradation of multilayer materials was evaluated by the TGA analysis, the multilayer samples in alumina crucibles were heated from 25 °C to 700 °C at 10 °C/min, by using a thermogravimetric analyser (TGA/SDTA 851e, Mettler Toledo, Schwarzenbach, Switzerland). All of the measurements were taken in triplicate using a nitrogen flow of 10 mL/min.

2.3.3. *Tensile properties*

The tensile properties of the films were measured using a Universal Machine (Stable Micro Systems, TA.XT plus, Haslemere, England). Equilibrated test specimens (25 mm x 100 mm) were mounted in the film extension grips with an initial separation of 50 mm and stretched at 50 $mm.min^{-1}$ until break, following the standard method, ASTM D882-02 (ASTM, 2002). Elastic modulus (EM), tensile strength (TS), and elongation at break point (E) were determined from the tensile stress (σ) vs. Hencky strain (εH) curves. The measurements were taken from eight samples of each treatment.

2.3.4. *Equilibrium moisture and barrier properties*

The moisture content of the previously conditioned films (53% RH; 25 °C) was analysed in triplicate using the gravimetric method. Four samples per treatment were dried in a convection oven (JP Selecta S.A., Barcelona, Spain) at 60 °C for 24 h and, subsequently, these were conditioned at 0% RH and 25 °C, until constant weight.

The water vapour permeability (WVP) was analysed in triplicate following a standard method, E96/E95M-05 (E. ASTM, 2003). In order to obtain a RH gradient of 53 to 100%, the samples were placed on Payne permeability cups (3.5 cm in diameter) (Elcometer SPRL, Hermelle/s Argentau, Belgium) containing 5 mL of distilled water, which were placed inside a desiccator at 25 °C with an oversaturated $Mg(NO_3)_2$ solution. The cups were weighed periodically every 1.5 h for 24 h using an analytical balance (0.00001 g). The slopes of the weight loss *vs.* time during the steady state period were determined by linear regression to calculate the water vapour transmission rate (WVTR). WVP was calculated as described by Cano, Jiménez, Cháfer, Gónzalez, & Chiralt (2014).

The oygen permeability (OP) in film samples (50 cm^2) was analysed in triplicate using an Ox-Tran system (Mocon, Minneapolis, US) at 23 °C and 53% RH, following a standard method, F1927-07 (F.-07 ASTM, 2004). The OP was calculated from the oxygen transmission rate multiplied by the average film thickness and divided by the partial pressure of oxygen. The film thickness was measured with a digital electronic micrometer (Palmer, COMECTA, Barcelona, Spain) to the nearest 0.001 mm at six random positions.

2.3.5. *Optical properties*

The CIE L∗a∗b∗ colour coordinates and the transparency of multilayer films were determined by measuring the reflectance spectra of the samples from 400 to 700 nm, on white and black backgrounds, by using a spectrocolorimeter (CM-3600d Minolta CO., Tokyo, Japan). The transparency was measured through the internal transmittance (Ti), applying the Kubelka-Munk theory for multiple scattering. The colour coordinates were obtained from the reflectance of a film of infinite thickness, as described by Sapper et al. (2018). Three measurements were taken from each film and three films were considered per formulation.

2.4. Packaging of beef in multilayer film bags.

Fresh beef fillets were purchased from a local supplier. To avoid cross contamination during sample preparation, all the materials and work surfaces were disinfected using 70% ethanol. Bags (40x60 mm) were made from multilayer films previously exposed to UV light for 15 minutes in a laminar flow cabinet (Airclean 600 STARLAB PCR Workstation, Airclean Systems, USA) to ensure the safety of the packaging. The samples (10 g) of beef fillet were individually placed into the bags and thermo-sealed. The packed samples were kept stored at 5 ^{o}C until their analyses. Three samples of each kind of multilayer bag were characterised as to the microbial counts and pH and colour coordinates at different times: 3, 7, 13 and 17days.

2.4.1. *Microbiological analysis of packaged beef*

The microbiological analysis of the packed beef fillets was carried out at different storage times: day 0 (before packaging) ,3, 7, 13 and 17. The meat sample was aseptically taken from the bag using sterile forceps and placed into a sterile stomacher strainer bag (Seward Limited, West Sussex, UK) to which 90 mL of buffered peptone water was added and mixed thoroughly for 3 minutes with a Masticator Paddle Blender (IUL S.A., Barcelona, Spain), to obtain a 10-fold primary dilution. From the homogenate, serial dilutions were made, using buffered peptone water, to enumerate the total viable count (TVC), psychrotrophic bacteria count (PBC), total coliform count (TCC) and *Escherichia coli* (*E. coli*) count. TVC and PBC were obtained on Plate Count Agar (PCA), after incubation at 37 °C or 4 °C for 48 h or 7 days, respectively. TCC and *E-coli* counts were evaluated on Coliform ChromoSelect agar plates after incubation at 37 °C for 48 h. The results of the counts were presented as Log_{10} colony-forming units per gram of sample (Log_{10} CFU/g).

2.4.2. *Determination of pH and colour parameters*

The pH of the beef samples was measured using a digital pH meter by direct insertion of the glass electrode probe into the beef fillet (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Six measurements were taken per sample (18 replicates).

The changes in colour of the packed beef with respect to the newly packaged sample, were determined from CIE-L*a*b* colour coordinates, using D65 illuminant/10° observer, at ten random points on the surface of the sample, using a portable Minolta CR-300 colorimeter (Minolta Camera Co., Osaka, Japan). The colour difference with respect to the newly packaged samples, $\Delta E^*_{a.b}$, was calculated using Equation (1), as described by (Rojas-Lema et al., 2020). The reported value is the average of 30 replicates.

$$\Delta E^*_{a.b} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

3. RESULTS AND DISCUSSION

3.1. Microstructure and crystallinity of the films

The layer assembly and microstructure of the developed multilayer materials can be observed through the cross-section images obtained by FESEM (**Figure 1**). FESEM images showed the different layers with thickness values of between 338 and 374 µm (**Table 1**), of which approximately 40% belong to the central PVA sheet (160 ± 11 µm average value), while the remaining 60% correspond to the two PLA external sheets (101 ± 5 µm average value). **Table 1** shows the theoretical (sum of the individual film thicknesses before compression) and measured thickness values of multilayer films, where the lowest measured values with respect to the theoretical values can be observed, due to the different radial flow of polymer sheets during thermocompression. This thickness reduction was more evident in the PVA layers than in the PLA sheets (being around 17% and 10%, respectively). Furthermore, the greatest reduction (20%) occurred in P-PVA_T and P-PVA_T-FA multilayers, which can be explained by the plasticising effect of glycerol in these PVA layers that favours the polymer flowability during thermo-compression.

The microstructural images (**Figure 1**) demonstrated a high degree of adhesion between the PLA and PVA sheets in all of the multilayers, which resist the mechanical impact that occurs in cryofracture, without a delamination effect. The effectiveness of the thermocompression method at obtaining laminated films, was also observed by other authors for PLA/cassava starch bilayers (Muller et al., 2017) or for PLA/PHBV/PEG blends with starch (Hernández-García et al., 2021; Requena et al., 2018).

As observed in previous studies into monolayers (Andrade et al., 2020c), the PVA sheets presented a smooth and homogeneous appearance, except when containing lecithin (P-PVA_C-L and P-PVA_C-LCA samples), where a dispersed phase was distributed in the hydrophilic PVA matrix. The lipid particles dispersed in PVA matrices must be mainly attributed to the lecithin lipid associations coming from the destructuring of incorporated liposomes during the film drying step, which can also entrap a part of the carvacrol in P-PVAC-LCA samples. A partition of carvacrol between the polymer chains and lecithin lipid associations could be assumed in the PVA, which may explain the microstructural differences found in the PVA layers from P-PVAC-L and P-PVAC-LCA samples.

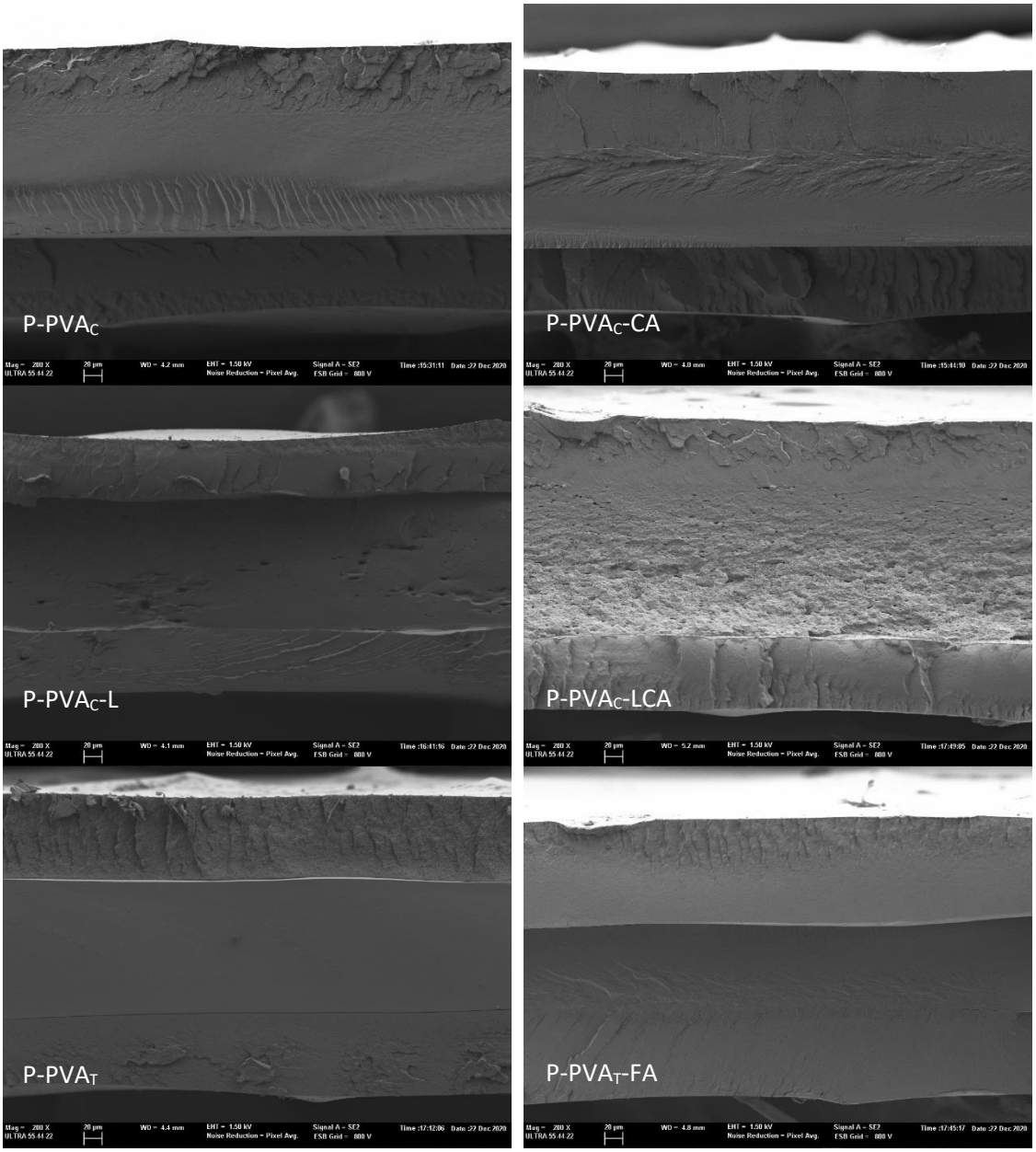

Figure 1. Field Emission Scanning Electron Microscope (FESEM) micrographs of the cross-section of the multilayer films with carvacrol (CA) or ferulic acid (FA) acid (right) and the corresponding control samples without (left) active compound (500X).

