DOCTORAL THESIS

INCORPORATION OF ACTIVE COMPONENTS IN BIOPOLYMER-BASED FILMS FOR FOOD USE

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Instituto Universitario de Ingeniería de Alimentos
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Hacen constar que:

La memoria titulada **"INCORPORATION OF ACTIVE COMPONENTS IN BIOPOLYMER-BASED FILMS FOR FOOD USE"** que presenta **Dª Emma Talón Argente** para optar al grado de Doctor por la Universitat Politècnica de València, ha sido realizada en el Instituto de Ingeniería de Alimentos para el Desarrollo (IuIAD – UPV) bajo su dirección y que reúne las condiciones para ser defendida por su autora.

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Fdo. Chelo González Martínez   Fdo. María Vargas Colás

A esos grandes y fuertes abuelos,

A mi familia,

Y en especial a Javi.

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"Lo peor ya ha pasado. Eso ya lo tienes!" "Es la etapa con más presión, pero es bonita también… Intenta disfrutar lo que puedas!" "Cómo quedaste ayer, bonica? Ya lo has subido?" "Me alegro Emmi!" "Carisol, los nervios son normales, pero ya verás como todo va a ir suuuper genial!" "Último esfuerzo! Tú puedes!"

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ABSTRACT

In the field of food packaging there is great interest in the development of biodegradable active materials, which extend the shelf-life of the food products and reduce synthetic plastic waste.

In the present Doctoral Thesis, different strategies have been used to incorporate non-volatile (polyphenols from thyme extract) and a volatile active compound (eugenol) into biodegradable films prepared with starch (S) of different sources or chitosan (CH) or with mixtures of CH:S. The functional properties and release kinetics of casted films prepared with pea starch or CH or CH:S blends were evaluated as affected by the incorporation of polyphenols from an aqueous thyme extract (TE) and tannic acid (TA), a polyphenol which was used as a cross-linking agent. Moreover, the functional properties and release kinetics of corn starch films, obtained by casting or by thermo-compression moulding, were evaluated as a function of the addition of eugenol (E) in free form or pre-encapsulated with different wall materials. In order to encapsulate E, whey protein isolate (WP) or soy lecithin (LE), as wall material, and maltodextrin (MD), as drying coadjuvant, were selected. Microencapsulate powders were obtained by spray-drying. The effect of the addition of oleic acid (OA), as eugenol carrier, and CH, as a potential capsule stabilizer, was also analysed. The different formulations were characterized before and after drying, in terms of encapsulation efficiency, thermal stability, release kinetics and antioxidant capacity and antimicrobial activity.

Results showed that TE provided pea starch and chitosan films with antioxidant activity. Polyphenols (TE and TA) interacted with CH chains and acted as cross-linkers, thus improving the tensile behaviour of films and reducing the release rate and the amount of polyphenol released from the films in water and ethanol aqueous solutions. The opposite effect was observed when TE was incorporated into the pure S matrix. All the films became darker, more reddish and less transparent when TE was incorporated, and this effect was more marked in pure S matrices, which suggests that the TE compounds were poorly encapsulated. Thus, S:TE films showed the fastest delivery rate and the highest delivery ratio of TE.

A good encapsulation efficiency (EE) of E was observed in the CH-free powders (87-98%). However, the use of CH provoked a marked EE decrease in both WP and LE powders (22% and 46%, respectively). The formulations exhibited similar E release behaviour in food simulants of different polarity, where practically the total E content was delivered at a similar rate. All the samples exhibited differing degrees of antioxidant and antimicrobial activities, according to the E content of the powder. The antibacterial effect of CH-free powders against *E. coli* was also coherent with their eugenol content, in line with the burst release of E to the culture medium, which exceeded the Minimum

Inhibitory Concentration of the bacteria with 15 mg powder/mL for OA-free powder. An additional positive effect of OA was detected in terms of the antilisterial action of this powder, which led to a similar antibacterial action in powders with and without OA (with different E load).

The process of thermo-compression moulding of the films led to important E losses (80-65%), which were minimised when using OA in the microcapsules (EOA-WP or EOA-LE). In the films containing non-encapsulated E, the retention of E was promoted due to the formation of E-starch complexes. The presence of microencapsulated eugenol increased the presence of discontinuities in the polymeric matrix, thus yielding films that were mechanically less resistant and stretchable, except for E-LE-S films, which became more resistant to break. The barrier properties were improved when adding microencapsulates to the films, except for OA-based microcapsules, due to the presence of large discontinuities and voids filled with OA. Eugenol release from the films was affected by microcapsule, their composition and the food simulant. Thus, the process of encapsulation promoted a faster E release rate and a greater amount of E released at the equilibrium, especially when using the most water soluble microcapsules (E-WP-S) and acetic acid as food simulant, in coherence with the hydrolysis of the starch matrix in the acidic environment.

In starch film obtained by casting, the addition of microencapsulated eugenol powder in starch film-forming dispersions modified film microstructure, yielding less resistant and less elastic films with reduced moisture content, transparency and oxygen permeability as compared to casted films formulated with non-encapsulated eugenol. The addition of eugenol microencapsulated with OA promoted the preservation of the antioxidant activity of the films, especially in less polar food simulants. To validate the functional activity of these films, a practical application of the active films to preserve sunflower oil was carried out. S-EOA-LE films were effective in preventing sunflower oil oxidation during 53 days of storage at 30ºC, maintaining low and almost constant values of peroxide index, conjugated dienes and trienes.

RESUMEN

Actualmente existe un gran interés en desarrollar materiales de envases activos biodegradables para poder alargar así la vida útil de los alimentos y reducir los residuos procedentes de plásticos sintéticos.

En la presente Tesis Doctoral, se han usado diferentes estrategias para incorporar compuestos activos no volátiles (polifenoles procedentes del extracto acuoso de tomillo) y volátiles (eugenol) en películas biodegradables a base de almidón procedente de diferentes fuentes, quitosano y mezclas quitosano-almidón. Para ello, se evaluó como se vieron afectadas las propiedades funcionales de las películas y las cinéticas de liberación del compuesto activo debido a la incorporación de los polifenoles del tomillo (TE) y del ácido tánico (TA), polifenol utilizado como agente de entrecruzamiento. Además, las propiedades funcionales y cinéticas de liberación de películas de almidón obtenidos por casting o por termoconformado también fueron evaluados en función de la adición de eugenol en forma libre (E) o previamente pre-encapsulado con diferentes materiales (proteína de suero de leche (WP) o lecitinas (LE) junto con maltodextrinas como agente coadyuvante). Los productos microencapsulados en polvo fueron obtenidos por atomización. El efecto de la incorporación de ácido oleico (OA) en los encapsulados, como soporte para el eugenol, y quitosano (CH), como estabilizador potencial de las capsulas también fue estudiado. Las diferentes formulaciones fueron caracterizadas antes y después del secado en términos de eficiencia de encapsulación, estabilidad térmica, cinéticas de liberación y capacidad antioxidante y antimicrobiana.

Los resultados mostraron que el extracto de tomillo (TE) confirió a las películas de almidón de guisante y quitosano actividad de antioxidante. Los polifenoles (TE y TA) interaccionaron con las cadenas de CH y actuaron como agentes de entrecruzamiento, mejorando el comportamiento mecánico de las películas y reduciendo la velocidad de liberación y la cantidad de polifenol liberado de las películas en agua y en disoluciones acuosas de etanol. El efecto opuesto fue observado cuando el TE fue incorporado en la matriz de S pura. Todas las películas fueron más oscuras, más rojizas y menos transparentes cuando el TE fue incorporado, y este efecto fue más marcado en las matrices de S puro, lo que sugiere que los compuestos de TE fueron escasamente encapsulados. Así, las películas S:TE mostraron la liberación más rápida y la mayor proporción de TE liberado.

Se observó una buena eficacia de encapsulación (EE) en los polvos sin CH (el 87-98 %). Sin embargo, el empleo de CH provocó una marcada disminución de la EE tanto en los polvos formulados con WP como en polvos de LE (22 % y 46 %, respectivamente). Las formulaciones mostraron un comportamiento similar en cuanto a la liberación de E en simulantes alimentarios de diferente polaridad, donde prácticamente el contenido de E

total fue liberado a una velocidad similar. Todas las muestras presentaron diferente grado de actividad antioxidante y antimicrobiana, según el contenido de E del polvo. El efecto antibacteriano de polvos sin CH contra *E. coli* fue también coherente con su contenido de eugenol, al igual que la brusca liberación de E al medio de cultivo, que excedió la Concentración Mínima Inhibitoria de esta bacteria con 15 mg polvo/mL para el polvo sin OA. Un efecto adicional positivo de OA se detectó con respecto a la actividad antilisteria de este polvo, que dio lugar a una actividad antibacteriana similar en polvos con y sin OA (con diferente carga de E).

El proceso de moldeo por termocompresión de las películas provocó importantes pérdidas de E (80-65%), que se minimizaron cuando se utilizó OA en las microcápsulas (EOA-WP o EOA-LE). En las películas con E no encapsulado, se promovió la retención de E debido a la formación de complejos almidón-E. La presencia de eugenol microencapsulado aumentó la presencia de discontinuidades en la matriz polimérica, produciendo películas que eran mecánicamente menos resistentes y elásticas, a excepción de las películas E-LE-S, que se volvieron más resistentes a la rotura. Las propiedades barrera se mejoraron al agregar microencapsulados a las películas, a excepción de las microcápsulas con OA, debido a la presencia de grandes discontinuidades y vacíos llenos de OA. La liberación de eugenol de las películas se vio afectada por la microcápsula, su composición y el simulante alimentario. Así, el proceso de encapsulación promovió una liberación de E más rápida y una mayor cantidad de E liberado en el equilibrio, especialmente para las microcápsulas más solubles en agua (E-WP-S) y en ácido acético, en coherencia con la hidrólisis de la matriz de almidón en medio ácido.

En la película de almidón obtenida por casting, la adición de polvo de eugenol microencapsulado en las dispersiones formadoras de película modificó la microestructura de las películas, que fueron menos resistentes y menos elásticas con un contenido reducido de humedad, y una menor transparencia y permeabilidad al oxígeno en comparación con las películas formuladas con eugenol no encapsulado. La adición de eugenol microencapsulado con OA promovió la actividad antioxidante de las películas, especialmente en los simulantes alimenticios menos polares. Para validar la actividad funcional de estas películas, se llevó a cabo una aplicación práctica de las películas activas para la conservación de aceite de girasol. Las películas S-EOA-LE fueron efectivas para prevenir la oxidación del aceite de girasol durante 53 días de almacenamiento a 30 °C, manteniendo valores bajos y casi constantes de índice de peróxidos, dienos conjugados y trienos.

RESUM

Actualment existeix un gran interès a desenvolupar materials d'envasos actius biodegradables per a poder allargar així la vida útil dels aliments i reduir els residus procedents de plàstics sintètics.

En la present Tesi Doctoral, s'han usat diferents estratègies per a incorporar compostos actius no volàtils (polifenols procedents de l'extracte aquós de timó) i volàtils (eugenol) en pel·lícules biodegradables de midó procedent de diferents fonts, quitosano i mescles quitosano-midó. Per a açò, es va avaluar com es van veure afectades les propietats funcionals de les pel·lícules i les cinètiques d'alliberament del compost actiu a causa de la incorporació dels polifenols del timó (TE) i de l'àcid tànic (TA), polifenol utilitzat com a agent d'entrecreuament. A més, les propietats funcionals i cinètiques d'alliberament de pel·lícules de midó obtinguts per càsting o per termoconformació també van ser avaluats en funció de l'addició d'eugenol en forma lliure (E) o prèviament pre-encapsulat amb diferents materials (proteïna de sèrum de llet (WP) o lecitines (LE) juntament amb maltodextrines com a agent coadjuvant). Els productes microencapsulats en pols van ser obtinguts per atomització. L'efecte de la incorporació d'àcid oleic (OA) en els encapsulats, com a suport per a l'eugenol, i quitosano (CH), com a estabilitzador potencial de les càpsules també va ser estudiat. Les diferents formulacions van ser caracteritzades abans i després de l'assecat en termes d'eficiència d'encapsulació, estabilitat tèrmica, cinètiques d'alliberament i capacitat antioxidant i antimicrobiana.

Els resultats van mostrar que l'extracte de timó va conferir a les pel·lícules de midó de pèsol i quitosano activitat d'antioxidant. Els polifenols (TE i TA) van interaccionar amb les cadenes de CH i van actuar com a agents d'entrecreuament, millorant el comportament mecànic de les pel·lícules i reduint la velocitat d'alliberament i la quantitat de polifenol alliberat de les pel·lícules en aigua i dissolucions aquoses d'etanol. L'efecte oposat va ser observat quan TE va ser incorporat en la matriu de S pura. Totes les pel·lícules van ser més fosques, més vermelloses i menys transparents quan TE va ser incorporat, i aquest efecte va ser més marcat en les matrius de S pur, la qual cosa suggereix que els compostos de TE van anar escassament encapsulats. Així, les pel·lícules S:TE van mostrar l'alliberament més ràpid i la major proporció de TE alliberat.

Es va observar una bona eficàcia d'encapsulació (EE) en les pols sense CH (el 87-98 %). No obstant açò, l'ocupació de CH va provocar una marcada disminució de la EE tant en les pols formulades amb WP com en pols de LE (22 % i 46 %, respectivament). Les formulacions van mostrar un comportament similar quant a l'alliberament d'eugenol en simulants alimentaris de diferent polaritat, on pràcticament el contingut d'eugenol total va ser alliberat a una velocitat similar. Totes les mostres van presentar diferent grau d'activitat antioxidant i antimicrobiana, segons el contingut de E de la pols. L'efecte

antibacterià de les pols sense CH contra *E. coli* va ser també coherent amb el seu contingut d'eugenol, igual que el brusc alliberament d'eugenol al medi de cultiu, que va excedir la Concentració Mínima Inhibitòria d'aquest bacteri amb 15 mg pols/ml per a la pols sense OA. Un efecte addicional positiu de OA es va detectar pel que fa a l'activitat antilisteria d'aquesta pols, que va donar lloc a una activitat antibacteriana similar en pólvores amb i sense OA (amb diferent càrrega d'eugenol).

El procés de modelat per termocompresió de les pel·lícules va provocar importants pèrdues d'eugenol (80-65%), que es van minimitzar quan es va utilitzar OA en les microcàpsules (EOA-WP o EOA-LE). En les pel·lícules amb E no encapsulat, es va promoure la retenció d'eugenol a causa de la formació de complexos midó-E. La presència d'eugenol microencapsulat va augmentar la presència de discontinuïtats en la matriu polimèrica, produint pel·lícules que eren mecànicament menys resistents i elàstiques, a excepció de les pel·lícules E-LE-S, que es van tornar més resistents al trencament. Les propietats barrera es van millorar en agregar microencapsulats a les pel·lícules, a excepció de les microcàpsules amb OA, a causa de la presència de grans discontinuïtats i buits plens de OA. L'alliberament de eugenol de les pel·lícules es va veure afectada per la microcàpsula, la seua composició i el simulant alimentari. Així, el procés d'encapsulació va promoure un alliberament d'eugenol més ràpida i una major quantitat d'eugenol alliberat en l'equilibri, especialment per a les microcàpsules més solubles en aigua (E-WP-S) i en àcid acètic, en coherència amb la hidròlisi de la matriu de midó al medi àcid.

En la pel·lícula de midó obtinguda per *casting*, l'addició de pols de eugenol microencapsulat en les dispersions formadores de pel·lícula va modificar la microestructura de les pel·lícules, que van ser menys resistents i menys elàstiques amb un contingut reduït d'humitat, i una menor transparència i permeabilitat a l'oxigen en comparació de les pel·lícules formulades amb eugenol no encapsulat. L'addició de eugenol microencapsulat amb OA va promoure l'activitat antioxidant de les pel·lícules, especialment en els simulants alimentosos menys polars. Per a validar l'activitat funcional d'aquestes pel·lícules, es va dur a terme una aplicació pràctica de les pel·lícules actives per a la conservació d'oli de girasol. Les pel·lícules S-EOA-LE van ser efectives per a prevenir l'oxidació de l'oli de girasol durant 53 dies d'emmagatzematge a 30 °C, mantenint valors baixos i quasi constants d'índex de peròxids, dienos conjugats i trienos.

PREFACE

DISSERTATION OUTLINE

This Doctoral Thesis was organized in five sections as described in **Figure 1**: Introduction, Objectives, Chapters, General Discussion and Conclusions. The **Introduction** section discusses the state of the art concerning the development of films based on biopolymers, such as starch and chitosan, for food packaging application, as well as the current strategies to incorporate active compounds from nature origin into films to obtain antioxidant and/or antimicrobial food packaging materials.

The **Objective** section presents the general and specific objectives of the Thesis. The obtained results were presented in two main blocks, which were divided into five **Chapters** as a collection of scientific publications with the usual sections: introduction, material and methods, results and discussion and conclusions.

The most relevant results obtained in the different chapters are now analysed together, from a global perspective, in the **General Discussion** section. Finally, the last section compiled the most relevant **conclusions** of the Thesis.

INTRODUCTION		
OBJECTIVES		
CHAPTERS	**CHAPTER 1** Incorporation of non-volatile active compounds in edible films	**I.** Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts.
		II. Release of polyphenols from starch-chitosan based films containing thyme extract.
	CHAPTER 2 Incorporation of volatile active compounds in edible films	**III.** Encapsulation of eugenol by spray-drying using whey protein isolate or lecithin. Release kinetics, antioxidant and antimicrobial properties.
		IV. Eugenol incorporation into thermoprocessed starch films, using different encapsulating materials.
		V. Starch-eugenol based films for sunflower oil preservation: release kinetics and functional properties.
GENERAL DISCUSSION		
CONCLUSIONS		

Figure 1. Scheme of the present Doctoral Thesis.

Active edible and biodegradable films show a high potential to contain ingredients such as antioxidants and antimicrobials that can help to extend the shelf life of food. However, the nature of the active compounds to be incorporated in the films and the matrix physical properties that contains the additives, may limit their functionality as a packaging use for foodstuff. According to the general objective of the thesis, different strategies have been analysed to incorporate effectively active compounds with different characteristics into biodegradable/edible films.

Thus, the **first chapter** covers the study of incorporation of non-volatile active compounds (such as polyphenols) in starch (S), chitosan (CH) based films and their mixtures. According to their structure, polyphenols can easily react with free radicals to exert their antioxidant activity. However, their incorporation into films could limit their potential action due to some losses during film development (oxidation) or by the establishment of interactions with the polymeric matrix. In this sense, the strategies followed to incorporate efficiently the polyphenols in films were on the way to improve the antioxidant capacity of the matrix. Thus, **Chapter 1.I**, entitled "Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts", evaluated the physical properties (barrier, mechanical and optical properties) and the antioxidant power of S, CH and S:CH blend films, obtained by casting and enriched with polyphenols from thyme extract. Moreover, the addition of tannic acid (TA) to the chitosan-based matrices, as a crosslinking agent, with also reported antioxidant properties, was studied as a reference compound. In **Chapter 1.II** entitled "Release of polyphenols from starch-chitosan based films containing thyme extract", the release kinetics of thyme extract polyphenols from chitosan-starch films in solvents with different polarity was evaluated. Moreover, the effect of the addition of TA into the polymeric matrix, as a cross-linking agent, was also studied.

On the other hand, **Chapter 2** includes the incorporation of volatile active compounds, such as eugenol, in edible films. The use of essential oils and other volatile compounds in active films for food preservation remained limited mainly due to their intense aroma, great losses during the film development and toxicity problems. Besides, many essential oils contains chemically labile components, which can suffer from oxidation processes, chemical interactions or volatilization. In this sense, the **Chapter 2.III,** entitled "Encapsulation of eugenol by spray-drying using whey protein isolate or lecithin. Release kinetics, antioxidant and antimicrobial properties", studied the eugenol encapsulation process by spray drying using WP or LE as wall-materials and the characterization of the different formulations used, before (emulsion properties) and after drying, in terms of the encapsulation efficiency, thermal stability, release kinetics, antioxidant and antimicrobial activities. The effect of the incorporation of both oleic acid (OA), as eugenol carrier, and of chitosan (CH) on the properties of the encapsulating systems was also analysed.

Once the microparticles obtained with eugenol were characterized, the formulations that presented the best functional properties were selected to follow with the next part of the work. Thus, the eugenol microencapsulated in WP and LE based microparticles containing or not OA were selected to be incorporated into starch-based films, processed by both casting and thermo-compression methods. This part of the work was analysed in the following chapters:

Chapter 2.IV, entitled "Eugenol incorporation into thermoprocessed starch films, using different encapsulating materials", studied the effect of the incorporation of eugenol microencapsulated in corn starch-based films obtained by thermo-compression moulding. These films were compared with those incorporating eugenol in a free form.

Finally, the **Chapter 2.V** "Starch-eugenol based films for sunflower oil preservation: release kinetics and functional properties" concluded the chapters section. This chapter evaluates the physical properties, the release kinetics and the antioxidant performance of corn starch-based films containing eugenol, which was added to the film-forming dispersions in the free form or previously microencapsulated with the different wall-materials (WP and LE). Taking into account the results obtained from these studies, a practical application of the best active film was carried out to enhance the preservation of sunflower oil during its storage.

DISSEMINATION OF RESULTS

INTERNATIONAL JOURNALS JCR

Published

"Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts." Emma Talón; Kata T. Trifkovic; Viktor A. Nedovic; Branko M. Bugarski; María Vargas; Amparo Chiralt; Chelo González-Martínez.
Carbohydrate Polymers 157 (2017) 1153–1161

"**Release of polyphenols from starch-chitosan based films containing thyme extract.**"
Emma Talón; Kata T. Trifkovic; María Vargas; Amparo Chiralt; Chelo González-Martínez.
Carbohydrate Polymers 175 (2017) 122–130

Submitted

"Encapsulation of eugenol by spray-drying using whey protein isolate or lecithin. Release kinetics, antioxidant and antimicrobial properties." Emma Talón; Anna-Maija Lampi; María Vargas; Amparo Chiralt; Kirsi Jouppila; Chelo González-Martínez.
Food Hydrocolloids

COMMUNICATIONS IN INTERNATIONAL CONGRESSES

Poster: **"Novel edible films containing thyme polyphenols".** Kata T. Trifković, Emma Talón, Maria Vargas, Verica B. Djordjević, Viktor A. Nedović, Branko M. Bugarski, Chelo González-Martínez. "1st Congress on Food Structure Design" Porto, Portugal (2014).

Poster: **"Physical properties of pea starch and pea starch:chitosan films as affected by tannic acid addition".** Emma Talón, Kata T. Trifković, Chelo González-Martínez, María Vargas, Amparo Chiralt. "International Conference on Food Innovation, FoodInnova" Concordia y Entre Ríos, Argentina (2014). Complete work published in the E-book: "International Conference on Food Innovation-FoodInnova 2014" (Vol. II). ISBN: 978-950-698-380-2

Poster: **"Release of Rosmarinic acid from edible films enriched with thyme extract".** Emma Talón, María Vargas, Chelo González-Martínez. "III Congreso Internacional de Calidad y Seguridad Alimentaria Acofesal 2015" Valencia, Spain (2015).

Poster: **"Release of polyphenols from chitosan-based films containing thyme extract".** Emma Talón, Kata T. Trifković, María Vargas, Chelo González-Martínez, Amparo Chiralt. "3rd International Meeting on Packaging Material/Bioproduct Interactions (MATBIM 2015)", Zaragoza, Spain (2015).

Poster: **"Characterization of microencapsulated eugenol for bioactive films".** Emma Talón, Kirsi Jouppila, María Vargas, Chelo González-Martínez. "Second International and third National Congress of Students of Science and Food Technology (AVECTA 2016)", Burjassot, Spain (2016).

Poster*: **"Microencapsulated eugenol by spray-drying: characterization and release studies"**. Emma Talón, Kirsi Jouppila, María Vargas, Chelo González-Martínez. "4th International ISEKI_Food Conference (ISEKI 2016)", Vienna, Austria, (2016).

*Award for the best poster presented in the PhD Workshop (Vienna, 2016) "Strategies and Actions for a Sustainable Food Chain Growth".

Poster: **"Antioxidant and antimicrobial properties of microencapsulated eugenol by spray-drying".** Emma Talón, Anna-Maija Lampi; María Vargas; Amparo Chiralt, Kirsi Jouppila; Chelo González-Martínez. International Conference on Food Innovation (FoodInnova 2017), Cesena (Emilia-Romagna), Italy (2017).

Oral communication: **"Functional properties of microencapsulated eugenol by spray-drying".** Emma Talón, Ann-Maija Lampi, Amalia Cano, María Vargas, Kirsi Jouppila, Chelo González-Martínez. 3rd International & 4th National Student Congress of Food Science and Technology (AVECTA 2017), Burjassot, Spain (2017).

Poster: **"Sodium caseinate and gelatin active films containing tannins: antimicrobial and antioxidant activity".** Amalia Cano, María Andrés, Carolina Ivone Contreras, Emma Talón, Amparo Chiralt, Chelo González-Martínez. 3rd International & 4th National Student Congress of Food Science and Technology (AVECTA 2017), Burjassot, Spain (2017).

Poster: **"Effect of microencapsulated eugenol incorporation on the properties of thermo-processed starch films".** Emma Talón; María Vargas; Amparo Chiralt; Chelo González-Martínez. 4th International Meeting on Material/Bioproduct Interaction (MATBIM 2017), Porto, Portugal (2017).

COMMUNICATIONS IN NATIONAL CONGRESSES

Oral communication: **"Properties of biopolymer-based films enriched with thyme extract as affected by tannic acid addition".** Emma Talón, María Vargas, Kata T. Trifković, Amparo Chiralt, Chelo González-Martínez. "VIII Congreso CYTA/CESIA", Badajoz, Spain (2015).

COMMUNICATION IN SCIENTIFIC EVENTS

Oral communication: **"Films a base de biopolímeros e ingredientes activos encapsulados para su aplicación a alimentos"** Emma Talón, María Vargas, Chelo González-Martínez. "Segundo encuentro de estudiantes de doctorado en la UPV", en Valencia, Spain (2015).

PREDOCTORAL STAYS AT FOREIGN INSTITUTIONS

Department of Food and Environmental Sciences, University of Helsinki (Helsinki, Findalnd). From 1st August 2015 to 31st October 2105 under the supervision of Professor Kirsi Jouppila.

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LIST OF ACRONYMS

%E: Percentage of elongation at break

AA: Antioxidant Activity

AA3%: 3% (w/v) Acetic acid solution

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

$C_{ab}*$: Chroma

CH: Chitosan

D: Diffusivity of Fick's second law

DPPH: 2,2-Diphenyl-1-pikryl-hydrazyl

DTG: Thermal weight loss derivate

E: Eugenol

EC_{50}: Half maximal effective concentration

EE: Encapsulation Efficiency

EM: Elastic modulus

EtOH10%: 10% (v/v) ethanol solution

EtOH20%: 20% (v/v) ethanol solution

EtOH50%: 50% (v/v) ethanol solution

F-C: Folin-Ciocalteu

FESEM: Field Emission Scanning Electron Microscopy

FFD: Film-forming dispersion

FT-IR: Fourier Transform Infrared

GAE: Gallic Acid Equivalent

GC: Gas Chromatography gas chromatography

GT: green tea

$h_{ab}*$: Hue

HDPE: high density Polyethylene

k: Rate constant of Korsmeyer-Peppas model

k_1: kinetic constant of Peleg model

k_2: Constant of the Peleg model

L*: Luminosity

LDPE: low density Polyethylene

LE: Lecithin

LSD: Least Significant Difference

M_∞: Amount of active compound released at equilibrium

MC: Moisture content

MD: Maltodextrin

MIC: Minimum Inhibitory Concentration

n: Difussional exponent of Korsmeyer-Peppas model

OA: Oleic cid

OP: Oxygen Permeability

PA: Polyamide

PBAT: Polybutyrate adipate terephthalate

PBS: Polybutylene succinate

PE: Polyethilene

PET: Polyethylenetetraphthalate

PHA: Polyhydroxyalkanoate

PLA: Polylactic acid

PUR: Polyurethane

PV: Peroxide Value

PVC: Polyvinylchoride

RA: Rosmarinic acid

RH: Relative Humidity

S: Starch

SEM: Scanning Electron Microscopy

SLC: Surface Lipid Content

T: Temperature

TA: Tannic Acid

TE: Thyme Extract

TEAC: Trolox equivalent antioxidant capacity

TGA: Thermogravimetric Analysis

Ti: Internal Transmittance

TLC: Total Lipid Content

TP: Total polyphenol

TS: Tensile strength

UV: Ultraviolet

WI: Whiteness Index

WP: Whey protein

WVP: Water Vapour Permeability

WVTR: Water Vapour Transmission Rate

INTRODUCTION

Every year, a third of the food products for human consumption, which is equivalent to about 1300 million tons per year, is lost or wasted around the world (FAO, 2011). In order to extend the shelf-life of food, all the degradation processes that they suffer must be delayed. Lipid oxidation together with microbial growth are the main causes of food deterioration (Gomez-Estaca *et al.,* 2014; Mozafari *et al.,* 2006; Quintavalla & Vicini, 2002). Oxidative reactions also promote the discoloration and the development of rancidity and of off-flavours, which negatively affects the appearance, nutritional value and quality of foodstuffs.

In the food industry, antioxidant compounds are commonly used to extend the shelf-life of food products. Oxidation is propagated by the formation of free radical intermediates. Antioxidants are substances that lend an electron to free radicals, thus oxidizing and reducing these radicals, preventing the formation of new free radicals (Zhou et al, 2005). Vegetable extracts from aromatic plants show antioxidant properties (Olmedo *et al.,* 2015), being polyphenols the most numerous group of antioxidants of plant origin. The potential antioxidant activity of these phenolic compounds makes them suitable to be used as possible substitutes for synthetic antioxidants in the food industry.

In general, most plant extracts consist of highly volatile substances that are chemically labile as a result of oxidation and chemical interactions. Therefore, the beneficial properties of these active compounds can be reduced by the presence of oxygen, humidity or by improper storage conditions (Fang & Bhandari, 2010).

An important challenge in food technology is the protection and conservation of these additives to promote their full effectiveness. For example, most of these problems can been overcome by using proper encapsulation techniques such as the formation of oil-in-water emulsions, liposomes and coacervation, or by spray-drying, thus allowing the easier handling of the active compound the protection during storage and transportation and its controlled release (Bae & Lee, 2008). By encapsulating such compounds, interactions among food components may be reduced such that the compounds can be added to foods without incurring losses of food quality attributes. On the other hand, these active compounds could also be incorporated within a film or coating applied to the food, which could release the antioxidant into the product or act on its surface, limiting the oxidative reactions of food components.

Currently, in the field of food packaging, there is great interest in the development of biodegradable active materials, which can reduce the use of synthetic plastic wastes and extend the shelf-life of the food products by means of the incorporation of active substances, such as antimicrobials and antioxidants. In this sense, biodegradable polymers such as chitin, lignin, cellulose, starch and proteins have been studied extensively to substitute total or partially the synthetic packaging materials for others more environmental friendly.

1. Current situation of food packaging

Edible films or coatings are defined as thin layers of material suitable for food consumption, which can act as a barrier towards different agents (water vapor, oxygen and aromas), thus increasing the shelf-life of the product. The material can be a complete food coating or can be disposed as a continuous layer between food components (Bourtoom, 2008; Guilbert *et al.,* 1996).

Currently, bioplastics represent about one per cent of the about 300 million tons of plastic produced annually, but their expected demand could grow by 50% in a few years (European Bioplastics, nova-Institute, 2016).

By definition, **bio-based plastics** are biopolymers of natural origin derived in whole or in part from biomass (Vert *et al.,* 2012). **Biodegradable plastics** are plastics susceptible to be degraded by biological activity, that is, under appropriate conditions of moisture, temperature, and oxygen availability. Biodegradation leads to the fragmentation or disintegration of the plastics with no toxic or environmentally harmful residue (Chandra Rustgi, 1998; Vert *et al.,* 2012). A **bioplastic** is a plastic with either bio-based or biodegradable properties or both of them (European Bioplastics, nova-Institute, 2016); they represent an alternative to the materials manufactured with synthetic polymers or petrochemical derivatives.

Bio-based, non-biodegradable plastics, such as polyurethanes, bio-based PE and bio-based PET, are the main drivers of the growth of bioplastics use in 2016, representing around 75% of the bioplastics production capacity worldwide. Production capacities of bio-based and biodegradable plastics, such as PLA, PHA, and starch blends, are also growing significantly (**Figure 1**). The application of bioplastics in packaging remains the largest field, representing the almost 40% of the total bioplastics market in 2016.
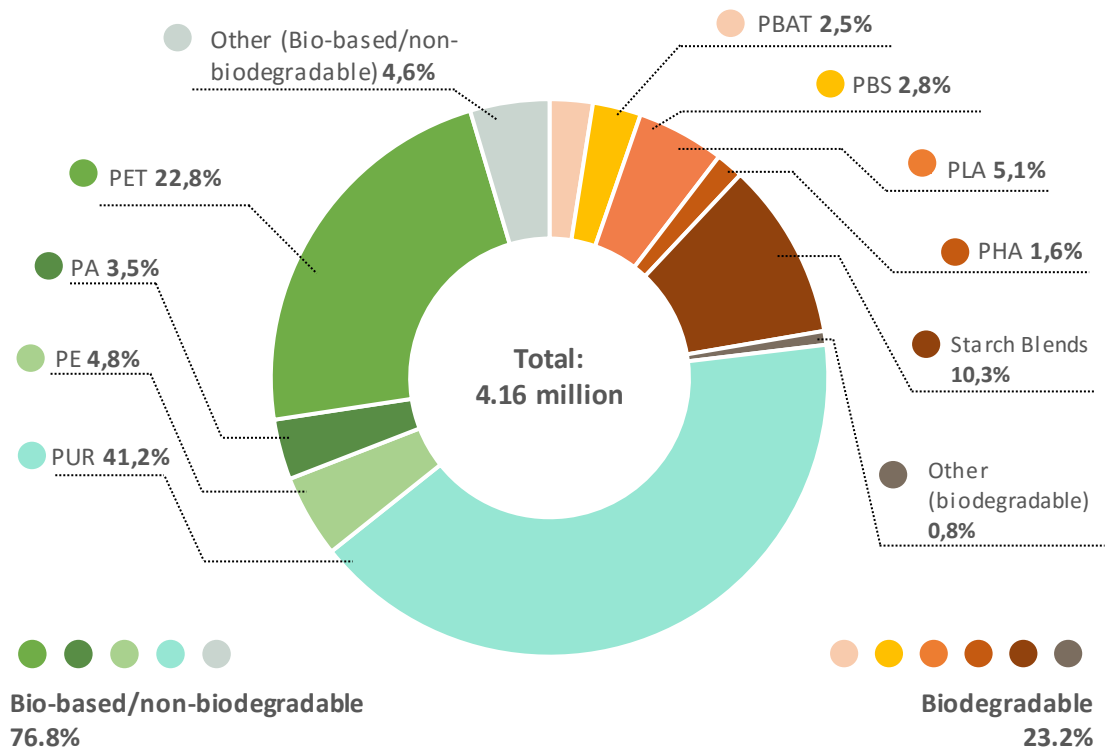
Figure 1. Global production capacities of bioplastics in 2016 (by material type). *Source: European Bioplastics, nova-Institute (2016)*

2. Polymer-based films for food packaging

There is a wide range of different types of polymers that can be used as packaging material. Polymers can be classified into four categories considering their biodegradability and source (most of them are obtained from natural origin and others from fossil origin and). **Table 1** shows the most used polymers for the development of films or coatings, classified as bio-based and biodegradability (Gennadios *et al.,* 1997, Bourtoom, 2008; Falaguera *et al.,* 2011).

Table 1. Classification of polymers (Iwata, 2015; Mülhaupt, 2013; Gennadios *et al.,* 1997; Bourtoom, 2008; Falaguera *et al.,* 2011).

Bio-based

Bio-based and non-biodegradable	**Bio-based and biodegradable**
Polyol-polyurethane Bio-polyethylene (bio-PE) Bio-poly(ethylene terephthalate) (bio-PET) Polyamide (PA) Poly(trimethylene terephthalate) (PTT) bio-poly(ethylene furanoate) (PEF) **Polysaccharides (high DS*):** *Cellulose acetate*	**Proteins:** *Gelatin, corn zein, wheat gluten, soy protein, casein, collagen, whey protein, egg white protein, sorghum protein, pea protein, rice bran protein, protein cottonseed, peanut protein, keratin* **Polysaccharides (low DS*):** *Cellulose, modified cellulose (CMC, MC, HPC, HPMC), chitin and chitosan, starch, modified starch, locust bean gum, guar gum, mesquite gum, gellan gum, xanthan gum, pectin, alginate, galactomannan, carrageenan, lignin* **Lipids:** *Waxes and paraffin (beeswax, paraffin wax, carnauba wax, candelilla wax, wax rice bran resins), acetoglyceride, shellac resins* Poly(lactic acid) (PLA) Polyhydroxyalkanoate (PHA) Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)
Non-Bio-based and non-biodegradable	**Non-Bio-based and biodegradable**
Polyethylene (PE) Polypropylene (PP) Polyestyrene (PS) Poly(ethylene terephthalate) (PET)	Poly(ϵ-caprolactone) (PCL) Poly(butylene succinate/adipate) (PBS/A) Poly (butylene adipate-co-terphthalate) (PBA/T)

Non Biodegradable — **Biodegradable**

Fossil-based

*DS: degree of substitution.

Fossil-based non-biodegradable plastics, such as PE, PP and PET, have extensively contributed to the development of today's society. Oil-based biopolymers such as polyesters (PCL, PBS-A, PBA-T) can be degraded by certain enzymes secreted by microorganisms, thus are considered biodegradable (Iwata, 2015).

Bio-based and biodegradable polymers such as chitosan, starch, celluloses and proteins have been widely studied (Versino *et al.,* 2016; Khalil *et al.,* 2016; Zink *et al.,* 2016). All these natural polymers have proved to be ideal raw materials for preparing biodegradable composites, hence may be an alternative to synthetic polymers. Besides, they are non-toxic and edible, and they can serve as carriers of antimicrobial and antioxidant agents (Quirós-Sauceda *et al.,* 2014). These edible films offer many advantages as active materials, due to their barrier properties to water and gases (oxygen and carbon dioxide) (Perdones *et al.,* 2012) and good mechanical response (Elsabee & Abdou, 2013; Galus & Kadzinska, 2015). These functional properties increase the shelf-life of the food product by protecting it from physical, chemical and biological

degradation. For example, Jridi *et al.* (2014) studied the physical, structural, antioxidant and antimicrobial properties of gelatin–chitosan composite edible films with high antioxidant activity and antimicrobial activity against Gram-positive bacteria.

2.1. Starch-based films

Starch (S) is a polymeric carbohydrate consisting of a large number of glucose units joined by glycosidic bonds (**Figure 2**). Of all the natural polysaccharides available, starch is one of the most abundant natural raw material, which is mainly obtained from corn, cereal grain, rice and potatoes. This polymer is a promising material in the bioplastics synthesis due to its biodegradability, availability, renewability and low cost (Moreno *et al.,* 2015). Native starch granules are water-insoluble and can vary in shape, size, structure, and chemical composition, depending on the origin of the starch (Smith, 2001). Chemically, native starch consists of two major components: amylose, a mostly linear alpha-D(I-4)-glucan and amylopectin, a highly branched polymer of short alpha-D-(I-4) glucan chain which has alpha-D(I-6) linkages at the branch point (Bastioli, 2002; Jimenez *et al.,* 2012).
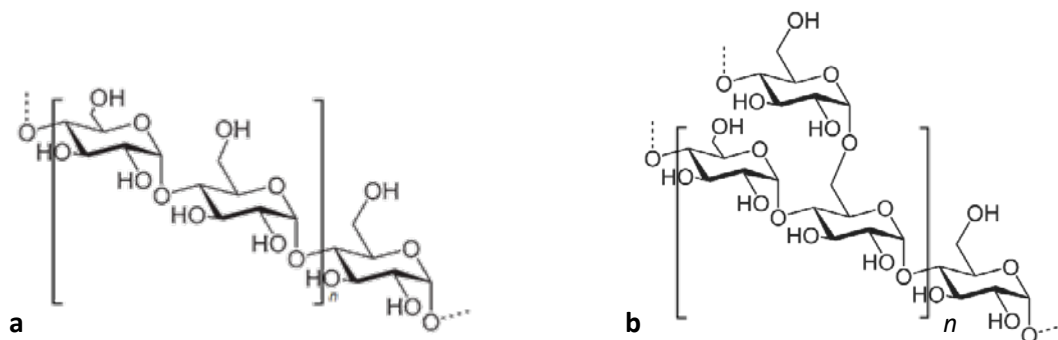
Figure 2. Amylose (a) and amylopectin (b) structures.

Both polymers are structured by hydrogen-bonding, containing crystalline and non-crystalline regions in alternating layers (Jenkins *et al.,* 1993). Amylose, which has a good film-forming ability, renders strong, isotropic, odourless, tasteless, and colourless films (Campos *et al.,* 2011). The branching points of amylopectin form the amorphous regions while the short-branched chains in the amylopectin are the main crystalline components. Thus, the higher content of amylopectin in native starch the greater the crystallinity. Based on X-ray diffraction studies, three types of starches, designated as type A, B, and C, have been identified, depending on the chain lengths of the amylopectin lattice, the density of packing within the granules, and the presence of water: Type A structure has amylopectin chain lengths of 23 to 29 glucose units with the formation of outer double helical structure between the hydroxyl groups of the chains of amylopectin. This pattern is very common in cereals. The type B structure consists of

amylopectin of chain lengths of 30 to 44 glucose molecules with water inter-spread. This is the usual pattern of starches in raw potato and banana. The type C structure is a combination of type A and type B, which is typical of peas and beans (Sajilata *et al.,* 2006).

The amylose:amylopectin ratio can vary depending of the source of the starch ranging from 15:85 to 35:65, except for waxy starch and high amylose corn starch with amylose content that is about 5% and 50–80%, respectively (Cano *et al.,* 2014).

The relative quantity of both polymers (amylose and amylopectin) and their physical organization within the starch granule confer different physico-chemical and functional properties on the starch film formation. **Table 2** shows the amylose and amylopectin content of starch obtained from different sources. Generally, legume starches are characterized by a high amylose content (24–65%) (Ratnayake *et al.,* 2002), thus conferring excellent film-forming ability. The amylose content of smooth pea, pea mutants and wrinkled pea starches range from 33.1–49.6%,8–72%, and 60.5–88%, respectively (Ratnayake *et al.,* 2002). Otherwise, common corn starch contains 25% in amylose (Sajilata *et al.,* 2006).

Table 2. Amylose and amylopectin contents (wt%) in starch from different sources. Sources: Carvalho et al. (2008); Sánchez-González *et al.,* 2015.

Starch type	Amylose (wt%)	Amylopectin (wt%)
Corn	28	72
Waxy corn	0 - <1	100 - >99
High-amylose corn	55-85	15-45
Cassava	9-22	91-78
Potato	20-30	80-70
Wheat	20-25	80-75
Rice	19	81
Pea	25-40	75-60
Tapioca	17	83

Native starch granules are not soluble in cold water due to the high number of intermolecular H-bonds. In order to obtain starch-based films, the granules have to be disrupted previously through a gelatinization process in an excess of water media, where they are gradually disrupted leading to a phase transition from the ordered granular structure into a disordered state (Jiménez *et al.,* 2012). Starch gelatinization is a process in which granules swell, depending on the available water, provoking the breakage of the amylopectin matrix and releasing the amylose (Carvalho, 2008). According to Ratnayake & Jackson (2007), the phase transitions of starch follow three stage processes: (1) water absorption by starch granules that facilitates the increased polymer mobility in the amorphous regions, (2) rearrange of starch polymers in the amorphous

regions often forming new intermolecular interactions, and (3) with increasing hydrothermal effects, the polymers become more mobile and lose their intermolecular interactions and overall granular structure. After the gelatinization, there is a spontaneous recrystallization process and the linear chains of amylose and amylopectin re-associate by hydrogen bonds (Cano *et al.,* 2017). Thus, although native starch is not a thermoplastic material, thermoplastic starch (TPS) can be obtained after gelatinization, acquiring similar properties to those of conventional plastics.

Consequently, TPS can be processed like conventional polymers, and starch films, with the addition of plasticizers and heat treatment, can be obtained by two main techniques: casting (wet method) or thermoprocessing (dry method) (Carvalho, 2008; Jiménez *et al.,* 2012).

Casting method is generally the most used to obtain edible preformed films or coatings by dipping or spraying onto food products. The casting process is divided into several steps: gelatinization and dispersion of the raw material, homogenization of the blends, casting on a mold, and drying under controlled temperature and relative humidity conditions. However, the casting method requires a high amount of solvent to complete the gelatinization of the starch (>63% w/w, Wang *et al.,* 1991), and involves drying times that are too long to permit large-scale manufacturing. Thus casting method is more applicable in labs than in the packaging industry, where the dry method is preferred. When the temperature increases, starch granules become progressively more mobile and the crystalline regions could melt showing the usual viscoelastic behavior exhibited by thermoplastic polymers (Wang *et al.,* 1991; Liu *et al.,* 2009). In other words, the process of gelatinization in lower moisture content presence could be defined as the melting of starch. By **thermoprocessing method**, starch gelatinization is typically achieved at low moisture content since the extrusion processing involves high-shear and high-pressure conditions, which physically tear apart the starch granules, allowing faster transfer of water into the interior molecules. During extrusion at low moisture content the loss of crystallinity is occurs due to the simultaneous gelatinization and melting of starch (Liu *et al.,* 2009). Nowadays, starch is processed thermally by dry techniques such as sheet/film extrusion, foaming extrusion, injection molding, compression molding, and reactive extrusion (a special type of extrusion in which chemical reaction and typical extrusion take place).

The thermal process of starch-based films usually includes two main steps: firstly, the starch is mixed with other additives and plasticizers to enhance their processability and to improve the properties of final products and it is extruded to disrupt the starch granules (normally at temperatures between 140°C and 160°C, Carvalho, 2008); secondly, the obtained paste (or pellets) is thermo-molded to form films. The most commonly used plasticizers are water and/or glycerol (Jiménez *et al.,* 2012). The sddition of plasticizers promotes the flowability of starch under milder conditions and reduces

degradation considerably. However, the thermomechanical stability of the obtained films is strongly reduced by the addition of plasticizers (Dufrense and Vignor, 1998).

Starch films have shown several advantages as low cost, biodegradability, flexibility, transparence, odorless and high oxygen impermeability (Acosta *et al.,* 2015; Cano *et al.,* 2016; Moreno *et al.,* 2015; Ortega-Toro *et al.,* 2015), being these properties affected by the type of starch, chemical modifications and processing conditions (Chaudhary *et al.,* 2008). However, starch-based material also exhibit some disadvantages, such as a high degree of water sensitivity which I turn negatively affects water barrier and mechanical properties as compared to synthetic polymers (Krochta & Mulder-Johnston, 1997; Petersson & Stading, 2005; Avérous & Boquillón, 2004). Improvement strategies are based on the mixture with other components, with better properties.

2.2. Chitosan-based films

Chitosan (CH) is a linear polysaccharide composed of randomly distributed chains of β-(1-4) D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated unit) (**Figure 3**). Chitosan is produced by the deacetylation of chitin, which is a structural element found it in the exoskeleton of crustaceans, such as crabs, prawns, lobsters, etc., as well as in insects (Kumar, 2000).
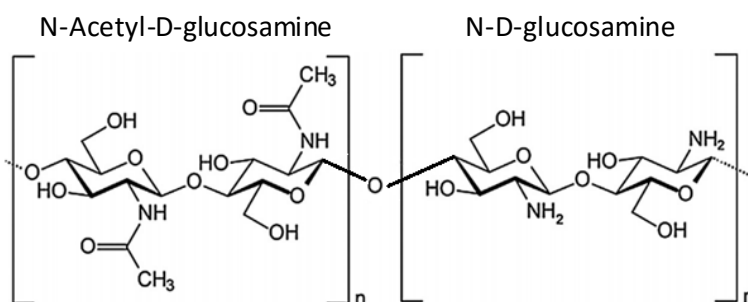
Figure 3. Chemical structure of chitosan.

This cationic polymer is the second most abundant polymer in nature after cellulose. The properties, biodegradability and biological role of chitosan often depend on relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues (George & Abraham, 2006).

In general, an N-deacetylation degree between 55 % and 70 % is considered as low (molecular weight: 50,000 - 190,000 Da), 70 to 85 % is considered as medium (molecular weight: 190,000 - 310,000 Da), 85 to 95 % is referred to as high (310,000 - 375,000 Da) and more than 95 % is called ultrahigh (>375,000 Da). Chitosan with 100% deacetylation

is called full chitosan which is difficult to produce in an industrial field (Shahidi & Abuzaytoun, 2005; He *et al.,* 2016).

Chitosan is insoluble in water and in most common organic solvents. The solubility is a very difficult parameter to control and it depends on the molecular weight, the ionic concentration, the pH, the nature of the acid used for protonation, and the distribution of acetyl groups along the chitosan chain, as well as the conditions of isolation and drying (Rinaudo, 2006). The semi-crystalline nature of chitosan is a function of the degree of deacetylation. Crystallinity is maximum for both chitin (0% deacetylated) and fully deacetylated (100%) chitosan. Thus, minimum crystallinity is achieved at intermediate degrees of deacetylation. Due to the stable and crystalline structure, chitosan is normally insoluble in aqueous solutions above pH 7 (Suh & Matthew, 2000). The solubility of chitosan is usually tested in acidic medium because of protonation of amine groups of the molecule. Neutralization of chitosan solutions (up to 6.2 pH) leads systematically to the formation of a hydrated gel-like precipitate (Nesic and Seslija, 2017). Besides, at relatively high concentrations (above >2 wt%) chitosan solutions become very viscous (Kaur & Dhillon, 2014; Yeul & Rayalu, 2013). Therefore, the properties of the solvent play a very important role in the behaviour of chitosan-based film-forming solutions or dispersions.

Chitosan presents excellent biocompatibility with other biopolymers and lipids and, as compared with starch, presents more hydrophobic character (Mendes *et al.,* 2016). Chitosan exhibits biodegradability properties, mucoadhesiveness and pH sensitiveness (George & Abraham, 2006). Chitosan also can act as chelating agent (Kumar 2000); and it is an excellent film forming material with no need for plasticizing compounds (Siripatrawan and Vitchayakitti, 2016; Aranaz *et al.,* 2016; Prateepchanachai *et al.,* 2017), yielding films with selective permeability to gasses (CO_2 and O_2) and good mechanical properties (Elsabee & Abdou, 2013).

Chitosan shows antimicrobial activity against bacteria, yeasts, moulds and fungi (Perdones et al, 2012; Chatterjee *et al.,* 2014; Tien *et al.,* 2014; Saharan *et al.,* 2013; Severino *et al.,* 2015; Kumar *et al.,* 2004; Dutta *et al.,* 2009; Xing *et al.,* 2015; Chien *et al.,* 2016; Bonilla *et al.,* 2014). The antimicrobial efficacy varies considerably as a function of the type of chitosan (Aider, 2010) and the microbial factors, related to microorganism species and cell age (Kong *et al.,* 2010). Moreover, environmental factors, such as the properties of the solvent play a very important role in the antimicrobial behaviour of chitosan (Hosseinnejad and Jafari, 2016).

There is no general agreement in the literature regarding the mechanisms of antimicrobial action o chitosan. It is believed that it originates from its polycationic nature. Briefly, the mode of antibacterial action is associated with its positive charge, which can interfere with the negatively charged residues of macromolecules on the microbial cell surface, causing membrane leakage (Siripatrawan & Harte, 2010). Thus,

for Gram-negative bacteria, positive chitosan chains can electrostatically interact with the negative charges of the cell membrane of the bacteria, and chitosan could also compete with the lipopolysaccharide and proteins of the surface for the divalent cations of the external membrane, both altering the cell function and provoking the cell lysis. The mechanism of action against Gram-positive bacteria could be the formation of a polymeric membrane of chitosan on the cell surface, which inhibits nutrients from entering the cell or the interaction with negative charges of phosphoryl groups of phospholipid components of cell membranes (Kong *et al.,* 2010; Perdones, 2016).

The potential of chitosan to act as a food preservative of natural origin has been widely reported both *in vitro* analysis and as well as through direct application on real complex matrix foods (Bourbon *et al.,* 2016; Gibis *et al.,* 2016; Sathiyabama & Parthasarathy, 2016; Munhuweyi *et al.,* 2017; Wang *et al.,* 2016). Chitosan has been applied as a component of active packaging material and also as a food additive or preservative to both retard microorganisms growth in food and improve the quality and shelf life of the product. Chitosan has a great potential for numerous applications due to its good properties that give it the ability to form films and gels, thicken emulsions or act as a coating agent (Vargas *et al.,* 2009). Positively charged chitosan molecules in acid media could enhance the stability of the dispersion by a viscous effect and electro-steric effect at the interface, thus promoting dispersion stability (Rodriguez *et al.,* 2002).

Chitosan-based matrices can be used as carrying materials to develop active films with bioactive substances or other antimicrobial agents, these gradually migrating from the packaging onto the surface of the food (Elsabee & Abdou, 2013). However, the fact that chitosan films are highly permeable to water vapour limits their use as food packaging and several strategies are required to improve the physical properties of biopolymer based films. Previous works have shown that the partial replacement of starch with chitosan in starch-based films can improve the mechanical response compared to pure starch films at the same time that the antimicrobial effect is promoted at low cost (Bonilla *et al.,* 2013a). In this sense, the incorporation of chitosan in films based on starch for food applications allows for the incorporation of active compounds that could confer an antioxidant and antimicrobial effect on the surface of the product, thus delaying its degradation. **Table 3** summarizes some examples of starch films obtained by different techniques (melting and thermoplastic processing) that incorporate chitosan and/or different active compounds.

2.3. Films based on mixtures of polymers

The development of film matrices based on the mixture/blend of different polymers allows for the obtention of films with improved properties.

In this sense, Acosta et al., 2015, have reported the benefits of the addition of lipids on the starch-gelatin blend films with different polymer ratios. Cassava starch films with 50% gelatin exhibited adequate properties for the purposes of food coating or packaging. Gelatin blends gave rise to more resistant, harder and more extensible films than pure starch material. Acosta et al. (2016), incorporated essential oils (cinnamon bark, clove and oregano) into cassava starch-gelatin films with that showed antifungal properties, even though, only the 35-45% of the initial essential oil was retained in the film matrix.

The combination of biodegradable polymers as bilayers films, can be an interesting approach to obtain new biodegradable films with antimicrobial activity. For example, Valencia-Sullca *et al.,* 2018 developed thermoplastic cassava starch-chitosan bilayer films containing essential oils (oregano and cinnamon leaf) that were effective in controlling bacterial growth in pork meat.

Muller et al. (2017a) obtained poly(lactic) acid (PLA) and starch bilayer films, containing cinnamaldehyde, by compression moulding. Despite the lower ratio of PLA sheet in the bilayer assembly, a great improvement in the tensile and water vapour barrier properties was achieved with respect to the pure starch films. When cinnamaldehyde was included in the cast PLA sheet, the improvement in the barrier properties was maintained but films exhibited lower mechanical resistance. In a later work, Muller *et al.,* 2017b studied the antimicrobial activity of such films against *Escherichia coli and Listeria innocua* and reported the amount of cinnamaldehyde released into the growth medium exceeded the minimum inhibitory concentration (MIC) of both bacteria. Moreover, the release kinetics of the active compound in different food simulants demonstrated that a part of the active was bonded to the PLA matrix.

Table 3. Examples of starch-chitosan films obtained by different techniques (casting and thermoplastic processing) incorporating chitosan and/or different active compounds.

Functional compound	Matrix material	Plastificant	Film formation method	Relevant results	Reference
–	corn starch/chitosan (chitosan content: 21, 41, 61 and 81 % dry weight)	glycerol (20 wt% with respect to the total polymer)	casting method	Good mechanical and water barrier properties.	Ren *et al.* (2017)
–	CS: CH (CH content: 0.37, 0.73, 1.09 and 1.45 %)	glycerol (25%)	extrusion method	Improvement water vapor and oxygen barrier properties and reduction of surface hydrophillicity to the film.	Dang & Yoksan (2016)
chitosan	WS with CH (CH content: 0, 10, 20, 30, 40, 50 and 100%)	glycerol (starch:glycerol 1:0.15)	casting method	Good mechanical and antimicrobial properties, but barrier properties slightly worsened. S:CH ratio of 80:20 was chosen as a potential food packaging.	Bonilla *et al.* (2013a)
glutaraldehyde as crosslinking agent	CS:PLA (7:3) + CH	glycerol (25-30%)	extrusion method combined with spray and coating chitosan solution	Improvement of water solubility, water vapor permeability and mechanical properties due to the croslinking effect. Coating by spraying was more effective than coating by immersion.	Sorares *et al.* (2013)
glutaraldehyde as crosslinking agent	cassava starch:PLA (7:3) + chitosan	glycerol (25-30%)	extrusion method combined with spray and coating chitosan solution	Effect on the water vapour barrier properties.	Soares *et al.* (2014)
oregano essential oil (0.1 - 1g /100g total solids)	cassava starch:chitosan (77:5)	glycerol (18%)	thermoprocessed method by extrussion	Good barrier, mechanical and antimicrobial properties. Possitive effect of chitosan addition into film matrix in WVP and mechanical properties.	Pelissari *et al.* (2009)

Functional compound	Matrix material	Plastificant	Film formation method	Relevant results	Reference
Grape fruit Seed Extract and Lemon Essential Oil (0.02-0.05 and 0.18-0.40 g active/g total solids, respectively)	corn starch:chitosan (0.4-0.66:0.14-0.08)	glycerol (25 %, w/w polymers)	casting method	Good functional properties and antibacterial activity.	Bof et al. (2016)
Oregano essential oil and Cinnamon leaf essential oil (0.25%; w/w)	cassava starch: chitosan (3:1)	glycerol:PEG (ratio 1:0.3) (30% with respect to the starch)	combitation of casting and melt blending and compression molding methods	Good antimicrobial activity in pork meat.	Valencia-Sullca et al. (2018)
Chitosan oligomers as antimicrobial agent	corn starch with chitosan oligomers (chitosan content: 0.12 g/g film)	glycerol (30%, w/w)	combitation of casting (monolayers) and compression molding (multilayer) methods	Notable antimicrobial activity against molds and yeasts, specially using active sachets.	Castillo et al. (2017)
Basil and thyme essential oils, α-tocopherol and citric acid (0.1 g/g of starch)	wheat starch:chitosan (4:1)	glycerol (starch:glycerol 1:0.15)	casting method	Effect on mechanical and barrier properties. Good antioxidant activity.	Bonilla et al. (2013b)
rosemary essential oil (0, 0.5, 1, 1.5 and 2%)	Potato starch:chitosan (chitosan content: 0, 2, 4, 6 and 8%)	glycerl (30% of starch weight)	casting method	Improvement of physical and microbial properties with rosemary addition and significant effect on physical properties with chitosan addition.	Sayyahi et al. (2017)
potassium sorbate (KS) (17 mg/g polymer)	tapioca starch:chitosan (ratio 2:1)	glycerol (0.21g/g polymer)	casting method	Notable antimicrobial activity against Zygosaccharomyces bailii and significant improvement on WVP and solubility with chitosan addition.	Vásconez et al. (2009)

3. Active compounds of natural origin for food preservation

In recent years, aromatic plants and their extracts have been examined for their effectiveness for food safety and preservation applications. Since ancient times, their compounds have been used for flavouring food, as traditional medicine, and as preservatives and nowadays, have received attention as growth and health promoters (Gyawali & Ibrahim, 2014; Calo *et al.,* 2015). Most of their properties are due to their essential oils and other secondary plant metabolite components (Sánchez-González *et al.,* 2011b). Besides, aqueous extraction of aromatic herbs, such as thyme, have been traditionally used as healthy food ingredients (Stojanovic *et al.,* 2012).

The potential antioxidant activity of these plant extracts makes them suitable to be used as possible substitutes for synthetic antioxidants in the food industry. These polyphenols could be incorporated within a film or coating applied to the food, which could release the antioxidant into the product or act on its surface, limiting the oxidative reactions of food components.

Oxidative reactions in foods represent a serious deteriorative process as they promote the discoloration and the development of rancidity and off-flavours, negatively affecting the appearance, nutritional value and quality of foodstuffs, leading to significant waste. To overcome these problems and prevent these undesirable processes, the food industry often uses synthetic antioxidants, with dubious health properties. In this sense, new natural antioxidant compounds are necessary to control oxidative reactions in foods. Current trends in new packaging technologies for food preservation are focused on the development of alternatives for replacing synthetic additives with natural antioxidant and antimicrobial compounds for two reasons; they are presumed to be safe because they are naturally occurring, and they are well accepted by consumers. In many cases, they are derived from plant sources, such as herbs and spices. Oxidation is propagated by the formation of free radical intermediates. Antioxidants are substances that release an electron to free radicals, oxidizing themselves and reducing said radicals, thus avoiding the formation of new free radicals (Zhou *et al.,* 2005). By definition, an antioxidant is a substance that is capable of inhibiting a specific oxidizing enzyme, or a substance that reacts with oxidizing agents prior to them causing damages to other molecules, or a substance that sequesters metal ions, or even a substance capable of repairing systems such as iron-transporting proteins (Miguel, 2010).

An antimicrobial is a substance that can eliminate microorganisms or inhibits their growth, such as bacteria, fungi or yeast. Antimicrobial compounds from plant sources have been widely studied, although the mechanism of action is not well known as it is not only explained by a single specific mechanism (Burt, 2004). Increasing interest has been shown in the extracts from aromatic plants, such as essential oils, due to their

antiseptic, bactericidal, antifungal and antioxidant properties (Bakkali *et al.,* 2008; Olmedo *et al.,* 2015; Sivakumar & Bautista-Banos, 2014; Sung *et al.,* 2013).

3.1. Polyphenols from plant extracts

Polyphenols are one of the most numerous and universal groups of natural antioxidants from plant sources, such as herbs and spices. They are organic compounds that contain at least one phenol group, that is, an aromatic ring attached to a hydroxyl (OH) group (**Figure 4**). In case of containing more aromatic rings they are called polyphenols.
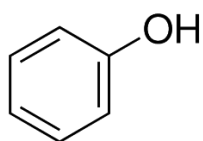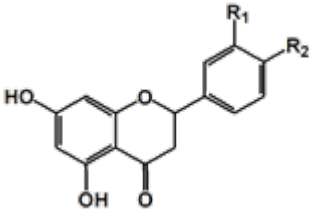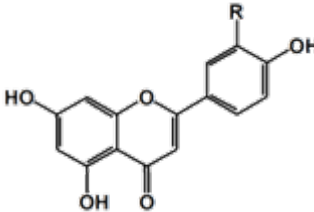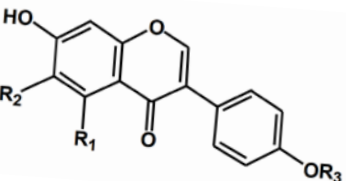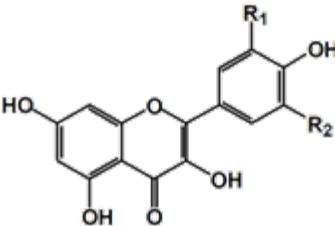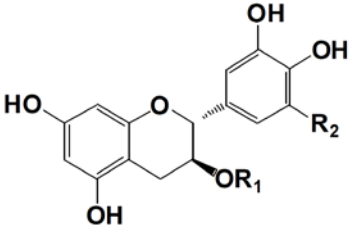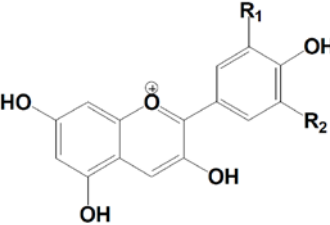
Figure 4. Chemical structure of fenol.
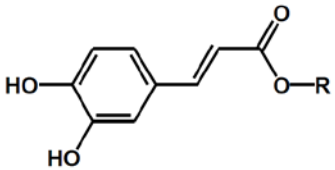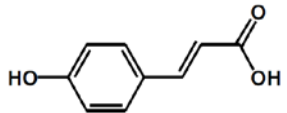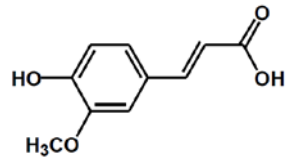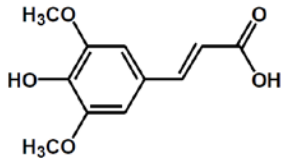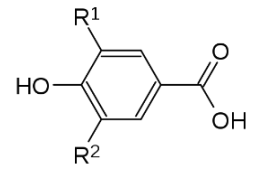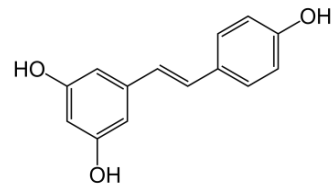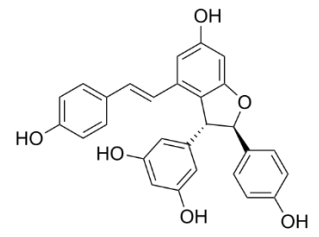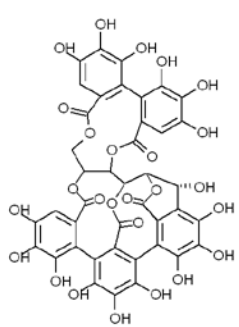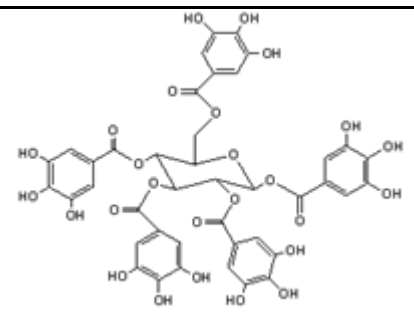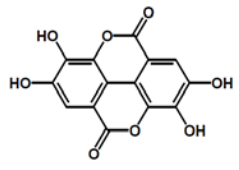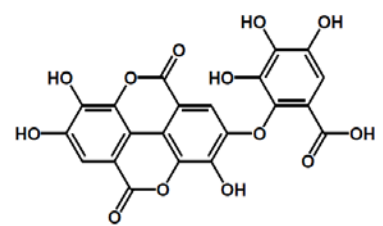
From the point of view of the chemical structure they are a very diverse group that ranges from simple molecules such as phenolic acids (caffeic, ferulic, coumaric and cinnamic, among others) to complex polymers. The antioxidant character of polyphenols is due to their ability to chelate metals, the inhibition of the activity of the lipoxygenase enzyme and their ability to act as a scavenger of free radicals (Garrido *et al.,* 2013). They possess a broad spectrum of biological activities, including antioxidants, anti-inflammatory, antibacterial and antiviral functions (Manach *et al.,* 2004, Fang and Bhandari, 2010).

According to their structure, polyphenols can be classified into two large groups: flavonoids and non-flavonoids. The flavonoids have a specific structure of three aromatic rings, where the central ring is a pyran since it has an oxygen in its structure (Manach *et al.,* 2004; Tsao, 2010). Although different classifications of the main polyphenols can be found, i.e. by their source of origin or biological function, in this work polyphenols have been grouped depending on their chemical structure (**Table 4**).

Table 4. Classification of polyphenols according their chemical structure (Source data: Manach *et al.,* 2004; Tsao, 2010; Waterhouse, 2002).

FLAVONOIDS		
Types	**Subtypes**	**Chemical structure**
Flavonones	Naringenin R_1=H, R_2=OH Hesperetin R_1=OH, R_2=OCH$_3$ Eriodictyol R_1=R_2=OH Pinocembrin R_1=R_2=H	
Flavones	Apigenin R=H Luteolin R=OH	
Isoflavones	Daidzein R_1=H; R_2=H; R_3=H Genistein R_1=OH; R_2=H, R_3=H Formononetin: R_1=H; R_2=H; R_3=OCH$_3$ Glycitein: R_1=H; R_2=OCH$_3$ Biochanin A: R_1=OH; R_2=H; R_3=OCH$_3$	
Flavonols	Kaempferol R_1=H, R_2=H Quercetin R_1=H, R_2=OH Myricetin R_1=OH, R_2=OH Isorhamnetin R_1=OCH$_3$, R_2=H	
Flavanols	Catechin: R_1=R_2=H; Catechin gallate: R_1=gallyl, R_2=H; Gallocatechin: R_1=H, R_2=OH; Gallocatechin gallate: R_1=gallyl,R_2=OH	
Anthocyanidins	Cyanidin R_1=OH, R_2=H Delphinidin R_1=OH, R_2=OH Pelargonidin R_1=H, R_2=H Malvidin R_1=OCH$_3$ R_2=OCH$_3$ Peonidin R_1=OCH$_3$ R_2=H Petunidin R_1=OH, R_2=OCH$_3$	

NON-FLAVONOIDS

Types	Subtypes	Chemical structure
Hydroxycinnamic acids (phenolic acids)	Caffeic acid, R=H Chlorogenic acid, R=5-quinoyl Cryptochlorogenic acid, R=4-quinoyl Neochlorogenic acid, R=3-quinoyl	

p-coumaric acid   ferulic acid   sinapic acid

Hydroxybenzoic acids (phenolics acids)	Gallic acid $R_1=R_2=H$ Syringic acid, $R_1=R_2=OCH_3$ Protocatechuic acid, $R_2=H$; Vanillic acid, $R_2=OCH_3$	
Estilbenes	 Resveratrol	 ε-viniferin
Hydrolysable tannins	 vescalagin (ellagitannin)	 Gallotannin

ellagic acid   Valoneic acid dilactone

Other non-flavonoids polyphenols

Rosmarinic acid

Gingerol

Tannic acid

Secoisolariciresinol

Curcumin
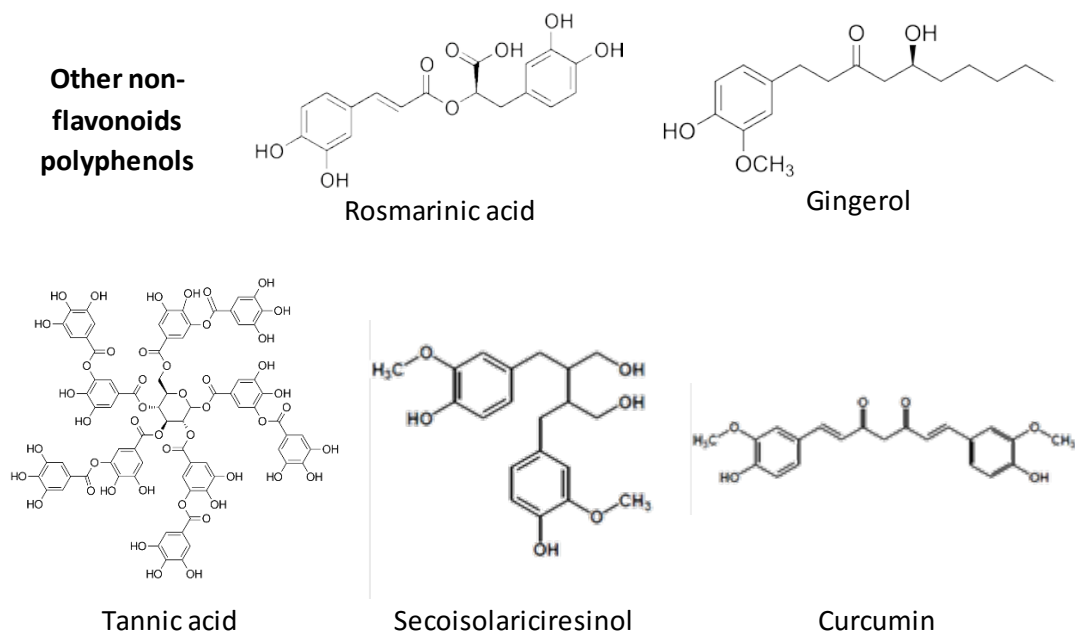
There is a wide range of polyphenol sources: several plants spices, fruits such as cherries and grapes, nuts, berries, tea, wine, olive oil, chocolate and leafy vegetables such as rosemary (Piñeros-Hernandez *et al.,* 2017), salvia (Atwi *et al.,* 2016) or thyme (Köksal *et al.,* 2017).