Additionally, some microstructural changes were observed in the PLA sheet after the layer assembly, which could be attributed to the interlayer migration of different components, introducing differences into their arrangement in the matrix. The increase in molecular mobility of the polymeric chains at temperatures above Tg during the thermo-compression process (Muller et al., 2017) will favour the compound diffusion associated with the mass transfer driving force (chemical potential gradients of compounds in the contact layers). The

PLA microstructural differences can be observed in **Figure 2,** where the micrographs of the PLA sheet before and after the assembly process are shown. These micrographs reveal that the structural changes suffered by the PLA sheet due to the thermo-compression assembly depended on the composition of the central PVA sheet with which it is in contact. Thus, the PLA sheets from multilayers containing carvacrol (P-PVA_C-CA and P-PVA_C-LCA) exhibited a more rubbery fracture appearance, whereas those coming from the glycerol-plasticised P-PVA_T and the P-PVAT-FA presented multiple pores in the matrix (Figure 2d). Interlayer compound migration could introduce water, active compounds or glycerol molecules into the PLA sheet, whereas PEG could migrate towards the PVA layer, depending on the specific diffusion coefficient and solubility (chemical affinity) in each polymeric matrix of each compound. Likewise, water migration into the PLA sheet could promote the hydrolysis phenomenon of polyester (Rocca-Smith et al., 2017). As concerns PEG-PLA interactions, other authors (Hu et al., 2003) have reported phase separation from a determined level of PEG concentration in the matrix. The observed voids in the assembled PLA with glycerol-plasticised PVA could be attributed to the separated PEG delivery to the PVA sheets containing glycerol, with a greater chemical affinity with PEG. Additionally, PLA could become more plasticised as a consequence of the migration of different compounds from PVA (included water molecules) into this polyester sheet. This hypothesis will be reinforced through the analyses of the mechanical and thermal behaviour of the films.

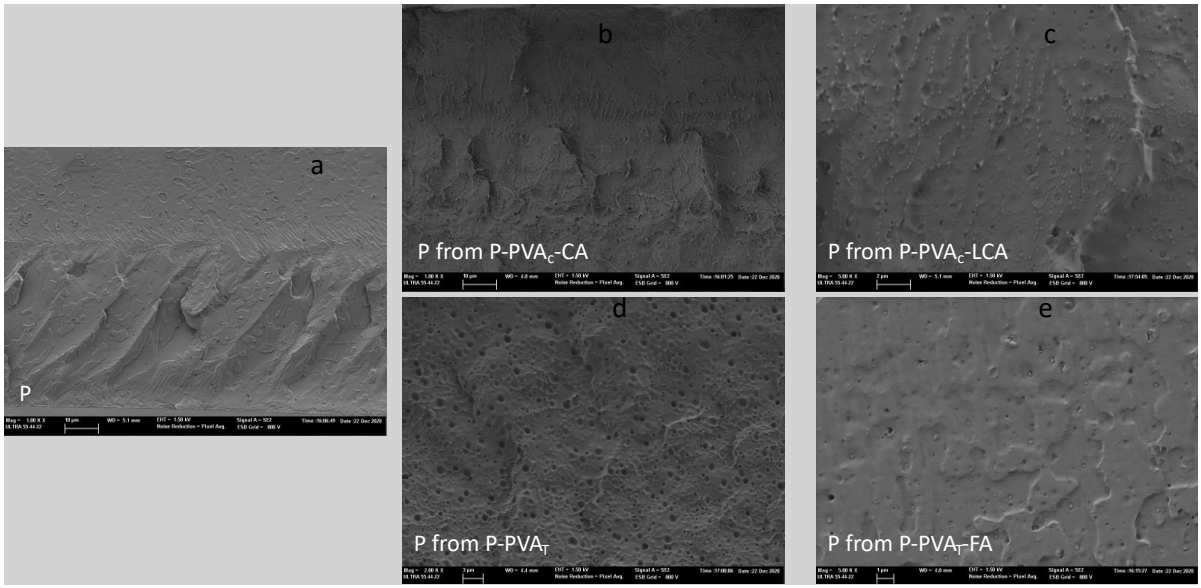

Figure 2. FESEM micrographs of the cross-section of the PLA layer (P) before (a) and after the assembly process for the P-PVA_C-CA (b), P-PVA_C-LCA (c), P-PVA_T (d) and P-PVA_T-FA (e) multilayers.

3.2. Thermal behaviour of mono and multilayers

The PLA and PVA sheets, both prior to the multilayer assembly and those separated from the multilayers, were analysed by DSC in order to determine if the assembly processes affected the thermal behaviour of the respective materials. The Tg values of amorphous PLA, and the Tg and Tm values of semicrystalline PVA before and after the assembly process are shown in **Table 2**. The thermo-assembly process, leading to layer adhesion, changed the thermal behaviour of the monolayers, due to the above-mentioned interlayer migration processes, which can cause plasticising or anti-plasticising effects in each polymer sheet. The Tg of PLA decreased when in contact with the lecithin-free PVA sheets, containing or not carvacrol, but did not significantly change when in contact with PVA films containing lecithin and increased when it was in contact with glycerol-plasticised PVA containing or not ferulic acid, this effect being less marked in the presence of ferulic acid. This suggests different mas transfer or reaction phenomena in the polymer layer responsible for distinct Tg changes. Water migration from PVA could promote partial PLA hydrolyses, reflected in the decrease in the Tg of samples in contact with lecithin-free PVA sheets. The water transfer could be limited in layers containing dispersed lecithin, amphiphilic in nature, which introduced a tortuous pathway for mass transfer. The Tg increase for layers in contact with glycerol-plasticised PVA could be attributed to the losses in PEG caused by the migration to the PVA sheet.

As concerns PVA sheets, no modifications in the Tm values were observed because of the thermo-assembly, which suggests that, as expected, compound migration/reaction mainly occurs in the amorphous phase. Nevertheless, a significant decrease in the Tg values were observed in every case, which was much more marked (a decrease of approximately 15^{o}C) in glycerol plasticised PVA, containing or not ferulic acid. The plasticising effect of carvacrol was observed in both cases (before and after assembly process), but more attenuated after the thermo-sealing, which suggests a partial migration of the compound towards the PLA sheets. In contrast, the marked decrease in the Tg of the glycerol plasticised PVA, containing or not ferulic acid, suggests the migration of PEG from the PLA layer, contributing to the PVA plasticisation. This agrees with the increase in the Tg of the PLA layer (because of the losses in plasticiser) and the observed voids in the PLA layer micrographs. The greater affinity of PEG with the glycerol plasticised PVA matrix could explain this higher degree of migration of the compound.

Therefore, the changes in the Tg values of the polymer matrices after thermo-sealing revealed the occurrence of different phenomena that occur to a different extent in each multilayer: the partial hydrolysis of the PLA brought about by water migration from PVA; the migration of PEG from PLA to PVA and the migration of carvacrol or ferulic acid from PVA to PLA. However, the analyses of the different values suggest the predominant migration of PEG from PLA, which was highly relevant when PVA contained glycerol as plasticiser and that was more attenuated when the matrix contained dispersed lecithin, probably due to the promotion of the matrix

hydrophobicity that reduced its chemical affinity with PEG, while the tortuosity factor introduced by the lipid dispersed phase limited the mass transfer rate.

The thermal degradation analysis (TGA) of the PVA sheets before and after thermo-assembly was also performed to verify the potential changes associated with the compound migration. No notable differences were observed in any case, except in that of glycerol-plasticised, thermo-processed PVA sheets, where the presence of migrated PEG from PLA could also be deduced from the new peak corresponding to PEG degradation (**Figure 3**). This was not observed in the other sheets, thus indicating that PEG migration occurred to a greater extent in these PVA sheets from PLA, as also deduced from the changes that occurred in the Tg values of polymers.

Table 2. Phase transition temperatures (glass transition temperature (Tg) and melting temperature (Tm)) of the PLA and PVA layers from the first thermal scan, before (b subscript) and after (a subscript) the multilayer thermo-assembly. Mean values and standard deviations in brackets.

Sample	PLA		PVA			
	Tg_b (ºC)	Tg_a (ºC)	Tg_b (ºC)	Tm_b (ºC)	Tg_a (ºC)	Tm_a (ºC)
P-PVA_C	36 (2)	33.2 (0.4) ^{a*}	53.8 (0.4) ^{c}	183 (1) ^{b}	49.2 (0.1) ^{c*}	184 (1) ^{b}
P-PVA_C-CA		33.7 (0.7) ^{a*}	49.9 (0.8) ^{b}	184 (1) ^{b}	47.7 (0.2) ^{c*}	185 (1) ^{b}
P-PVA_C-L		36.3 (0.3) ^{b}	54.3 (0.7) ^{c}	186 (1) ^{c}	51.4 (0.1) ^{d*}	186 (1) ^{b}
P-PVA_C-LCA		36.7 (0.1) ^{b}	48.9 (0.4) ^{b}	186 (1) ^{c}	47 (1) ^{b*}	187 (3) ^{b}
P-PVA_T		43.1 (0.4) ^{d*}	32 (3) ^{a}	175 (2) ^{a}	17.2 (0.4) ^{a*}	172 (2) ^{a}
P-PVA_T-FA		39.6 (0.6) ^{c*}	34 (1) ^{a}	172 (3) ^{a}	16.6 (0.3) ^{a*}	175 (1) ^{a}

Different superscript letters within the same column indicate significant differences between samples, while asterisks indicate significant differences between the Tg or Tm of the sample after multilayer lamination ($p < 0.05$).

Thermal stability is one of the most dominative properties for polymeric materials, since it generally governs the durability, shelf life and life cycles of polymers (Xia et al., 2005). **Figure 3** shows the DTGA curves of the different multilayers that exhibited a similar degradation pattern. The degradation mechanisms of PVA (Andrade et al., 2020a, 2020b; Cristancho et al., 2013) and PLA (Hassouna et al., 2011) generated overlapped patterns, due to the fact that the degradation temperature ranges were very similar. This behaviour was also observed in PVA-PLA bilayers obtained from PVA solutions cast on thermoformed PLA sheets (Tampau, González-Martínez, Vicente, et al., 2020). The multilayers presented a main degradation event at between 180 °C and 390 °C, followed by two subsequent thermal events at above 400 °C; the degradation continued up to 650 °C, from which no residue was observed. Despite all the films following a similar degradation pattern, it should be noted that the onset degradation temperature of multilayer films containing a glycerol-plasticised PVA sheet (P-PVA_T and P-

PVA_T-FA) was about 20 °C lower than the rest of the samples probably due to the early degradation of glycerol, as observed by other authors (Sreekumar et al., 2011). The degradation peak of films containing lecithin was also slightly displaced towards higher temperatures due to the previously observed protective effect of lecithin on PVA thermo-degradation (Andrade et al., 2020c).