Polyphenols from plant extracts are of special interest due to the synergistic action of different polyphenolic compounds (Dai, Chen, & Zhou, 2008; Trifkovic´ *et al.,* 2014). They have been found to be strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. Polyphenols may terminate the propagation reactions, induced by free radical intermediates, by reacting either directly with the free radical o by preventing hydroperoxides from decomposing into free radicals by acting as chain-breaking (Zhou *et al.,* 2005; Mozafari *et al.,* 2006; Garrido and Borges, 2013). In addition, polyphenols are also known as metal chelators. Chelation of transition metals such as Fe^{2+} can directly reduce the rate of Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals (Tsao, 2010). Tea preparations have been shown to trap reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide and peroxynitrite, reducing their damage to lipid membranes, proteins and nucleic acids in cell-free systems (Khan and Mukhtar, 2007).

Flavonoids are one of the most studied groups within the phenolic compounds They consist of two phenyl rings (A and B), bound by a pyran ring (C) as shown in **figure 5** and in **table 4.**
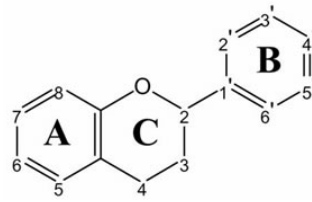
Figure 5. Basic structure of the flavonoids.

Flavonoids high antioxidant capacity is caused by their ability to reduce the production of free radicals, either by inhibiting the enzymes involved, or by chelation with the transition metals responsible for the generation of free radicals. In addition, due to their low redox potential, they are capable of reducing highly reactive oxygen species (such as oxygen ions, free radicals and peroxides) (Garrido *et al.,* 2013). In general, polyphenolic compounds as antioxidants are multifunctional and can act according to most of the above mentioned mechanisms. Procházková *et al.* (2011) review the main mechanisms of the antioxidant and prooxidant effect of flavonoids:

- Direct scavenging of reactive oxygen species by hydrogen atom donation.
- Activation of antioxidant enzymes. Flavonoids are able to induce phase II detoxifying enzymes, which are the major defense enzymes against electrophilic toxicants and oxidative stress.
- Metal chelating activity,
- Reduction of α-tocopheryl radicals. Flavonoids can act as hydrogen donors to α-tocopheryl radical, which is a potential prooxidant.
- Inhibition of enzymes responsible for superoxide ($O_2 \bullet^-$) production.
- Mitigation of oxidative stress caused by nitric oxide
- Increase in uric acid levels.
- Increase in antioxidant properties of low molecular antioxidants (Several authors described prooxidative activity of ß-carotene under certain conditions and suggested that its combination with an antioxidant may have preventive effect).

The antioxidant properties of foodstuffs rich in flavonoids depend not only on the polyphenol content, but also on their type. For example, quercetin and catechin showed the greatest antioxidant properties *in vitro* (Kozlowska & Szostak-Wegierek, 2014).

Regarding the non-flavonoid group, phenolic acids (hydroxycinnamic and hydroxybenzoic acids) have been shown interesting antimicrobial and antioxidant activity as shown by several authors (Calabriso *et al.,* 2016; Escriche *et al.,* 2014; Heleno *et al.,* 2015; Rice-Evans *et al.,* 1996; Proestos *et al.,* 2006). Cinnamic, ferulic, caffeic, sinapic, salicylic and vanillic acids, among others, are some examples of polyphenols with strong antioxidant activity.

Thyme (*Thymus serpyllum L.*) is an aromatic plant from the family *Lamiaceae* that has been traditionally used as a medicinal plant or as a condiment in food. It is a rich source of polyphenols, especially its leaves (Gallego *et al.,* 2013; Stojanovic *et al.,* 2012). The quality of the thyme is generally determined by its essential oil content. The lipid extract from thyme herb mainly contains large amount of p-cymene and thymol and to a lesser extent carvacrol, linalool, borneol, among others (Grigore *et al.,* 2010). Several *in vitro* experiments carried out in recent decades reported the antibacterial, antifungal, and antioxidant effects of both the essential oils and the extract of thyme (Safaei-Ghomi *et al.,* 2009; Trifkovic´ *et al.,* 2014). Roby *et al.,* (2013) evaluated the antioxidant capacity and the total amount of phenolic compounds of thyme, and showed that the extract with methanol from thyme had better antioxidant activity as compared to α-tocopherol and butylated hydroxyanisole, which is a synthetic antioxidant used as a food additive.

In spite of the great potential of thyme essential oil, its uses in food preservation are limited mainly due to potential toxicity problems and its intense aroma and flavour i, which led in many cases to remarkable changes in the sensory attributes of the food products (Sánchez-González *et al.,* 2011). In this sense, the use of aqueous thyme extract could be especially desirable because it has an acceptable flavour and limited aroma. . The hydrophilic extracts of thyme contain interesting compounds, such as caffeic acid and its oligomers, flavonoid glycosides, hydroquinone derivatives, terpenoids and biphenyl compounds, among others (Fecka & Turek, 2008). Rosmarinic acid, which is a caffeic acid oligomer, is the main phenolic compound found in the aqueous infusion (Mihailovic-Stanojevic *et al.,* 2013) with remarkable biological activities, e.g. antiviral, antibacterial, anti-inflammatory and antioxidant (Petersen and Simmonds, 2003).

Thyme extract polyphenols, have been also used as crosslinkers, being the main chemical pathway related with the oxidization of diphenyl moieties of phenolic acids or other polyphenols, producing quinone intermediates that react with nucleophiles (mainly amino or sulfhydryl side chains) to form covalent C-N or C-S bonds with the phenolic ring (Azeredo and Waldron, 2016).

3.2. Polyphenols from essential oils

Essential oils (EOs) are aromatic oily liquids obtained from plant material (i.e. flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Burt, 2004). Numerous studies reported the its antioxidant and antimicrobial effect of essential oils against the oxidation and growth of pathogens in numerous foods such as cheese, meat or nuts (Olmedo *et al.,* 2012).

Terpenoids and phenylpropanoids (cinnamic acid derivatives) are the main constituents of the essential oils. In addition, few aromatic and aliphatic constituents are also present. Monoterpenes, sesquiterpenes (two and three isoprene units, respectively)

and oxygenated derivatives of these two are the largest group of chemical entities in EOs (Carson *et al.,* 2006; Raut & Karuppayil, 2014). The isoprene units can make connections to form rings, thus important terpenoids can derived from monocyclic terpenes such as menthol, thymol, carvacrol and many others.

Pure components from essential oils, commonly used as flavouring agents in the food industry, also present interesting antibacterial, antifungal and antioxidant properties. Numerous studies have reported their bioactive nature including antiviral, antimutagenic, anticancer, anti-inflammatory, immunomodulatory, and antiprotozoal activities (Bakkali *et al.,* 2008; Jayasena & Jo, 2013; Ramos *et al.,* 2016; Sokolik *et al.,* 2018; Sivakumar & Bautista-Baños, 2014; Stratakos *et al.,* 2018). Although all the components of essential oils may present activity, some studies try to determine which compounds are responsible for the major antioxidant or antimicrobial effect. Carvacrol, thymol, eugenol are the main components responsible for the antioxidant activity of basil and thyme oils (Sánchez-González *et al.,* 2011). Sometimes the main components of EOs are not responsible for the activity since the bioactive effect is explained by the presence of a combination of other minority molecules (Hyldgaard *et al.,* 2012; Raut & Karuppayil, 2014). In this sense, Ismam et al. (2008) observed that the effectivity of rosemary oil against *epidopteran larvae* was a consequence of the possibly synergistic effects of several chemical constituents, and not because of the individual main compounds.

Eugenol (4-allyl-2-methoxyphenol) is a natural phenolic substance found as a major compound in different plant essential oils, such as clove, nutmeg, cinnamon or basil. It is a very versatile molecule, of liquid and oily consistency, of a light yellow color, sparingly soluble in water and soluble in alcoholic solutions. Eugenol has traditionally been widely used in cosmetics, perfumery, foodstuffs or pharmaceutical products (Bullerman *et al.,* 1977; Chatterjee & Bhattacharjee, 2013; Friedman *et al.,* 2000) for different purposes (flavoring in alcoholic beverages, ice creams, candies, mouth rinses, etc.). In dentistry, for example, it is used for its analgesic and antiseptic properties. In combination with zinc oxide, eugenol forms a polymerized cement used for surgical bandages, temporary fillings, coating or coating agents (Shinde & Nagarsenker, 2011).

The interest of eugenol in food technology it is related with its antioxidant and antimicrobial capacity (Devi *et al.,* 2010; Woranuch & Yoksan, 2012). The antimicrobial activity of eugenol against Gram positive and Gram negative bacteria (*Bacillus subtilis*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*, (Dorman & Deans, 2000; Cetin-Karaca & Newman, 2015; Burt, 2004)), fungi (*Aspergillus carbonarius*, *Penicillium roqueforti* and *Microsporum gypseum* (Šimović *et al.,* 2014; Lee *et al.,* 2007) and yeast (Saccharomyces cerevisiae and Candida (Bennis *et al.,* 2004; Pinto *et al.,* 2009) has been described. The antioxidant capacity of eugenol have also been studied by several authors (Gülçin, 2011; Kamatou *et al.,* 2012; Nagababu & Lakshmaiah, 1992; Ogata *et*

al., 2000; Scherer & Godoy, 2009). Mayonnaise formulated with eugenol had significantly higher antioxidant activity and phenolic content and better maintained its properties for a longer period of time than commercial samples (Chatterjee & Bhattacharjee (2015). Cortes-Rojas et al. (2014) produced antioxidant powder products with solid lipid nanoparticles (SLN) containing eugenol.

Recently, eugenol has been investigated for active packaging Sanla-Ead *et al.,* (2012) reported a positive activity of cinnamaldehyde and eugenol against *Aeromonas hydrophila* and *Enterococcus faecalis in* cellulose-based packaging films.

As a result of its wide range of pharmacological and biological applications and in food technology, eugenol and clove remain a source of research of interest, as shown in **Table 5.**

Table 5. Applications and properties of eugenol (source: Katmatou *et al.,* 2012)

Pharmacologic properties	Anti-Infective Activity:	Antibacterial Activity	Dorman & Deans (2000)
		Antifungal Activity	Cheng *et al.* (2008)
		Antiplasmodial Activity	Van Zyl *et al.* (2006)
		Antiviral Activity	Astani *et al.* (2011)
		Anthelmintic Activity	Pessoa *et al.* (2002)
	Anti-Inflammatory Activity		Daniel *et al.* (2009)
	Analgesic Activity		Guenette *et al.* (2006)
	Antioxidant Activity		Mastelic *et al.* (2008)
	Anticancer Activity		Bayala *et al.* (2014)
	Antimutagenic and antigenotoxic Activities		Rompelberg *et al.* (1995)
	Modulatory Effects		Manikandan *et al.* (2011)
	Other Pharmacological Properties:	Anti-ulcerogenic effects	Katmatou *et al.* (2012)
		Spasmolytic and relaxant effects	Katmatou *et al.* (2012)
		Antipyretic activity	Katmatou *et al.* (2012)
Agricultural applications: Fungicidal effect			Abbaszadeh *et al.* (2014)
Insecticides and Fumigant properties			Huang *et al.* (2002)
Improvement of skin permeation			Zhao & Singh (1998)
Toxicity and allergenicity: as a function of the concentration and its application			Katmatou *et al.* (2012)

Eugenol is very volatile, unstable and sensitive to oxygen, light and heat (Choi *et al.,* 2009) and has an intense aroma, which is an important drawback in terms of its potential application as antioxidant agent.

A major challenge in food technology is related with the protection and conservation of volatile additives to promote their full effectiveness and to reduce the high impact in the flavor products. One interesting option to preserve eugenol activity would be the use of edible coatings as vehicles, which provide protective mechanisms that can maintain the activity until the time of application or consumption. Also, there are recent studies that study the encapsulation of active compounds as a method to preserve their functional properties. In this way, the retention of the volatile compounds is promoted at the same time that the doses of active are reduced, controlling its release.

4. Development of films with active compounds

The direct addition of active compounds to the food surface can cause neutralization or rapid diffusion in the food thus reducing their effectiveness (Mastromatteo *et al.,* 2010; Ubbink and Krüger, 2006). In particular, the direct food application of polyphenols is limited by their relatively rapid actuation (Perazzo *et al.,* 2014). Therefore, active compounds require protective mechanisms that can maintain the activity until the time of application or consumption. One interesting option would be the use of edible coatings as vehicles of these natural compounds. The combined use of natural antioxidants and antimicrobials and packaging materials could contribute to increase their effectiveness of their food application on, thus limiting the oxidative reactions and extending their action during a longer time.

Edible and biodegradable films show a high potential to contain active ingredients, such as antioxidants, dyes, flavorings, nutrients, spices and antimicrobial agents that can help to extend the shelf life of the food and reduce the risk of pathogen growth on the surface thereof (Antunes & Cavaco, 2010; Gonzalez-Aguilar *et al.,* 2008; Oms-Oliu *et al.,* 2010; Zúniga *et al.,* 2012. The use of edible films incorporating active compounds, such as essential oils or extracts from plant origin, has been particularly prominent in recent years due to its potential applications in food preservation (Sung *et al.,* 2013). These active natural compounds are known for their antimicrobial and/or antiviral activity, insecticide and antioxidant properties (Sivakumar & Bautista-Banos, 2014). Among these active ingredients, both essential oils and some of their main constituents have been widely studied, such as carvacrol, menthol, thymol or eugenol due to their antimicrobial and antioxidant power.

Most of the studies on the incorporation of active ingredients into packaging materials are focused on their antimicrobial activity (Siripatrawan & Vitchayakitti, 2016; Clarke *et al.,* 2016; Yemenicioğlu, 2016) and few studies used these active agents as antioxidants. Bonilla *et al.,* (2013) evaluated the effect of the incorporation of basil and thyme essential oils on the physical, mechanical, structural and antioxidant properties of blend-

films based on wheat starch and chitosan. The addition of these essential oils improved the oxygen barrier properties. Peng & Li (2014) added three essential oils (lemon, thyme or cinnamon) in chitosan films and reported that when these oils are combined the water barrier properties of the films improved with few synergistic antimicrobial effects against the bacterial strains studied.

The incorporation of antioxidants in films is difficult due to its instability under the processing conditions and storage (e.g. temperature, oxygen and light), which limits their potential antioxidant activity Previous studies have shown that the incorporation of polyphenols in films led to some antioxidant capacity (Gómez-Guillén *et al.,* 2007), which depends on the encapsulating power of the polymer matrix.

Many studies have been conducted on the improvement of film functional properties by the incorporation of natural antioxidant compounds. Siripatrawan & Vitchayakitti (2016) incorporated propolis extract, high in polyphenols, into chitosan films that showed better antioxidant and physical properties and an improvement in the barrier and antimicrobial properties. Piñeros-Hernandez *et al.* (2017) obtained similar results in terms of antioxidant activity when incorporating polyphenol-rich rosemary extracts within cassava starch films.

The choice of the biodegradable polymers used as carriers of the active compounds could be determinant in the final properties of the film. Corn starch as a biodegradable biopolymer, exhibits a greater potential to obtain edible films due to its easy availability and low cost. Several authors have shown the possibility to obtained starch-based films by thermo-processing techniques under destructuring and plasticization conditions (Averous *et al.,* 2000; Jiménez *et al.,* 2012; Muller *et al.,* 2017; Park *et al.,* 2002). Nevertheless, the incorporation of different active compounds to starch films could have an impact on their physical properties which can affect their functionality as a packaging material (Sánchez-González *et al.,* 2015; Cano *et al.,* 2016a). Moreover, the antioxidant and antimicrobial properties of the active compound could be affected by the kinetics of their release to the food. These parameters have been little studied and may depend on several factors such as the characteristics of the food or the structure of the film.

In hydrocolloid-based films, crosslinking is an important step to ensure their stability and mechanical resistance (Mathew & Abraham, 2008; Rivero *et al.,* 2010). A cross-link is a bond that links one polymer chain to another. They can be covalent bonds or ionic bonds. In this sense, genipin was employed to stabilize gelatin films (Bigi *et al.,* 2002); citric acid has been tested to cross-link starch-based films (Reddy & Yang, 2010); gelatin films were cross-linked, respectively, by ferulic acid and tannin acid by Cao *et al.,* 2007; proanthocyanidin crosslinked gelatin/chitosan films (Kim *et al.,* 2005).

Tannic Acid (TA) is a polyphenol naturally found in some green leaves, which exhibits interesting properties due to its multiple phenolic groups that can interact with macromolecules, such as chitosan (Aelenei *et al.,* 2009). Thus, TA can act as a cross-

linker, leading to a more rigid and compact chitosan matrix and improving the films' physical properties. Rivero et al. (2010) studied the capacity of tannic acid in plasticized chitosan films. The addition of tannic acid to the chitosan matrix led to form a more rigid structure because it acted as a crosslinking agent. The increase of tensile strength and the decrease of elongation as well as WVP indicated the compaction of the chitosan matrix. The presence glycerol, showed a synergic effect in combination with tannic acid, and the effect of the storage was also relevant leading to a more stable structure due to the reorganization toward an anhydrous conformation.

When developing films with essential oils it is important to take into account the losses of the volatile compounds from the oil. In this sense, many current works are oriented towards improving the retention of the compound in the matrix by encapsulation techniques.

4.1. Encapsulation of the incorporated active ingredients

Encapsulation can be defined as the process by which the active compounds are surrounded by another coating substance (Dickinson, 1992; McClements, 2005; Desai & Park, 2005; Gharsallaoui *et al.,* 2007; Fang & Bhandari, 2010). Until now this technique has been used mainly in the pharmaceutical field. However, currently its application in new food products is increasing. The main advantages in the food industry of having an encapsulated active compound, whether they are antioxidant compounds or microorganisms, are the ease of handling, the protection during storage and transport and their better control in the release towards food (Bae & Lee, 2008). The reduction of the size of the particles to nano (less than 100 μm) or micro scale would allow the direct addition of the encapsulates to a multitude of foods (Hansen *et al.,* 2002).

The encapsulation is developed through a process divided mainly in two stages. First, active compounds and encapsulants (wall-materials) dissolve homogeneously in an aqueous phase. In the case of using hydrophobic compounds, the dispersion formed will be an emulsion, generally of the oil-in-water (O/W) type.

As seen in **Figure 6**, depending on the encapsulation technique that is applied, different procedures will be required. Some of the most important and usual microencapsulation methods to produce humid and dry capsules are discussed below.
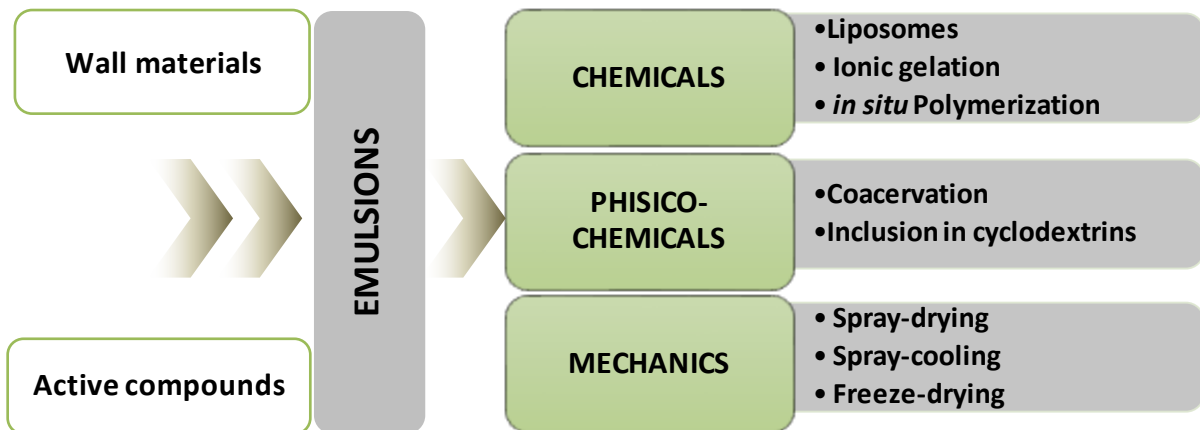
Figure 6. Encapsulation methods, according to their nature.

Liposome formation

Lecithins are used as an encapsulating agent, both for hydrophilic and lipophilic components (Taylor *et al.,* 2005). The major source of lecithin are soy beans, eggs, milk, sunflower seeds and rapeseed (Szuhaj, 2005). Commercial soy lecithin contains about 65-75% phospholipids as i.e phosphatidylcholine and phosphatidylethanolamine, 34% triglycerides, and smaller amounts of carbohydrates, pigments, sterols, and sterol glycosides (Dickinson, 1993). The chemical structure of lecithins allows the formation of liposomes which can entrap different kinds (more or less polar) of active compounds (Liolios *et al.,* 2009).

Liposomes, obtained by chemical processes, are spherical bilayer vesicles composed of polar lipids from lecithin dispersed in a polar medium such as water. At neutral pH, phosphate and carbonyl groups from phosphatidylcholine and phosphatidylethanolamine components in lecithin contribute to the negative charge of the particles in the emulsion, thus contributing to emulsion stability by charge (Dickinson, 1993; Wang and Wang, 2008). Liposomes have been used to encapsulate both soluble and insoluble compounds in water as antimicrobials, flavours, antioxidants and dyes. Liposomes have also been widely used in the encapsulation of essential oils (Sebaaly *et al.,* 2015). Active compounds can interact with liposomes in several different ways depending on their solubility and polarity characteristics. Thus, they can be inserted in the lipid chain bilayer region, intercalated in the polar head group region or adsorbed on the membrane (Grabielle-Madelmont *et al.,* 2003) (**Figure 7**).

The release of the active compound is determined by the nature of the liposome. Thus, when the lipid reaches the gel to liquid transition temperature, the active compound will be released. For example, Sebaaly *et al.,* (2015) encapsulated clove essential oil in liposomes formed from soybean phospholipids, being effective against the degradation induced by exposure to UV light, also maintaining the antioxidant properties stable after 2 months of storage at 4°C.
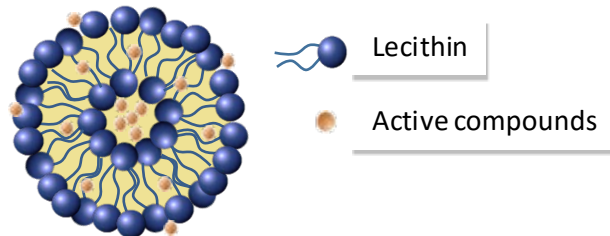
Figure 7. Schematic representation of liposome and possible positions of active compounds.

The deposition of several polymer layers on the liposome capsules reduces the accessibility to the active compounds and their reactivity. The technique of electrostatic deposition of polymers has proved to be very effective in the encapsulation and can offer physical and chemical protection to the encapsulated compounds. This approach has been studied by Gibis *et al.,* (2012) in dispersions based on grape seed extract, where the encapsulation was performed by liposomes and the formation of multilayers was achieved by the addition of chitosan and pectins, as a second and third layer, respectively. The liposomes are negatively charged, which interact with the positive charges of the chitosan, thus being covered by a second layer. In turn, the pectin, with a negative charge, is deposited on the particle previously coated with chitosan, which now has a positive charge, giving rise to a multilayer. The results showed that the multiple layers formed with the biopolymers that coated the liposomes decreased the amount of polyphenols that reacted with the Folin-Ciocalteu reagent, which suggested that the polyphenols were not exposed to the aqueous phase. In addition, the microcapsules coated with polymers were very effective encapsulation systems that could contribute to reduce the interactions of the polyphenols with the rest of the food ingredients, thus maintaining their functional properties intact.
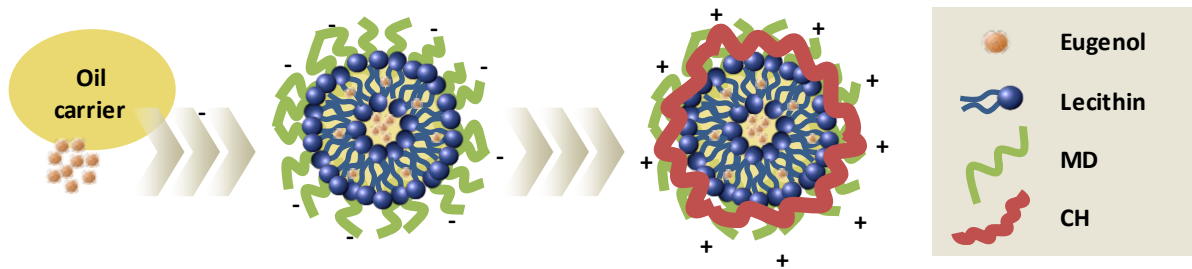
Figure 8. Schematic overview of possible interactions between active compounds (especially essential oils), lecithin as wall material, maltodextrins (MD) as coadjuvant agent and chitosan (CH) as oppositely charged polymer.

Coacervation

Coacervation is a physicochemical process that involves the separation of one or more hydrocolloids and the deposition of the coacervate phase over the active ingredient. In the food field, biopolymers of different load have been used to form the coacervate complex. The strength of the interaction between the two polymers will depend on the type of polymer, molar mass, pH and charge. The characteristics of the biopolymers will also determine the formation of a one-phase or two-phase system (Augustin & Hemar, 2009). Microcapsules produced by coacervation are water-insoluble and heat-resistant, possessing excellent controlled release characteristics based on mechanical stress, temperature and sustained release (Sánchez *et al.,* 2016). Coacervation encapsulation can be achieved simply with only one colloidal solute such as gelatin, or through a more complex process, for example, with gelatin and gum acacia (Fang & Bhandari, 2010). A graphic representation of the formation of coacervation complexes from whey protein or gelatin with gum arabic is shown in **Figure 9.**
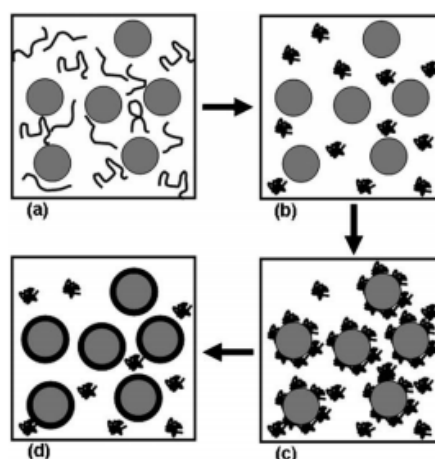
Figure 9. Active compound dispersión (a), initial coacervation of gelatin as a concequence of coacervant agent (for example, absolute ethanol) (b), gelatin coacervation around the core (c) and calpsule formation (d). Source: Augustin & Hemar, 2009.

Ionic gelation

The ionic gelation technology has attracted great interest due to its highly compatible, non-toxic, organic solvent free, convenient and controllable benefits. The technique is based on the subjection of polysaccharides to ionic gelation, which precipitates to form spherical microbeads as a result of the complexation between oppositely charged compounds. After the dispersion of the alginate into droplets, the next step is to gel the droplets. This is typically achieved by crosslinking the alginate polymer chains with divalent cations, which forming and "egg-box" structure (**figure 10**) (Zhang *et al.,* 2017). It has been applied using different polysaccharides as encapsulating materials such as alginate, k-carrageenan, gellan gum, xanthan, pectin, chitosan and gelatin (Abdel-Hafez *et al.,* 2014; Benavides *et al.,* 2016). This is the most popular method for humid microcapsules production and has been used successfully for microencapsulation of several essential oils (Valencia-Sullca, 2017).
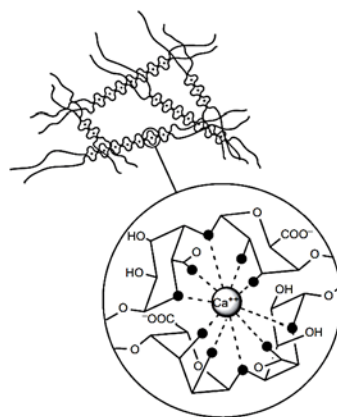
Figure 10. Egg-box structure of alginate after gelation. Source: Selimoglu & Elibol, 2010.

Spray-drying

Although there are other techniques, spray drying is the most widely used technique in encapsulation, being economical and with good quality results. Spray drying is especially useful for the encapsulation of thermo-sensitive food ingredients, since it is a quick drying process and the temperatures reached are relatively low. In addition, the particle size (10-50 μm) and the regular microstructure obtained by this technique are appropriate (Barrow *et al.,* 2013). The first step to spray-drying is to prepare an emulsion with the active compound and the encapsulating materials, until oil drops of 1-3μm are formed. Once the emulsion is obtained, it is atomized in the equipment (**Figure 11**). The dispersion is atomized with hot air to facilitate the rapid elimination of water while the drops are mixed with hot air in the drying chamber. Once the capsules are obtained, they are collected from the bottom of the dryer (Augustin and Herman, 2009; Gibbs *et al.,* 1999). In food technology, the spray drying technique is used to encapsulate vitamins, polyunsaturated oils, enzymes and probiotics and mask flavors, among others

(Augustine & Hemar, 2009). For example, Rodea-Gonzalez et al. (2012) efficiently encapsulated chia essential oil in a matrix of whey protein and polysaccharides, using the spray technique. In the food industry it is very common to work with dispersions of the aqueous type, therefore, the materials that make up the matrix must be very water-soluble. In addition, the glass transition temperature must be high enough not to obtain gummy dust. The active compounds that will become part of the nucleus of the new microcapsules can be both hydrophobic and hydrophilic. In the case of a hydrophobic active compound, an oil-in-water (O/W) emulsion will be formed prior to drying. On the other hand, those hydrophilic active compounds will be dispersed in the aqueous phase directly (Augustin & Hemar, 2009).
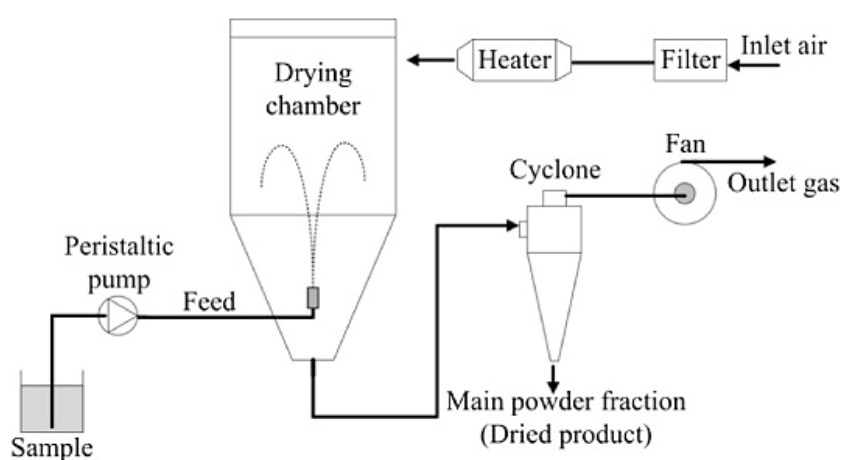
Figure 11. Schematic diagram of a spray dryer. Source: Khuenpet *et al.,* 2016.

Freeze-drying

Freeze-drying is used for the dehydration of almost all heat-sensitive materials and aromas such as oils. Besides protecting heat-sensitive core materials, freeze-drying is simple and easy to operate (Bakry *et al.,* 2016). Also, is widely used for microencapsulation, especially in probiotics encapsulation, in order to increase the bacteria resistance to freezing and freeze drying of the food (Heidebach *et al.,* 2010; Semyonov *et al.,* 2010). It is a multistage operation stabilizing material through four main operations such as freezing, sublimation, desorption and finally storage (Ray *et al.,* 2016). Freeze-drying is a drying process that enables the long-term preservation of several heat sensitive food and other biological materials. Freeze-dried materials seem to have higher retention of volatile compounds in comparison to that of spray-drying (Krokida & Philippopoulos, 2006). Some examples of successfully active compounds encapsulation are some oils such as fish, flaxseed, walnut and olive (Calvo *et al.,* 2011; Tamjidi *et al.,* 2013), and some main compounds from oils such as thymol and cinnamaldehyde (Ponce Cevallos *et al.,* 2010).

The major disadvantages of freeze-drying are the high energy input and long processing time. In addition, during processing, a barrier with an open porous structure between the active agent and its surroundings is formed, offering poor protection when prolonged release of an active is required (Zuidam & Shimoni 2010).

Spray cooling/chilling

Spray cooling/chilling is a similar process as spray-drying, but no water is evaporated, and the air used is cold, which enables particle solidification (Đorđević *et al.,* 2015). It is based on the atomization of a mixture of the active substance and a melted lipid material (usually melting fats) in a cold chamber. The, the fat solidifies, and the core is immobilised (Augustin & Hemar, 2009). These processes, which have high process costs and require special handling and storage conditions, are suitable to encapsulate water soluble materials (i.e.mineral salts, enzymes, flavours, food acids and protein hydrolysates andthey are also used for encapsulation of aroma compounds to improve heat stability and control release (Gavory *et al.,* 2014; Lopes *et al.,* 2015; Zuidam & Shimoni, 2010).

Spray-freeze drying

Spray–freeze drying (SFD) is an effective alternative to spray-drying and freeze-drying techniques. SFD is a two-step process where the first step involves spray freezing, and the second one involves freeze drying (Hundree *et al.,* 2015). Currently, there are three types of techniques being used for spray freezing: spray freezing into vapour, spray freezing into vapour over liquid, and spray freezing into liquid (Anandharamakrishnan *et al.,* 2010).

SFD was used for the first time for producing docosahexaenoic acid (DHA) in microencapsulated form. The main advantages of spray-freeze-drying techniques are controlled size and high capsules surface area. SFD technique may be an effective alternative to conventional freeze drying (needs long drying time) and spray drying techniques (required higher temperatures) for microencapsulation of active compounds (Karthik & Anandharamakrishnan, 2013).

This technique has some disadvantages including the high use of energy and the cost, which is 30-50 times higher than spray drying (Zuidam & Nedovic, 2010).

Fluid bed coating

Fluid bed (**figure 12**) coating is an encapsulation technique where a coating is applied onto powder particles in a batch processor or a continuous set-up. The powder particles

are suspended by an air stream at a specific temperature and sprayed with an atomized, coating material (Nedovic *et al.,* 2011). This type of encapsulation process is capable of efficiently coating dry solid particles (powders), where the particles can be surrounded by a uniform layer or multiple layers of coating materials (Teunon & Poncelet, 2002). This technique allows for the improvement of the barrier properties and increases the protection of sensitive ingredients (Coronel-Aguilera & San Martín-González, 2015).

Fluid bed technology is a batch process that is expensive and time-consuming. However, the fluid bed system is the most adequate for particle coating, especially in pharmaceutical and cosmetic industries where the cost of the process is compensated by the high price of their final product (Teunon & Poncelet, 2002).
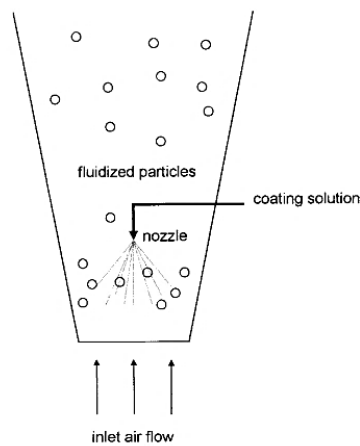
Figure 12. Top-spray fluidized bed coating. Source: Dewettinck & Huyghebaert (1999).

Electrospinning

Electrospinning is a technique that uses electrostatic forces to create polymer fibres. The equipment (**figure 13**) consists of an electrically conductive needle tip, a grounded collector and a power supply, responsible for generating the high voltage (around 5-50 kV) (Tampau *et al.,* 2017). A high electric field is applied to a fluid, which comes out from the tip of a die that acts as one of the electrodes. This leads to droplet deformation, leading to a structure known as a Taylor cone, and finally to the ejection of a charged jet from the tip towards the counter electrode, hence to the formation of continuous capsules, in a cylinder shape (Ghorani & Tucker, 2015).

The good results obtained by several authors (Fabra *et al.,* 2014; Rieger & Schiffman, 2014) in terms of encapsulation efficiency and improvement of the physical properties of multilayer systems for food packaging applications, show the potential of this method (Agarwal *et al.,* 2008).
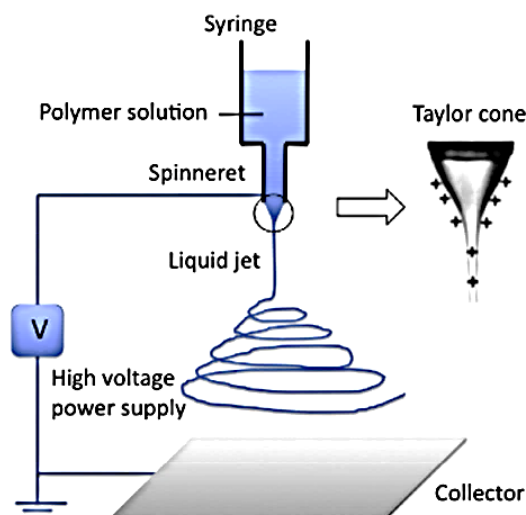
Figure 13. Schematic diagram of the electrospinning setup used during the experiment. Source: Ghorani & Tucker (2015).

4.2. Encapsulation agents (wall materials)

Depending on the physico-chemical properties of the core, the wall composition, and the microencapsulation technique used, the types, size and shape of particles can be modulated. In addition, all these conditions could also affect the functional properties and the potential applications of the encapsulated components (Bakry *et al.,* 2016; Dias *et al.,* 2015; Fang & Bhandari, 2010).

The wall material determines the stability of microparticles, the process efficiency and the degree of protection for the active compound (Bakry *et al.,* 2016). The encapsulated agent can be released by several mechanisms such as mechanical action, heat, diffusion, pH, biodegradation and dissolution. The selection of the technique and wall material depends on the final application of the product, considering physical and chemical stability, concentration, required particle size, release mechanism and manufacturing costs (Martins *et al.,* 2014; Dias *et al.,* 2015). For instance, the choice of the materials used as coating in the encapsulation can be decisive in the production of microparticles by spray-drying, since they have a great influence on the properties of the emulsion before drying and on the retention of the active compound and shelf life of the product in powder after drying (Botrel *et al.,* 2014).

The materials used for encapsulation of ingredients have to be food-grade (Augustin & Hemar, 2009). Due to legal restrictions, different compounds widely accepted for drug encapsulation but not certified as GRAS, cannot be used in the food industry.

When selecting the wall material, some criteria must be taken into account, such as its physico-chemical properties (solubility, molecular weight, glass/melting transition,

crystallinity, diffusivity), film-forming and emulsifying properties, mechanical strength, compatibility with the food product, appropriate thermal or dissolution behaviour, release profile and particle size. Moreover, the costs should be also considered (Madene *et al.,* 2006).

For example, the microencapsulation process by the spray-drying method makes possible the transformation of the mixture of active compound and encapsulating agents into a powder, where small droplets of emulsion are transformed into dehydrated microparticles. The effectiveness of the process will be determined by the properties of the emulsion to be atomized and, consequently, by the materials used as a wall system to encapsulate the active compounds. Thus, an encapsulating material must be soluble in water, be a good emulsifying agent to stabilize the emulsion, have low viscosity and must act as a coating and barrier, so it has to be similar to the compound to be encapsulated.

During the drying process, small droplets surrounded by wall materials are placed in contact with hot air. When relatively high temperatures are used, thermal degradation of thermosensitive products can occur, so the material must have stable thermal properties (Bae and Lee, 2008; Kagami *et al.,* 2003; Ré, 1998).

Maltodextrins, gums and some proteins have been found to be highly effective compounds for atomization encapsulation. The choice of a material capable of encapsulating active compounds of interest is therefore decisive since they have a great influence on the properties of the emulsion before drying, on the retention of the active compound during the spray drying and on the duration of the products in powder once they are obtained (Botrel *et al.,* 2014).

Whey protein isolate (WPI) belongs to the group of milk proteins that show emulsifying and gelling properties (Turgeon *et al.,* 2001). In general, WPIs have good compatibility with certain polysaccharides under neutral or acidic conditions and are widely used to stabilize emulsions. The effectiveness of WPU for the encapsulation of volatile fats and oils have been reported (Bae & Lee, 2008; Kagmi *et al.,* 2003). For the production of powdered products, proteins have been used mainly in combination with carbohydrates such as maltodextrins (Baik *et al.,* 2004, Kagami *et al.,* 2003, Keogh & O'Kennedy., 1999).

Maltodextrin (MD) is a hydrolysed starch widely used in food technology. Its relatively low cost, its neutral aroma and taste, its low viscosity at high concentrations and its good protection against oxidation gives it great potential for many uses (Sheu & Rosenberg, 1998). Spray encapsulation studies have shown that maltodextrins improve the properties of the capsules during the drying stage due to the formation of a larger crust around the drops. In this way, oxidative stability is increased since oxygen permeability is reduced (Kagami *et al.,* 2003). However, the main drawback of this encapsulating material is its low emulsifying capacity, which is why it has to be used in combination with other polymers (Fernandes *et al.,* 2008). For example, mixtures of WPI with MD

have been used in the encapsulation of different components of avocado, ginger, oregano and essential oils (Shah *et al.,* 2012). Akhtar & Dickinson, (2007) developed mixtures of WPI and conjugated MD (Maillard reaction) to improve the emulsifying properties of protein dispersions and orange essential oil in water.

Lecithin (LE) is a polar lipid compound, extracted mainly from soy or egg, which is used to encapsulate by liposomes, as previously mentioned. LEs are used as emulsifiers and texture modifiers of some foods and, more currently, as an encapsulating agent, both for hydrophilic and lipophilic components (Taylor *et al.,* 2005). LEs together with maltodextrin can be also a good anionic wall system that is able to stabilise in oil-in-water emulsions as microcapsules (Karadag *et al.,* 2013).

Chitosan (CH) has been used as an encapsulating material. Being a positively charged polymer, the molecule reacts easily with negatively charged substances (Kosaraju *et al.,* 2006), forming a film around it.

Polar lipids, such as glycerides, phospholipids or fatty acids, can be used as carriers of lipophilic compounds in aqueous media, favoring their dispersion, prior to their encapsulation by spray drying. They have been extensively studied for the controlled release of substances of poor water solubility, mainly in cosmetic and pharmaceutical applications (Tamjidi *et al.,* 2013; Woo *et al.,* 2014). Oleic acid in combination with other solid lipids has been successfully used to prepare micro and nanostructured lipid carriers with improved drug delivery properties (Chen *et al.,* 2010; Souto *et al.,* 2004; Woo *et al.,* 2014). The presence of oleic acid in the dispersed phase of the film-forming dispersion increased the retention and activity of the active compound (essential oil of cinnamon leaf) in films based on of chitosan (Perdones *et al.,* 2014).

Table 6 shows some examples of the incorporation of active ingredients in biodegradable packaging materials by means of different encapsulation methods.

Table 6. Effect of the incorporation of encapsulated active ingredients in biodegradable packaging materials.

Active ingredient	Wall material	Polymer/plasticizer/ surfactant	Encapsulation Method	Film-formation method	Main results	Reference
n-hexanal (10,000 ppm/film)	Fat: Grinsted Barrier System 2000 (GBS) (2,4 g/film)	ι-Carrageenan (6 g/film)/ glycerol (1.8g/film)/Sodium dodecyl sulfate (SDS)	emulsion	casting	Fat was capable of protecting aroma compounds and the ι-carrageenan matrix.	Hambleton et al. (2008)
D-Limonene and carvone (0.5 mL aroma/g fat)	Fat (2,4 g/film): Grinsted Barrier System 2000 (GBS) and Glycerol monostearate (GMS) (90:10, w/w)	-ι-Carrageenan or wheat gluten (6 g/film) / glycerol (1.8g/film)/-	emulsion	casting	Good retention of D-limonene in the gluten-based film, with better protection from oxidation, and controlled release. .	Marcuzzo et al. (2012)
Ethyl acetate, Ethyl butyrate, Ethyl hexanoate, 2-Hexanone, 1-Hexanol, Cis-3-hexenol D-limonene and n-hexanal (10,000 ppm)	Fat (2,4 g/film): Grinsted Barrier System 2000 (GBS) and glycerol monostearate (GMS) (90:10, w/w)	Alginate (6 g/film)/ glycerol (1.8g/film)/-	emulsion	casting	Good protection of encapsulated aroma compounds the films.	Hambleton et al. (2009)
ethyl acetate, ethyl butyrate, ethyl hexanoate, 2-hexanone, 1-hexanol and cis-3-hexenol (ratio lipid:polymer, 0.4:1)	Beeswax (BW) and oleic acid (OA) (mixtures OA:BW, 70:30, 50:50 and 30:70) (ratio lipid:polymer, 0.4:1)	i-Carrageenan (6 g/film)/ glycerol (polymer:glycerol ratio 1:0.3)/-	emulsion	casting	Interactions between aroma compounds-hydrocolloid and aroma compound-lipid, which induce structural changes and modify film permeability.	Fabra et al. (2009)
Orange essential oil and limonene (essential oil:soy/rapeseed 1.1)	Soy and rapeseed (10 g of nanoliposome in 90 g of hydrocolloid dispersions)	Corn starch and sodium caseinate (50:50) (88.84 g of solids/m²)/ glycerol (hydrocolloid:glycerol ratio 1:0.25)/-	nanoliposomes	casting	Liposomes decreased the mechanical resistance and extensibility of the films.	Jiménez et al. (2014)
Cinnamon leaf oil and eugenol (0.5 g of active compound/film)	Sunflower seed lecithin (10 g of nanoliposome in 90 g of chitosan dispersions)	chitosan (1 g of solids/film) -/-	nanoliposomes	casting	Encapsulation of eugenol or CLEO promoted retention of the active ingredients in the film and improved the film extensibility and water vapour barrier capacity.	Valencia-Sullca et al. (2016)

Active ingredient	Wall material	Polymer/plasticizer/ surfactant	Encapsulation Method	Film-formation method	Main results	Reference
Cinnamon (0.5-3 g)	β-cyclodextrin (β-CD) (1-3 g)	Poly Vynil Alcohol (6-10 g) -/-	electrospinning	electrospun nanofibrous	Film maintainedthe volatile antimicrobial agent in the nanofilm and had antimicrobial activity against Gram+ and Gram- bacteria, thus prolonging the shelf-life of strawberry.	Wen et al. (2016)
Ascorbic acid (30%)	Lauric acid (LA) and oleic acid (OA) (mixtures: 70/30 and 80/20 w/w) (ratio core:carrier 70/30, 75/25, w/w)	Unripe banana starch (4%)/ glycerol (22%)/Polyglycerol polyricinoleate (5%)	double fluid pressurized atomizer	casting	Microcapsules modified the physical properties of the films and protect ascorbic acid from oxidation.	Sartori & Menegalli (2016)
Rosemary leaves (10 g leaves/100 mL ethanol) (5-20 g rosemary extract/film)	-	Cassava starch (5.0 g/film)/ glycerol (1.5 g/film)	Solvent displacement method	casting	Rosemary nanoparticles modified the mechanical properties of the films and promoted a. controlled release of actives in fatty food simulants during 7-days	López-Córdoba et al. (2017)
Nisin Z (1 mg mL⁻¹)	Soybean lecithin (2.5% w/v) (nano-vesicles:HPMC FFS, 1:1)	Hydroxypropyl methylcellulose (HPMC) (6% w/v)/ glycerol (30% w/w)	Nanoliposomes	casting	Nanoliposomes affect the physicochemical properties and nisin Z release. Films were effective against Listeria monocytogenes.	Imran et al. (2012)
Rose hip seed oil (perfused into the inner needle of the coaxial system at flow rate 0.1 mL/h)	Zein prolamine (ZP) (25, 30 and 35 w/v %)	Zein prolamine (25, 30 and 35 w/v %)/-/-	Electrospinning	electrospun fibrous	Optimal loading capacity and encapsulation efficiency were 12.24% and 90.16%, respectively, using a ZP concentration of 35 w/v%.	Yao et al. (2016)
Nisin (1.0 mg/ml)	Phospholipon* (6.5% v/v)	Gelatin (40 g/l) and casein (30 g/l)/ glycerol (1%, v/v)/nanoclays* (0.5 g/l) (as reinforcer)	liposomes	casting	The barrier properties of halloysite favoured thecontrolled release of nisin.	Boelter & Brandelli (2016)
Thyme oil 0.5% (w/w)	Trehalose (0.2, 0.4, 0.6 and 0.8%, w/w), β-cyclodextrin (0.1, 0.15, 0.2 and 0.25%, w/w) and Tween 20 (1, 1.5, 2 and 2.5%, w/w).	Sodium alginate (1%, w/w)/ sorbitol (1%, w/w), calcium carbonate (0.02%, w/w)	encapsulating agents	casting	Tween 20 led to more interactions between components and the lowest particle size.	Navarro et al. (2016)

Active ingredient	Wall material	Polymer/plasticizer/ surfactant	Encapsulation Method	Film-formation method	Main results	Reference
eugenol and carvacrol (5%, w/w)	α-, β-, and γ-cyclodextrins (CD) (50% capacity.)	whey protein isolate (WPI, 10% w/w)/ glycerol (5%, w/w)/-	cyclodextrines and spray-drying	casting	Incorporation of β-CD to the film resulted in a decreased release rate in a lipid food simulant, when there is an effective interaction between β-CD and the active.	Barba et al. (2015)
E. coli O157:H7 phages (10^{11} PFU/mL) (20%, v/v)	soybean lecithin and cholesterol (5:1, w/w) (liposome:chitosan ratios 8:2, 6:4, 4:6 and 2:8, v/v)	chitosan (1%, w/v)/ glycerol (0.5%, v/v) / polyvinylpyrrolidone* (1.0 mg/mL, w/v) a macroviscosity agent	liposomes	casting	High antibacterial activity against Escherichia coli O157:H7 of chitosan films with liposome-encapsulated phages applied to beef.	Cui et al. (2017)
tea polyphenols (TP) (51.3%, 83.3% and 96.9%)	chitosan hydrochloride (CSH) and sulfobutyl ether-β-cyclodextrin sodium (SBE-β-CD)	gelatin (Type B) (8%, w/v)/glycerol (20% w/w of gelatin)/-	ionotropic gelation	casting	Good controlled-release as a function of the different ratios of nanoencapsulated TP and fatty food simulant- and concentration-dependent.	Liu et al. (2017)
Trans-anethole (1 g/7.67 g β-CD)	β-cyclodextrin (β-CD); dialdehyde carboxymethyl cellulose (solution 1%, w/v) as crosslinking reagent (DCMC, 6 wt%)	gelatin (Type B)/glycerol (20 wt%)/-	cyclodextrines and freeze-dried	casting	Trans-anethole conferred the gelatin films with good antimicrobial activity, which was concentration-dependent.	Ye et al. (2017)
2-Nonanone (2-NN) (β-CD:2-NN ratio 1:0.5)	β-cyclodextrin (β-CD) (10 g/100g polymer)	polylactic acid and low density polyethylene/-/-	cyclodextrines dried in a convection oven	extrusion	Great growth inhibition of phytopathogenic fungus B. cinerea (36% and 60% of growth reduction for PLA and LDPE, respectively).	Abarca et al. (2017)
ferulic acid (FA-CTS ratio 1-1)	ferulic acid-coupled chitosan (FA-CTS) (0.02, 0.04, 0.08 and 0.16 g/100 g polymer)	poly(lactic acid), cassava starch and poly(butylene adipate-co-terephthalate)/glycerol (35g/100 g starch)	carbodiimide-mediated coupling reaction	blown film extrusion	Improvement of oxygen barrier property and antioxidant activity.	Woranuch et al. (2015)

5. Effectiveness of the incorporation of the active compounds

Edible films based on biopolymers incorporating active compounds, such as antioxidants or antimicrobials, can control the release of the incorporated active compounds, thus maintaining effective doses of the additive along the storage period (Fernández-Pan *et al.,* 2015). However, the effectiveness of the development of active packaging depends not only on the nature and type of the biopolymer used as structural matrix and the incorporation of the active substances into the materials, but also on the antioxidant and antimicrobial type, its retention and release, the physicochemical characteristics of the product to be protected, and all their interactions and storage conditions (Atarés & Chiralt, 2016; Fernández-Pan *et al.,*2015; Lee & Yam, 2013; Requena *et al.,* 2017).

It must be taken into account that the initial incorporation of the bioactive compounds in the film matrix does not assure food protection. A small amount of active ingredients in the film, or a low release rate could not be enough for food preservation; in the same way, an excessive amount or a fast delivery rate could result in the production of dangerous compounds due to the destruction of favorable bioactive compounds, or to the overcoming of the toxicity limit (Mihaly-Cozmuta *et al.,* 2017).

The potential sensory impact of the active ingredients on the coated or packaged product and the losses of the volatile compounds during the film preparation represent an important problem in terms of film effectiveness (Valencia-Sullca *et al.,* 2016).

The retention of the active compound in the film will be influenced by the processability of the biopolymers and of the active ingredients. Essential oils are usually incorporated into hydrophilic biopolymer films by casting, which is a technique y used for food coating or as a part of a food thermoplastic package (Alvarez *et al.,* 2014; Galus & Kadzińska, 2015; Kashiri *et al.,* 2017). However, during the film drying step, oil droplets could flocculate, coalesce and cream to the top of the drying film, where oil components volatilize together with water at a lower temperature than their boiling point (Valencia-Sullca *et al.,* 2016), thus compromising the retention of the active ingredient in the film matrix and thus its effectiveness.

The previous encapsulation of the compounds before film preparation could mitigate the losses of the actives and thus could maintain the antioxidant or/and antimicrobial film activity. Valencia-Sullca *et al.* (2016) studied the effect of eugenol and cinnamon leaf essential oil encapsulation in lecithin liposomes on their losses during chitosan-based film formation process by casting. The results showed a significant retention of the essential oils (40% - 50%) when the actives were previously encapsulated as compared to the direct incorporation of the actives in the polymer matrix (1% – 2%).

Thermoplastic processing is potentially used in industrial scale-up (Moreno *et al.,* 2016). The biopolymer is blended with a plasticizer and the active ingredients by using a hot

mixer and, then, pressed at high temperature to get the thermoformable packaging. The incorporation of active compounds by thermal processing could suppose an advantage since no volatilization of the compounds by steam distillation occurs due to the low water content required in the pellets. However, the antioxidant or antimicrobial activity of the films could be compromised by the high temperatures used that can evaporate or decompose the labile compounds.

The alternatives to overcome the losses of the active volatile compounds could be the production of bilayer or multilayer films that could promote the retention of the active ingredients in the matrix during the film formation. For example, good results in terms of antimicrobial activity against *E. coli* and *L. innocua* were obtained by Requena *et al.* (2017) when incorporating oregano and clove essential oils and their main compounds (carvacrol and eugenol, respectively) at the interface of poly[(3-hydroxybutyrate)-co-(3-hydroxyvalerate)] bilayer films produced by compression molding.

5.1. Antioxidant activity

The effective retention of the active ingredients into the film could be measured based on its final antioxidant effect. The antioxidant properties of different compounds of natural origin, such as plant extracts, essential oils and pure compounds, and their antioxidant power after incorporating into film matrices can be evaluated using various assays.

The most common phenolic quantification method used is the **Folin-Ciocalteu method,** which is based on the phenolic compounds reaction with the Folin-Ciocalteu reagent, in a basic pH, giving rise to a blue coloration susceptible to be determined spectrophotometrically at 765 nm (Atarés & Chiralt, 2016; Miguel, 2010). Several authors have been determined the antioxidant activity of films containing polyphenols based on their quantification by this Folin-Ciocalteu method. For example, Piñeros-Hernandez et al. (2017) obtained cassava starch films containing rosemary extracts and observed an increase in their antioxidant activity as the polyphenol content increased. Also, Siripatrawan & Vitchayakitti, (2016) obtained an improvement in the antimicrobial and antioxidant activities of chitosan films containing propolis extract, high in polyphenols.

The **2,2-diphenyl-1-picrylhydrazyl (DPPH) radical** was one of the earliest synthetic radicals used to test the activity of phenolic antioxidants. The DPPH method (Brand-Williams *et al.,* 1995) is based on the reduction of the DPPH˙ radical in an alcoholic solution by a hydrogen-donor antioxidant. In the radical form, this molecule shows absorbance at 515 nm of wavelength, which disappears after accepting an electron or hydrogen radical from antioxidant compounds. Siripatrawan & Harte (2010) produced chitosan films incorporated with green tea extract, and used DPPH test to show that

green tea extract enhanced polyphenolic content and antioxidant activity of the films. Liu et al. (2017) formulated gelatin films with free and nanoencapsulated polyphenols from tea extracts and the antioxidant properties of the films were investigated by monitoring their DPPH radical scavenging activity. The results showed that free tea polyphenols could maintain the short-term antioxidant properties, whereas the encapsulated tea polyphenols released could maintain the intermediate and/or long-term antioxidant properties.

Other method based on the electron transfer reaction that is used to determine the antioxidant power of films is the **FRAP** (ferric-reducing antioxidant power) **assay**. This method measures directly the ability of antioxidants to reduce a ferric (Fe^{3+}) tripyridyltriazine complex to the blue coloured ferrous complex at low pH. The spectrophotometric measurement at 593 nm is linearly related to the total reducing capacity of electron donating antioxidants (Benzie & Szeto, 1999). **Ferrous ion chelating activity (FIC) assay** measures how effectively the compounds in the antioxidant sample can compete with ferrozine for ferrous ion (Fe^{2+}) (Atarés & Chiralt, 2016; Ruiz-Navajas et al. 2013). The antioxidant causes the inhibition of the Fe^{2+}-ferrozine complex, which is spectrophotometrically quantified at 562 nm. Ruiz-Navajas et al. (2013) evaluated the antioxidant properties of chitosan edible films incorporated with *Thymus moroderi* and *Thymus piperella* essential oils by using three different antioxidant methods: DPPH, FRAP and FIC. The results showed great antioxidant activity that depended on essential oil content.

The **Trolox Equivalent Antioxidant Capacity (TEAC)** is based on comparing the antioxidant activity of the analysed substance with that of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), derived from vitamin E. The substrate used is ABTS (2'2-azino-bis- [3-ethylbenzothiazole-6-sulphonic acid]), which is previously oxidized to the cationic radical $ABTS^{\bullet+}$, of blue coloration. An aqueous solution of 7 mM ABTS and 2.45 mM potassium peroxodisulfate remained in the dark for about 16 h, obtaining the coloured cationic radical. This solution is diluted in ethanol to an initial absorbance of 0.70 (± 0.02) at 734 nm, and mixed with the antioxidant sample. The absorbance reduction is then registered over a 6 min period and compared to a calibration curve obtained with trolox solutions. Bonilla et al. (2013) measured the antioxidant activity by TEAC method in wheat starch-chitosan films containing basil and thyme essential oils, citric acid and α-tocopherol reported the greatest antioxidant capacity in films containing α-tocopherol.

5.2. Antimicrobial activity

The antimicrobial activity of plant aqueous extracts or essential oils incorporated in film matrices can be measured *in vitro* using a variety of methods. The most frequently methods used to determine the antibacterial activity of essential oils are disk diffusion,

agar wells, agar dilution and broth dilution methods (Atarés & Chiralt, 2016). In this sense it is important to take into account that the diffusion of the antimicrobial compounds in the product can be reduced when they are included in the film matrix due to interactions with film-forming materials (Ruiz-Navajas *et al.,* 2013).

The most common *in vitro* method used to test the antibacterial activity of films containing antimicrobial ingredients is the disk diffusion method, where a film disk is laid on top of an inoculated agar plate. Genskowsky et al. (2015) used this method to evaluate the antibacterial properties of chitosan films incorporated with maqui berry extract (*Aristotelia chilensis*) against *Listeria innocua, Serratia marcescens, Aeromonas hydrophila, Achromobacter denitrificans, Alcaligenes faecalis, Pseudomonas fluorescens, Citrobacter freundii and Shewanella putrefaciens*, Alboofetileh et al. (2014) developed antimicrobial alginate/clay nanocomposite films enriched with essential oils (clove, coriander, caraway, marjoram, cinnamon, and cumin essential oils) and studied their antibacterial activity against *Escherichia coli, Staphylococcus aureus,* and *Listeria monocytogenes,* by application of agar diffusion assay. The antibacterial activity of the essential oils was maintained when incorporated into the nanocomposite film, being films containing marjoram essential oil the ones with the highest antimicrobial activity.

Ortega-Toro et al. (2017) evaluated the antifungal activity of starch-based edible films containing *Aloe vera* gel. Films effective at controlling the growth of several fungi (*Fusarium oxysporum*, *Alternaria alternate*, *Colletrotrichum gloesporoides*, *Bipolaris spicifera*, *Curvularia hawaiiensis* and *Botryotinia fuckeliana*). Moreover, The films with the highest *Aloe vera* gel ratio were effective at controlling fungal decay and weight loss in cherry tomatoes.

5.3. Release studies

The potential antioxidant and antimicrobial effect of the active compounds present in bioactive films are affected by the kinetics of their release into the food surface, which in turn depends on several factors such as the food product characteristics and film microstructure. Therefore, migration studies are required to determine the rate of release of the active agents from the film to the product where it is applied.

The release of active compounds from a polymeric matrix can be influenced by several phenomena that occur in successive steps: polymeric swelling, which allows the water diffusion, macromolecular matrix relaxation, and active compounds diffusion through the polymeric matrix into the outer solution until thermodynamic equilibrium is achieved (Buonocore *et al.,* 2003; Sánchez-González *et al.,* 2011a).

The compound migration from films to the food system will be influenced by several factors such as the physicochemical properties of the migrant to be released, the hydrophobic nature of the film material, the composition and physical properties of the

film and their storage conditions. Besides, the concentration of the active compound in the film, the interactions between the actives and the matrix components, and the chemical compatibility between the active compound and the foodstuff will affect the release kinetics (Requena *et al.,* 2017).

Foods are complex structures that involve complicated release studies. For this reason, the Commission regulation (EU) 10/2011 (14 January 2011) have established different food simulants, which can imitate the behaviour of real food systems. These solvents should represent the main physicochemical properties that the food that they simulate shows. When using food simulants, test duration and temperature conditions should reproduce, as far as possible, the conditions that may occur when the active ingredient is released from the film to the food.

To describe the phenomena that occur during the release by means of mathematical models allow us for simulating similar behaviours in new delivery systems (Buonocore *et al.,* 2003). In this sense, different theoretical, empirical and semi-empirical mathematical models have been used to describe the release kinetics, such as Ritger-Peppas power law equation, Fick's second law of diffusion, Higuchi equation, Peppas and Sahlin, Berens-Hopfenberg differential equation, Burst model, and Weibull and Peleg model (Siepmann & Peppas, 2012; Trifković *et al.,* 2015).

The generalized expression (**equation 1**) of the **Rigter-Peppas model** (Ritger & Peppas, 1987) is used to investigate the possible superposition of two mechanisms of compound transport: swelling and relaxation of the polymer in contact with the solvent and diffusion of the compound through the polymer matrix. This equation can be used to analyse the first 60% of a release curve.

$$\frac{M_t}{M_\infty} = kt^n \qquad \qquad \textbf{Equation 1}$$

Where M_t is the amount of compound released at time t, M_∞ is the amount of compound released as time approaches infinity, t is the release time, k the is kinetic constant and n is the diffusional exponent characteristic of the release mechanism.

The diffusion coefficients within the polymeric systems can be determined by fitting an analytical solution of **Fick's second law** of diffusion to experimentally determined model compound release kinetics. The fickian process for a thin film is described by the following equation (Crank, 1979):

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

Where, M_t is the mass of active compound released at time t, M_∞ is the mass of active compound released at equilibrium and L is the half thickness of film.

Peleg's model is described by the following equation (Peleg, 1988):

$$M_t = M_0 + \frac{t}{k_1 + k_2 t}$$ **Equation 3**

where M_t is the mass of active compound released at time t, M_0 is the initial active compound content, k_1 is the kinetic constant of the model that is inversely related to the mass transfer rate at the beginning of the process, and k_2 is a constant that is related to the asymptotic value, which can be related to the equilibrium value. Peleg's model has been used so far mainly to describe sorption processes in real food matrices. However, this model can be very useful to predict the release kinetics of films since it can predict the value at equilibrium when time tends to infinite, and thus, this model can estimate long range values from experimental data obtained in tests of relatively short duration.

The **Weibull's model** is an alternative empirical equation that can be used to describe the entire set of experimental data. The tree-parameters that Weibull's model is given by the following equation (Costa & Lobo, 2001):

$$\frac{M_t}{M_\infty} = c(1 - \exp(-(k_{10}t)^d))$$ **Equation 4**

Weibull's equation expresses the accumulated fraction of the released ingredient, M_t/M_∞, in a solution at time, t. The location parameter t represents the lag time before the onset of the dissolution or the release process.

The kinetics of release of active ingredients in hydrocolloid-based films has been little explored and it is necessary to increase the knowledge in this field. In order to design efficient active films and to increase their functionality, more release studies are required.

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OBJECTIVES

The **general objective** of this Doctoral Thesis is to establish effective strategies for the incorporation of different types of active compounds (i.e. polyphenols from thyme extracts and eugenol) into biodegradable films based on hydrocolloids and to study the effect of the active ingredients on the functional properties of the films, as well as the release kinetics.

Specific objectives

1. To incorporate phenolic compounds from the aqueous extract of thyme in films based on pea starch, chitosan and their mixtures (**Chapter 1**).

> **1.1.** To analyse the effect of the incorporation of polyphenols, and the use of tannic acid as a crosslinking agent, on the physicochemical and antioxidant properties of the films (**I**).

> **1.2.** To study the release kinetics of polyphenols from the films in different food simulants (**II**).

2. To encapsulate eugenol with lecithin or whey protein isolate as wall materials by spray-drying, and to study the effect on the microcapsule properties of the use of oleic acid as eugenol carrier and of chitosan as a capsule stabilizer (**Chapter 2**).

> **2.1.** To analyse the functional properties of the emulsions and the spray dried product (**III**).

> **2.2.** To analyse the physicochemical and functional characteristics (antimicrobial and antioxidants) of the encapsulated compounds (**III**).

> **2.3.** To study the release kinetics of eugenol from spray-dried microcapsules in different food simulants (**III**).

3. Analysis of the effect of the incorporation of the selected microcapsules on the physicochemical and antioxidant properties of films based on corn starch obtained by casting or by thermo-compression moulding (**Chapter 2**).

> **3.1.** To analyse the effect of microencapsulated eugenol incorporation on the physicochemical and antioxidant properties of corn starch films obtained by compression moulding (**IV**).

> **3.2.** To study the release kinetics of eugenol from thermoplastic starch films in different food simulants (**IV**).

> **3.3.** To analyse the effect of eugenol incorporation in free or pre-encapsulated form in the physicochemical and antioxidant properties of corn starch films obtained by casting (**V**).

3.4. To study the release kinetics of eugenol from casted starch films in different food simulants (**V**).

3.5. To apply the selected film, according to its functional properties, to prevent sunflower oil oxidation (**V**).

CHAPTERS

CHAPTER 1

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Incorporation of non-volatile active compounds in edible films

I. Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts

II. Release of polyphenols from starch-chitosan based films containing thyme extract

CHAPTER 2

....

Incorporation of volatile active compounds in edible films

III. Encapsulation of eugenol by spray-drying using whey protein isolate or lecithin. Release kinetics, antioxidant and antimicrobial properties.

IV. Eugenol incorporation into thermoprocessed starch films, using different encapsulating materials.

V. Starch-eugenol based films for sunflower oil preservation: release kinetics and functional properties.

Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts

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ABSTRACT

The aim of this study was to analyse the antioxidant activity of different polymeric matrices based on chitosan and starch, incorporating a thyme extract (TE) rich in polyphenols. TE provided the films with remarkable antioxidant activity. When mixed with chitosan, the polyphenols interacted with the polymer chains, acting as crosslinkers and enhancing the tensile behaviour of films. The opposite effect was observed when incorporated into the starch matrix. All the films became darker, more reddish and less transparent when TE was incorporated. These colour changes were more marked in starch matrices, which suggests that TE compounds were poorly encapsulated. The use of chitosan-based matrices carrying TE polyphenols is recommended as a means of obtaining antioxidant films, on the basis of their tensile response and greater antioxidant activity, which could be associated with the development of polyphenol-chitosan interactions, contributing to a better protection of the functionality of polyphenols during film formation and conditioning.

Keywords: tensile properties, colour, gloss, DPPH method, microstructure.

1. INTRODUCTION

Oxidative reactions in foods represent a serious deteriorative process, leading to significant waste. Oxidative reactions promote the discoloration and the development of rancidity and off-flavours, negatively affecting the appearance, nutritional value and quality of foodstuffs. To overcome these problems, the food industry often uses synthetic antioxidants, with dubious health properties, to prevent these undesirable processes. So, new natural antioxidant compounds are necessary to control oxidative reactions in foods. More and more attention is being paid to natural antioxidants for two reasons; they are presumed to be safe because they are naturally occurring and they are well accepted by consumers. In many cases, they are derived from plant sources, such as herbs and spices. Polyphenols in plant extracts are of special interest due to the synergistic action of different polyphenolic compounds (Dai, Chen & Zhou, 2008; Trifkovic et al., 2014). Phenolic compounds may terminate the propagation reactions, induced by free radical intermediates, by reacting either directly with the free radical or by preventing hydroperoxides from decomposing into free radicals (Zhou, Wu, Yang & Liu, 2005; Mozafari et al., 2006).

Thyme (*Thymus serpyllum L.*) is a rich source of polyphenols, especially its leaves (Gallego, Gordon, Segovia, Skowyra & Almajano, 2013; Stojanovic et al., 2012). The quality of this genus of the family laminaceae is generally determined by its essential oil content. Many studies have been carried out in the field of films and coatings using essential oils as antioxidant agents, being the results usually limited by the intense aroma and flavor impaired by them, which led in many cases to remarkable changes in the sensory attributes of the food products. In this sense, the use of aqueous thyme extract could be especially desirable because it has an acceptable flavor and limited aroma for food products. The hydrophilic extracts of the herb contain interesting compounds, such as caffeic acid and its oligomers, flavonoid glycosides, hydroquinone derivatives, terpenoids and biphenyl compounds, among others (Fecka & Turek, 2008). Rosmarinic acid (caffeic acid oligomer) is the main phenolic compound found in the aqueous tea infusion (Mihailovic-Stanojevic et al., 2013). The potential antioxidant activity of these thyme extracts makes them suitable to be used as possible substitutes for synthetic antioxidants in the food industry. These polyphenols could be incorporated within a film or coating applied to the food, which could release the antioxidant into the product or act on its surface, limiting the oxidative reactions of food components. However, no previous studies about the aqueous thyme extract incorporation into film formulations have been found.