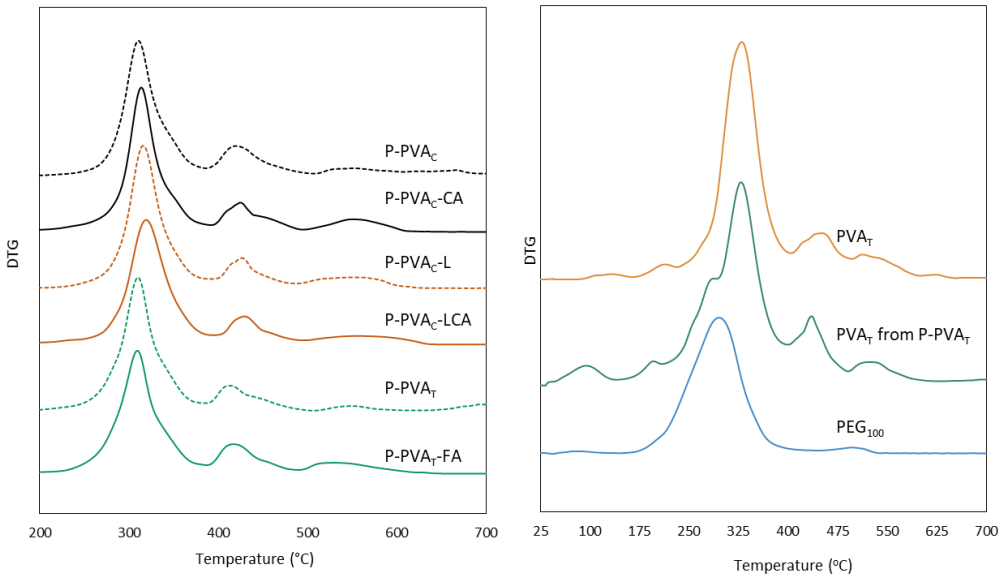

Figure 3. DGT curves from the thermogravimetric analysis (TGA) of multilayer films of PLA and PVA, obtaining by casting (C) or thermo-processing (T), with and without carvacrol (CA), lecithin-encapsulated carvacrol (LCA) or ferulic acid (FA) (left). DGT curves from the TGA of thermo-processed PVA monolayer (PVAT) before and after thermo-assembly and PEG_{1000} (Right).

3.3. Tensile properties

The tensile parameters, tensile strength (TS), elongation at break (E) and elastic modulus (EM) of the multilayers and of the corresponding PVA sheets are presented in **Figure 4**, where the values of the PLA monolayers are also indicated. As expected, the strength of multilayers was improved with respect to the monolayer PVA films due to the contribution of the stiffer PLA sheets, which also provided the multilayers with brittleness. Thus, although the values of the elastic modulus and tensile strength at break were closer to those of the PLA sheets (60 wt. % of the multilayer), they were lower (reductions of around 30% and 13-35%, respectively). This effect can be attributed to the above-mentioned changes in the PLA sheet composition during thermo-assembly (partial hydrolysis and compound migration) that provoke modification in the tensile properties. Likewise, the interlayer adhesion forces, and the stretching delamination will also contribute to the tensile properties of the materials. As reported by

other authors (Kotsilkova et al., 2016) interfacial delamination during elongation is one of the major failures affecting the mechanical response of the interconnected layers. Nevertheless, the multilayers were significantly more stretchable ($p<0.05$) than the non-laminated PLA monolayers, the stretchability values ranging between 8 and 43%, depending on the multilayer. The greater multilayer extensibility enhanced the values of resistance to break, but in some cases were lower than those of highly extensible PVA films. The greatest elongation values were found in P-PVA$_C$ and P-PVA$_C$-CA films, where a marked decrease in the Tg of the PLA sheet was observed after thermo-assembly. This plasticisation of PLA, attributed to a partial chain hydrolysis provoked by the migrated water, led to more extensible laminates, whose stretchability was, in part, limited by the brittleness of PLA layers. In contrast, multilayers containing lecithin, where no significant changes in the Tg values of PLA were observed, exhibited brittleness levels closer to those of the PLA monolayers. In general, multilayers containing active compounds (carvacrol or ferulic acid) tend to be more extensible than active-free laminates, which may be due to the partial migration of active compounds into the extension-limiting PLA layer, which favours the multilayer stretchability.

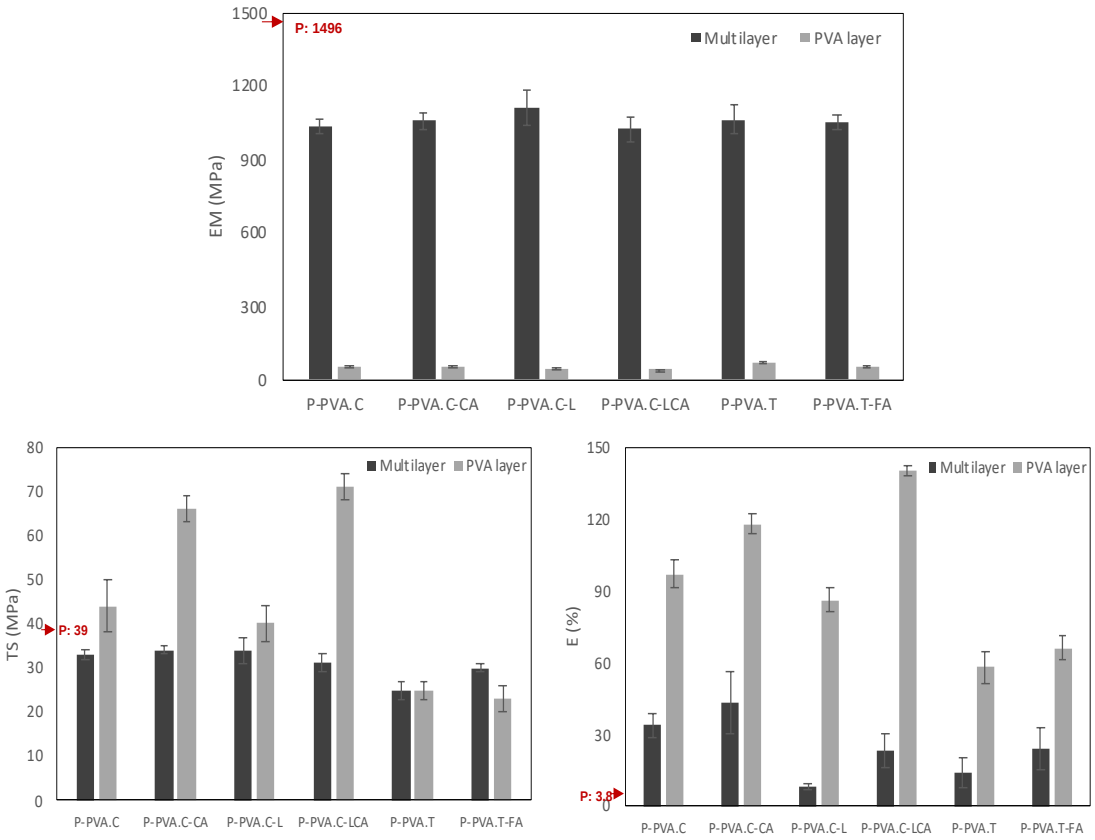

Figure 4. Tensile parameters (Elastic modulus EM (a), tensile strength TS (b), elongation E (c)) of multilayer films and the corresponding PVA layers with and without carvacrol (CA), lecithin-encapsulated carvacrol (LCA) or ferulic acid (FA).

3.4. Equilibrium moisture and barrier properties

The equilibrium moisture, water vapour permeability (WVP) and oxygen permeability (OP) of multilayer films are compared with the corresponding values of PVA sheets and shown in **Figure 5.** As can be observed, the equilibrium moisture contents of the multilayer were very similar (2.7-3.6%) and coherent with the respective values of the corresponding monolayers and their mass fraction in the laminate (0.4 for PVA). The equilibrium moisture of PVA monolayers ranged between 5 and 7%, and it was 0.4% in PLA films, depending on the different water affinity or polarity of the polymer chains. The assembly of the PVA sheet between two PLA sheets led to an equilibrium moisture content that was coherent with the mass fraction and the equilibrium moisture content of each sheet.

Undesirable changes in the physicochemical and nutritional quality of foods are largely related to moisture absorption and oxygen availability during storage (Sousa Gallagher et al., 2011). Therefore, improving the barrier properties against water vapour and oxygen is desirable in the development of useful materials in the food industry. The lamination of two or more materials in a single structure has been used as a successful strategy with which to obtain high barrier capacity materials (Tampau, González-Martínez, Vicente, et al., 2020). In the current study, PLA and PVA sheets, with a high barrier capacity as regards water vapour and oxygen, respectively, were arranged in parallel to form a three-layer film with adequate barrier capacity. The values of water vapour permeability (WVP) and oxygen permeability (OP) of the multilayers and their corresponding sheets were also shown in **Figure 5**.

The WVP of multilayers was approximately 2.5 x 10^4 g/m.h.Kpa, in line with the high water barrier capacity of the external PLA layers (1.7 x 10^4 g/m.h.Kpa), with no significant differences between different multilayers ($p<0.05$). This is coherent with the limiting effect on the water vapour transfer of the PLA layers, which was similar in every case. The OP values of the multilayers were significantly lower ($p<0.05$) than those of the PLA sheets (598 x 10^8 cm^3/m.h.Kpa), in agreement with the barrier effect of the PVA inner layer, but exhibited greater variability (from 25 to 178 x 10^8 cm^3/m.h.Kpa). Although variability in OP was also observed in the OP values of the PVA layer, depending on the formulation and processing conditions, this was enhanced in the multilayer assemblies. This indicates that both the interactions of sheets at interfacial level and compound migration negatively affect the expected oxygen barrier capacity of PVA in multilayers. In fact, the obtained values were higher than those expected, according to the ideal laminate theory, ILT, law (Mrkić et al., 2006; Siracusa, 2012). This theory relates the permeability and thickness of each sheet with the corresponding global values of the multilayer. Thus, the central PVA sheet, responsible for the high oxygen barrier capacity, probably increased the molecular mobility and the free volume as a consequence of the above- mentioned PEG migration, thus increasing the OP of this layer, which ends up influencing the barrier capacity of the multilayer. In general, multilayer films with active compounds, carvacrol or ferulic acid, exhibited a greater oxygen barrier capacity

than the corresponding film without active, which could be attributed to the oxygen-blocking effect exerted by the antioxidant capacity of these compounds. This effect was also observed by Tampau et al. (2020) in bilayers containing carvacrol.

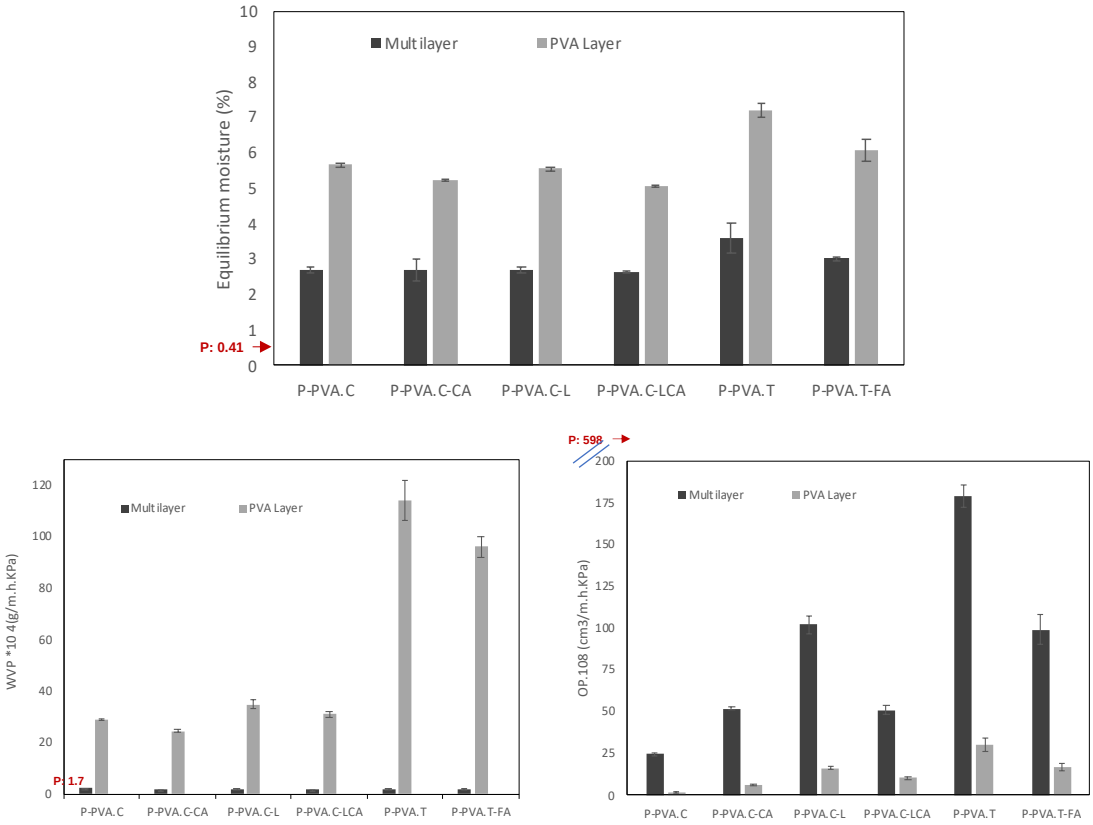

Figure 5. Equilibrium moisture content, water vapour permeability (WVP) and oxygen permeability (OP), of PLA and PVA multilayer films with and without carvacrol (CA), lecithin-encapsulated carvacrol (LCA) or ferulic acid (FA).

3.5. Optical parameters

The values of the colour coordinates (L*, lightness; C_{ab}*, Chroma; h_{ab}*, hue) of the multilayer films and the spectral distribution curves of the Internal transmittance (Ti), are shown in **Table 3** and **Figure 6**, respectively. There were some differences observed between the colours of the films, which exhibited high transparency or Ti values. The hue values of the multilayers markedly decreased ($p<0.05$) in the yellowish films containing ferulic acid and lecithin due to the contribution of the typical colour of these components. The chrome values of the formulations were also similar, except for films with ferulic acid, which exhibited the greatest colour saturation. The typical light absorption of coloured compounds (ferulic acid and lecithin) also affected the internal transmittance of the films, slightly reducing the internal

transmittance mainly in the low wavelength range. Likewise, the thermo-processing of PVA in the P-PVAT and P-PVAT-FA samples also promoted colour development, reducing internal transmittance and increasing the colour saturation. Nevertheless, as observed in **Figure 6**, every multilayer had a very good appearance, with adequate transparency.

Table 3. Lightness (L*), Chroma (Cab*) and hue (hab*) of multilayer films of polyester (P) and PVA layers, without and with phenolic compounds, carvacrol (CA) or ferulic acid (FA), obtaining by casting (C) or thermo-processing (T).

Sample	L*	Cab*	hab*
P-PVA_C	89 (1) ^{b}	3.1 (0.1) ^{a}	101.7 (0.6) ^{d}
P-PVA_C-CA	89 (2) ^{b}	3.23 (0.07) ^{a}	100.1 (0.2) ^{bc}
P-PVA_C-L	88 (1) ^{b}	8.1 (0.6) ^{d}	96.1 (0.3) ^{b}
P-PVA_C-LCA	89 (1) ^{b}	6.9 (0.2) ^{c}	96.7 (0.3) ^{b}
P-PVA_T	88 (1) ^{b}	5.0 (0.2) ^{b}	99.8 (0.9) ^{c}
P-PVA_T-FA	86 (1) ^{a}	15 (2) ^{e}	94.2 (0.3) ^{a}

Different superscript letters indicate significant differences between formulations within the same processing method, while different numbers indicate significant differences between formulations with equivalent mass fractions but processed by another method ($p < 0.05$).

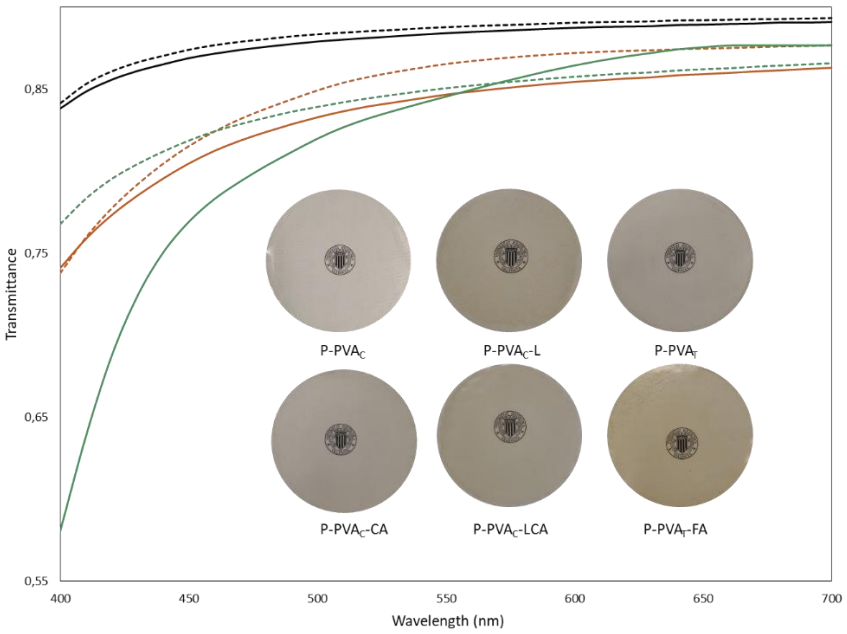

Figure 6. Internal transmittance spectra of multilayer PLA and PVA films with (dashed lines) and without (continuous lines), carvacrol (CA, black lines), lecithin- encapsulated carvacrol (LCA, orange lines) or ferulic acid (FA, green lines).

3.6. Analyses of multilayer films as to their preservation capacity of beef fillets.

3.6.1. *Antimicrobial activity of the packaging systems*

The microbial deterioration of meat is the result of various metabolic pathways, such as those involved in energy production or the synthesis of molecules essential for bacterial growth, which compromise the safety, causing odour, colour or texture defects. The mechanism involved in the bacterial spoilage of meat is a complex process that depends both on the microorganisms initially present and on their biotic and abiotic interactions (Zagorec & Champomier-Vergès, 2017). Kerry & Tyuftin (2017) stated that abiotic conditions, such as the storage temperature or the packaging system, exert a selective pressure on microbial growth in meat products. Then, active packaging can extend the shelf life of meat due to the action of the incorporated antimicrobial compounds and/or through the modification of the film barrier properties, thus reducing oxygen penetration through packaging materials and subsequently, depriving microbes of the availability of the gas (Hunt et al., 2004).

Figure 7 shows the microbial counts of psychrotrophic bacteria (PBC), total viable bacteria (TVC) and coliforms (TCC) and *E. coli* obtained in packaged beef meat samples and stored at 5 °C for 17 days. Count values for each active film and the respective control films can be observed. In general, every sample packaged with the active laminates presented lower counts ($p<0.05$) than the controls, regardless of the antimicrobial compound used. Therefore, the antibacterial activity of the films with active compounds could be mainly attributed to the action of the active compounds. The initial TVC was 5.5 Log, these microorganisms growing slowly in the samples packed in active materials (they did not exceed 6 Log at the end of storage), whereas the samples packaged in control bags exhibited higher growth rates, reaching 6 Log (acceptability limit) from the third storage day onwards. The growth pattern of TCC showed a similar trend to TVC in every case. *E-coli* is found among the main coliform bacteria that cause foodborne illnesses, hence the importance of controlling the growth of this microorganism. In this study, *E-coli* counts from samples stored in the active packages were below the detection limit, while counts between 1.5 and 1.8 Log were detected from 7 storage days onwards in the control samples. The results were coherent with the high sensitivity of *E. coli* to these phenolic compounds (Pires et al., 2013; Requena et al., 2018). The psychrotrophic bacteria exhibited accelerated growth in every sample, except in those packaged with P-PVA_C-CA, whose counts were lower; this was probably due to the inhibitory effect of CA, especially noticeable after 7 storage days, exhibiting a decrease of 0.6 Log with respect to its corresponding control.

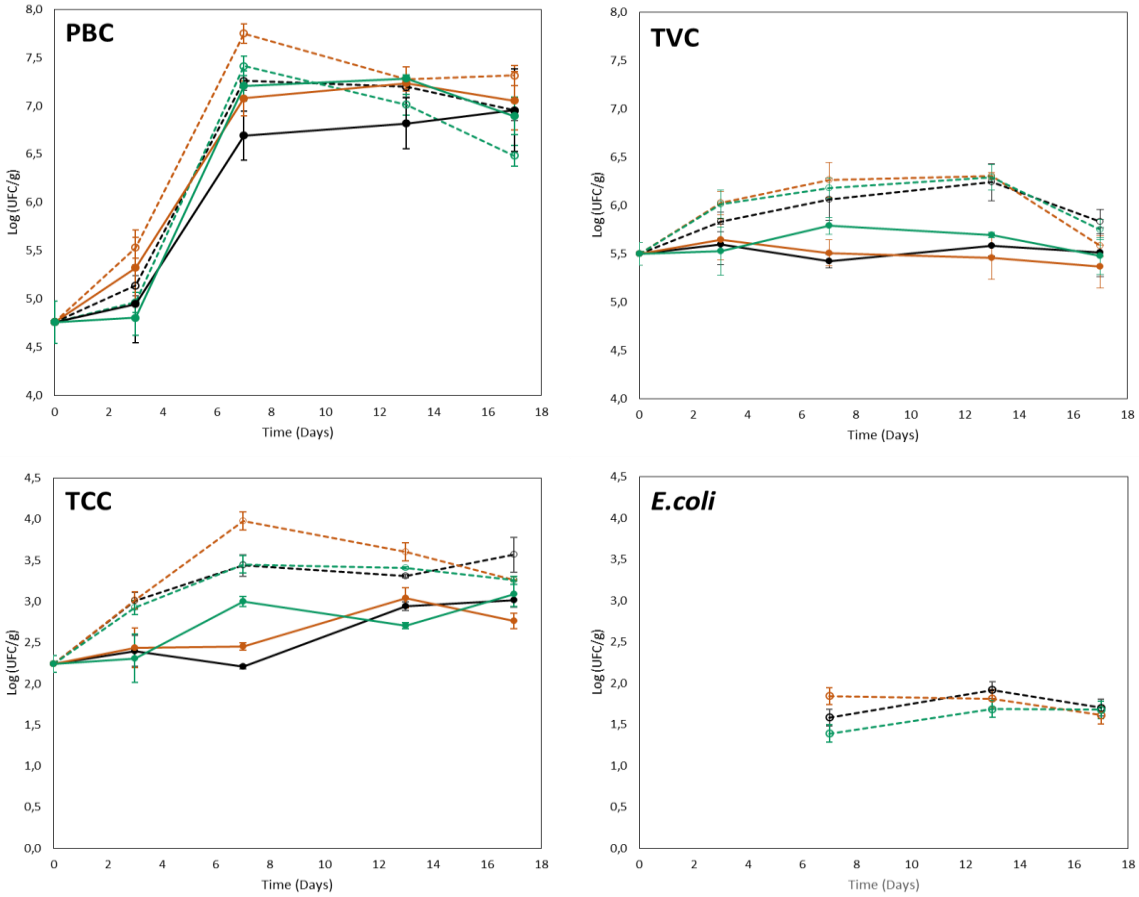

Figure 7. Average values (and 95% LSD intervals) of microbial counts obtained for the beef samples in packaging systems based on PLA-PVA multilayer films with (full lines) and without (dashed lines) active compounds: carvacrol (CA), lecithin- encapsulated carvacrol (LCA) or ferulic acid (FA), for 17 storage days at 5 °C. a: psychrotrophic bacteria; b: total viable bacteria; c: coliforms; d: *E-coli.*

The growth inhibition effects of the studied active films on the beef meat samples, in terms of the reduction in log CFU with respect to the corresponding control, are shown in **Figure 8**. In general, the carvacrol-loaded films (encapsulated or not) exhibited significantly ($p<0.05$) greater antimicrobial efficiency. The maximum antimicrobial reduction was up to 1.5 Logs, the active packaging multilayers being less efficient against psychrotrophic bacteria. In general, this maximum inhibition capacity was found after seven storage days.