Currently, in the field of food packaging, there is great interest in the development of biodegradable active materials, which contribute both to a reduction in the use of synthetic plastic wastes and to a longer shelf-life of the food products by means of the incorporation of active substances, such as antioxidants and antimicrobials. Of the

biodegradable polymers, polysaccharides such as starch or chitosan have good film-forming capacity (Bergo, 2008). Starch (S) films are low cost, flexible, transparent and highly impermeable to oxygen, but exhibit a high degree of water sensitivity which negatively affects barrier and mechanical properties. Improvement strategies are based on the mixture with other components, which allows films with better properties to be obtained. Chitosan (CH) is a cationic hydrocolloid with interesting film-forming properties (Elsabee & Abdou, 2013). It presents a great potential for a wide range of food applications due to its biocompatibility, non-toxicity and low cost of application (Sánchez-González, et al. 2011; Krochta & Mulder-Johnston, 1997). Furthermore, numerous studies have shown the antioxidant capacity (Yen, Yang & Mau, 2008) and the antimicrobial activity of chitosan (Dutta, Tripathi, Mehrotra & Dutta, 2009; Kumar, Muzzarelli R., Muzzarelli C., Sashiwa & Domb, 2000), associated both with its positive charges, which can interfere with the negatively charged residues of macromolecules on the microbial cell surface, causing membrane leakage (Siripatrawan & Harte, 2010). Previous studies have reported that the S-CH blends exhibited an improved mechanical response compared to pure S films while providing antimicrobial activity at low cost (Bonilla, Atarés, Vargas & Chiralt, 2013). Tannic Acid (TA) is a polyphenol naturally found in some green leaves, which exhibits interesting properties due to its multiple phenolic groups that can interact with macromolecules, such as chitosan (Aelenei, Popa, Novac, Lisa & Balaita, 2009). Thus, TA acts as a cross-linker, leading to a more rigid and compact chitosan matrix and improving the films´ physical properties (Rivero, García & Pinotti, 2010).

The aim of this study was to evaluate the physical and antioxidant properties of biodegradable films composed of different matrices made up of chitosan and pea starch enriched with thyme extract. Moreover, the addition of TA to the chitosan matrices, as a crosslinking agent, with reported antioxidant properties, was studied as a reference compound.

2. MATERIALS AND METHODS

2.1 Raw materials

High molecular weight chitosan (Batch MKBH57816V) and tannic acid from Sigma-Aldrich, Madrid, Spain, pea starch (Batch W469V) supplied by Roquette Laisa SA Benifaió, Valencia, Spain, glacial acetic acid and glycerol from Panreac Química SLU, Castellar del Vallès, Barcelona, Spain, were used to prepare the films.

Methanol and 2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) from Sigma-Aldrich, Madrid, Spain and phosphorus pentoxide (P_2O_5), sodium carbonate (Na_2CO_3) and sodium chloride (NaCl), provided by Panreac Química SLU, Castellar del Vallès, Barcelona, Spain, were used to carry out the different analyses.

2.2 Thyme extract preparation

Thyme extract (TE) was prepared by a conventional water extraction method with boiling distilled water. 10 g of dry thyme leaves, provided by Serpylli herba (Serbia) (Batch 29660913), were added to 200 mL of hot water (100°C), stirring for 30 minutes. The resulting infusion was vacuum filtered using filter paper (Stojanovic et al., 2012) and lyophilized in a freeze dryer Alpha 1–2 (Martin Christ, GmbH, Osterode am Harz, Germany) under vacuum pressure. Thyme extract powders were kept packaged under vacuum and refrigeration conditions.

2.3. Experimental design and preparation of the films

Films containing TE were produced by means of a casting method. Two different film forming dispersions (FFDs) based on pure biopolymers (CH and S) and a mixture of both polymers (CH:S) were obtained. In those formulations based on chitosan, tannic acid was also added as a cross-linking agent (Rivero et al., 2010). As controls, films with no added TE were prepared (CH, CH:TA, S, CH:S, CH:S:TA) (**Table 1**).

CH (2 % w/w) was dispersed in an aqueous solution of acetic acid (2 % v/w) under magnetic stirring at 40°C and 150 rpm for 24 h. Glycerol was added in a polymer:glycerol ratio of 1:0.2, (w/w). To obtain the CH:TA film forming dispersion, TA was added to the CH dispersion in a polymer:TA ratio of 1:0.04.

2 % (w/w) pea starch solution in distilled water was prepared to obtain S dispersions by heating at 95°C for 30 min to gelatinize the starch, under continuous stirring. Afterwards, glycerol was added in the same ratio as that commented on above.

The CH and S dispersions prepared above were mixed in a CH:S ratio of 1:4 (w/w). In formulations with TA, this was added in a polymer:TA ratio of 1:0.04 (w/w).

Dispersions containing TE were added using a polymer:TE ratio of 1: 0.15. All of the dispersions were homogenized using rotor-stator equipment (Ultraturrax Yellow Line DL 25 Basic, IKA Janke and Kunjel, Germany) for 4 min at 13500 rpm and degassed by means of a vacuum pump. In order to obtain the films, these FFDs were poured into Teflon plates (15 cm diameter) or petri dishes (8.7 cm diameter) resting on a level surface. The amount of polymer always remained constant, the density per unit area being 56.62 g of polymer/m^2.

After drying for 48 hours under controlled conditions (T=25°C and RH=50 %) and prior to further analysis, the films were conditioned in a desiccator at 25°C with a supersaturated NaCl solution (aw= 0.75) till they reached constant weight.

Table 1. Total solids amount (g) per 100 g of the different film forming dispersions and nomenclature used.

Formulation	CH	S	TA	TE	Glycerol
CH	1	-	-	-	0.2
CH:TA	1	-	0.04	-	0.2
S	-	1	-	-	0.2
CH:S	0.2	0.8	-	-	0.2
CH:S:TA	0.2	0.8	0.04	-	0.2
CH:TE	1	-	-	0.15	0.2
CH:TA:TE	1	-	0.04	0.15	0.2
S:TE	-	1	-	0.15	0.2
CH:S:TE	0.2	0.8	-	0.15	0.2
CH:S:TA:TE	0.2	0.8	0.04	0.15	0.2

2.4 Characterization of the films

2.4.1 Barrier properties

Water vapour permeability (WVP) was determined gravimetrically at 25°C and a RH gradient of 75-100 %, using a modification of the ASTM E96-95 gravimetric method (1995) for hydrophilic films. Payne permeability cups, 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium), were filled with 5 mL of distilled water (100 % RH). Six circular samples of each formulation (3.5 cm in diameter), without visible defects, were secured in the cups. The cups were placed in pre-equilibrated cabinets containing saturated solutions of sodium chloride to generate 75 % RH inside the cabinet and with a fan on the top of the cup in order to reduce the resistance to water vapour transport. The shiny side of the films was exposed to the atmosphere at the lowest RH (75 %). The cups were weighed periodically using an analytical balance (ME36S, Sartorius, Germany) (±0.00001 g) at intervals of 1.5 h for 24 h after the steady state had

been reached. The slope of the weight loss versus time was plotted and the water vapour transmission rate (WVTR) and water vapour permeability were calculated according to Vargas, Albors, Chiralt & González-Martínez (2009).

The oxygen permeability (OP) was determined by following the ASTM Standard Method D3985-05 (2010). Three replicates of each formulation were measured using Ox-Tran equipment (Model 1/50, Mocon, Minneapolis, USA), where sample areas of 50 cm^2 were exposed to nitrogen and oxygen flows at 25°C and 75 % of RH. Oxygen permeability was calculated by dividing the oxygen transmission by the difference in oxygen partial pressure between the two sides of the film, and multiplying it by the average film thickness.

2.4.2 Tensile properties

Mechanical properties were determined using a Universal Testing Machine (Stable Micro System TA. XT plus, Haslemere, England) following the ASTM standard method (D882.ASTM D882, 2001). Twelve pre-conditioned film pieces were cut (25 mm × 100 mm) and mounted in the film's extension grip of the equipment and stretched at 50 mm·min^{-1} until breaking. Force-distance curves were obtained and transformed into stress-Hencky strain curves. The mechanical behaviour was analysed in terms of: elastic modulus (EM), tensile strength (TS) and percentage of elongation at break (%E).

2.4.3 Optical properties: colour, internal transmittance and gloss

The optical properties (transparency and colour coordinates) were determined in triplicate by measuring the reflection spectrum of the samples from 400 to 700 nm using a MINOLTA spectrocolorimeter, model CM-3600d (Minolta CO., Tokyo, Japan). Measurements were taken on the side of film which was in contact with air during the drying.

The transparency was measured by means of the internal transmittance (T_i), applying the Kubelka-Munk theory of the multiple dispersion of reflection spectrum (Hutchings, 1999) given the reflection spectra of both black and white backgrounds.

The CIEL*a*b* colour coordinates were obtained from the reflectance of an infinitely thick layer of the material by considering illuminant D65 and observer 10°. Psychometric coordinates Chroma (C_{ab}*) and hue (h_{ab}*) were also determined (Bonilla, Talón, Atarés, Vargas & Chiralt, 2013).

Finally, gloss was determined at six random points of two films following the ASTM method ASTM D523 (1999), using a flat surface gloss meter at an angle of 60° (Multi-Gloss 268, Konica Minolta, Langenhagen, Germany). Gloss measurements were taken

over a standard black matte surface in duplicate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a value close to 100.

2.4.4 Microstructural evaluation

The microstructure of the films, previously conditioned at 25°C and 75 % RH, was analysed in a JEOL JSM-5410 electron microscope (Japan) by using the scanning electron technique (SEM). SEM observations were carried in the cross-sections of the films. Thus, samples were previously dehydrated in a desiccator with phosphorus pentoxide, immersed in liquid N_2 and cryofractured. SEM micrographs were obtained in duplicate by mounting the samples on copper stubs and gold coating, using an accelerating voltage of 15 kV (x1500).

2.4.5. Fourier transform infrared (FT-IR) analysis

FT-IR spectroscopy of CH; CH:TE; CH:TA and CH:TA:TE films was obtained by using a Thermo Nicolet 5700 Fourier transform infrared spectrometer. The average spectra were collected from 64 accumulations with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} range, from five different locations of the same specimen.

2.4.6 Antioxidant activity

The antioxidant capacity of the films was determined by using a 2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) reduction method, following the methodology described by Brand-Williams, Cuvelier, & Berset (1995). In this method, the stable free radical, DPPH$^{\cdot}$, is directly obtained by dissolving the compound in an organic medium. In the radical form, this molecule shows absorbance at 515 nm, which disappears after accepting an electron or hydrogen radical from antioxidant compounds (Özcelik, Lee & Min, 2003).

For this purpose, films containing TE (8,7 cm diameter) were dissolved in 150 mL of 1 % acetic acid solution using the Rotor Stator (2 min at 8000 rpm) and magnetic stirring for 20 min at 25°C in dark bottles. Blank films with added TA were also evaluated, as it is a naturally occurring polyphenol with reported antioxidant activity (Lopes, Schulman & Hermes-Lima, 1999); Ferguson, 2001; Wu et al., 2004). In every case, 0.05 to 0.3 mL of the different appropriately diluted samples were mixed with a methanol solution of DPPH· (0.022 g·L^{-1}) to a final volume of 4 mL.

The decrease in absorbance at 25°C was determined in triplicate by using a spectrophotometer (ThermoScientific spectrophotometer Evolution 201 UV-visible) at 515 nm. Measurements were taken every 15 min until the reaction reached a plateau. The DPPH$^{\cdot}$ concentration (mM) in the reaction medium was calculated from the

calibration curve (**equation 1**) determined by linear regression (R^2 = 0.999). The percentage of remaining DPPH˙ (%DPPH˙$_{rem}$) was calculated following **equation 2**.

$$Abs_{515nm} = 12.21604 \cdot [DPPH^{\bullet}] + 0.00336 \qquad \textbf{(Equation 1)}$$

$$\%[DPPH]_{rem} = \frac{[DPPH^{\bullet}]_{t=30}}{[DPPH^{\bullet}]_{t=0}} \cdot 100 \qquad \textbf{(Equation 2)}$$

Where, $[DPPH˙]_{t=30}$ is the concentration of DPPH˙ at steady state and $[DPPH˙]_{t=0}$ is the concentration at the beginning of the reaction.

From these values, the parameter EC_{50} was determined so as to measure the antioxidant activity of the films. The EC_{50} antioxidant parameter indicates the amount necessary to reduce the initial concentration of DPPH˙ to 50 % once the stability of the reaction was reached. Thus, the EC_{50} values were obtained by plotting % [DPPH˙]$_{rem}$ *versus* the mass ratio of film to DPPH˙ (kg film/mol DPPH˙).

The antioxidant activity of the pure lyophilized thyme extract (TE), TA and CH was also determined, using the same methodology, in order to evaluate the contribution of these compounds to the total antioxidant activity of films.

2.4.6 Statistical analysis

Statistical analyses of the results were performed through an analysis of variance (ANOVA) using Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.). Fisher's least significant difference (LSD) procedure was used at the 95 % confidence level.

3. RESULTS AND DISCUSSION

3.1 Physical properties of films

3.1.1 Barrier properties

The water vapour and oxygen permeabilities of the films are shown in **Table 2.** Pure chitosan and CH:TA films presented greater values of WVP and OP than the starch films, in agreement with the results reported by Kowalczyk, Kordowska-Wiater, Nowak & Baraniak (2015) and Bonilla et al. (2013). Blend CH:S films exhibited intermediate behaviour. The effect of TA and TE incorporation on the barrier properties of the films followed the same trends. These properties were affected by the water plasticisation level of the matrix due to its high water affinity, as is commented on below.

Tannic acid (TA) has been reported to act as a cross-linker in the presence of chitosan (CH), providing enhanced mechanical and water barrier properties. Thus, Rivero et al. (2010) reported that the addition of TA to CH films decreased the WVP values of the CH films conditioned at 20°C and 65 % RH. This was not observed in the films conditioned at 75 % RH, probably due to their greater equilibrium moisture content (around 22 %, data not shown). In this case, the water plasticization effect of the polymeric matrix seems to mitigate the cross-linking effect of TA.

The addition of TE significantly decreased the WVP values of the films with lower water content that is, pure starch films and starch blends, around 14 % (g water/100 g film), where the effect of the water plasticization was less notable. No effect was observed when incorporated in pure CH and CH-TA films, in all likelihood due to its high water content (around 22 %) and to the low concentration of TE incorporated (ratio of CH:TE was 1:0.15). Siripatrawan & Harte (2010) reported a significant decrease in the WVTR of chitosan films, equilibrated at 25°C and 50 % RH, when adding green tea (GT), also rich in polyphenolic compounds, but only when incorporated at high concentrations (CH:GT ratio of 2:5). As regards the oxygen permeability (OP), CH based films showed the highest OP values, probably due to the greater water uptake capacity of chitosan versus starch, which gave rise to a more plasticized, open matrix (Bonilla et al., 2013).

The addition of TA improved the oxygen barrier property of the films, which is consistent with the development of a tighter more closed structure. Nevertheless, the incorporation of TE was only effective ($p < 0.05$) when incorporated into low moisture content films (pure S and S blends).

Table 2. Water vapour and oxygen permeability and water content of the films with or without thyme extract (TE) equilibrated at 25°C and 75 % RH.

Formulation	WVP [g·mm/(h·m²·KPa)]		OP [cc·µm/(h·m²·KPa)]	
	Control Films	Films + TE	Control Films	Films + TE
CH	11.3 (1.7)[b,1]	11.6 (1.8)[c,1]	11.95 (1.14)[c,1]	14 (4)[b,2]
CH:TA	12 (2)[b,1]	11.1 (1.3)[c,1]	8.6 (0.4)[b,1]	11 (2)[b,2]
S	8.3 (1.5)[a,2]	6.9 (0.4)[a,1]	4.2 (1.4)[a,1]	4.1 (0.3)[a,1]
CH:S	9.6 (0.3)[a,2]	8.8 (1.3)[b,1]	6.6 (1.9)[ab,2]	4.3 (1.7)[a,1]
CH:S:TA	9.3 (0.4)[a,2]	7.27 (1.09)[ab,1]	4.8 (1.6)[a,2]	3.9 (0.5)[a,1]

[abc] Different letters in the same column indicate significant difference among formulations (p<0.05).
[12] Different numbers in the same row indicate significant difference between the film with and without TE (p<0.05).

3.1.2 Tensile properties

The tensile strength (TS) and the deformation at break (%E) are the maximum stress and elongation that a film can withstand before it breaks up, respectively. The elastic modulus (EM) is related to the stiffness of the material at low strains. These mechanical parameters are useful for the purposes of describing the mechanical properties of the film, and are closely related to its structure (Mc Hugh & Krochta, 1994). **Figure 1** shows the typical stress–strain curves of pure S and CH films and the S:CH blend, where the effect of both the different polymeric matrix (**Figure 1A**) and the addition of TE on the mechanical behaviour of the samples (**Figure 1B**) can be observed. The incorporation of TA led to mechanical changes similar to those produced by TE, but more pronounced (**Figure 1**). Pure chitosan behaved as a rigid material, while pure S exhibited more viscoelastic behaviour (**Figure 1**). The CH:S blend exhibited a better mechanical response than pure starch, becoming harder and more stretchable. In every case, the incorporation of TE markedly decreased the elongation at break of the films.

Table 3 shows the values of the mechanical parameters obtained. The mechanical behaviour of the films greatly depended on the type and concentration of active incorporated, the kind of polymeric matrix and the specific interactions between components (Ahmad, Benjakul, Prodpran & Agustini, 2012), which determine the effective attraction forces between polymeric chains. Pure CH based films presented significantly (p <0.05) greater extensibility (higher %E) than pure starch films, coinciding both with other studies (Silva-Weiss, Bifani, Ihl, Sobral & Gómez-Guillén, 2013; Siripatrawan & Harte, 2010) and with its higher plasticisation level.

The addition of TA to pure CH-based films produced a significant increase in the elastic modulus (EM) and the resistance to break (TS) due to the reinforcement effect of the crosslinker (Rivero et al., 2010), resulting in stiffer and stronger films but, consequently,

slightly less stretchable (lower %E). The same effects were observed when TE was added to chitosan based films due to the presence of polyphenols which can establish interactions with the chitosan chains, similarly to what occurred in the case of TA. These mechanical effects were more marked in films incorporating both TE and TA. Interactions between chitosan and polyphenolic compounds from green tea (Siripatrawan & Harte, 2010) and Indian gooseberry extract (Mayachiew & Devahastin, 2010), as well as catechin (Zhang & Kosaraju, 2007) and gallic acid (Curcio et al., 2009), have also been observed. It has been reported that the interaction mechanisms of different polyphenols with chitosan are due to electrostatic interactions (ionic complexations in acidic conditions), ester linkages and hydrogen bonds (Silva-Weiss et al., 2013). Silva-Weiss et al., (2013) reported similar mechanical behaviour when working with chitosan films incorporating murta leaf extracts, rich in polyphenolic compounds (Hauser et al., 2016; Silva-Weiss, Bifani, Ihl, Sobral & Gómez-Guillén, 2014).

On the other hand, when TE was incorporated into pure starch films, they became less rigid (lower EM) and less resistant to fracture (lower TS) and the extensibility was not affected (p>0.05). CH blend films (CH:S and CH:S:TA) presented similar behaviour to pure CH films, but the tensile strength was not enhanced, in all likelihood due to the presence of starch which limits phenol-CH interactions.

Figure 1. Typical stress-Hencky strain curves of films without (A) and with (B) thyme extract. S (⋯), CH (- - -), CH:TA (grey - - -), CH:S (—), CH:S: TA (grey —).
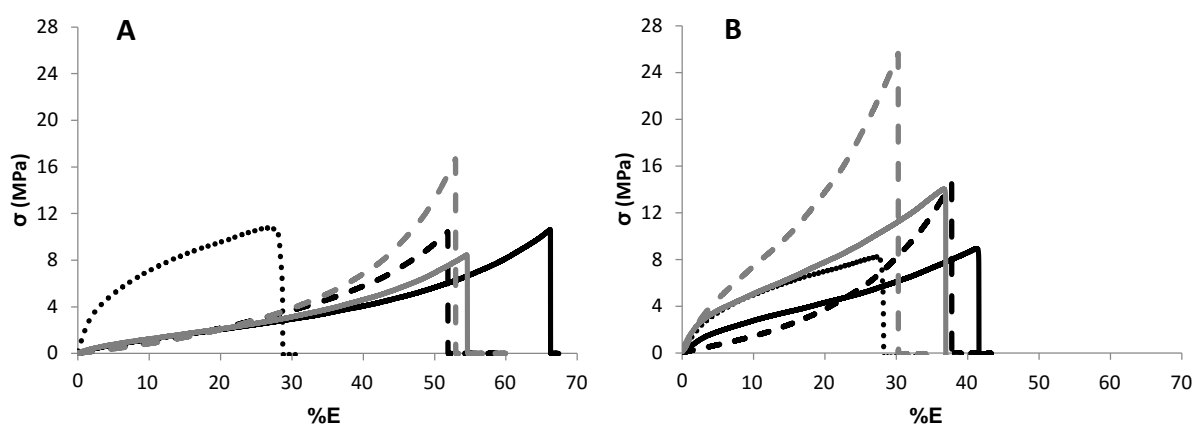
Table 3. Elasticity modulus (EM), Tensile strength (TS) and percentage of elongation (%E) of films containing or not thyme extract (TE). Mean and (standard deviation).

	EM		TS (MPa)		%E	
Formulation	**Control Films**	**Films + TE**	**Control Films**	**Films + TE**	**Control Films**	**Films + TE**
CH	8.1 (0.5)[a,1]	14 (3)[a,2]	11 (3)[a,1]	13 (4)[c,1]	70 (4)[c,2]	39 (9)[b,1]
CH:TA	8.4 (0.5)[a,1]	102 (17)[d,2]	15 (3)[b,1]	23 (6)[d,2]	64 (4)[b,2]	35 (4)[ab,1]
S	147 (51)[b,2]	103 (36)[d,1]	9.8 (0.9)[a,2]	7.3 (0.9)[a,1]	29 (4)[a,1]	30 (9)[a,1]
CH:S	17 (3)[a,1]	51 (9)[b,2]	9.5 (1.7)[a,2]	8.2 (1.2)[ab,1]	90 (11)[e,2]	47 (6)[c,1]
CH:S:TA	18 (2)[a,1]	82 (31)[c,2]	10 (2)[a,1]	11 (3)[bc,1]	79 (7)[d,2]	36 (6)[ab,1]

[abcde] Different letters in the same column indicate significant difference among formulations ($p < 0.05$).
[12] Different numbers in the same row indicate significant difference between the film with and without TE ($p < 0.05$).

3.1.3. Infrared spectroscopy analysis

In order to obtain evidences about the molecular interactions between chitosan and phenolic compounds deduced from the mechanical behaviour, infrared spectroscopy analysis has been performed. **Figure 2** shows FT-IR spectra of chitosan based matrices containing or not polyphenols (CH, CH:TA, CH:TE and CH:TA:TE). Pure CH samples exhibit characteristic bands at 3253 cm^{-1} (st. vibration of N-H, O-H), 1635 cm^{-1} (C-N, N-H) and 1552 cm^{-1} (C-N, N-H), as previously observed by other authors (Sionkowska et al., 2015; Rivero et al., 2010). When polyphenols were added, the spectra were similar to those obtained for polyphenol free chitosan and no characteristic bands of polyphenols were appreciated. Nevertheless, the absorption peaks decreased in intensity at constant wavenumber, as previously described by Rivero et al. (2010) for chitosan-tannic acid films at similar weight ratio. This indicates that the concentration of these phenol functional groups in the blend is not enough to give a sensitive response in the spectra and that their low ratio also contribute to the lack of notable changes in the chitosan bands, such as other authors (Aelenei et al., 2009; Sionkowska et al., 2015) observed for higher TA:chitosan ratios (2 to 20). However, despite the lack of FTIR evidences, mechanical response of the films reveals the establishment of molecular interactions between chitosan and tannic acid or thyme extracts as proved by different authors (Silva-Weiss et al., 2013; Sionkowska et al., 2015). All films containing these compounds were stiffer and less stretchable than the net chitosan films, which put in evidence the crosslinking effect in the polymer network.

Figure 2. FT-IR spectra of different chitosan based films: CH (—), CH:TA (···), CH:TE (—) and CH:TA:TE (···).
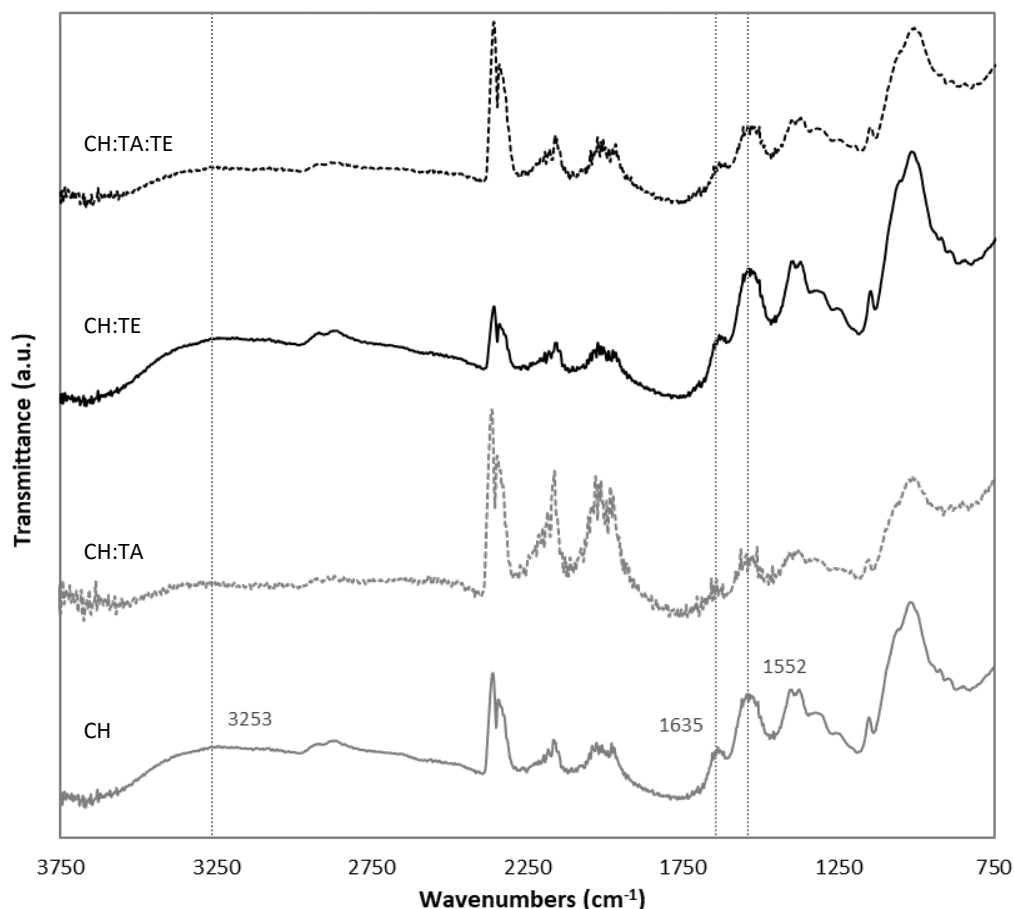
3.1.4 Optical properties

Table 4 shows the colour parameters of lightness (L*), chrome (C*) and hue (h*) and the values of the internal transmittance at 460 nm (Ti) of the different films, related to the transparency of the films. Pure chitosan matrices exhibited similar lightness values to starch but lower internal transmittance values, in agreement with their denser packed chain structure (Sánchez-González, Cháfer, Chiralt & González-Martínez, 2010).

The effect of the addition of polyphenols (both from TA and TE) on the optical properties of the films can also be observed in **Table 4**. Their incorporation provoked significant ($p<0.05$) changes, leading to darker, more reddish (lower L* and h* values, respectively) and more opaque films (lower Ti values) than the control. Similar results were found by Siripatrawan & Harte, (2010), when working with chitosan based films and green tea extract. These effects can be explained by the light selective absorption of polyphenols (from TE and TA) at low wavelengths that imparts a reddish colour to the films, thus decreasing the hue and Ti values at low wavelengths. In the case of the chitosan films, the crosslinking process in the presence of polyphenols significantly reduced the

luminosity values. In general, the addition of polyphenols from TA or TE gave rise to a more saturated colour in the films (higher C*).

On the other hand, pure starch films containing TE presented the lowest luminosity, Ti and hue values in coherence with the development of more brownish colours, related to the oxidation of polyphenols during the drying and storage of the films. This underlines the protective effect of the chitosan matrix against the oxidation of TE polyphenols, which were less affected by light oxidation. Several authors previously reported the formation of reversible polyphenols-chitosan complexes by means of the establishment of interactions between the hydroxyl groups of the polyphenols and the amine functionality of the chitosan molecule (Popa, Aelenei, Popa, & Andrei, 2000; Liang et al., 2011; Dudhani & Kosaraju, 2010; Kosaraju, D'ath & Lawrence, 2006), leading to the encapsulation of these compounds. Because of the encapsulation process, the core material is usually protected against the undesirable effects of light and oxygen, among others (Shahidi & Han, 1993; Desai & Jin-Park, 2005; Fang & Bhandari, 2010).

In general, gloss values at 60° of all the films were very similar, with an average value of 28 ± 7. No significant changes ($p > 0.05$) were observed when incorporating the polyphenols (data not shown).

Table 4. Luminosity (L*), chroma (C*) and hue (h*) and internal transmittance at 460 nm (Ti) values of the films containing or not thyme extract (TE).

Formulation	L*		Cab*		hab*		T_i (460nm)	
	Control Films	Films + TE	Control Films	Films + TE	Control Films	Films + TE	Control Films	Films + TE
CH	72 (2)[bc,2]	48 (1)[b,1]	21.3 (0.3)[e,1]	31.9 (0.3)[c,2]	90.0 (0.3)[d,2]	73.7 (0.8)[c,1]	0.771 (0.003)[b,2]	0.31 (0.05)[b,1]
CH:TA	61 (3)[a,2]	49 (2)[b,1]	15.9 (0.7)[d,1]	30 (1)[b,2]	73.8 (1.7)[b,1]	72 (3)[b,1]	0.706 (0.012)[a,2]	0.38 (0.03)[c,1]
St	76 (8)[c,2]	37 (2)[a,1]	1.2 (0.3)[a,1]	20 (2)[a,2]	61 (9)[a,1]	59 (3)[a,1]	0.856 (0.004)[e,2]	0.18 (0.04)[a,1]
CH:St	80 (1)[d,2]	55 (1)[c,1]	10 (1)[b,1]	34 (1)[d,2]	95.8 (0.8)[e,2]	79.0 (0.7)[d,1]	0.832 (0.005)[d,2]	0.540 (0.014)[d,1]
CH:St:TA	71 (2)[b,2]	56 (1)[c,1]	14.2 (0.3)[c,1]	31 (1)[c,2]	80.1 (1.6)[c,2]	78,1 (0.4)[d,1]	0.786 (0.005)[c,2]	0.534 (0.018)[d,1]

[abcde] Different letters in the same column indicate significant difference among formulations (p<0.05).
[12] Different numbers in the same row indicate significant difference between the film with and without TE (p<0.05).

3.2 Film microstructure

The microstructure of the films is affected by the spatial organization of their different components and by how they interact during the drying process. Their microstructure provides information about the arrangement of the different compounds and permits a better understanding of the film's physical properties (Vargas, Perdones, Chiralt, Cháfer & González-Martínez, 2011).

Figure 3 shows the cross-section SEM micrographs of the films, where remarkable differences can be observed. Thus, chitosan films presented a heterogeneously-fractured surface, probably due to the presence of more ordered and crystalline regions. On the other hand, although starch films exhibited a smoother appearance, the presence of crystalline regions at the top and bottom of the films can also be observed. The S-CH blend presented a more homogenous, smoother structure, indicating a good compatibility between both polymers. The reduction in the amount of crystalline regions in the chitosan films when mixed with gelatinised starch has been previously reported by Xu, Kim, Hanna & Nag (2005).

In pure chitosan films, polyphenols (TA or TE) led to a more dense structure with a more regular packaging of polymer chains, which could be related with the described interactions between chitosan and polyphenols. This contributes to the formation of a more compact matrix, with enhanced mechanical resistance in agreement with the results obtained from the mechanical assays. In the blended films, the microstructure was only modified when using TA. In this case, the film structure became less smooth, exhibiting areas with more brittle fracture in agreement with the crosslinking action. The incorporation of TE into starch or CH-S films was observed to have no notable effect on the film microstructure.

Figure 3. SEM micrographs of the cross-sections of the films containing or not thyme extract (TE) (magnification 1500x) and camera pictures.
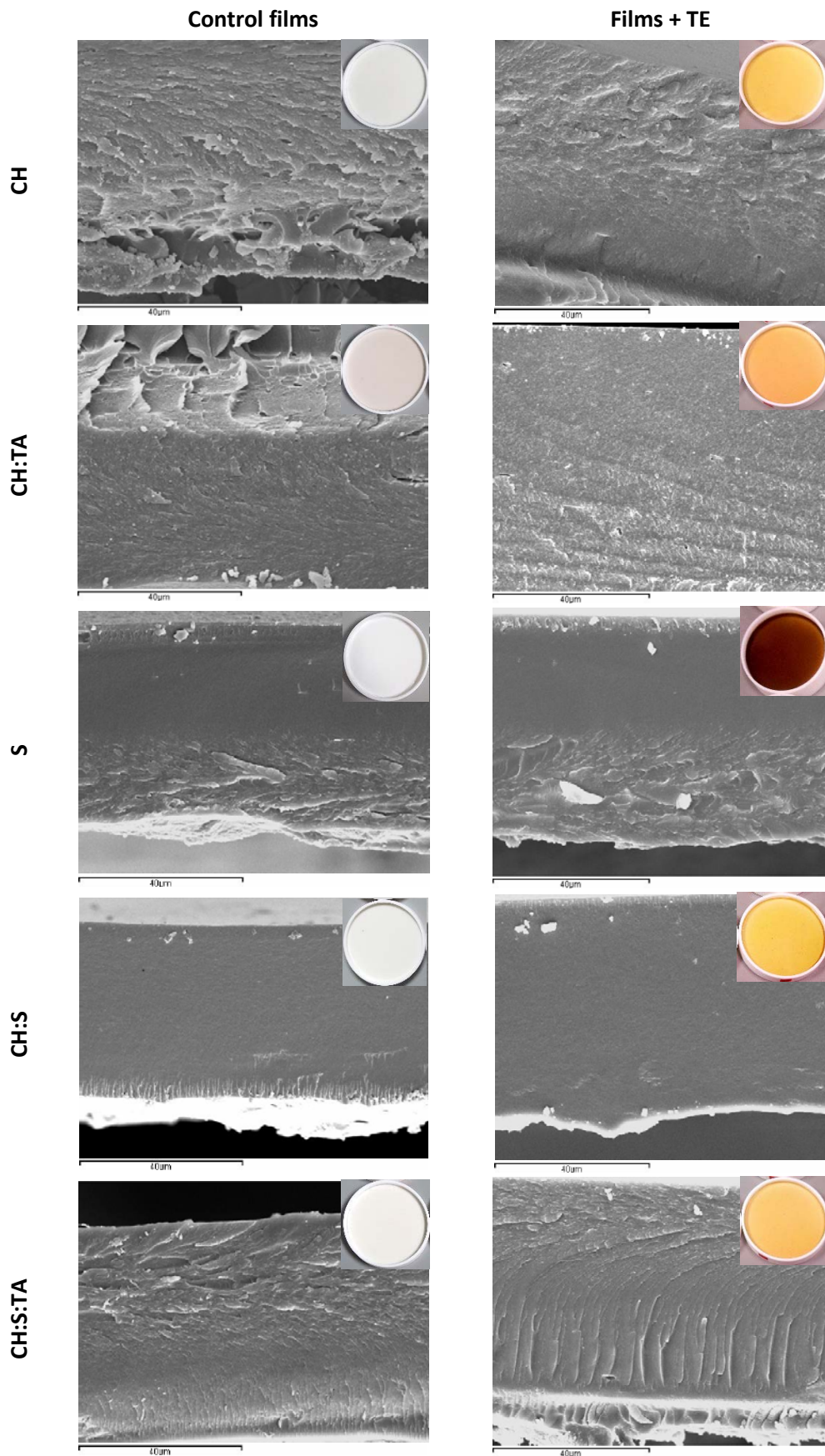
3.3. Antioxidant activity

From the absorbance data both at zero reaction time and after 30 minutes (when the steady state of the reaction was achieved), the EC_{50} parameter (mass of sample/mol DPPH, necessary for the purposes of reducing the initial DPPH concentration to 50 %) was calculated for every sample. Thus, the lower the EC_{50} values, the greater the antioxidant activity of the tested sample.

This value was also determined for the phenolic ingredients (tannic acid and lyophilized thyme extract) so as to take into account the contribution of these components to the total antioxidant activity of the films. The EC_{50} value for TA was 0.022 ±0.002 kg TA/mol DPPH (or 0.0131 ±0.0013 mol TA/mol DPPH). This pointed to its high antioxidant activity, more potent than resveratrol, ascorbic acid, caffeic acid, or BHT, but close to that of gallic acid (0.7, 0.27, 0.1, 0.08 mol antioxidant/mol DPPH, respectively) (Brand-Williams et al., 1995; Pastor, Sánchez-González, Chiralt, Cháfer & González-Martínez, 2013). This high antioxidant capacity has been correlated with its multiple phenolic groups (Aelenei et al., 2009). On the other hand, thyme extracts exhibited a remarkable antioxidant capacity (of around 0.26 ± 0.02 kg TE/mol DPPH), but lower than that of TA. Although CH has been reported to exert some antioxidant activity (Yen et al., 2008; Vargas & González-Martínez, 2010), it was not able to reduce the initial DPPH concentration to 50 %. Both tannic acid and thyme extract were found to react quickly with DPPH (fast kinetic behaviour), taking around 30 minutes to reach the steady state.

In **Table 5**, the EC_{50} values of the films with and without polyphenols are shown. In films without TE, DPPH reductions of only 50 % were obtained in those films containing tannic acid, in agreement with its potent antioxidant activity. In these films, the DPPH reductions were more marked in the formulations with chitosan-starch mixture, probably because tannic acid is more available as there is less chitosan to interact with, compared to the pure CH formulation. The EC_{50} values found were in range of those reported by Pastor et al. (2013) for methylcellulose and chitosan based films containing resveratrol (of around 2-20 kg film/mol DPPH).

All of the films containing TE exhibited antioxidant activity, although as shown in **Table 5**, the formulations were found to have significant differences (p <0.05). In accordance with previous data, the films containing both tannic acid and TE (CH:TA:TE and CH:S:TA:TE) showed the greatest antioxidant power (0.7 and 0.8 kg film/mol DPPH, respectively). TA-free films (CH, S and CH:S) with TE also exhibited remarkable antioxidant activity, especially those films formulated with chitosan. Taking into account that all the films contained the same amount of TE, these differences in the films' antioxidant activity once again underline the protective effect of the chitosan matrix against polyphenol oxidation during the drying/storage of the films, as commented on above. The blend film (S-CH with TE) exhibited a lower antioxidant capacity than the CH

film with TE, coinciding with the less effective encapsulating ability of the blend than the pure CH films.

The values obtained for EC_{50} were also expressed in terms of the g of polyphenols per DPPH moles (by taking into account the amount of TA and TE in each film sample). These values reflect that, in pure starch films, some losses of antioxidant activity occurred, since these values are significantly higher ($p<0.05$) than the ones obtained for pure TE (0.26 kg TE/mol DPPH). This could be due to some degradation of TE during the drying of the films caused by the poor protective effect of starch. On the other hand, in CH+TE films, the EC_{50} values were lower ($p<0.05$) than in pure TE. This could indicate that the chitosan is preventing the oxidation of polyphenols occurring during the handling of the compound since, when TE powder was dissolved, degradation occurred to some extent. In order to clarify this hypothesis, the EC_{50} value of a water solution containing the same amount of chitosan and TE as CH+TE films (0.22 % and 0.034 %, respectively) was measured. The EC_{50} value obtained for this system was 0.148 kg TE/mol DPPH; this is very close to that of the CH+TE film, thus pointing to the protective effect of CH against phenol oxidation. Nevertheless, the fact that chitosan can also contribute to the total antioxidant activity of the film or dispersion cannot be neglected.

Table 5. Antioxidant activity of films containing or not thyme extract (TE), expressed in terms of EC_{50} values.

	EC_{50} (kg film/mol DPPH)		EC_{50} (kg polyphenol/mol DPPH)
Formulation	**Control Films**	**Films + TE**	**Films + TE**
CH	-	1.56 (0.03)[b]	0.141 (0.003)[b]
CH:TA	1.31 (0.07)[b,1]	0.713 (0.017)[a,2]	0.081 (0.002)[a,2]
S	-	3.5 (0.3)[d]	0.42 (0.05)[d]
CH:S	-	2.07 (0.06)[c]	0.227 (0.005)[c]
CH:S:TA	1.061 (0.008)[a,1]	0.90 (0.03)[a,2]	0.121 (0.004)[b,2]

[abcd] Different letters in the same column indicate significant difference among formulations ($p<0.05$).
[12] Different numbers in the same row indicate significant difference between the film with and without TE ($p<0.05$).

4. CONCLUSIONS

The use of thyme extract (TE) was analysed as a source of natural polyphenols for the purposes of the development of antioxidant films for food preservation. This extract was efficiently incorporated into starch and chitosan film matrices. The films presented some changes in their microstructural and physical properties due to the incorporation of TE, which depended on the type of matrix. The best results were obtained when thyme extract was added to chitosan based films, due to the cross-linking effect that occurs among the polyphenols and the chitosan, promoting a better tensile response (greater resistance at break and higher degree of stiffness). Nevertheless, films became less stretchable and more opaque due to the structural changes provoked by polyphenols in the matrix. TE improved tensile properties of the films in the same way as TA and the ratio polyphenol-chitosan seems to play an important role in this response. Although the antioxidant activity of TA was higher than of TE, the obtained films also exhibited remarkable antioxidant activity after film formation and conditioning. The results also highlighted the possibility of using these antioxidant films for coating purposes in order to promote the shelf life of the products sensitive to oxidative processes.

Acknowledgements

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II

Release of polyphenols from starch-chitosan based films containing thyme extract

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ABSTRACT

The release kinetics of thyme extract polyphenols (TE) from chitosan (CH), pea starch (S) and CH:S blend films in different solvents was evaluated, as well as their antioxidant activity in each release media. Pure starch films showed the fastest delivery rate, the highest delivery ratio of polyphenols and the highest antioxidant capacity. TE provided CH based films with remarkable antioxidant activity, despite the lower polyphenol release obtained in all solvents, due to the strong polyphenols-chitosan interactions. The maximum amount of polyphenols delivered was found in the acetic acid solution, due to the high solubility of CH. The incorporation of tannic acid (TA) into CH films promoted cross-linking effect, which delays the TE release rate in water and ethanol aqueous solutions, except for CH:S:TA films. Thus, the polarity of the solvents and the polyphenols-matrix interactions markedly affected the polyphenol release and the antioxidant activity of the films.

Keywords: starch, chitosan, thyme, tannic acid, antioxidant activity, kinetics.

1. INTRODUCTION

Microbiological degradation and oxidation are the most common causes of food deterioration (Mozafari et al., 2006; Quintavalla and Vicini, 2002). Current trends in new packaging technologies for food preservation are focused on the development of alternatives for replacing synthetic additives with natural antioxidant and antimicrobial compounds.

Polyphenols are one of the most numerous and universal groups of natural antioxidants from plant sources, such as herbs and spices. The antioxidant nature of polyphenols is related with their ability to chelate metals, inhibit lipoxygenase enzyme activity and act as free radical scavenger (Garrido and Borges, 2013).

Aromatic herbs, such as thyme, have been traditionally used as healthy food ingredients obtained through an aqueous extraction process (Stojanovic et al., 2012). Thyme serpyllum L. is a rich source of polyphenols with reported antibacterial, antifungal, and antioxidant effects (Safaei-Ghomi, Ebrahimabadi, Djafari-Bidgoli and Batooli, 2009; Trifkovic et al., 2014). Despite the great potential of thyme essential oil, its uses in food preservation are limited mainly due to its intense aroma, toxicity problems and possible changes in the organoleptic properties of the food (Sánchez-González, Vargas, González-Martínez, Chiralt and Cháfer, 2011). Thus, the attention has shifted towards the use of hydrophilic extracts, which contain phenolic acids (rosmarinic acid, caffeic acid and its oligomers), flavonoids, hydroquinone derivatives, terpenoids and biphenyl compounds (Fecka and Turek, 2008; Mihailovic-Stanojevic et al., 2013).

The direct food application of polyphenols is limited by their relatively rapid actuation (Perazzo et al., 2014). The combined use of natural antioxidants and packaging materials could contribute to increase their effectiveness of their food application on, thus limiting the oxidative reactions and extending their action during a longer time.

Starch is one of the most abundant low-cost hydrocolloids with recognized film-forming ability. In spite of the biodegradability, flexibility and transparency of starch films, their lack of adequate mechanical and barrier properties. Improvement strategies are based on the mixture of starch with other polymers (e.g. chitosan), which allow for producing films with better properties. Chitosan is a cationic hydrocolloid with interesting film-forming properties (Elsabee and Abdou, 2013). Chitosan presents a great potential for a wide range of food applications due to its biocompatibility and non-toxicity (Krochta and Mulder-Johnston, 1997; Sánchez-González et al. 2011). Numerous studies have shown the antioxidant and antimicrobial activity of chitosan (Dutta, Tripathi, Mehrotra and Dutta, 2009; Kumar, 2000; Yen, Tseng, Li and Mau, 2007; Yen, Yang and Mau, 2008). The antibacterial activity of chitosan can be related with the interaction between the positive charges of chitosan and the negatively charged residues of macromolecules on the microbial cell surface (Siripatrawan and Harte, 2010). Chitosan films have good

mechanical properties (Butler, Vergano, Testin, Bunn and Wiles, 1996), but their application is limited by their high water vapor permeability (Caner, Vergano and Wiles, 1998; Vargas, Albors, Chiralt and González-Martínez, 2011).

Previous works have shown that the partial replacement of starch with chitosan in starch-based films can improve the mechanical properties of starch-based films at the same time that the antimicrobial effect is promoted (Bonilla, Atarés, Vargas and Chiralt, 2013). Moreover, the physical properties of chitosan based-films can be improved by the addition of tannic acid (TA), a natural polyphenol with antioxidant and crosslinking capacity (Aelenei, Popa, Novac, Lisa and Balaita, 2009; Rivero, García and Pinotti, 2010). The use of TA as a crosslinking agent has been related with electrostatic interactions (ionic complexations in acidic conditions), ester linkages and hydrogen bonds with chitosan chains (Silva-Weiss, Bifani, Ihl, Sobral and Gómez-Guillén, 2013).

The postulated main chemical pathway of phenolic compounds involves oxidization of diphenol moieties of phenolic acids or other polyphenols, under alkaline conditions, producing quinone intermediates which react with nucleophiles (mainly amino or sulfhydryl side chains of proteins) to form covalent C-N or C-S bonds with the phenolic ring (Azeredo and Waldron, 2016). In this sense, Talón et al. (2017) showed that tannic acid interacted with chitosan chains, acting as a crosslinking agent and enhancing the tensile behaviour of chitosan-based films.

Tannic acid shows antioxidant and antimicrobial properties due to its multiple phenolic groups (Cao, Fu and He, 2007; Rivero et al., 2010). The potential antioxidant effect of the active compounds present in bioactive films are affected by the kinetics of their release into the food surface, which in turn depends on several factors such as the food product characteristics and film microstructure. In order to design efficient active films and to increase their functionality, release studies are required. However, to the best of our knowledge, the kinetics of release of active ingredients in hydrocolloid-based films has been little explored. Moreover, to define the phenomena that occur during the release by means of mathematical models will allow for simulating similar behaviours in new delivery systems (Buonocore, Del Nobile, Panizza, Corbo and Nicolais, 2003).

The aim of this work was to evaluate the kinetics of the release of thyme extract polyphenols from chitosan-pea starch films in solvents of different polarity. Moreover, the effect of the addition of tannic acid into the polymeric matrix was also studied.

2. MATERIALS AND METHODS

2.1 Raw materials

To prepare the films, high molecular weight chitosan (Batch MKBH57816V, Sigma-Aldrich, Madrid, Spain), tannic acid (Sigma-Aldrich, Madrid, Spain), pea starch (Batch W469V, Roquette Laisa SA, Valencia, Spain,), glacial acetic acid (Panreac Química SLU, Barcelona, Spain) and glycerol (Panreac Química SLU, Barcelona, Spain) were used.

Folin-Ciocalteu reagent was obtained from Sigma-Aldrich (Madrid, Spain) whereas phosphorus pentoxide (P_2O_5), sodium carbonate (Na_2CO_3) and sodium chloride (NaCl) were provided by Panreac Química SLU (Castellar el Vallés, Spain).

Thyme (Thymus serpyllum L.) was kindly provided by The Institute of Medicinal Plant Research ''Dr. Josif Pančić'' (Belgrade, Serbia). The aqueous extract was prepared by a conventional water-extraction method. In this way, 200 mL of boiled distilled water was poured over 10 g of dry thyme herb. The extraction process was carried out for 30 min, at room temperature, and with intermittent stirring. The obtained extract was filtered through a medical gauze (Stojanovic et al., 2012; Trifkovic et al., 2014). After filtration, the extract was lyophilized in a freeze drier under vacuum pressure (Alpha 1–2, Martin Christ, GmbH, Osterode am Harz, Germany). Thyme extract powder was kept stored under vacuum and refrigeration conditions.

2.2. Measurement of thyme extract polyphenols

The total content of polyphenols was determined by the Folin-Ciocalteu method (Stojanovic et al., 2012). To perform this analysis, 0.5 mL of Folin-Ciocalteu reagent was mixed with 1.5 mL of Na_2CO_3, 0.1 mL of sample and distilled water to complete 10 mL. After 2 hours, the absorbance of the samples was measured at 765 nm, in triplicate, by means of a UV-vis spectrophotometer (Evolution 201, Thermo Scientific). Gallic acid was used as a standard and the results were expressed as mg·L−1 of Gallic acid equivalents (GAE).

Rosmarinic acid (RA) is the predominant phenolic compound present in thyme extract (Mihailovic-Stanojevic et al., 2013). In this study, the amount of RA was obtained by means of high-performance liquid chromatography (HPLC) performed with a Waters 2695 Alliance HPLC System (Alliance, USA) equipped with a photodiode detector (Waters 2996, Alliance). The concentration of RA was determined by using a Brisa LC2 column (C18, 250 × 4.6 mm, 5μm) and a C18 pre-column. 20 μL of each sample, previously filtered through a 0.45 μm filter and injected in the HPLC system. The elution was performed with the following solvents: 0.2% (v/v) formic acid (solvent A) and pure methanol (solvent B) with a flow rate of 1 mL/min. The gradient used was: 26% solvent B (in the first minute); in the second minute, a linear gradient from 26% to 50% solvent

B was used and it was kept constant until during 2 minutes; from minute 4 to minute 7 a linear gradient from 50% to 95% solvent B was used and it was kept constant until minute 19; the last minute was used to return to the initial conditions. The chromatograms were recorded at 278 nm and rosmarinic acid peak was confirmed by comparing the retention times and the UV-spectra with the HPLC standard. The integrated peak areas of the standard solutions were obtained and graphs representing concentration versus area was prepared. Lineal regression was used to calculate the corresponding concentrations of samples (R2=0.998). All analyses were performed in triplicate.

2.3. Film preparation and characterization

Films were produced by means of casting method. Different film-forming dispersions (FFDs) based on pure chitosan (CH) or pure starch (S) or a mixture of both polymers (CH:S) were obtained. In FFDs based on chitosan, tannic acid (TA) was also added as a cross-linking agent (Rivero et al., 2010).

Chitosan (2% w/w) was dispersed in an aqueous solution of acetic acid (2% v/w) under magnetic stirring at 40°C and 150 rpm for 24 h. Glycerol was added in a polymer:glycerol ratio of 1:0.2 (w/w). To obtain the CH:TA film forming dispersion, TA was added to the CH dispersion in a CH:TA ratio of 1:0.04 (w/w).

Starch dispersions were prepared by dissolving 2% (w/w) of pea starch in distilled water, while heating at 95°C for 30 min to promote starch gelatinization, under continuous stirring. Afterwards, glycerol was added in a polymer:glycerol ratio of 1:0.2 (w/w).

Chitosan and starch dispersions were mixed in the adequate proportion to obtain a CH:S ratio of 1:4 (w/w). TE was incorporated to the FFDs at a polymer:TE ratio of 1: 0.15. As control samples, films without TE were also prepared (CH, CH:TA, S, CH:S, CH:S:TA). All FFDs were homogenized by using a rotor-stator (Ultraturrax Yellow Line DL 25 Basic, IKA Janke and Kunjel, Germany) for 4 min at 13500 rpm, and degassed by means of a vacuum pump. The FFDs were poured into levelled Petri dishes (8.7 cm in diameter) and the amount of polymer remained constant (56.62 g of polymer/m2). After drying for 48 hours under controlled conditions (T=25°C and RH=50%) and prior to further analysis, the films were conditioned in a desiccator at 25°C with a supersaturated NaCl solution (aw= 0.75) until they reached constant weight.

2.3.1. Water content, solubility and film thickness

Conditioned films were cut into small pieces to measure the moisture content in triplicate by means of a gravimetric method. The samples were desiccated 48 hours in a vacuum oven (Vacio TEM-T) at 60°C and then were stored in a desiccator with phosphorus pentoxide until they reached constant weight.

Solubility test was carried out with the films that were dehydrated to measure their water content. Distilled water was added in a film:water ratio of 1:25 (w/v). Samples were and kept for 24 h at 25°C. After that, water was removed and the samples were placed in a convection oven (J.P. Selecta, S.A., Barcelona, Spain) at 60°C for 24h. Afterwards, samples were stored in a desiccator with P_2O_5 for two weeks until constant weight. The solubility of the films was obtained from the difference between the initial and final dry weight of the films (Ortega-Toro, Morey, Talens and Chiralt, 2015). The test was performed in triplicate.

For the measurement of film thickness, three samples of all formulations conditioned at 25°C and 75% RH were used. Thickness was measured in six random points of each sample by means of a digital electronic micrometer with an accuracy of 0.001 mm (Palmer model COMECTA, Barcelona).

2.3.2. Antioxidant activity of films

The antioxidant capacity of the films with thyme extract was expressed as Trolox equivalent antioxidant capacity (TEAC), which was estimated by the ABTS radical cation decolorization assay. This method is based on the scavenging of stable blue–green ABTS radical cation ($ABTS^{\bullet+}$), which is formed by chemical oxidation of ABTS (Re et al., 1999). The amount of ABTS radical cation scavenged by antioxidants was measured by monitoring the decrease in absorbance of ABTS radical cation. Briefly, in order to oxidize ABTS to $ABTS^{\bullet+}$, 5 mL of ABTS water stock solution (7 mM) was mixed with 88 µL of potassium persulfate (140 mM) and incubated in the dark for 12-16 h, at the room temperature. Prior to analysis, the $ABTS^{\bullet+}$ solution was diluted with ethanol to absorbance value of 0.70 (± 0.02), measured at 734 nm. Films antioxidant activity was measured in solvents of different lipophilic-hydrophilic nature: water, 3% (w/v) acetic acid solution, 10% (v/v) ethanol solution and 20% (v/v) ethanol solution. Film specimens (59.4 cm2) were cut in small bits and placed into 50 mL vials containing 25 mL of each of the four different solvents. After 24 hours, an aliquot of 10 µL of sample was withdrawn and added to 2.0 mL of the diluted $ABTS^{\bullet+}$ solution. The absorbance of the obtained sample was measured after exactly 6 min. Absorbance values were compared to the blank sample, which was prepared by adding the 10 µL of ethanol to 2 mL of the diluted $ABTS^{\bullet+}$ solution. The analysis were performed in triplicate and the results, were expressed as Trolox equivalents.

2.4. Kinetics of the polyphenols release

Four different solvents were used to perform the release studies: water, 3% (v/v) acetic acid solution (AA3%), 10% (v/v) aqueous ethanol solution (E10%) and 20% (v/v) aqueous ethanol solution (E20%). A piece of film of 59.4 cm2 was cut in small bits and placed into 50 mL vials containing 25 mL of each of the four different solvents. Release studies were carried out during 48 h at 25°C. Successively, aliquots of 100 µl of sample were taken at different film-solvent contact times and the total phenolic content was determined in triplicate as previously described in Section 2.2. The results were expressed as amount of gallic acid equivalents per gram of polymer (mg GAE/g polymer).

2.4.1. Mathematical modelling of polyphenols release

Two empirical models were applied to determinate the release profiles of experimental data. The generalized expression (**equation 1**) of the Korsmeyer-Peppas model (Siepmann and Peppas, 2012) was used to investigate the possible coupling of the relaxation of the polymer in contact with the solvent with the diffusion of the active compound through the polymer matrix.

$$\frac{M_t}{M_\infty} = kt^n$$

(**Equation 1**)

where M_t/M_∞ represents the fraction of active compounds releases at time t, k is the rate constant of the film, related to the diffusion process and n is the diffusional exponent that provides information about the mechanisms involved in the release process. Thus, a n value of 0.5 means that the release takes place through Fickian diffusion, whereas if the n value is higher than 0.5, known as anomalous transport, the diffusion and the polymer relaxation rates are coupled. If the n value is lower than 0.5, a quasi-Fickian diffusion for the active release can be considered (Siepmann and Peppas, 2012) describing the transport mechanism.

Peleg's model (Peleg, 1988), described by **equation 2**, was applied to experimental data in order to predict the release kinetics.

$$M_t = \frac{t}{k_1 + k_2 t}$$

(**Equation 2**)

where, M_t is total phenolic content at time t, k_1 is the kinetic constant of the model that is inversely related to the mass transfer rate at the beginning of the process, and k2 is a constant of the model that is related to the asymptotic value, which can be related to the equilibrium value ($1/M_\infty$ where M_∞ is the amount of active compound released at equilibrium).

2.5. Statistical analysis

Results were submitted to analysis of variance (ANOVA) using Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.). Fisher's least significant difference (LSD) procedure was used at the 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Water content, solubility, thickness and antioxidant activity of the films

The equilibrium moisture content of the films stored at 75% RH - 25°C is shown in **Table 1**, together with the thickness, solubility and antioxidant activity.

In order to understand the possible interactions of polyphenols from thyme extract with the polymeric matrix, pure polymer films, without polyphenols (TE), were also evaluated. In these films, the highest equilibrium moisture content were obtained for pure CH and CH:TA films (0.242 and 0.22 g water/g dry solids, respectively). This was explained by the higher hydrophilic character of chitosan film as compared to starch-based films (Bonilla et al., 2013), and it can be associated with the higher water binding capacity of chitosan molecules. Chitosan-starch films showed a lower water content (0.142 g water/g dry solids) and similar solubility values to that of pure chitosan or pure starch films (6.8 g film/L). The incorporation of tannic acid into the polymeric matrix reduced the average water content of chitosan-starch films (0.131 g water/g dry solids), without affecting its solubility. In a similar way, the addition of thyme extract polyphenols to the polymeric matrix, containing or not tannic acid, promoted a slight decrease in the water content of the films, which can be explained by the interactions between these hydrocolloids with polyphenols (Chung, Wong, Wei, Huang, Lin, 1998). A similar effect was observed by Wang, Dong, Men, Tong and Zhou (2013) when incorporating tea polyphenols into chitosan-based films at the same equilibrium conditions.

As shown in **Table 1**, significant differences ($p < 0.05$) were detected in terms of film thickness depending on the formulation. Film thickness depends on the organization of the polymer chains, on the interactions between the polymer chains and on the polyphenol and water content of the film matrix. Pure chitosan films showed the highest thickness values (91 ± 11 µm), and presented a more open structure (Talón et al., 2017), which is also consistent with their greater moisture content, as commented on above. Starch-based films showed lower thickness values (55 ± 11 µm), and a more compact organization of the polymer chains (Talón et al., 2017). The mixture of the two polymers yielded intermediate thickness values (58 ± 2 µm). In general, two opposite trends were observed in terms of the thickness of films containing polyphenols; in pure chitosan films the incorporation of polyphenols led to a significant decrease in the thickness of the films whereas in CH:S films the addition of polyphenols yielded thicker films. Polyphenols (TE and TA) and chitosan have opposite charges, and therefore their mixture may cause interactions that lead to a compaction of the polymer chains and hence a reduced thickness. This was not observed in CH:S mixtures, possibly due to the screening effect of CH surface charge.

Table 1 shows the antioxidant activity of the released active compounds after 24 h of contact with solvents of different polarity. The antioxidant activity was expressed as Trolox equivalent antioxidant capacity (mM TEAC). The higher the values, the higher the antioxidant activity. Both factors, type of solvent and film had a significant effect on the antioxidant activity of the film, being the interaction between these two factors also significant ($p < 0.05$). The effect of TA addition on the film antioxidant activity depended both on the type of solvent and on the polymer matrix. In CH:S:TE films and in ethanolic solvents (E10% and E20%) the addition of TA significantly increased the antioxidant activity of the films ($p < 0.05$). However, the opposite effect was observed in solvents of high polarity (water and AA3%). In CH:TE films, for all types of solvent, the addition of TA did not have significant effect on the film antioxidant activity. The highest antioxidant activity regardless the solvent polarity was obtained for S:TE films, especially in least polar solvent (E20%). On the contrary, the lowest antioxidant activity was obtained when using CH:S films containing TE (without TA). Differences in the polyphenol release from the different films into the simulants and potential oxidation of the compounds during the film processing can explain the different antioxidant activity observed in each case.

Table 1. Moisture content, water solubility, thickness and antioxidant activity in different solvents (H$_2$O, AA3%: 3% (w/v) acetic acid aqueous solution, E10%: 10% (v/v) ethanol aqueous solution, E20%: 20% (v/v) ethanol aqueous solution). Mean values and standard deviation, in brackets.

Film	MC % (g water/100 g dry solids)	Water solubility (g solubilized film/100 g film)	Thickness (mm)
CH:TE	21.61 (0.09)[c]	21.1 (1.1)[ab]	87 (4)[c]
CH:TA:TE	20.9 (0.4)[c]	22.7 (1.0)[bc]	84 (3)[c]
S:TE	12.4 (0.3)[a]	23.7 (1.3)[c]	50 (3)[a]
CH:S:TE	14.0 (0.6)[b]	19.3 (1.4)[a]	71 (5)[b]
CH:S:TA:TE	14.6 (0.7)[b]	20.2 (0.3)[a]	70.3 (1.4)[b]

Film	Antioxidant activity (mM TEAC)			
	H$_2$O	**AA3%**	**E10%**	**E20%**
CH:TE	0.5 (0.2)[ab,1]	1.3 (0.2)[b,2]	0.69 (0.09)[ab,1]	0.69 (0.12)[ab,1]
CH:TA:TE	0.76 (0.05)[b,12]	0.64 (0.07)[a,1]	0.68 (0.07)[ab,1]	0.95 (0.07)[b,2]
S:TE	1.70 (0.19)[c,12]	1.45 (0.07)[b,1]	2.1 (0.4)[c,3]	2.26 (0.04)[d,3]
CH:S:TE	0.69 (0.09)[b,1]	0.65 (0.10)[a,1]	0.53 (0.07)[a,1]	0.51 (0.04)[a,1]
CH:S:TA:TE	0.41 (0.09)[a,1]	0.58 (0.05)[a,1]	0.90 (0.14)[b,2]	1.3 (0.3)[c,3]

[abcd] Different letters in the same column indicate significant difference among formulations ($p < 0.05$).
[123] Different numbers in the same row indicate significant difference among solvents ($p < 0.05$).

3.2. Polyphenols release kinetics

The total polyphenolic content (TPC) of pure thyme extract incorporated to the films was 44.845 ± 3.696 mg GAE/g polymer. Tannic acid (TA), which was incorporated to the polymer matrix to act as a crosslinking agent (Rivero *et al.,* 2010), is also a polyphenolic compound. However, in the release study, the absorbance values obtained in the release studies performed with films containing TA (without TE) were subtracted from the absorbance obtained in the release studies carried out with the corresponding films containing both TA and TE. Therefore, the reported results (**Table 2**) show the polyphenols released from thyme extract (without considering the release of polyphenols from TA).

The release of active compounds from a polymeric matrix can be influenced by several phenomena that occur in successive steps: polymeric swelling, which allows the water diffusion, macromolecular matrix relaxation, and active compounds diffusion through the polymeric matrix into the outer solution until thermodynamic equilibrium is achieved (Buonocore *et al.,* 2003; Sánchez-González *et al.,* 2011). In the present study, the release of polyphenols was affected by different factors, such as solvent migration to starch, chitosan or chitosan-starch matrices, the polymer solubility and the diffusion of the polyphenols to the food simulant.

Table 2 shows the total polyphenol (TP) from TE released to the solvent after 24 hours, when equilibrium was assumed on the basis of kinetic curves. Two trends, which were not affected by the type of solvent were observed; the highest amount of polyphenols was delivered from pure starch films, which is coherent with the above mentioned higher antioxidant activity observed in these films. In this matrix, the values of total polyphenols released ranged from 22.3 to 23.8 mg GAE/g polymer, which represented 49.7 – 53.0% of the total polyphenols incorporated in the films after 180 min of release. This can be explained by the high solubility of starch matrix, which leads to the rapid release of polyphenolic compounds.

Table 2. Total polyphenols (TP) released (mg GAE/g of polymer), total rosmarinic acid (RA) released (mg RA/g of polymer), percentage RA of released, total polyphenols retained (TPR) in the matrix (mg GAE/g of polymer) and percentage of polyphenols retained in the matrix, as a function of different solvents (H_2O, AA3%: Acetic acid aqueous solution (3%, w/v), E10%: Ethanol aqueous solution (10%, v/v), E20%: Ethanol aqueous solution (20%, v/v).

Solvent	Film	TP (mg GAE released /g polymer)	RA (mg RA / g polymer*)	% RA released from film	TPR (mg GAE/ g polymer)	% TP retained in the matrix**
H_2O	CH:TE	4.68 (0.17)[b,2]	-	-	40.16	89.55
	CH:TA:TE	2.4 (0.5)[a,2]	-	-	42.47	94.71
	S:TE	20.3 (0.8)[d,1]	3.8 (0.3)[b,1]	49.10	24.56	54.77
	CH:S:TE	7.7 (0.5)[c,2]	0.05 (0.00)[a,1]	0.66	37.18	82.90
	CH:S:TA:TE	4.8 (0.3)[b,1]	-	-	40.09	89.39
AA3%	CH:TE	7.46 (0.16)[b,3]	-	-	37.32	83.21
	CH:TA:TE	7.4 (0.5)[b,3]	-	-	37.39	83.37
	S:TE	20.0 (0.3)[d,1]	-	-	24.86	55.43
	CH:S:TE	11.7 (0.5)[c,3]	-	-	33.12	73.86
	CH:S:TA:TE	4.6 (0.4)[a,1]	0.57 (0.00)[a,1]	7.39	40.24	89.72
E10%	CH:TE	4.35 (0.10)[b,1]	-	-	40.50	90.30
	CH:TA:TE	1.1 (0.5)[a,1]	-	-	43.78	97.62
	S:TE	20.5 (0.7)[e,1]	3.70 (0.13)[b,1]	47.97	24.37	54.35
	CH:S:TE	6.9 (0.2)[d,12]	0.70 (0.04)[a,2]	9.11	37.92	84.56
	CH:S:TA:TE	5.1 (0.2)[c,1]	0.43 (0.00)[a,1]	5.53	39.74	88.61
E20%	CH:TE	5.1 (0.3)[a,12]	0.13 (0.00)[a,1]	1.64	39.72	88.57
	CH:TA:TE	-	0.06 (0.02)[a,1]	0.80	44.85	100.00
	S:TE	21.1 (0.5)[c,2]	4.8 (0.8)[d,2]	61.67	23.72	52.88
	CH:S:TE	6.6 (0.2)[b,1]	2.23 (0.02)[c,3]	28.90	38.25	85.30
	CH:S:TA:TE	6.5 (0.3)[b,2]	0.98 (0.16)[b,1]	12.74	38.37	85.55

* Total amount of rosmarinic acid in the thyme extract is 7.7 ± 0.8 mg RA/ g of polymer.

** Total amount of polyphenols in the thyme extract is 45 ± 3 mg of GAE/ g of polymer.

[abcd] Different letters in the same column indicate significant difference among formulations ($p<0.05$).

[123] Different numbers in the same row indicate significant difference among solvents ($p<0.05$).