The antimicrobial activity exhibited by laminates points to an effective migration of the active through the external plasticised PLA sheet, which seems to reach a maximum after approximately 7 storage days. When plasticised with hydrophilic PEG (P sheet), the PLA polymer matrix seems to favour the mobility of the active compounds through the matrix, increasing the level of relaxation in contact with food and contributing to the migration of the

antimicrobials. Similar results were found by other authors (Essa et al., 2010) when studying the compound release kinetics in plasticised and non-plasticised PLA.

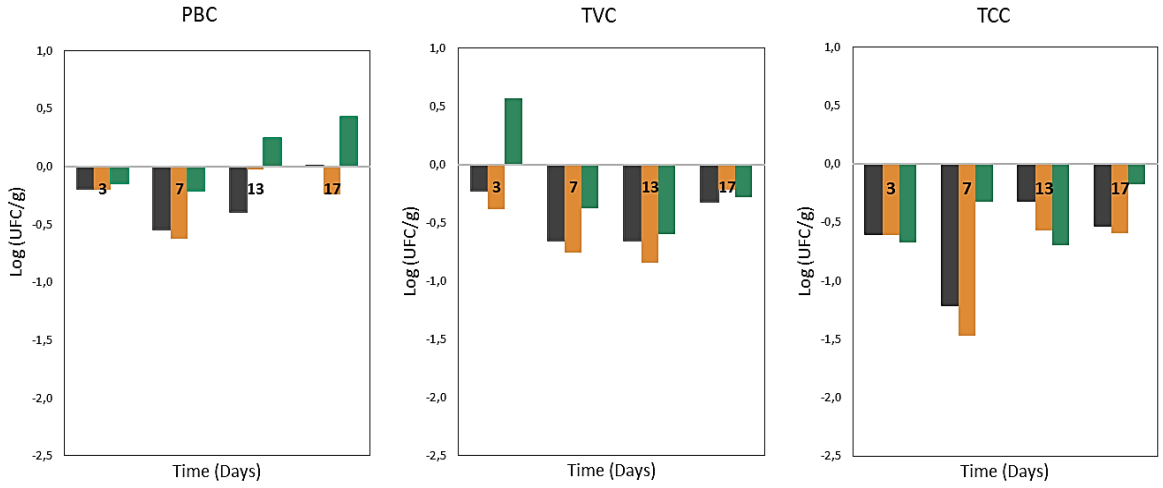

Figure 8. Count reduction (Log CFU difference), with respect to the corresponding control films, in beef meat samples storage at 5 °C at different storage times, for psychrotrophic bacteria (PBC), total viable bacteria (TVC) and total coliforms (TCC), obtained with multilayer films containing carvacrol (black), lecithin encapsulates carvacrol (orange) and ferulic acid (green).

3.6.2. *pH and colour of the packaged beef*

The pH development of the packaged beef is shown in **Figure 9**. The samples were kept within the normal pH range of fresh beef [5-5.8] (Borch et al., 1996) during the evaluation time. The pH value at the beginning of storage was 5.6, a value that decreased throughout storage time in every sample. This has been related to the accumulation of lactic acid, generated during the glycolysis of muscle glycogen, in response to the growth of lactic acid bacteria and other lactose-fermenting bacteria, such as coliforms (Han et al. (2014). Thus, beef packed in materials containing carvacrol (CA or LCA) presented a lower acidification rate than their respective controls, between 7 and 13 storage days, which would have been related to the antimicrobial effect of CA. The samples packaged in the bags containing ferulic acid presented the lowest pH values, probably due to the progress of the migration of the active compound towards the beef surface, thus contributing to the decrease in pH.

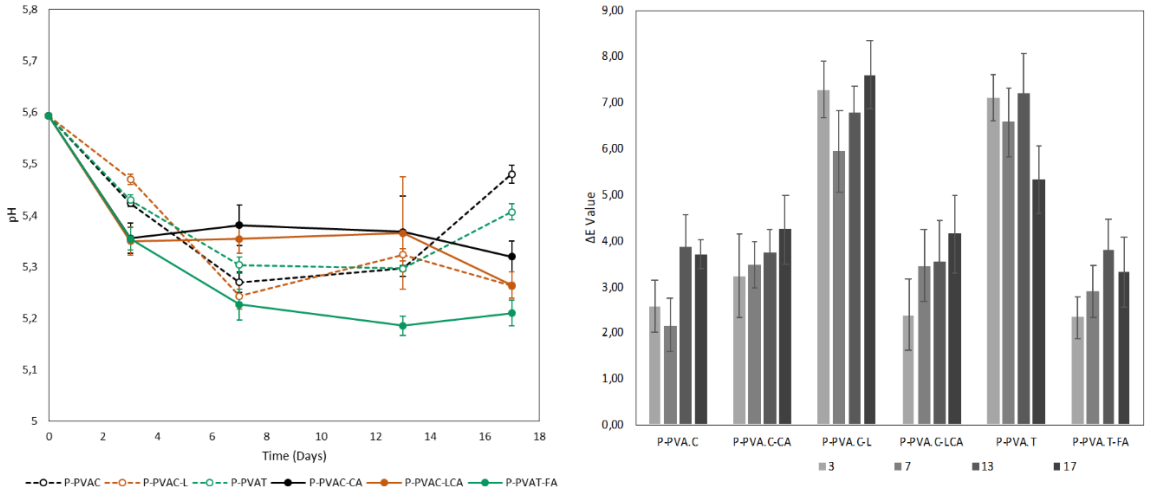

Figure 9. pH (left) and the total colour difference (ΔE) with respect to the initial sample (right) of the packaged meat samples during storage.

The colour of the meat is an important sensory attribute for the consumer, who associates it with quality and freshness (Faustman et al., 2010; Kanner, 1994). Myoglobin is the heme protein responsible for meat colour. The oxidation of the central iron atom within the heme group is responsible for discoloration: a change from red OxyMb to brownish MetMb. Lipid oxidation and myoglobin oxidation often appear to be linked and the oxidation of one of these leads to the formation of chemical species that can exacerbate the oxidation of the other. Thus, several studies reported the preservation of the fresh meat colour following the inclusion of antioxidant ingredients (Faustman et al., 2010). The colour changes in the packaged beef over time was monitored through the $L^*h_{ab}^*C_{ab}^*$ coordinates at different storage times (0, 3, 7, 13, and 17 days). The film packaging slightly affected the colour of fresh meat, decreasing Lightness (from 38 to 36) and chrome (from 22 to 12) and increasing hue (from 40 to 42-45) depending on the kind of film. After 3 days, the sample lightness increased (up to 38-42) mainly in samples packaged in lecithin containing laminates or thermo-processed PLA without ferulic acid. The hue values oscillate, depending on the packaging material, whereas smaller variations were observed in chrome. To simplify the analyses, the total colour difference with respect to the newly packaged meat (ΔE) was determined and shown in **Figure 9**. It is remarkable that, in almost every case, the main colour change occurred very soon after packaging (3 d) and did not markedly progress during storage, which could be attributed to the oxygen restrictions inside the package that limit the OxyMb formation responsible for the typical red colour of the meat. In the meat samples packaged in active films (CA and FA), the colour differences were smaller than 3.5 units until day 7, with a slight increase in the remaining storage time, but without exceeding the usual tolerance limit for food products (ΔE <5) (Hutchings, 1999; Rojas-Lema et al., 2020). In contrast, the samples stored in control packages (except P-PVAc with the lowest OP value (**Figure 5**)) showed a

slightly more marked colour change. The antioxidant action of active compounds or the low oxygen permeability of the package, could limit the oxidation of myoglobin and of the unsaturated fatty acids, responsible for the colour changes in the meat.

4. CONCLUSION

Three-layer films with PLA (external layers) and PVA (internal sheet) were successfully obtained by thermocompression, carrying carvacrol, lecithin-encapsulated carvacrol or ferulic acid in the central PVA sheet, with good layer adhesion. The process promoted the inter-sheet migration of plasticisers and active compounds to a different extent, which slightly affected the functional properties of the threelayers as packaging material. All of the multilayers exhibited tensile properties close to those of the PLA films but with enhanced stretchability (8 - 43%), due to the plasticisation of PLA sheets promoted by compounds migrated from PVA, including water molecules that could enhance partial hydrolyses of polyester. The barrier capacity of multilayers was greatly improved with respect to the monolayers, by combining polyester and PVA hydrophilic layers, which provide the laminate with water vapour and oxygen barrier capacity, respectively. The actives containing multilayers were effective at controlling the microbial growth of beef meat during cold storage and in no case was the legal limit for total viable counts reached during the 17 cold-storage days, whereas this limiting time was reached after 3 days in the control samples. Therefore, the developed active multilayer materials had adequate functionality for food packaging purposes and successfully promoted the meat preservation and shelf-life extension. Further studies into the antioxidant capacity of the materials must be carried out to discover the full potential of the packaging multilayer.

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IV. GENERAL DISCUSSION

Advances in plastic packaging systems have allowed the food industry to guarantee the delivery of products with high quality and safety standards, since it protects them from their environment and makes it possible to slow down the physicochemical and microbiological processes involved in the food deterioration. Nevertheless, the accelerated generation of plastic waste has triggered an eminent environmental imbalance, mainly in marine ecosystems, where most of the plastic waste accumulates, whose resistance to degradation provoke their persistence for many years in the ecosystems. This serious problem makes the transition towards the development and use of biodegradable packaging necessary, as a strategy to significantly reduce the pollution generated by traditional plastics.

However, biodegradable packaging systems have several challenges, since they must meet specific requirements in terms of high barrier properties, mechanical resistance and the effective sealing, among others. To meet these requirements, combination of different materials through laminated assemblies or blending of different polymers and additives are necessary, since one material by itself does not meet all the packaging requirements of a determined target food. Likewise, the incorporation of active compounds of natural origin into the biodegradable packaging systems would allows the protection and extension of the shelf life of food, adding value to the developed materials. Of the potentially active compounds, essential oils or their major compounds has been extensively studied, due to their remarkable activity as antimicrobials and antioxidants. Nevertheless, their application is limited by inherent aspects such as volatility, heat sensitivity and organoleptic impact on the food. In this sense, different methods of encapsulation have been studied to protect them, to promote their adequate incorporation and to control their retention in polymeric matrices, as well as their controlled release. Likewise, the search for other natural active compounds, with less sensory impact on food, is a potential issue on which it must be deepened.