Figure 1 shows the percentage of TP polyphenols released as a function of time in the 4 evaluated solvents. In S:TE films, polyphenols release was reduced after 180 minutes in all solvents, which can be related with the fast oxidation of polyphenols. A similar effect was observed by Perazzo et al., (2014) when incorporating a high content of green tea polyphenols into cassava starch films. This suggests that polyphenols should be incorporated in starch-based matrices at limited concentrations in order to preserve their effectiveness. As regards the release of polyphenols from pure CH films, no significant differences in the amount of released polyphenols were noticed; and lower amounts of polyphenols were released in all solvents, as compared to starch-based films. This can be explained by hydrogen bonding and ionic interactions between the amine groups of chitosan and phenolic groups (Mayachiew and Devahastin, 2010;

Kanatt, Chander and Sharma, 2008; Aelenei et al., 2009; Trifkovic et al., 2015), which led to the retention of high amounts of polyphenols into the CH matrix. The highest amount of polyphenols released was obtained in the 3% acetic acid solution (7.46 mg GAE/g polymer), due to the higher solubility of chitosan polymer in acid media, in accordance with the high antioxidant activity observed in these films (1.3 mM TEAC). The addition of TA into chitosan and chitosan-starch films led to a reduction in the amount of polyphenols released probably due to the previously reported crosslinking effect of TA (Talón et al., 2017). A similar effect when incorporating TA in chitosan films where TA acted as a cross-linker and led to a more rigid closed and compact matrix was shown by Rivero et al. (2010). These results were consistent with the lower water content of the films containing tannic acid as compared to pure CH films (**Table 1**).
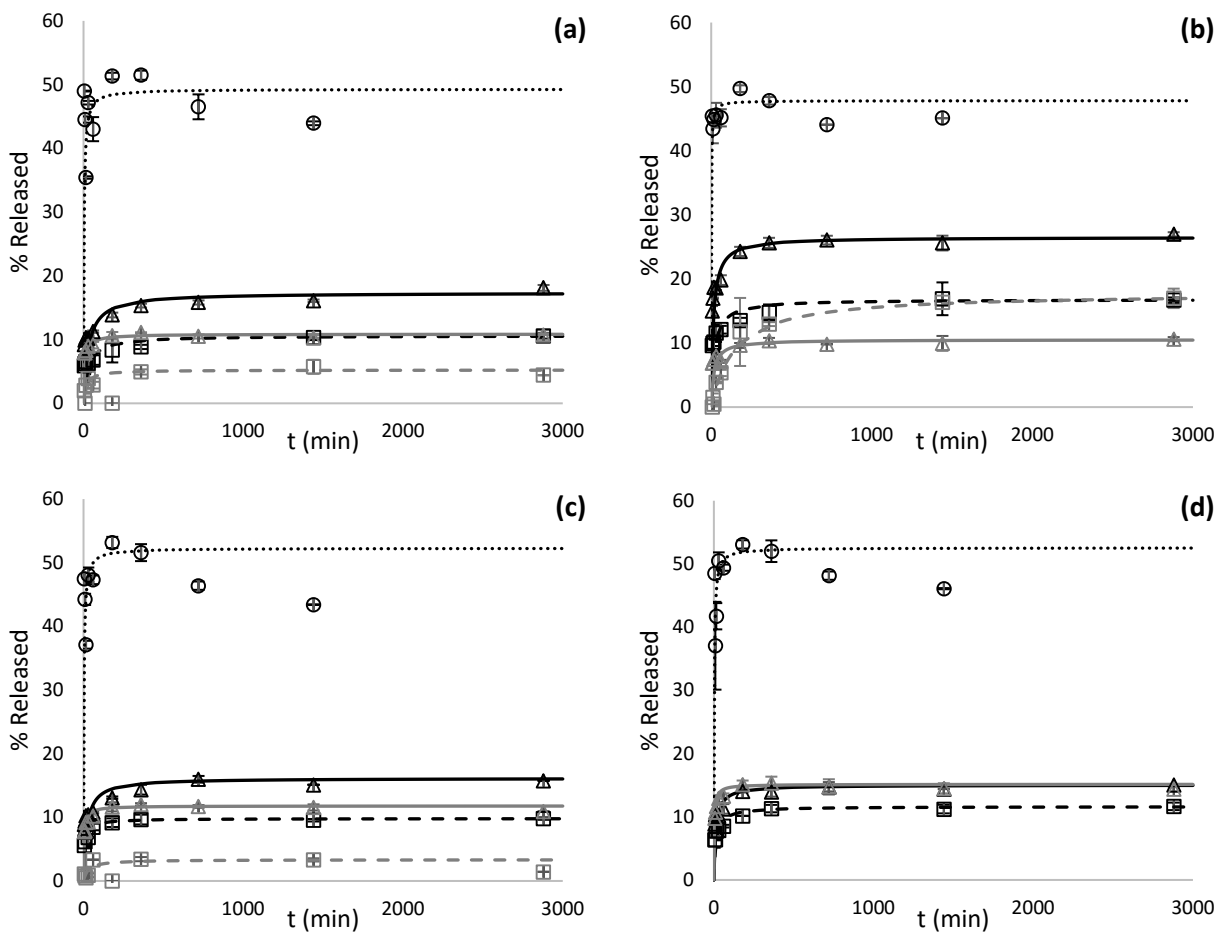
Figure 1. Percentage of total polyphenol released at 25°C as a function of time in different solvents (a: H_2O; b: Acetic acid aqueous solution (3% w/v); c: Ethanol aqueous solution (10% v/v); d: Ethanol aqueous solution (20% v/v); experimental data (□ CH:TE; ▣ CH:TA:TE; ○ S:TE; △ CH:S:TE; △ CH:S:TA:TE) and fitted Peleǵs model (- - - CH:TE; - - - CH:TA:TE; ⋯ S:TE; —— CH:S:TE; —— CH:S:TA:TE).

Polyphenols release from chitosan-starch films was affected by the polarity and pH of the solvent. The amount of polyphenols in the acetic acid solution showed a maximum, due to the high solubility of CH in presence of acetic acid, and a more open structure, which governs polyphenols release. This is in accordance with the above-mentioned highest antioxidant activity observed in these films in this solvent. The opposite trend was observed in CH:S:TA:TE films, where the TP amount showed a minimum value in the acetic acid aqueous solution.

Table 2 shows the amount of rosmarinic acid released (mg/g of polymer) from the different film formulations after 24 hours in the four different solvents. The amount of rosmarinic acid (RA) becoming from the pure thyme extract was 7.7 ± 0.8 mg/g polymer. The total release of RA depended on both film composition and the solvent. The highest amount of RA was released from pure starch films, which is in agreement with the above-mentioned higher release of polyphenols from these films. On the other hand, the release of RA was inhibited in pure CH films, while S:CH films showed intermediate results. The increase in RA release with the increase in the ethanol content of the food simulant is in agreement with the decrease in solvent polarity and the subsequent increase of the chemical affinity and solubility of RA with the solvent (Zibetti, Aydi, Claumann, Eladeb and Adberraba, 2016).

Table 2 shows total polyphenols retained (TPR) considering the theoretical content in the different matrices at the end of the release process. Two different trends were observed: the lowest retention of polyphenolic compounds in pure starch films and the enhancement effect of tannic acid on the TPR values of chitosan-based films. Polyphenols retention in pure starch based films after the release process was very similar in the four solvents and represent about 50% of the incorporated amount. The highest solubility of the pure starch matrix (**Table 1**), provided a greater exposure of polyphenols thus promoting athe rapid diffusion of active compounds into the food simulants andthe highest antioxidant activity.

In order to evaluate the tannic acid release from films containing this compound, CH:TA and CH:S:TA films were submitted to a release study for 48 h. TA was quantified as the total polyphenol content by using the same procedure by the Folin-Ciocalteu method. The TP content of pure tannic acid was 0.889 mg GAE/mg TA. This value agrees with that reported by Sahiner, Sagbas and Aktas (2015) and Sahiner, Sagbas, Aktas and Silan (2016), which found 0.820 mg GAE/ mg of TA. The total percentage of TA released after 24 hours (when the steady state was reached) from the CH:TA films was 10.7%, 37.5%, 16.8%, 26.5% in water, 3% acetic acid solution, 10%, and 20% ethanol aqueous solutions, respectively.

A remarkable increase in the amount of TA released with the increase in ethanol concentration was observed, but the maximum amount released was obtained for the acetic acid medium due to the high solubility of the chitosan matrix in this solvent. For

CH:S:TA films, the amount of TA released was 13.2%, 27,1%, 19.0%, 24.2% in water, 3% acetic acid solution, 10% and 20% ethanol aqueous solutions, respectively. No significant differences in the TA retention in the films were observed for CH:S and CH films, except in acetic acid, where CH solubilisation implied a greater release of TA.

The addition of TA in CH:TE films promoted the retention of the polyphenols in the films matrix in the ethanol solutions, ranging from 94.7 to 100% of polyphenols retained. Although this effect was mitigated in the acetic acid medium (83.4 % of retention) due to the CH dissolution (Table 2). In addition, polyphenols retention ranged between 85.6% and 89.7% when adding TA to CH:S:TE films. In this case, no significant TA retention effect of polyphenols was observed in the 20% ethanol aqueous solution, which can be attributed to the greater solubility of phenols, which greatly affects partition coefficient of the compounds between the matrix and the solvent.

The analysis of results indicates that partition of phenols between the polymer matrix and solvent was greatly affected by the presence of CH and TA incorporation. CH bonded polyphenols to a greater extent than starch (Talón et al., 2017), but TA crosslinking of CH chains also contribute to enhance the polyphenol retention in the film, thus limiting their release. Nevertheless, in acid media, both TA and TE phenols are easily delivered to the solvent from CH containing films, according to the water solubility promotion and the partial matrix disruption. Nevertheless, even in acid media the total polyphenol delivery was lower than that obtained in CH free starch matrices.

The experimental data obtained in the release studies were fitted to different models. Generally, the process of diffusion of compounds from swelling equilibrium systems can be fitted to Fick's law. However, phenomena of swelling process may produce a non-Fickian migration mechanism of compounds. To assess the pertinence of the Ficks' law fitting, the data were fitted to Korsmeyer-Peppas model for the sort time range of the process (driving force, $1-M_t/M_\infty$, lower than 5). For a thin film, according to this model, a value of n equal to 0.5 means that the release is related to a Fickian diffusion mechanism of first order; on the contrary, when n=1, polymer relaxation or degradation occurs, leading to zero-order release. An anomalous Fickian diffusion occurs when n values are between 0.5 and 1 (Mandal, Mann and Kundu, 2009; Huang, Yu and Xiao, 2007). As shown in Table 3, the values of coefficient n were far from the Fickian behaviour except for the release of polyphenols from CH:TA:TE films in acetic acid, where the behaviour was Fickian (n = 0.506). The low values of n can be attributed to the partial films solubilisation in the solvents which contribute to the compound delivery in a non-Fickian process.

Table 3. Kinetic constants of Korsmeyer-Peppas model (rate constant, k, h^{-1} and difussional exponent) at 25°C in different simulants (H2O, AA3%: Acetic acid aqueous solution (3%, w/v),

E10%: Ethanol aqueous solution (10%, v/v), E20%: Ethanol aqueous solution (20%, v/v). Mean values and standard deviation, in brackets.

Film		H_2O	AA3%	Et10%	Et20%
CH:TE	k	0.45 (0.03)[b,1]	0.483 (0.010)[b,12]	0.55 (0.07)[c,2]	0.48 (0.07)[a,12]
	n	0.101 (0.013)[b,1]	0.097 (0.008)[b,1]	0.09 (0.02)[ab,1]	0.101 (0.017)[b,1]
CH:TA:TE	k	0.35 (0.13)[a,2]	0.03 (0.02)[a,1]	0.31 (0.05)[a,2]	-
	n	0.16 (0.05)[c,1]	0.51 (0.10)[c,2]	0.16 (0.03)[c,1]	-
CH:S:TE	k	0.43 (0.04)[b,1]	0.52 (0.03)[b,2]	0.45 (0.02)[b,12]	0.53 (0.03)[a,2]
	n	0.111 (0.002)[b,1]	0.093 (0.003)[b,1]	0.112 (0.013)[b,1]	0.094 (0.013)[ab,1]
CH:S:TA:TE	k	0.68 (0.03)[c,2]	0.56 (0.04)[b,1]	0.64 (0.05)[d,12]	0.69 (0.03)[b,2]
	n	0.055 (0.003)[ab,1]	0.075 (0.012)[b,1]	0.072 (0.007)[ab,1]	0.062 (0.004)[ab,1]
S:TE	k	0.84 (0.07)[d,2]	0.87 (0.07)[c,2]	0.74 (0.02)[e,1]	0.72 (0.05)[b,1]
	n	0.24 (0.014)[a,1]	0.023 (0.009)[a,1]	0.050 (0.003)[a,1]	0.049 (0.004)[a,1]

[abcde] Different letters in the same column indicate significant difference among formulations (p<0.05).
[123] Different numbers in the same row indicate significant difference among solvents (p<0.05).

Experimental data in terms of total released polyphenols as a function of time were also fitted to Peleg's model, and the obtained parameters are shown in **Table 4**. Unlike Korsmeyer- Peppas model, which is only valid for the first 60% of compound released, Peleg model can predict the value at equilibrium when time tends to infinity, thus, this model can estimate long range of values from experimental data obtained in tests of relatively short duration (Botelho, Corrêa, Martins, Botelho and Oliveira, 2013; Hines and Kaplan, 2011; Peleg, 1988). In the present study, high correlation coefficients were obtained for Peleg's model fit, as seen in **Figure 2.** However, in S:TE films, the model was fitted only in the first section of the release due to rapid oxidation of the polyphenols, as observed in **Figure 1**, where the released amount of polyphenols tends to decrease after the equilibrium was reached.

Table 4. Parameters of Peleg´s model at 25ºC in different simulants (H_2O, AA3%: Acetic acid aqueous solution (3% w/v), E10%: Ethanol aqueous solution (10% v/v), E20%: Ethanol aqueous solution (20% v/v). Constant k_1 (min·mg^{-1} GAE·g^{-1} polymer) is related to the release rate at the beginning of the process. k_2 (g polymer·mg^{-1} GAE) relates to the asymptotic value which can be related to the equilibrium value. M_∞ is the inverse of k_2 and is the amount of polyphenols released at equilibrium (mg GAE·g^{-1} polymer). Mean values and standard deviation, in brackets.

Film		H_2O	AA3%	E10%	E20%
CH:TE	k_1	6.2 (1.6)[ab,2]	2.8 (1.6)[a,1]	2.1 (1.2)[a,1]	2.8 (1.7)[c,1]
	k_2	0.210 (0.008)[c,3]	0.123 (0.006)[c,1]	0.227 (0.003)[a,4]	0.192 (0.010)[c,2]
	M_∞	4.76 (0.19)[b,1]	7.5 (0.3)[b,3]	4.40 (0.06)[b,1]	5.2 (0.3)[a,2]
CH:TA:TE	k_1	11 (5)[b,12]	25 (14)[b,2]	20 (12)[b,2]	-
	k_2	0.43 (0.08)[d,2]	0.123 (0.013)[c,1]	0.67 (0.05)[b,3]	-
	M_∞	2.4 (0.5)[a,1]	8.2 (0.8)[b,2]	1.503 (0.112)[a,1]	-
S:TE	k_1	0,08 (0.08)[a,1]	0.05 (0.03)[a,1]	0.12 (0.03)[a,1]	0.10 (0.05)[a,1]
	k_2	0.046 (0.004)[a,12]	0.047 (0.002)[a,2]	0.0427 (0.0012)[a,1]	0.042 (0.001)[a,1]
	M_∞	21.7 (1.6)[d,12]	21.1 (0.9)[d,1]	23.4 (0.6)[e,2]	23.6 (0.7)[c,2]
CH:S:TE	k_1	4.0 (1.8)[ab,2]	1.2 (0.6)[a,1]	2.9 (0.6)[a,12]	1.9 (0.3)[bc,1]
	k_2	0.128 (0.008)[b,2]	0.084 (0.002)[b,1]	0.138 (0.003)[a,2]	0.149 (0.006)[b,3]
	M_∞	7.8 (0.5)[c,2]	11.9 (0.3)[c,3]	7.24 (0.15)[d,12]	6.7 (0.3)[b,1]
CH:S:TA:TE	k_1	1.7 (1.5)[ab,12]	3.59 (1.18)[a,2]	1.1 (0.5)[a,1]	0.5 (0.3)[ab,1]
	k_2	0.205 (0.007)[c,3]	0.212 (0.009)[d,3]	0.1876 (0.0009)[a,2]	0.147 (0.007)[b,1]
	M_∞	4.87 (0.17)[b,1]	4.7 (0.2)[a,1]	5.33 (0.02)[c,2]	6.8 (0.3)[b,3]

[abc] Different letters in the same column indicate significant difference among formulations (p<0.05)
[123] Different numbers in the same row indicate significant difference among solvents (p<0.05)

Table 4 shows the parameters of Peleg's model, where k_1 is related to the inverse of the initial velocity of the release process and k_2 is the inverse of the total concentration released at equilibrium (M_∞), which reflects the total amount released at equilibrium in mg GAE/g polymer. The M_∞ values coincide with the experimental determination at 24 h of film-solvent contact time, reported in **Table 2.** In pure CH films the greater release occurred in acid media, with similar rate as in the ethanol solutions, whereas slower release occurred in pure water, where the final release was similar as in the ethanol solutions. When CH films contained TA, the release rate was greatly reduced in all solvents showing similar values in all cases, but the final release was enhanced in acid media. This is consistent with the crosslinking effect of TA in CH films, which delays the release rate according to the higher tortuosity factor for mass transfer in the films, although the enhancement of CH solubility in acid medium let to a greater delivered amount of actives. In pure starch films the fastest delivery rate and highest delivery ratio were found, regardless the solvent, according to the weaker interactions of polyphenols with the matrix. In starch films, a decrease in the phenol content released

was observed after the maximum release that was observed at 180 min (Figure 1). This can be explained by the above mention fast oxidation of polyphenols. In spite of this reduction in TP content after 24h, the films exhibited the maximum antioxidant activity (**Table 1**).

In CH:S blend films, release rate slightly increased with respect to CH films, as well as the total released amounts, showing similar effects of the solvents as observed for CH films. This behaviour suggests that the greatest part of the active compounds interact mainly with the CH fraction, despite the highest proportion of starch in the films. Finally, the incorporation of TA to CH:S films, provoked some similar effects to those observed in pure CH films, but the acid medium did not promote an increase in the final total amount released, which was similar to that occurred in water. However, the amount of polyphenols release in these films was higher in ethanol solutions, where the release rate was promoted, with respect to neutral and acid aqueous solutions. Therefore, as expected, TA mainly interact with CH and the crosslinking effect occur to lesser extent in CH:S matrix, being less limiting for the polyphenol release.

The obtained results for CH and films containing TA are in agreement with that reported by Trifković et al. (2015), who shown an incomplete release of polyphenols from chitosan beads with a crosslinker agent (glutaraldehyde) in acid medium (pH=2.20). Likewise, polyphenol also strongly interact with the TA free CH matrix, which limit their release to the aqueous media, as compared with the starch matrix.
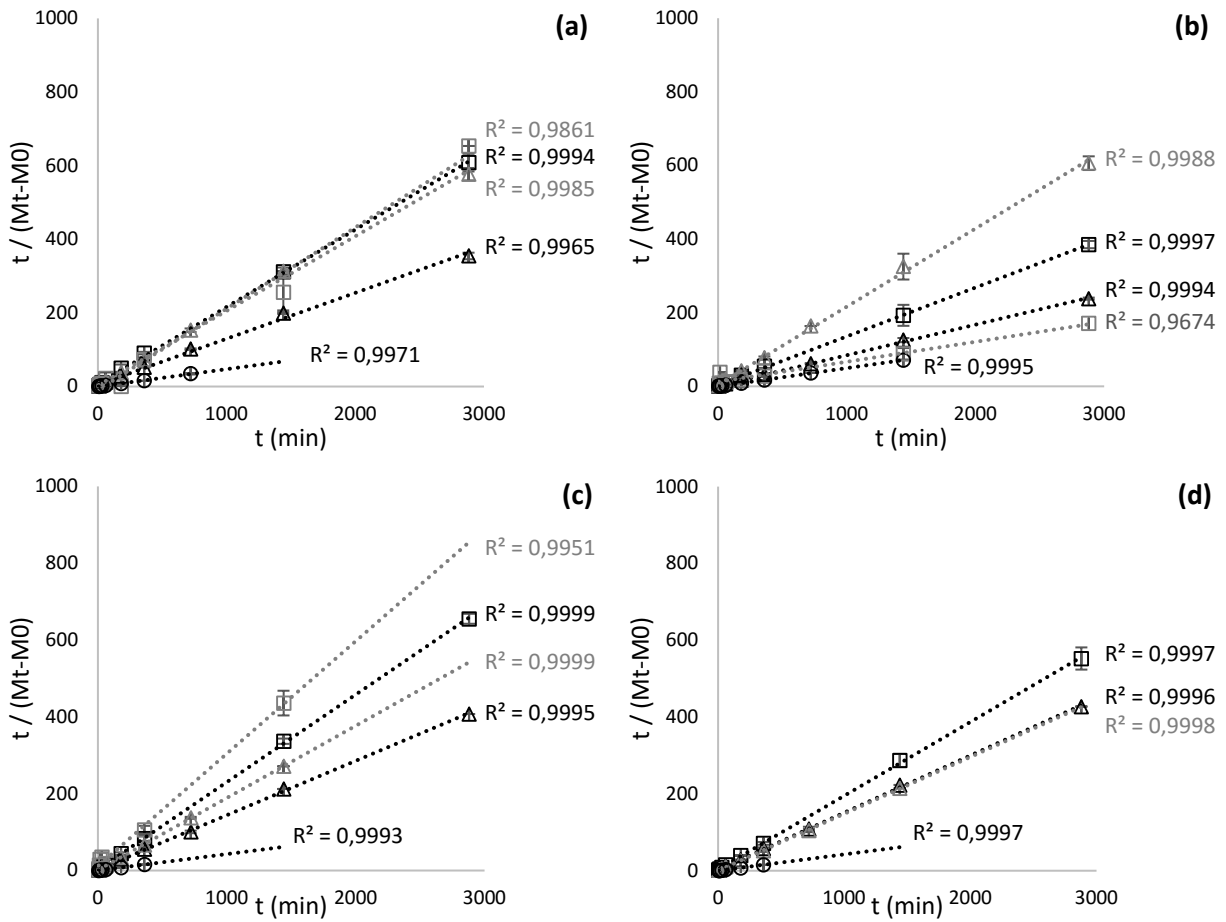
Figure 2. Application of Peleg equation to experimental data of all the films (□ CH:TE; □ CH:TA:TE; ○ S:TE; △ CH:S:TE; △ CH:S:TA:TE) in different solvents (a: water; b: 3% acetic acid; b: 10% ethanol and d: 20% ethanol).

4. CONCLUSIONS

Polyphenols from thyme extract strongly interact with chitosan chains in both pure chitosan matrix and chitosan-starch blend films. These interactions led to a reduction of polyphenols release rate in aqueous media, even at low pH where, despite the increase in the total delivered amount, this was much lower than that occurred in starch films. Incorporation of tannic acid to the chitosan films provoked the matrix crosslinking, to a greater extent in pure chitosan films, which greatly reduced the ratio and rate of polyphenol release, although in the acid medium, the increase in the chitosan solubility enhanced the total polyphenol delivery. However, in chitosan:starch blends, the cross-linking effect of tannic acid inhibited the release promotion of the acid medium, while both release rate and ratio were enhanced in ethanol solutions, thus indicating the role of chitosan-starch interaction in the blend matrix rearrangement. In fact, the total delivery of polyphenols in CH:S blend films was nearer to that occurred in chitosan films rather than to the delivery in starch films, in spite of the greater starch ratio in the blend.

Acknowledgements

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Encapsulation of eugenol by spray-drying using whey protein isolate or lecithin.
Release kinetics, antioxidant and antimicrobial properties.

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GRAPHICAL ABSTRACT

Encapsulation of eugenol by spray-drying using whey protein isolate or lecithin. Release kinetics, antioxidant and antimicrobial properties

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ABSTRACT

The encapsulation of eugenol (E) by spray-drying using whey protein isolate (WP) or soy lecithin (LE), as wall material, and maltodextrin, as drying coadjuvant, has been carried out in order to obtain antioxidant and/or antimicrobial powders to be used in food applications. The effect of incorporating oleic acid (OA), as eugenol carrier, and chitosan (CH), as a potential capsule stabilizer, has also been analysed. A good encapsulation efficiency (EE) of E was observed in the powders with only WP or LE (95-98%), whereas the incorporation of OA reduced EE to 87% in WP systems and the use of CH provoked a marked EE decrease in both WP and LE powders (22, 46 %, respectively). The formulations exhibited similar eugenol release behaviour in the food simulants of different polarity, where practically the total content was delivered at a similar rate. All the samples exhibited differing degrees of antioxidant and antimicrobial activities, according to the E content in the powder. The antibacterial effect of CH-free powders against *E. coli* was also coherent with their eugenol content, in line with the burst release of E to the culture medium, which exceeded the minimum inhibitory concentration (MIC) of the bacteria with 15 mg powder/mL for OA-free powder. An additional positive effect of OA was detected in the powder's antilisterial action, which led to a similar antibacterial action in powders with and without OA, with different E contents.

Keywords: encapsulation efficiency, release kinetics, oleic acid, chitosan, antioxidant capacity, antibacterial properties.

1. INTRODUCTION

Over the last few years, substantial efforts have been focused on making use of natural compounds to develop novel health-promoting ingredients for use in the food industry. In this sense, increasing interest has been shown in the extracts from aromatic plants, such as essential oils, due to their antioxidant and antimicrobial properties (Mozafari et al., 2006; Olmedo, Asensio & Grosso, 2015). Eugenol (E) is a natural phenolic substance found as a major compound in different plant essential oils, such as clove, nutmeg, cinnamon or basil, and has been widely used in foodstuffs, pharmaceutical products or cosmetics (Bullerman, Lieu & Seier, 1977; Chatterjee & Bhattacharjee, 2013; Friedman, Kozukue & Harden, 2000) for different purposes (flavoring, antioxidant, antimicrobial,...). Several bioactive properties have been reported for E, including antibacterial and antioxidant activities. Particular antimicrobial activity for E has been described by different authors against Gram positive and Gram negative bacteria (*Bacillus subtilis, Clostridium sporogenes, Enterococcus faecalis, Lactobacillus plantarum, Listeria monocytogenes, Escherichia coli* and *Salmonella typhimurium,* (Burt, 2004; Cetin-Karaca & Newman, 2015; Dorman & Deans, 2000)), fungi (*Aspergillus carbonarius, Penicillium roqueforti* and *Microsporum gypseum* (Lee et al., 2007; Šimović, Delaš, Gradvol, Kocevski & Pavlović, 2014)) and yeast (*Saccharomyces cerevisiae* and *Candida* (Bennis, Chami, F., Chami, N., Bouchikhi & Remmal, 2004; Pinto, Vale-Silva, Cavaleiro & Salgueiro, 2009)). Its effective antioxidant capacity has also been studied by several authors (Kamatou, Vermaak & Viljoen, 2012; Nagababu & Lakshmaiah, 1992; Ogata, Hoshi, Urano & Endo, 2000; Scherer & Godoy, 2009; Gülçin, 2011). Chatterjee & Bhattacharjee (2015) studied the use of eugenol-rich clove extract in mayonnaise as a flavoring agent and as a source of natural antioxidants to improve its shelf-life and functional value. Mayonnaise formulated with eugenol had a significantly higher antioxidant activity and phenolic content and better maintained its properties for a longer period of time than commercial samples. Cortes-Rojas, Souzca & Oliveira (2014) also produced antioxidant powder products with solid lipid nanoparticles (SLN) containing eugenol.

Nevertheless, the beneficial properties of these active compounds can be reduced by inadequate storage conditions (Fang & Bhandari, 2010) due to their volatility and sensitivity to oxygen, light or heat. Moreover, their incorporation into aqueous systems, such as most foods, is limited by their low water solubility and impact on flavor (Choi, Soottitantawat, Nuchucha, Min & Ruktanonchai, 2009; Woranuch & Yoksan, 2012). Most of these problems can be overcome by using encapsulation techniques, allowing for the easier handling of the active compound, a better protection during storage and transportation and a better control in the release (Bae & Lee, 2008). Of all the encapsulating methods, spray-drying is the most feasible from the industrial point of view and, so, is the most commonly-used microencapsulation method. Nevertheless, the composition of the aqueous phase, where eugenol is initially dispersed, must be

optimized in order to ensure the formation of a good shell material, entrapping the active compound in the core, after the drying process; this allows for its controlled release when the powder is incorporated into a determined matrix, when novel compound interactions will take place, with notable alterations in the product quality attributes. Spray drying has been extensively used for the encapsulation of different bioactive ingredients, including vitamins, polyunsaturated oils, phenolic compounds, enzymes, probiotics or some other compounds with an undesirable flavor, for masking purposes (Augustin & Hemar, 2009). The effectiveness of the encapsulation process is greatly affected by the properties/stability of the initial dispersion/emulsion of the active compound and, consequently, by the wall materials used in their formulation (Bae & Lee, 2008; Kagami et al., 2003; Ré, 1998). In addition to the encapsulating efficiency, the antimicrobial or antioxidant properties of the encapsulated compound in the final dried capsules is affected by its total load in the powder (active/support compounds ratio) and its release kinetics into a determined target medium into which it could be incorporated. All of these factors define the effective concentration on the target point, which must be studied to ensure the required functionality.

The components of encapsulation matrices for food application purposes are limited to edible, preferably inexpensive, materials, biopolymers being the ideal candidates meeting these requirements. Proteins, polysaccharides and polar lipids such as lecithin have been proposed as promising vehicles for the protection and/or delivery of bioactive ingredients. Proteins, such as whey protein and lecithin, are usually incorporated to promote emulsion formation and interfacial stabilization in the capsule-forming dispersions. In these dispersions, polysaccharides act as stabilizers by increasing the viscosity of the continuous phase or by means of the development of electrostatic interactions at the oil-water interface. In this sense, positively charged chitosan molecules in acid media could enhance the stability of the dispersion by means of a viscous electro-steric effect at the interface, thus promoting dispersion stability (Rodríguez, Albertengo & Agullo, 2002). Maltodextrins can improve the properties of the capsules during the drying stage due to the formation of a larger crust around the drops (Kagami et al., 2003), thus providing good protection against oxidation (Sheu & Rosenberg, 1998).

Lecithins are commonly used as emulsifiers and texture modifiers of some foods and, currently, as an encapsulating agent, both for hydrophilic and lipophilic components (Taylor, J., Taylo, JRN., Dutton & De Kock, 2005). The chemical structure of lecithins allows the formation of liposomes which can entrap different kinds (more or less polar) of active compounds (Liolios, Gortzi, Lalas, Tsaknis & Chinou, 2009). Commercial soy lecithin contains about 65-75% phospholipids as i.e phosphatidylcholine and phosphatidylethanolamine, 34% triglycerides, and smaller amounts of carbohydrates, pigments, sterols, and sterol glycosides (Dickinson, 1993). At neutral pH, phosphate and carbonyl groups from phosphatidylcholine and phosphatidylethanolamine

components in lecithin contribute to the negative charge of the particles in the emulsion, thus contributing to emulsion stability by charge (Dickinson, 1993; Wang & Wang, 2008). Whey protein isolate (WP) or LE together with MD could form good wall systems able to stabilise in oil droplets in the oil-water emulsions, favouring the formation microcapsules during the emulsion spray drying (Karadag et al., 2013).

On the other hand, other polar lipids, such as glycerides, phospholipids or fatty acids, can be used as carriers of lipophilic compounds in aqueous media, favoring their dispersion, prior to their encapsulation by spray drying. They have been extensively studied for the controlled release of substances of poor water solubility, mainly in cosmetic and pharmaceutical applications (Tamjidi, Shahedi, Varshosaz & Nasirpour, 2013; Woo, Mirsan, Lee & Tan, 2014). Oleic acid (OA) in combination with other solid lipids has been successfully used to prepare micro and nanostructured lipid carriers with improved drug delivery properties (Chen, Tsai, Huang & Fang, 2010; Hu et al., 2005; Souto, Wissing, Barbosa & Müller, 2004; Woo et al., 2014). Perdones, Vargas, Atarés & Chiralt (2014) observed an increase in the retention and activity of the cinnamon essential oil when using OA as a carrier material in biopolymer matrices for film development.

The aim of this study was to encapsulate eugenol by spray drying using WP or LE as wall-materials and to characterize the different formulations before (emulsion properties) and after drying, in terms of the encapsulation efficiency, thermal stability, release kinetics and antioxidant and antimicrobial activities. The effect of the incorporation of both oleic acid (OA), as eugenol carrier, and of chitosan (CH) on the properties of the encapsulating systems was analysed.

2. MATERIALS AND METHODS

2.1. Raw materials

Soy lecithin (LE) Lipoid S45 from Lipoid GmbH (batch 574510, Ludwigshafen, Germany); whey protein isolate (WP) Prodiet 90S (95% whey and 1.5% fat) from Ingredia (batch 131848, France); maltodextrin (MD) Kyrosan E18 1910 QS (DE19.2, batch 02157372, Emsland Group, Germany); purified oleic acid (OA) (77% C18:1; 11% C18:2; 4% C16:0; 1% C16:1; 3% C18:0) from VWR Chemicals (Germany) and high molecular weight chitosan (CH) from Sigma-Aldrich (Madrid, Spain) were used to encapsulate pure eugenol (E), also from Sigma-Aldrich (batch STBD6235V, Madrid, Spain).

Sodium hydroxide (Merck, Darmstadt, Germany), boron trifluoride in methanol and sodium chloride (Sigma–Aldrich, Steinheim, Germany), sodium sulphate (purity 99%, VWR International, West Chester, PA, USA), C19:0 methyl ester and a GLC-63 mixture of fatty acid methyl esters (Nu-Check Prep, Elysian, MN, USA) as reagents and heptane and 2-propanol (Rathburn Chemicals Ltd., Walkerburn, Scotland) as HPLC grade solvents were used for the chromatographic fatty acid analysis. Glacial acetic acid, absolute ethanol and methanol and diphosphorus pentoxide (P_2O_5) were purchased from Panreac AppliChem (Barcelona, Spain) and 2,2-Diphenyl-1-pikryl-hydrazyl and Folin-Ciocalteu reagent were obtained from Sigma-Aldrich (Madrid, Spain), in order to determine the other assays.

2.2. Emulsion preparation

Whey Protein Isolate (WP) or Lecithin (LE) were mixed with Maltodextrin in a WP/LE:MD ratio of 1:42 (w/w) to obtain aqueous dispersions (43g solids/100g). After leaving these aqueous solutions overnight under stirring, 3% eugenol (w/w) was added, obtaining the formulations E-WP and E-LE (**Table 1**). 7 wt. % of oleic acid was added in formulations EOA-WP, EOA-LE, EOA-WPCH and EOA-LECH (**Table 1**). All of the dispersions were homogenized with a Rotor Stator (Ultra-Turrax T 25 Basic, IKA Werke GmbH & Co. KG, Germany) at 11,000 rpm for 6 minutes and microfluidized (three cycles) with the high-pressure homogenizer (Microfluidics M-110Y, Newton, Massachusetts, USA) at 15,000 psi pressure (103,42 MPa). Formulations with CH (EOA-WPCH and EOA-LECH) were obtained by previously dispersing 1% (w/w) chitosan (CH) in 1% (v/v) acetic acid solution for 14 h, under stirring at 150 rpm. The chitosan solution was added to formulations in a CH solution:emulsion ratio of 1.5:10.

Table 1. Mass fraction of each component (g/g total solids) of the different dried formulations.

Formulation	WP	LE	MD	Eugenol	OA	CH
E-WP	0.022	-	0.913	0.065	-	-
EOA-WP	0.019	-	0.792	0.057	0.132	-
EOA-WPCH	0.019	-	0.790	0.056	0.132	0.003
E-LE	-	0.022	0.913	0.065	-	-
EOA-LE	-	0.019	0.792	0.057	0.132	-
EOA-LECH	-	0.019	0.790	0.056	0.132	0.003

2.3. Spray-drying

All of the emulsions were spray dried by a Mobile Minor TM spray-dryer (GEA Niro, GEA Process Engineering A/S, Søborg, Denmark) with a two fluid atomizer (co-current two-fluid nozzle system). Samples were introduced into the drying chamber at an initial flow rate of 20 mL/min and an inlet air temperature adjusted to 180 °C. The outlet temperature was kept at 80 ± 2 °C by controlling the feed rate using peristaltic pump (Watson Marlow 520s IP31, head type 314, Watson-Marlow Bredel Pumps, Cornwall, UK). During spray-drying the fan speed was set to 2,800 rpm and the atomization air flow pressure, 1.9 bar.

2.4. Characterization of the emulsions

2.4.1. Z-potential

Z-Potential of emulsions was measured in triplicate by using a dynamic light scattering instrument capable of measuring electrophoretic mobility (Zetasizer nano ZS, Malvern Instruments, Worcestershire, UK). The E-LE formulation was measured without dilution. The rest of the emulsions were diluted to reach a final concentration of 1% (w/w) so as to prevent multiple scattering effects.

2.4.2. Particle size

The technique of laser diffraction was used to determine the size of particles in emulsions (Mastersizer 3000, Malvern Instruments). The Mie theory was applied by considering refractive and absorption indexes of 1.48 and 0.01, respectively. Samples were diluted in de-ionised water at 2500 rpm until an obscuration rate of 10% was obtained. D_{32} (surface weighted mean diameter) and D_{43} (volume weighted mean diameter) parameters were obtained. Light microscopy images of the emulsions were taken using a light microscope (Olympus, GWB MTV-3, Japan) with a digital camera.

2.4.3. Rheological behaviour

The rheological behavior of emulsions by six-fold at 20°C were characterized. The flow curves (apparent viscosity as a function of shear rate) of emulsions were determined by ThermoHaake Rheostress 600 rheometer (Thermo Electron GmbH, Dreieich, Germany) equipped with rotating cone of 35 mm in diameter and cone angle of 1°, over a shear rate range of 0.03–100–0.03 s^{-1}. Ostwald model was fitted to the flow curves.

2.5. Characterization of the spray-dried powders

2.5.1. Particle size and microstructure (SEM)

The particle size of the spray-dried powder formulations was measured by the laser diffraction technique (Mastersizer 3000, Malvern Instruments, UK), equipped with a dry dispersion unit. A refractive index of 1.48 and an absorption of 0.01 was also considered. Samples were fed into the system at a feed rate of 60% and a pressure of 2.2 bar until an obscuration rate was obtained within the range of 0.5-6%. The parameters, D_{32} and D_{43}, were obtained.