In this context, this doctoral thesis proposed the development of biodegradable multilayer films with active compounds incorporated, using polymers with complementary barrier properties for their application as active food packaging. This general objective was addressed through the following strategies: in **Chapter I**, the liposomal encapsulation of carvacrol, an active compound with high antimicrobial and antioxidant capacity, was evaluated. In **Chapters II**, **III** and **IV**, biodegradable films based on poly (vinyl alcohol) (PVA) with different active phenolic compounds were developed and characterized, and finally in **Chapter V**, the active PVA films with the greatest potential, selected on the basis conclusions of the previous chapters, were assembled in multilayer systems with PLA for developing active biodegradable laminates with adequate properties for food packaging, which were tested in beef meat preservation.

In the **Chapter I,** the ability of different liposomal systems, obtained with soy lecithin enriched in phosphatidylcholine (SL-PC), soy lecithin (SL) and sunflower lecithin (SFL) to encapsulate carvacrol, and to promote their retention in PVA films, was analysed. To this end, two types of poly (vinyl alcohol) PVA were used: high molecular weight, fully hydrolysed (PVA_F) and low

molecular weight, partially hydrolysed (PVA_P). The results showed that the encapsulation efficiency of liposomal systems to carvacrol mainly depended on the lipid composition of the liposomal structure. Thus, SL-PC liposomes, rich in phosphatidylcholine, were the most effective at maintaining the stability of the carvacrol emulsion during film formation, leading to its highest retention (between 54 and 74%) in the PVA films. The membrane composition of this type of vesicles was more homogeneous, more cohesive and less fluid, therefore being less susceptible to the destabilization phenomenon and structural modifications (lyotropic mesomorphism) associated with water loss during the drying step of the cast films. Likewise, the degree of carvacrol retention depended not only on the liposomal encapsulation systems, but also on the interaction of the active compound released on the polymeric matrix. PVA_P, with partially acetylated molecular chains, had greater chemical affinity with carvacrol, which contributed to a higher retention of carvacrol in the films than PVA_F. In conclusion, the SL-PC liposomes were the most efficient at retaining carvacrol in the films, especially when they were incorporated into partially acetylated PVA matrix. Therefore, these were used in developing active PVA films.

In **chapters II, III** and **IV,** different types of active PVA-films with carvacrol or phenolic acids (ferulic and cinnamic acids) were developed and characterised. Films containing carvacrol were obtained by casting the aqueous polymer solutions, incorporating emulsified or lecithin encapsulated carvacrol at 5 or 10 %. Films with phenolic acids (at 1 and 2 %) were obtained by both casting and thermoprocessing (only PVA_P) by adding glycerol (10%) as plasticizer. Glycerol favoured the acid solubility in the film forming aqueous solution of polymer for cast films and the thermoprocessing in PVA_P films. The specific composition, the retention degree of the active compounds and crystallinity degree in different studied films are summarized in **Table 1**.

Poly (vinyl alcohol) is a semi-crystalline biodegradable polymer that is characterized by its high oxygen barrier capacity, in line with its polar nature, rich in hydroxyl groups, where oxygen exhibited low solubility, with a great capacity for the hydrogen bonds formation between neighbouring polymer chains, which reduces the mobility of molecular chains, also limiting molecular diffusion. PVA is obtained from the controlled hydrolysis of poly (vinyl acetate) (PVAc), which allows the PVA to present different molecular weights (Mw) and degrees of hydrolysis (DH). Considering the relevance of the molecular characteristics on the properties of the developed materials, two types of PVA were used to obtain the films, fully hydrolysed PVA (PVA_F) (Mw 89,000–98,000; 99-99.8% hydrolysed) and partially hydrolysed PVA (PVA_P) (Mw 13,000–23,000; 87–89% hydrolysed).

Chapter II and **Chapter III** deal with characterization of PVA_F and PVA_P films obtained by the casting method, with emulsified carvacrol and liposome encapsulated carvacrol, respectively. The applied encapsulation conditions were those optimized in chapter I. These studies demonstrated that both the molecular weight (Mw) and the degree of hydrolysis (DH) of the PVA matrices affected the structural arrangement of the films (degree of crystallinity) and,

consequently, the functional properties of the materials, as well as the thermal behaviour and the carvacrol retention capacity. As shown in **Table 1**, the carvacrol-free PVA_F films presented a higher degree of crystallinity (54%) than the PVA_P films (44%) because of the more homogeneous molecular chain structure, without side acetyl groups, which enhances the ability to form inter-chain hydrogen bonds, generating a more cohesive and highly ordered structural arrangement. Whereas PVA_P, with partially acetylated chains, has steric hindrance that limits the crystalline order to a greater extent. The degree of crystallinity, as well as the chain length, affected the functional properties of the materials. Thus, the PVA_F matrix generated more cohesive films, with higher mechanical performance (higher values of elastic modulus (EM), tensile strength (TS) and elongation (E), at break) (**Figure 1 and 2**) and slightly better barrier capacity against oxygen (OP) and water vapor (WVP) (**Figure 3**).

As reported in **Chapter I**, the degree of CA retention depended to a great extent on the type of PVA. The residual acetyl groups (12%) in the polymer chain of PVA_P promoted structural resonance, providing negative charge to the chains and electron pair donor character, which enhanced the Lewis base nature of PVA_P chains. In this way, the formation of Lewis adducts with carvacrol (Lewis acid) may explain the homogenous structure of carvacrol containing films and the high retention levels of this compound in the matrix, despite its volatility. In this sense, the acetyl groups in the molecular chain of PVA_P promoted the chemical affinity with carvacrol, favouring the effectiveness for obtaining active films for food packaging. This same trend persisted when carvacrol was previously encapsulated in liposomes (**Chapter III**), since retention was significantly higher in PVA_P (67% and 74%) than in the PVA_F matrix (55% and 57%). As can be seen in **Table 1**, encapsulation gave rise to about 15% increase in the degree of CA retention in the films, with respect to the films with CA directly emulsified in the PVA matrices, thus demonstrating that the liposomal system enhanced the retention of non-polar compounds, such as carvacrol in hydrophilic polymeric matrices of PVA, despite the restructuring of liposomes and carvacrol delivery to the matrix during film drying.

Table 1. Composition, final content of active compounds, degree of crystallinity and nomenclature of all films developed in this doctoral thesis.

CHAPTER	DESCRIPTION	Nominal mass fraction							FINAL AC CONTENT (%)	CRYSTALLINITY DEGREE (%)	PROCESSING METHOD	NOTATION
		$x_{F\text{-}PVA}$	$x_{P\text{-}PVA}$	x_{CA}	x_{L}	x_{CNA}	x_{FA}	X_{G}				
II	Films based on fully hydrolysed (F) and partially hydrolysed (P) PVA containing carvacrol (CA)	1								54	Casting	PVA_F
		0,95		0,05					48	58		PVA_F-CA5
		0,91		0,09					51	59		PVA_F-CA10
			1							44		PVA_P
			0,95	0,05					58	51		PVA_P-CA5
			0,91	0,09					63	53		PVA_P-CA10
III	Films based on fully hydrolysed (F) and partially hydrolysed (P) PVA containing carvacrol (CA) encapsulated in lecithin liposomes (L)	0,91			0,09					33	Casting	PVA_F-L
		0,87		0,04	0,09				55	25		PVA_F-LCA5
		0,84		0,08	0,08				57	29		PVA_F-LCA10
			0,91		0,09					35		PVA_P-L
			0,87	0,04	0,09				74	43		PVA_P-LCA5
			0,84	0,08	0,08				67	41		PVA_P-LCA10
IV	Films based on fully hydrolysed (F) and partially hydrolysed (P) PVA containing cinnamic (CNA) or ferulic acid (FA)	0,91						0,09		58	Casting	PVA_{FG}
		0,9				0,01		0,09	96	46		PVA_{FG}-CNA1
		0,89				0,02		0,09	78	21		PVA_{FG}-CNA2
		0,9					0,01	0,09	83	54		PVA_{FG}-FA1
		0,89					0,02	0,09	76	62		PVA_{FP}-FA2
			0,91					0,09		26		PVA_{PG}
			0,9			0,01		0,09	91	27		PVA_{PG}-CNA1
			0,89			0,02		0,09	86	28		PVA_{PG}-CNA2
			0,9				0,01	0,09	78	32		PVA_{PG}-FA1
			0,89				0,02	0,09	77	31		PVA_{PG}-FA2

Table 1. Composition, final content of active compounds, degree of crystallinity and nomenclature of all films developed in this doctoral thesis.

CHAPTER	DESCRIPTION	Nominal mass fraction							FINAL AC CONTENT (%)	CRYSTALLINITY DEGREE (%)	PROCESSING METHOD	NOTATION
		$x_{F\text{-}PVA}$	$x_{P\text{-}PVA}$	x_{CA}	x_{L}	x_{CNA}	x_{FA}	X_{G}				
IV	Films based on partially hydrolysed (P) PVA containing cinnamic (CNA) or ferulic acid (FA)		0,91					0,09		35	Melt blending and compression moulding	PVA_{PGT}
			0,9			0,01		0,09	83	22		PVA_{PGT}-CNA1
			0,89			0,02		0,09	72	24		PVA_{PGT}-CNA2
			0,9				0,01	0,09	20	30		PVA_{PGT}-FA1
			0,89				0,02	0,09	27	30		PVA_{PGT}-FA2
V	Multilayer films obtained by assembly of PLA (plastisised with 10% PEG_{1000}) and active PVA sheets		1								Multilayer assembly by thermocompression of PVA and PLA sheets	PLA/PVA_{P}
			0,91	0,09								PLA/PVA_{P}-CA
			0,91		0,09							PLA/PVA_{P}-L
			0,84	0,08	0,08							PLA/PVA_{P}-LCA
			0,91					0,09				PLA/PVA_{PGT}
			0,89				0,02	0,09				PLA/PVA_{PGT}-FA

Figure 1 shows the map tensile strength (TS) vs elongation (E) at break where the location of all studied films, together with some usual plastics, can be observed. The incorporation of carvacrol, both emulsified and encapsulated, into the PVA_P matrix increased the film's resistance and elongation capacity, whereas in PVA_F matrix this compound decreased the film resistance to break, which could be attributed to the different chemical affinity of carvacrol with the polymer matrix. **Figure 2** shows the resistance to break (TS) *vs*. the elastic modulus (EM) of the films, showing that the liposomal incorporation into both PVA_P and PVA_F matrices reduced the film stiffness, which can be attributed to the greater amount of lipid dispersed phase (lecithin and carvacrol) that reduced the chain polymer attraction forces and matrix cohesiveness. This also affected the film microstructure and the crystallinity degree.

Figure 3 shows the map of barrier properties (oxygen permeability vs. water vapour permeability) with the location of the developed films. This figure shows that the water vapour barrier capacity was only significantly reduced in PVA_F films with encapsulated carvacrol, probably due to lower matrix compactness associated with the interruption of the polymer chain bonding provoked by the dispersed lipid fraction. Likewise, the incorporation of carvacrol, emulsified or encapsulated, reduced the oxygen barrier capacity in both PVA_F and PVA_P films, probably due to its plasticization effect and the introduction of less polar regions in the PVA matrix where oxygen would be more soluble.

Summarising, PVA_P and PVA_F were useful for obtaining active films with carvacrol, encapsulated or not, with some differences in their functional properties. PVA_P better retain carvacrol, but exhibited lower mechanical performance and barrier capacity. The liposomal encapsulation improved the retention of the compound in the films, although the associated microstructural changes generated a reduction in the oxygen barrier capacity and in the stiffness mainly in PVA_F materials. Therefore, their application in food packaging, is subjected to the specific needs of the packaged food.

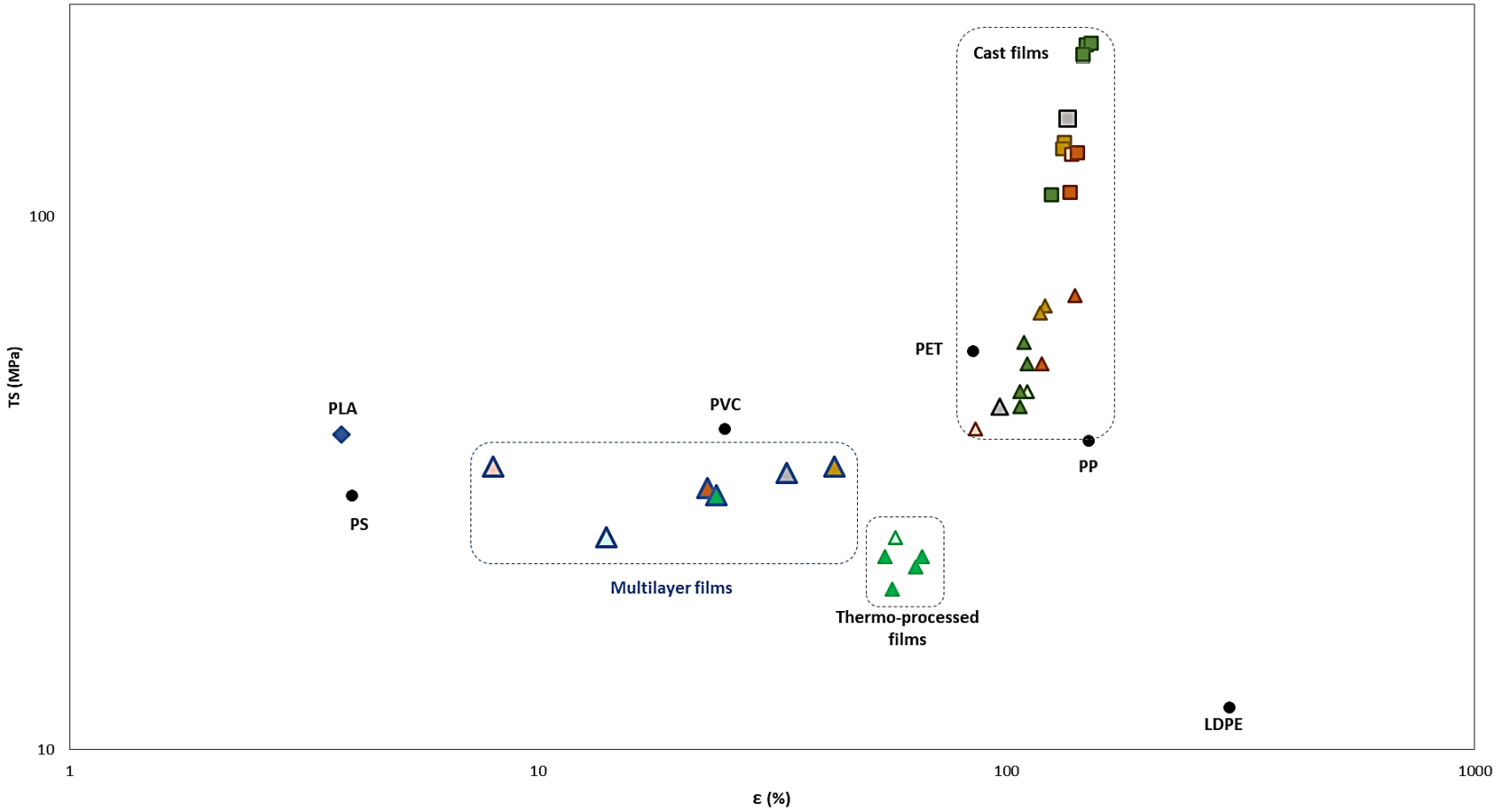

Figure 1. Map of mechanical properties (Tensile Strength vs. stretchability) showing the location of the different film formulation and some synthetic plastics commonly used in food packaging (PS, PVC, PET, PP, LDPE) (Plackett, 2012). Squares: PVA_F, Triangles: PVA_P, incorporating emulsified carvacrol (yellow), lecithin encapsulated carvacrol (orange), phenolic acids (green) incorporated by casting or thermo-processing method.

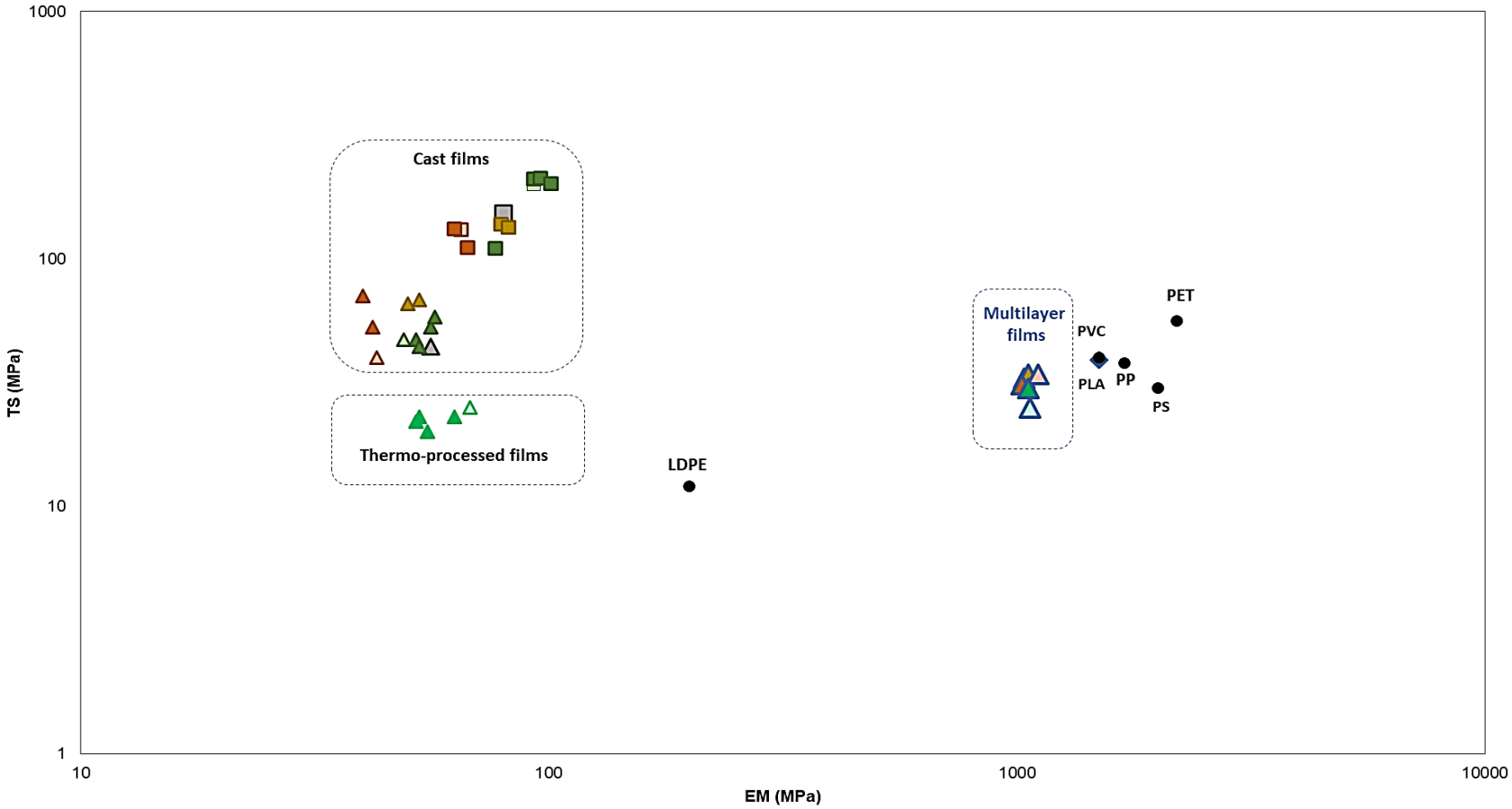

Figure 2. Map of mechanical properties (Tensile Strength vs. Elastic Modulus) showing the location of the different film formulation and some synthetic plastics commonly used in food packaging (PS, PVC, PET, PP, LDPE) (Plackett, 2012). Squares: PVA_F, Triangles: PVA_P, incorporating emulsified carvacrol (yellow), lecithin encapsulated carvacrol (orange), phenolic acids (green) incorporated by casting or thermo-processing method.

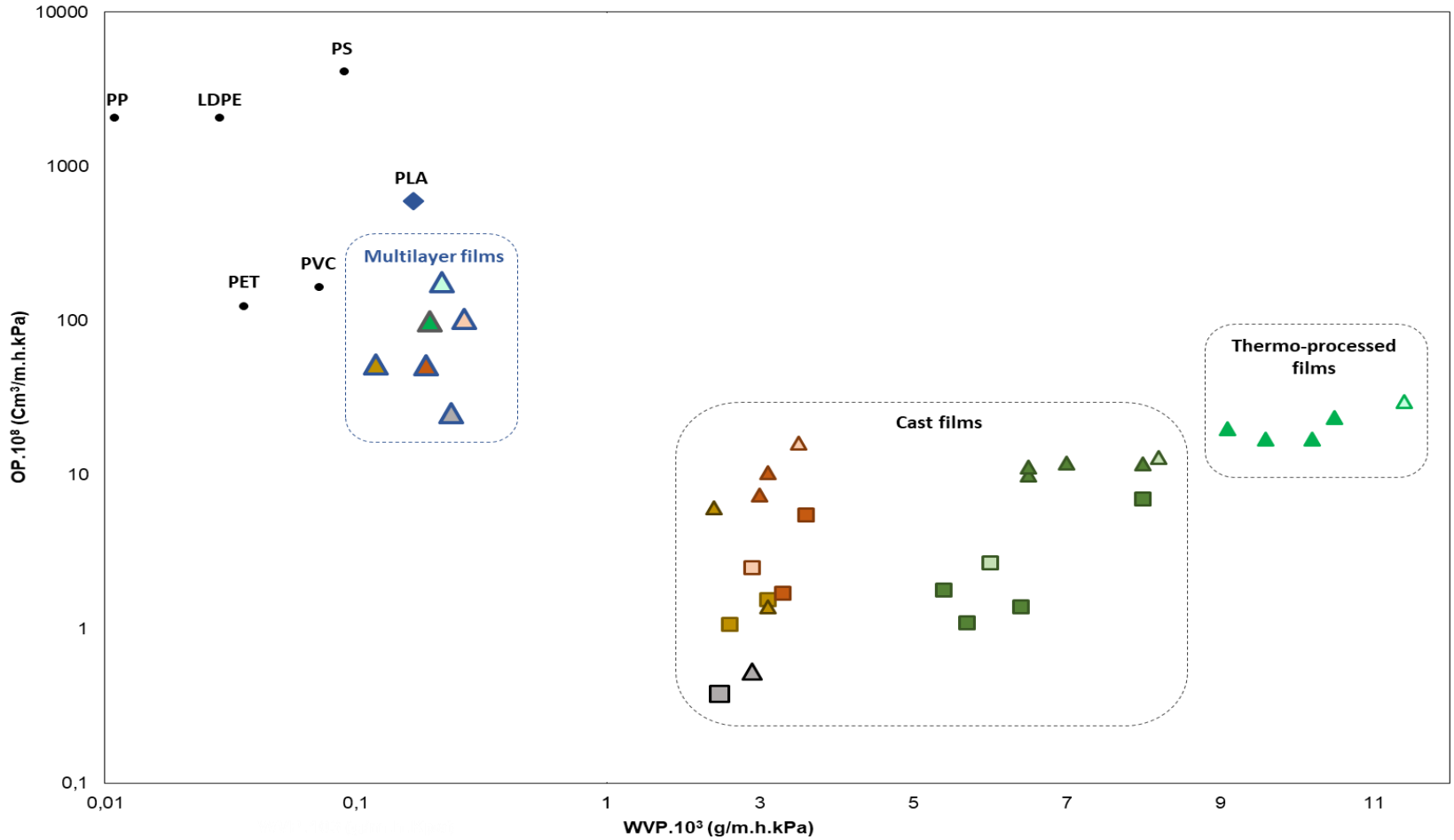

Figure 3. Map of barrier properties showing the location of the different film formulations and some synthetic plastics commonly used in food packaging (PS, PVC, PET, PP, LDPE) (Plackett, 2012). Squares: PVA_F, Triangles: PVA_P, incorporating emulsified carvacrol (yellow), lecithin encapsulated carvacrol (orange), phenolic acids (green) incorporated by casting or thermo-processing method.

Chapter IV explored the development of active films with lower sensory impact than that provoked by CA, using phenolic acids (PA). Cinnamic acid (CNA) and ferulic acid (FA), were incorporated into polymeric solutions of PVA (PVA_F and PVA_P) to obtain films by the casting. To improve the solubility of the PA, the polymer solution was incorporated with glycerol, which gave rise to plasticized films (PVA_{FG} and PVA_{PG}). The PVA_F matrix showed more favourable interactions with glycerol, probably due to the greater capacity to establish hydrogen bonds between glycerol and highly hydroxylated molecular chains, which provoked a slight increase the film stiffness, resistance to break and stretchability (**Figures 1 and 2**). However, both plasticized films, PVA_{FG} and PVA_{PG}, exhibited higher values of permeability to water vapor and oxygen (**Figure 3)** due to the promotion of molecular mobility induced by plasticizer.

The incorporation of ferulic acid promoted greater changes in the films than cinnamic acid, which was attributed to the presence of the phenolic hydroxyl group in the aromatic ring that promoted the formation of interchain hydrogen bonds (crosslinking effect). Therefore, the films containing ferulic acid exhibited a higher degree of crystallinity (**Table 1**) and were stiffer (**Figure 2**). Coherently, the greater compactness of the matrix reduced the molecular free volume and mobility, thus limiting the number of permeation pathways through the polymer network and improving the barrier capacity of the films to water vapor and oxygen (**Figure 3**).

The PVA_{FG} matrices generated plasticized films with better mechanical and barrier properties than the PVA_{PG} matrices, due to the inherent molecular characteristics (higher molecular weight and higher degree of hydrolysis). Therefore, the incorporation of phenolic acids, especially ferulic acid, in this matrix could have great potential for the developing active packaging materials for food preservation. However, these materials could not be obtained by the usual thermal processes of industry because of the polymer onset temperature of thermodegradation is very close to its melting temperature. In contrast, PVA_{PG} films can be obtained by thermoprocessing due to the thermoprotective effect of the residual acetyl groups in their polymer chains, which shift the start of polymer degradation (T_{donset}: 220 °C) above the melting point (T_m: 170 °C).

On this basis and considering the higher thermal stability of cinnamic (Td_{onset}: 230 °C) and ferulic acid (Td_{onset}: 210 °C) with respect to carvacrol (Td_{peak}: 180 °C), in the second section of **Chapter IV,** PVA_{PG} films with phenolic acids were obtained by melt blending and compression moulding and compared with those obtained by casting. In this study it was found that the processing method had a significant influence on the microstructural arrangement of the matrix, crystallinity, tensile behaviour and barrier capacity. Thus, as shown in **Figure 1**, the cast films were more stretchable (E) and more resistant to break (TS), whereas the stiffness was not markedly affected by the type of processing (**Figure 2**). On the other hand, the thermoprocessed films exhibited values of oxygen permeability (OP) and water vapor permeability (WVP) significantly higher than those obtained by casting (**Figure 3**). It is highlighted that the incorporation of phenolic acids, especially ferulic acid, improved the

barrier capacity to water vapor of the plasticized PVA_P films obtained both by thermoprocessing and casting. Furthermore, in vitro studies demonstrated that cast and thermoprocessed films containing phenolic acids exhibited antioxidant and antimicrobial activity, especially when incorporating ferulic acid.

In conclusion, PVA_{PG} films obtained by casting exhibited better functional properties than those obtained by thermoprocessing. However, casting is not a scalable process at industrial level, whereas thermoprocessing are the usual manufacture process industrially applied.

Chapters II, III and **IV** showed the ability of PVA to form films with high barrier capacity to oxygen, carrying active compounds such as carvacrol, cinnamic or ferulic acids, with potential application in active food packaging. However, due to the highly hydroxylated molecular chains, the materials have high moisture sensitivity and permeability to water vapor, while are water soluble. These characteristics limit its use for direct contact with high moist surface food, as well as for controlling the water vapor exchanges between food and environment. To mitigate these drawbacks, lamination of PVA with a hydrophobic polymer, such as poly (lactic acid) PLA, was the alternative that **Chapter V** focused on. Poly (lactic acid) was chosen on the basis of its high moisture barrier capacity, mechanical resistance and biocompatibility. In **Chapter V,** developing and characterization of multilayer films composed of a central layer of PVA loaded with active compounds (carvacrol: CA, lecithin encapsulated carvacrol LCA or ferulic acid: FA) and two external layers of PLA were carried out. Polymer sheets were assembled by thermo-compression to obtain active multilayer materials with improved barrier capacity. The thermocompression of the preformed sheets of PLA and PVA was effective for the interlaminar adhesion, providing the laminate with layers of regular thickness, factors closely linked to the functional properties of the multilayer assemblies.

As shown in **Figure 2,** the tensile behaviour of the multilayer films was mainly determined by the mechanical characteristics of the most resistant/stiffer layer (PLA). In fact, the multilayers presented values of EM (1024 - 1111 MPa) and TS (25 and 34 MPa) very close to those reported for PLA films (EM: 1496 MPa; TS: 39 MPa). Nevertheless, the elongation at break (E) of the multilayers (8-43%) (**Figure 1**) was higher than that of PLA, despite that the PLA sheet exhibited the first break during film stretching. This indicates that PLA was effectively plasticized in contact with PVA layers during the thermocompression. This plasticization indicates the migration of PVA sheet compounds to the PLA layer or a partial hydrolyses of the polymer in contact with water becoming from PVA layer. Different levels of plasticization and film stretchability was observed, depending on the multilayer specific composition (**Table 1**). The high temperature and pressure during thermocompression promoted the molecular mobility in the film layers, enhancing the partial inter-sheet migration of compounds such as water, plasticizers and/or active compounds. **Figure 3** shows that all multilayer films presented improved water vapor permeability with respect to the PVA films and improved oxygen permeability with respect that PLA layers, globally exhibiting more adequate barrier capacity for food applications. The WVP in the multilayers was $2.5x10^{-4}$ g/m.h.Kpa, close to

that of PLA, without significant differences between the samples, in line with the barrier effect of the outer PLA layers. The OP values of all multilayers (50-100x10^{-8} cm^3/m.h.Kpa) were significantly lower than of the PLA film (600x10^{-8} cm^3/m.h.Kpa), due the barrier effect of PVA, although the values were higher than that of PVA monolayers.

The developed active multilayers were applied in the packaging of beef and their ability to preserve the product against microbial growth, throughout cold storage was analysed. The results showed that the incorporation of carvacrol, directly emulsified or lecithin encapsulated, and ferulic acid into the multilayer materials was a successful strategy to control microbial growth (TVC, CTT, PBC and E-coli) in packaged beef, extending its shelf-life. The presence of antioxidant compounds reduced the meet colour changes during storage time, favouring the beef colour preservation.

The obtained results showed that multilayer PVA and PLA materials have a high potential to be used in food packaging, since they combine good passive barrier properties of each of the polymers with the active function of phenolic compounds, which together guarantee the quality of packaged food and extend its shelf life. The material with the greatest potential for microbial control was PLA/PVA_P-CA probably because probably the carvacrol migration to food would have been more accelerated than that of encapsulated carvacrol or ferulic acid, which could be attributed to its greater volatility.

A global analysis of all the films developed in this thesis proved that the lamination of PVA and PLA in multilayer systems was a successful strategy to obtain films with functional properties closer to those of some synthetic plastics commonly used in food packaging (polystyrene PS, poly vinyl chloride PVC, poly ethylene terephthalate PET, polypropylene PP and low-density polyethylene LDPE). **Figure 2** indicates that all PVA films were considerably more flexible (lower EM) than synthetic plastics and presented high tensile strength at break (TS). Regardless of the type of active compound incorporated into the PVA matrices, the tensile strength of PVA_P was comparable to that of synthetic plastics, while PVA_F films were significantly more resistant at break. Meanwhile, the lamination of PVA and PLA generated stiffer films than PVA monolayers, mainly due to the mechanical characteristics of PLA, resulting in multilayer materials with TS and EM close to those reported for traditional plastics, especially PVC and PP. As concerns the barrier properties, all the PVA developed films exhibited better oxygen barrier capacity than the synthetic materials considered in **Figure 3**. This favourable aspect contributed to enhance the oxygen barrier capacity of the multilayer materials compared with PS, PP and LDPE, this being close to that of PET or PVC. Additionally, lamination of PLA and PVA sheets, made the water vapour permeability of multilayers lower than that of PVA films, although the moisture barrier capacity of the traditional materials, such as PP, LDPE or PS was not still reached.

As is known, packaging materials must meet a series of requirements depending on the characteristics of the food to be packaged, such as its water activity, composition, pH, hygroscopicity, etc. Thus, the multilayer materials developed, with a high oxygen barrier and

incorporating active compounds, could be applied to the packaging of foods highly susceptible to oxidation and microbial pollution, such as cheeses and meats, as well as oils and creams, mainly affected by oxidation. However, further antioxidant and antimicrobial evaluations in different food matrices, and migration tests, are necessary to validate the safe use of these materials as food packaging. On the other hand, considering the potential of PVA films as oxygen barrier, good mechanical properties and high capacity to load active compounds, they could be further explored as part of other multilayer materials or as food coatings.

References

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V. CONCLUSIONS

Low molecular weight, partially acetylated poly (vinyl alcohol) is a polar polymer that exhibited high chemical affinity with phenolic active compounds, such as carvacrol or ferulic and cinnamic acids. Therefore, it could be used as an effective carrier of these compounds to obtain active packaging materials, allowing for a sustained release of the actives into food substrates. Likewise, these materials exhibited very high oxygen barrier capacity and can be thermo-processed by the usual techniques of the plastic industry. These characteristics make this polymer an excellent candidate for obtaining laminates with a non-polar polymer, such as poly (lactic acid), with complementary properties, that provide the laminate with high water vapour barrier capacity and mechanical performance. These active laminates have been effective at preserving microbiological quality of high moisture foods, such as beef meat, and could be used for active packaging of many other food products with similar characteristics, extending their shelf life. However, deepening into antioxidant and antimicrobial evaluations in different food matrices, and carrying out migration tests, are necessary to validate the safe use of these materials as food packaging.