The microstructure of the microcapsules was evaluated by means of scanning electron microscopy (SEM) (JEOL, JSM-5410, Japan). The powders were previously conditioned in a desiccator with diphosphorus pentoxide (P_2O_5) and they were mounted on copper stubs with double-sided adhesive carbon tape and gold coated. The images were captured by using an acceleration voltage of 15kV at 1,500 magnifications.

2.5.2. Thermogravimetric analysis

To evaluate the thermal stability of the samples, both powders and pure compounds, a thermogravimetric analysis (TGA) (StareSystem, Mettler Toledo Inc., Switzerland) was performed. The TGA was carried out from 50°C to 600°C at a heating rate of 10°C/min under a nitrogen atmosphere (20 mL/min). Sample weight versus temperature curves were recorded using the STARe software of (Version 9.01, Mettler Toledo) in triplicate. The samples were previously conditioned in a desiccator with P_2O_5 until constant weight.

2.5.3. Concentration of eugenol in the powders. Encapsulation efficiency

Spectrophotometric analysis was used to analyze the concentration of encapsulated eugenol in the dried formulations, previously submitted to methanol extraction. 0.1 g of sample were weighed using an analytical balance (ME36S, Sartorius,Germany; ±0.00001 g) and extracted in 100 mL of methanol under constant stirring for 24h (previously

determined maximum time of extraction). Then, the absorbance of the filtered samples was measured in triplicate, by using a spectrophotometer (ThermoScientific spectrophotometer Evolution 201 UV–vis) at 282 nm (maximum eugenol absorption in methanol). The extract of the corresponding control without eugenol was used as a blank in each case. The calibration curve (y=0.018·x; R²=0.998) was obtained from the absorbance measurements of standard solutions of eugenol and was used to determine the concentration of eugenol in the samples.

The encapsulation efficiency (%EE) was calculated by using **Equation 1**, where CE was the amount of eugenol determined by methanol extraction and $C_{theoretical\ E}$ was the theoretical eugenol content.

$$\%EE = \frac{C_E}{C_{theoretical\ E}} \cdot 100$$ **Equation 1**

2.5.4. Extraction and quantification of the lipid content in the whole particles and on their surface.

The surface and total lipids were extracted using the methodology described by Damerau et al. (2014). First, samples (0.3 g) were washed with 5 mL of heptane by means of a mild shaking in an orbital shaker for 15 min and then centrifuged at 2,000 rpm for 2 min (Baik et al., 2004). For the extraction of total lipids, 0.3 g of sample were re-suspended in 3 mL of water at 40°C and vortexed. The lipids were extracted by shaking in an orbital shaker for 15 min using 10 mL of a heptane/2-propanal mixture (3:1, v/v). After shaking, the mixture was centrifuged at 3000 rpm for 2 min and the organic phase was collected.

The fatty acid composition of the lipid extracts (both surface and total lipids) was analysed by using the method described by Damerau et al. (2014). This method is based on the saponification of the sample, followed by the methylation of the liberated fatty acids in the presence of boron trifluoride (Soupas, Huikko, Lampi & Piironen, 2005). An aliquot of each lipid extract (4 mL), containing an internal standard C19:0 (1 mL), were mixed and evaporated under nitrogen atmosphere at 37°C until total evaporation. The residue was saponified with 1 mL of NaOH (0.5 M methanol solution) in a water bath in order to liberate the fatty acids. Then, the liberated fatty acids were methylated with 2 mL of boron trifluoride-methanol solution (BF3CH3OH) at 100°C for 5 minutes. 1mL of saturated NaCl and 1mL of heptane were added and the samples were stirred for one minute. After that, anhydrous Na_2SO_4 was added to eliminate residual water. The organic phase was separated and transferred to GC vials for analysis. All of the samples were analyzed by using a Hewlett Packard 5890 Series II GC (Karls-ruhe, Germany) equipped with an automated on-column injection system and a flame ionization detector (FID) (Soupas, Juntunen, Lampi & Piironen, 2004). The conditions were as

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follows: column, 60 m × 0.32 mm i.d., 0.10 µm, Rtx-5 w/ Integra Guard (crossbond 5% diphenyl-95% dimethyl polysiloxane) capillary column (from Restek); carrier gas, helium (>99.996%) at a constant flow of 1.4 mL/min; temperature program, 70 °C (1 min), 60 °C/min to 245 °C (1 min), 3 °C/min to 275 °C (32 min); detector temperature, 300 °C. The fatty acid methyl esters were identified through the retention times by comparison to a standard GLC-63 mixture of fatty acid methyl esters and quantified through the peaks' areas by means of the internal standard method, (C19:0 methyl ester as the internal standard). The content of each fatty acid was determined, and referred per g of solid powder, and the total lipid content was estimated from the total sum of all of the determined fatty acids.

2.6. Release Kinetics of eugenol from powders into food simulants

Four different food simulants were used for the release studies: 3% (w/v) acetic acid (B); 10% (v/v) (A), 20% (v/v) (C) and 50% (v/v) (D1) ethanoic solutions. 0.1 g of each sample was placed into flasks containing 100 mL of each simulant. Release studies were carried out throughout 90 minutes at 25°C, using a spectrophotometric method, at 282 nm of wavelength, to determine the released E at different times (1, 3, 5, 7, 10, 15, 20, 30 and 90 minutes), as described by Pramod, Ansari & Ali (2013). The assay was performed in triplicate. The results were expressed as the amount of eugenol per gram of dried powder (mg /g powder). The amount of eugenol released at each time (Mt) was fitted to Peleg's model (Peleg, 1988), described by **Equation 2**, and parameters k_1 (inverse of the initial release rate) and k_2 (inverse of the asymptotic value) were obtained. The delivered amount at equilibrium (M_∞) was deduced from k_2 (**Equation 3**). M_0=0, since no E was initially present in the simulants.

$$M_t = M_0 + \frac{t}{k_1 + k_2 t}$$
Equation 2

$$M_\infty = \frac{1}{k_2}$$
Equation 3

2.7. Antioxidant activity

The antioxidant capacity of the powders was determined by using a 2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) reduction method, following the methodology described by Brand-Williams, Cuvelier & Berset (1995). In this method, the stable free radical, DPPH·, which absorbs at 515 nm, disappears after accepting an electron or hydrogen radical from the antioxidant compounds (Özcelik, Lee & Min, 2003). For this purpose, 0.1 g of powder was dispersed in 100 mL of methanol under stirring for 30 minutes. Different volumes of the dispersions were reacted with a 0.06 mM methanol solution of DPPH·.

The absorbance measurements were taken in triplicate at 25°C after 2 hours, when the reaction (absorbance at 515 nm) reached a plateau by using a spectrophotometer (Thermo-Scientific Spectrophotometer Evolution 201 UV-visible). The DPPH· concentration (mM) in the reaction medium was determined from the calibration curve (**Equation 4**) determined by linear regression (R2 = 0.997). The reduction percentage in DPPH· concentration (%DPPH·$_{rem}$) was calculated using **Equation 5**.

$$Abs_{515nm} = 11.793 \cdot [DPPH^\bullet]$$
Equation 4

$$\%[DPPH]_{rem} = \frac{[DPPH^\bullet]_{t=2h}}{[DPPH^\bullet]_{t=0}} \cdot 100$$
Equation 5

where, [DPPH·]$_{t=2h}$ is the concentration of DPPH· at the equilibrium time and [DPPH·]$_{t=0}$ is the initial concentration. From these values, the parameter EC$_{50}$ (the antioxidant concentration required to reduce the initial [DPPH] concentration to 50%: efficient concentration) was determined through the relationship between the %[DPPH·]$_{rem}$ and the mass ratio of powder to DPPH· (mg powder/mg DPPH). Thus, a low value of EC$_{50}$ is related to a higher antioxidant activity of the analysed sample. The antioxidant activity of the pure eugenol was also determined, using the same method.

2.8. Antimicrobial activity

The antimicrobial effectiveness of powders was evaluated by using an in vitro method adapted from Sánchez-González, González-Martínez, Chiralt & Cháfer (2010), and Cano, Cháfer, Chiralt & González-Martínez (2015). Two bacteria, *Listeria innocua* as Gram+ and *Escherichia coli* as Gram-, were used. The bacteria were regenerated by transferring a loopful into 10 mL of TSB and incubating at 37°C overnight. A 10 µL aliquot from the overnight culture was again transferred to 10 mL of tryptic soy broth (TSB) and grown at 37°C to the end of the exponential growth phase. These cultures were diluted to approximately 5.0–6.0 log CFU/mL. Different amounts of each powder were added to test tubes containing 9 mL of TSB and 1 mL of the inoculum; the final E concentration (from the powder) ranged from 0.5 to 1.75 g Eugenol/L. The mixtures were vortexed and kept under stirring for 30 min at the optimum growth temperature. A bacterial suspension sample of 1 mL was serially diluted in water peptone and 1 mL of the dilutions were inoculated into Petri dishes in duplicate by using Violet Red Bilis agar (Sharlab S.A., Barcelona, Spain) in the case of *E. coli* cultures, and Palcam Agar Base (Sharlab S.A., Barcelona, Spain) supplemented with Palcam Selective Supplement (Sharlab S.A., Barcelona, Spain) in the case of *L. innocua*. Plate samples were incubated for 24 or 48 h at 37 °C for Listeria or E. coli, respectively, and then counted as CFU/mL.

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2.9. Statistical analysis

Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.) for Windows 5.1 (Manugistic Corp. Rockville, MD, USA) was used to carry out a statistical analysis of data through an analysis of variance (ANOVA). Fisher´s least significance difference (LSD) was used at the 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Emulsion characterization

The particle size distribution of the different formulations can be observed in **Figure 1**. All of the dispersions exhibited multimodal distributions with droplet diameters ranging from 0.1 to 100 μm, except the EOA-WP formulation, which exhibited monomodal behaviour. The E-WP based emulsion had particle size distributions between 0.5 and 100 μm, with the main peak at 10 μm. Similar particles sizes have been found by other authors using whey protein-oil-water emulsions homogenized at similar homogenization pressures (100 MPa) (Hebishy, Zamora, Buffa, Blasco-Moreno & Trujillo, 2017). However, the E-LE based emulsion showed the formation of smaller particles (main peak around 0.1 μm), which indicates the formation of lecithin nanoliposomes, although some bigger particles appeared at around 100 μm, which may be due to the formation of either some lamellar forms or some clusters of maltodextrins as a result of their high concentration in the emulsion. In fact, Gibis, Thellmann, Thongkaew & Weis (2014) obtained monomodal distributions (0.1 μm peak) using lecithin and different plant extracts submitted to higher homogenization pressures (155 MPa). The incorporation of oleic acid notably reduced ($p<0.05$) the particle sizes and promoted narrower particle size distributions in systems with WP, although the curve shifted to higher size values in the LE liposome systems, probably due to the OA interactions with the lipid associations of lecithin, which modify the aggregation number of the lipid association structure. The amphiphilic nature of OA favors the emulsification process and the reduction in the droplet particle size, as previously reported by other authors (Vargas, Albors, Chiralt & González-Martínez, 2009), but the OA interactions with other polar lipids, such as lecithin compounds, affect the final lipid rearrangement both on the lipid-water interface or in the lipid association of micellar structures. OA interactions with WP can also imply differences in the amphiphilic layer adsorbed on the lipid (E) droplets, even provoking the displacement of protein from the interface due to the lower surface tension of the surfactant.

The incorporation of CH to WP or LE systems provoked particle flocculation, especially in the WP systems, as revealed by the shift of the particle sizes towards multimodal distributions with bigger particles (peaks near 100 μm, in both WP and LE systems). This effect could be due to the emulsion depletion associated with the exclusion effect (McClements, 2005). However, the positive charge of the polymer could also provoke an entanglement effect on the negatively charged droplets revealed by their zeta potential (**Table 2**). In lecithin-based formulations, attractive interactions between the positively-charged chitosan and the negatively-charged groups of phospholipids (PK_a values of anionic phosphatidic groups are typically around 1.5; Israelachvili, 1992; Ogawa, Decker & McClements et al., 2004), at an emulsion pH of nearly 4 (Table 2), were expected, leading to the formation of larger particles. In fact, the zeta potential

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(**Table 2**) of CH-free EOA-LE system was -45.7 mV at the emulsion's natural pH (**Table 2**), as reported by Gibis, Vogt & Weiss (2012) at pH 3.8. This charge was inverted when CH was incorporated, leading to a zeta potential of +61.5 mV.

In WP systems, electrostatic interactions between whey protein and chitosan were not expected, since the isoelectric point (IP) of whey protein is around 4-5 (Giese, 1994) and, although the zeta potential of the WP emulsions at their natural pH (nearly 6) was negative, the incorporation of a CH solution decreased the pH to about 4 and the zeta potential became positive. The CH-free WP systems also exhibited positive zeta potential at this pH (4) (**Table 2**), according to the IP of the protein. Moreover, at pH values close to the WP isoelectric point, the solubility of protein is limited which can lead to emulsion flocculation by solvent effect (McClements, 2005). Therefore, the use of chitosan promoted a greater polydispersity in the particle size distributions and the formation of bigger particles, associated with different aggregation phenomena, especially in WP-based dispersions. Light microscopy images in **Figure 1** show the different droplet sizes in the emulsions, coherent with the distributions commented on above. The flocculated particles and large lipid droplets can be clearly observed, reflecting the occurrence of coalescence, associated with the emulsion destabilisation provoked by CH addition in both WP and LE systems.
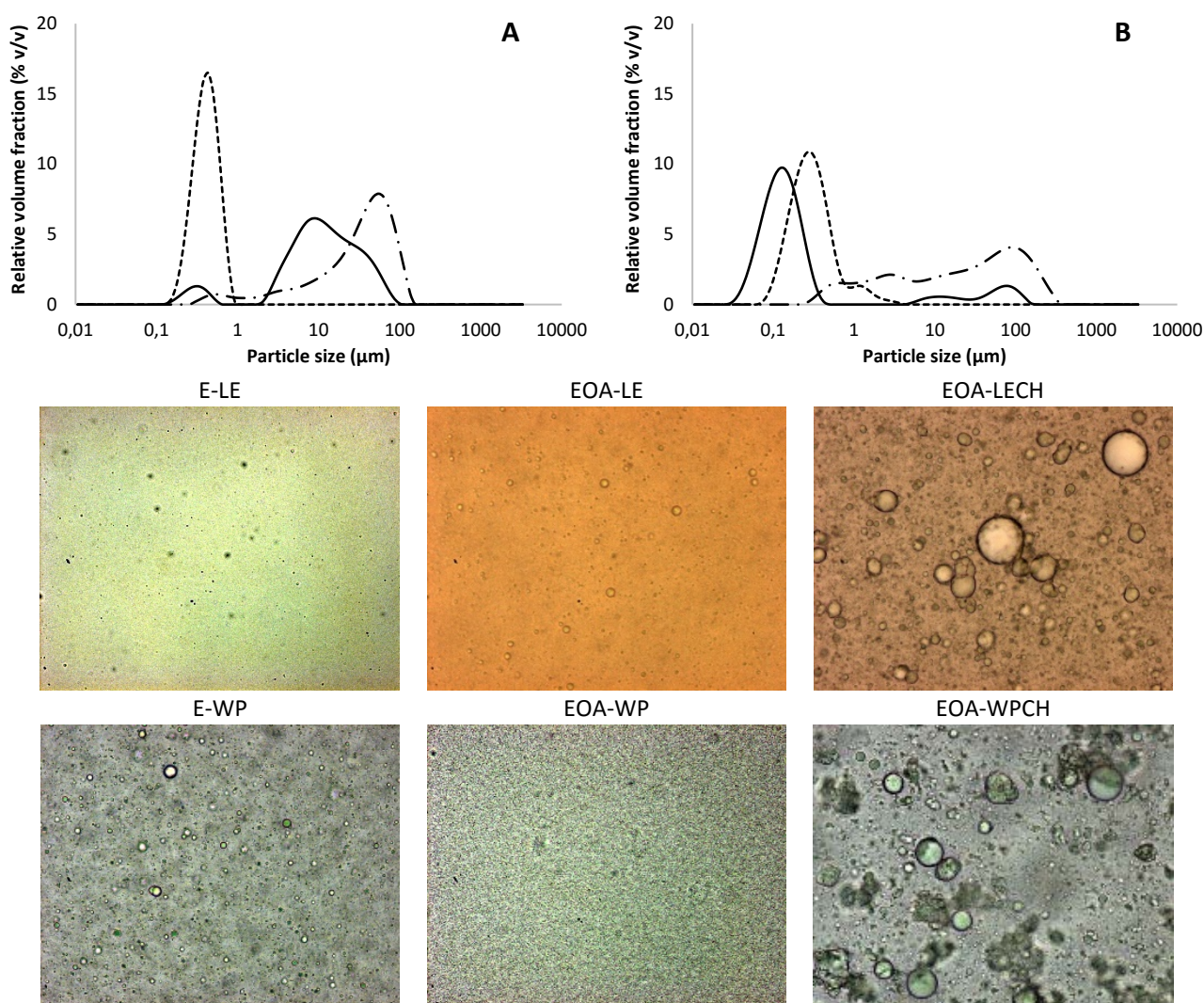
Figure 1. Typical particle size distributions of eugenol (E) emulsions using whey protein (A) or lecithin (B) as wall materials, incorporating or not oleic acid (OA) and chitosan (CH) (——E; - - - EOA; — . — . EOA-CH). Light microscopy images (x40) of the different emulsions are also shown.

All of the emulsions exhibited pseudoplastic rheological behaviour. **Table 2** also shows the values of the rheological parameters (flow index: n and consistency index: K) and the apparent viscosity of the different emulsions at 50 s^{-1}. All of the CH-free dispersions almost presented Newtonian behaviour (n close to 1), whereas the incorporation of CH promoted a more pseudoplastic pattern. The incorporation of OA did not produce significant changes in the rheological behaviour or viscosity of the dispersions (**Table 2**) ($p>0.05$). An increase in the emulsion's consistency could be expected in line with the higher volume fraction of the dispersed phase, but the reduction in the particle size promoted by OA or its efficient incorporation into WP micelles or LE-liposomes could mitigate this effect. The dispersions turned more shear-thinning and viscous with the addition of CH, in agreement with the formation of large aggregates whose coarse

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structure would be more sensitive (e.g. disaggregation or deformation of large droplets) to the shear rate.

Table 2. Zeta potential, pH, rheological parameters and apparent viscosity at 50 s^{-1} of the different emulsions.

| Formulation	pH	Z-Potencial (mV)*	Z-Potencial (mV) pH=4	K (Pa·s^n)*·100	n	η50 (mPa·s)
E-WP	6.6	-28.9 (1.5)[b]	+19. (0.4)[a]	5.81 (0.18)[a]	0.993 (0.004)[cd]	56.2 (1.9)[a]
EOA-WP	5.7	-32.5 (0.9)[a]	+12 (2)[a]	6.95 (0.15)[a]	0.995 (0.005)[d]	68.0 (1.3)[ab]
EOA-WPCH	4.1	+40 (3)[c]	+40 (3)[b]	41.65 (11.13)[b]	0.836 (0.018)[b]	219 (46)[c]
E-LE	4.3	-46.6 (0.5)[d]	-	73 (0.9)[a]	0.988 (0.005)[cd]	69 (8)[ab]
EOA-LE	4.4	-45.7 (0.5)[d]	-	90 (10)[a]	0.982 (0.004)[c]	84 (9)[b]
EOA-LECH	4.0	+61.5 (0.9)[e]	-	69 (17)[c]	0.726 (0.020)[a]	214 (11)[c]

[abcd] Different letters in the same column indicate significant differences among formulations (p<0.05).
*at the pH of the emulsion

3.2. Powder encapsulate characterization

The morphology of the particles obtained by spray-drying depends on several factors, such as the drying kinetics and the liquid phase composition. Al the beginning of the drying process, the surface of the atomized droplets begins to dry, forming a crust, then bubble nucleation occurs and bubbles grow, enlarge and burst out through the surface until most of the internal moisture has evaporated (Rosenberg, Kopelman & Talmon, 1990). Since the drying conditions were constant for every formulation, the different morphology observed for dried particles (**Figure 2**) would only be affected by their composition. Factors, such as the film forming properties of the drying carrier and the interactions of the wall material with the active substance (eugenol), could affect the morphology of the solid particles. Eugenol encapsulated in LE or WP (no OA or CH present) produced particles with irregular surfaces over a wide range of sizes, typical of low-loaded capsules. Surface irregularities suggest the swelling of the rubbery particle surface in the initial drying stages due to the internal pressure of the water vapour, which collapses when the internal vapour pressure decreases as a result of the lower volume of the internal lipid. Ré (1998) associated these particle shapes with a slow surface film formation during drying in the atomized droplet. Similar morphological characteristics were found by Carneiro, Tonon, Grosso & Hubinger (2013), Soottitantawat et al. (2005) and Tonon, Grosso & Hubinger (2011). In contrast, when the formulations contained OA as eugenol carrier, the particles became more spherical in shape with fewer surface irregularities, due to the presence of OA inside the particles (0.134 mass fraction in the powder, against 0.06 of E), which limits the further shrinkage

of the non-lipid shell. As expected, bigger particles and large agglomerates were observed in systems containing chitosan. No notable differences in the particle appearance were observed when using LE or WP as wall materials, although in the WP systems a slightly higher degree of particle aggregation could be appreciated in the powder, thus indicating greater attractive forces between dry particles.
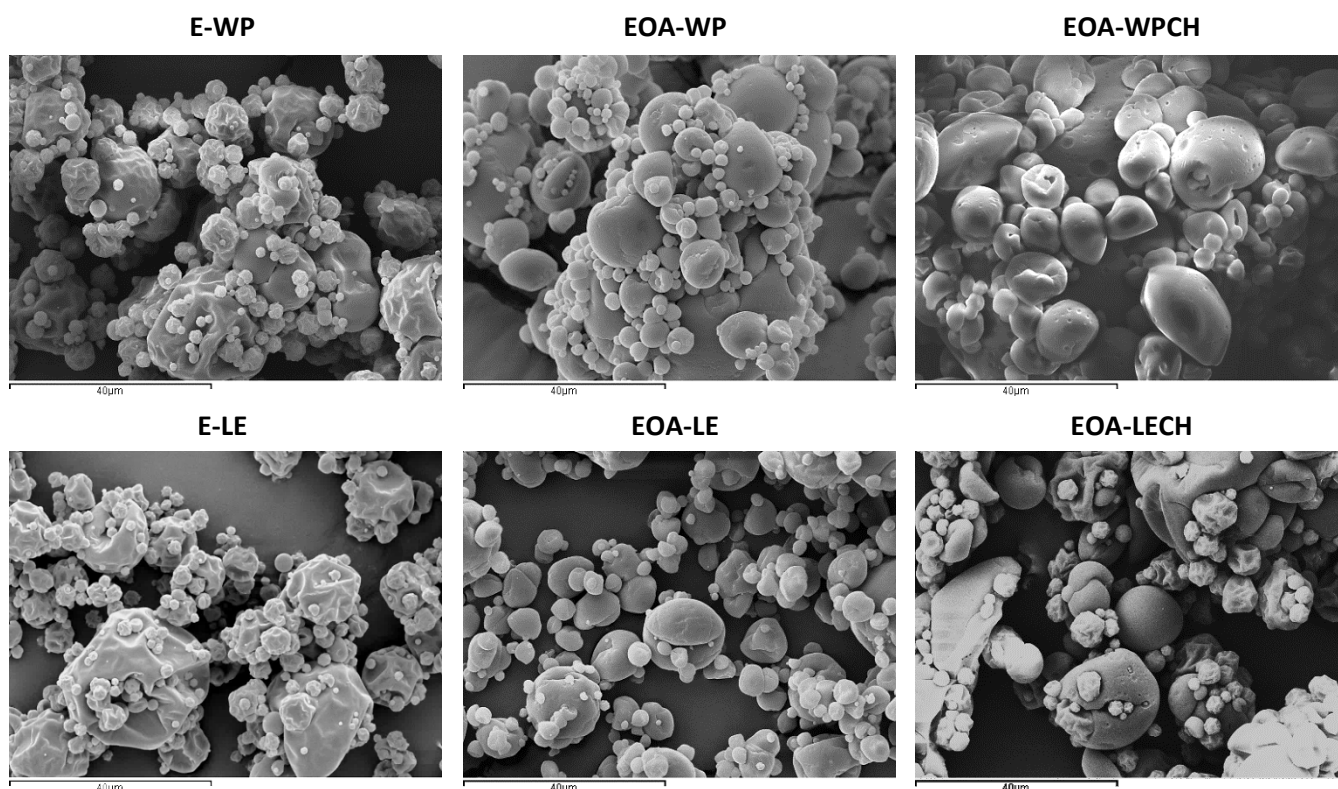
Figure 2. SEM micrographs and of the different encapsulated eugenol particles (x1500).

The particle size distributions of the different powder formulations can be observed in **Figure 3**. As can be observed, all chitosan-free formulations exhibited very similar, "almost" monomodal, distributions with a mean particle diameter of around 15 μm, regardless of the wall material (WP or LE). A very small shoulder, corresponding to the finest particles (around 0.5 μm), was also observed in both systems. This is particularly interesting in the case of powders, as the population of smaller particles can penetrate the spaces between the larger ones, thus giving rise to powders with higher apparent density during the powder compaction (Carneiro et al., 2013).

The addition of chitosan shifted the particle size distributions towards larger sizes, exhibiting a multimodal pattern, as was also observed in SEM micrographs. Two main populations, showing peak values of 20 and 170 μm for EOA-WPCH and of 30 and 150 μm for EOA-LECH formulations, were observed. The high viscosity and larger particles of these emulsions could limit the jet disruption in smaller droplets during the spray drying

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process. Several authors (Masters, 1991; McClements, 2005) reported that the atomized droplet size depends directly on the emulsion viscosity at a constant atomization speed. The greater the emulsion viscosity, the larger the droplets formed during atomization, and consequently, the larger the particles in the obtained powder.
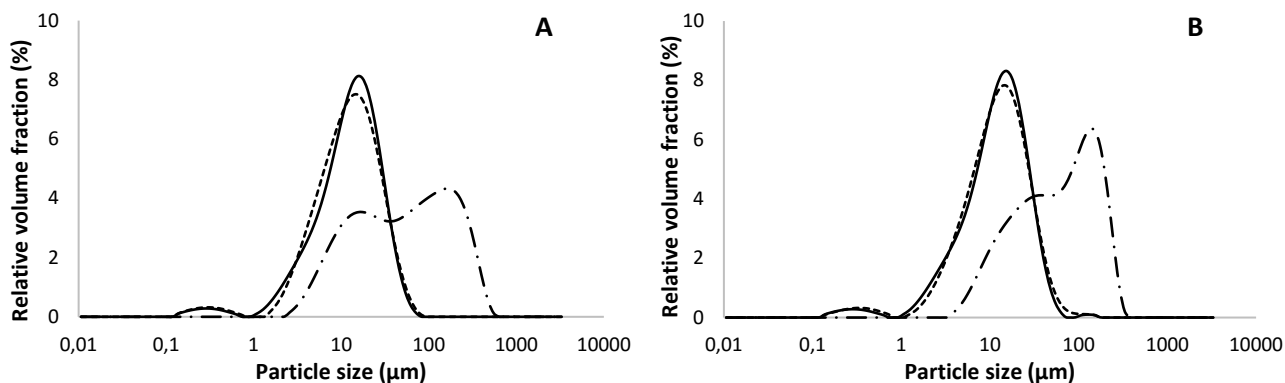
Figure 3. Typical particle size distributions of powders prepared using whey protein (A) and lecithin (B) as wall materials, incorporating or not oleic acid and chitosan (⎯ E; ₋₋₋ EOA; ₋.⎯. EOA-CH).

Table 3 shows the moisture content and onset and peak temperatures from the TGA analysis of powder encapsulates. The different formulations exhibited moisture contents ranging between 1.7 to 3 g water/100 g dry powder.

The TGA and DTGA curves of the different samples are shown in **Figure 4**. Two different weight loss steps were observed below 250°C. The first one, below about 100°C, must be attributed to the evaporation of the powder's water content (He et al., 2016), while the small peaks (shoulders) in DGTA curves, at about 200-250°C, reflect the evaporation of eugenol (254°C boiling point) from the powder. The main thermodegradation step corresponds to the thermal degradation of the major compounds in the matrix (maltodextrins: 0.8-0.9 mass fraction in the powder), affected by their interactions with the other minor, non-volatile components (WP, LE, OA or CH). In **Figure 4**, the thermal degradation behaviour of pure components was also shown to facilitate the analysis of the component interaction effect on the thermal degradation of the different encapsulates. In the case of maltodextrins, the peak temperature of the maximum degradation rate is at 286°C, whereas in the powder encapsulates, these temperatures were about 283 and 260°C, respectively, for matrices containing WP and LE. No practical effect of WP was observed on the thermal behaviour of maltodextrin matrices, whereas LE notably decreased the thermal stability of the powder. The WP powders degraded at a higher temperature than the LE, due to the proteins' contribution to the increase in

the mean molecular weight of the maltodextrin matrix and the subsequent enhancement of the cohesive forces through the entanglement effect of the protein chains. In contrast, the LE lipids reduce the thermal stability of the matrix, probably due to the plasticizing effect of the lipids, which reduce the attractive forces between the carbohydrate chains, weakening the network cohesion. OA or CH slightly affected the thermal degradation temperature of the WP powders, but the only significance is to be found in the small decrease provoked by OA, which could also be associated with its plasticizing effect in the matrix (Fabra, Talens & Chiralt, 2010). In the LE based systems, the CH or OA incorporation did not have a significant effect on the thermal stability of the material.

As regards the loss of eugenol from encapsulant matrix, associated to its thermal release, the behaviour of the powders was remarkably different. A clear peak (maximum evaporation rate) was observed at about 200°C for samples containing CH, whereas the compound thermal release appeared at about 240°C in WP systems with and without OA (respective shoulders in DGTA curves). In LE powders, the E thermal release overlapped with the degradation temperature range of the matrix and no specific E weight loss event was observed in the DGTA. In contrast, for free eugenol submitted to the same thermal test, the maximum evaporation rate occurred at 175°C. These results reflect the different protective effect of the encapsulates when it is a matter of limiting the loss of E from the powder, the LE systems without CH being the most effective at retaining E in the matrix. The incorporation of CH into the encapsulates implied less protection against the evaporation of E, which suggests a poor inclusion of the compound in the particle core, but probably a greater presence on the particle surface. Additionally, the thermal stability of the encapsulated materials allows for their incorporation into different products submitted to thermal processing, involving temperatures lower than 175°C or 200°C, for powders with or without CH, preventing the potential thermal release of eugenol, as previously observed by other authors (He et al., 2016; Hundre, Karthik & Anandharamakrishnan, 2015).

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Table 3. Moisture content and thermal degradation temperatures (onset values, T_{onset} and value at maximum degradation rate, T_{max}) of the particles using whey protein and lecithin as wall materials, incorporating or not oleic acid and chitosan. Mean values and (standard deviation).

Formulation	% MC (dry weight basis)	T_{max} (°C)	T_{onset} (°C)
E-WP	3.01 (0.02)[d]	283.7 (0.8)[c]	221.4 (0.7)[ab]
EOA-WP	2.74 (0.09)[c]	282 (2)[b]	222 (2)[ab]
EOA-WPCH	1.50 (0.06)[b]	284.2 (1.0)[c]	227 (13)[b]
E-LE	2.84 (0.05)[c]	259.8 (0.6)[a]	214.8 (0,9)[a]
EOA-LE	1.77 (0.03)[a]	258.6 (0.3)[a]	224.1 (1.6)[ab]
EOA-LECH	2.97 (0.14)[c]	259.4 (0.3)[a]	226.4 (0.5)[b]

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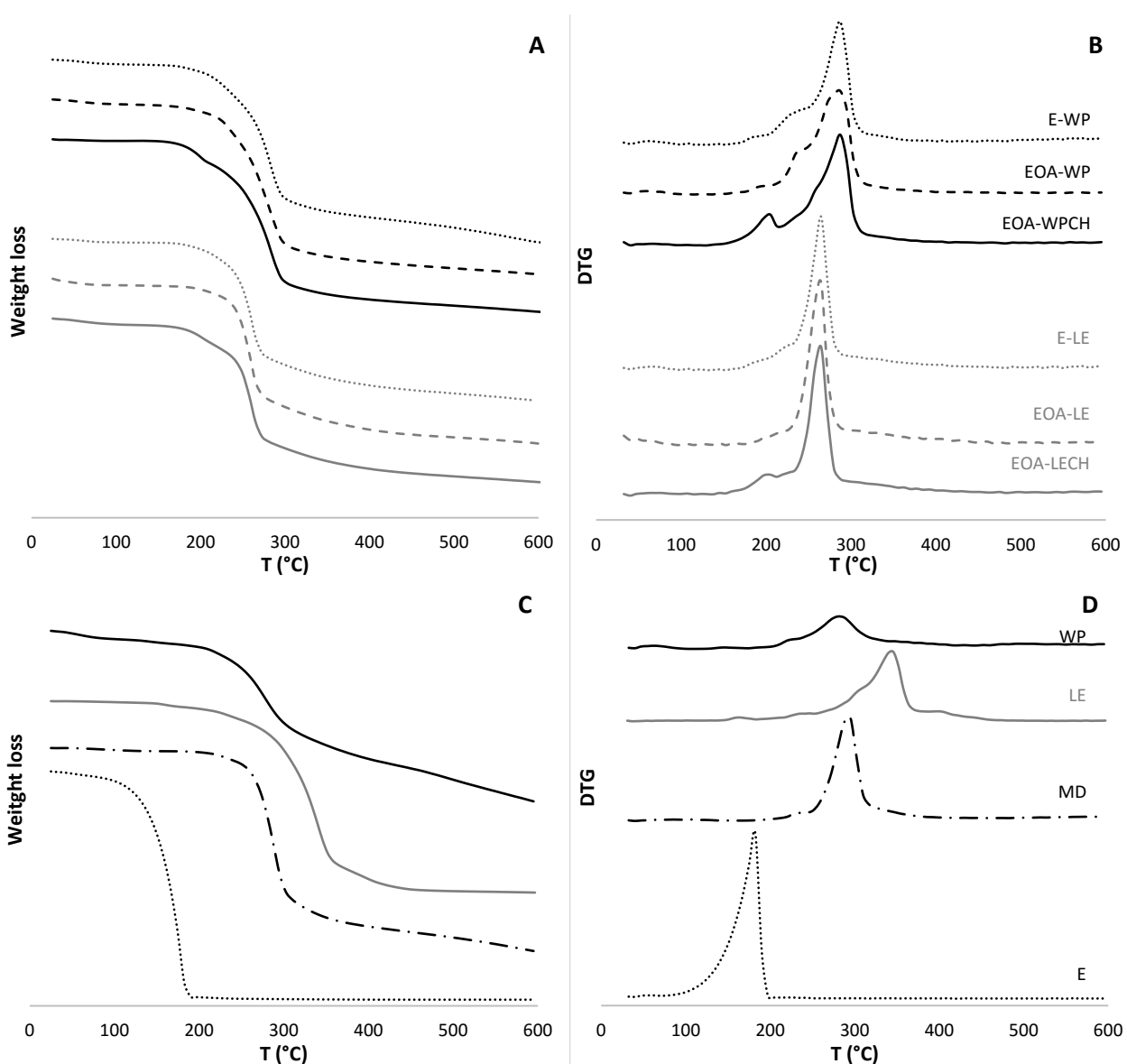

Figure 4. Weight loss curves (A and C) and derivative curves (B and D) from TGA analysis from 25°C to 600°C of encapsulated samples (A and B) and different pure components (C and D).

In order to know the encapsulation efficiency (EE) of eugenol, its total content in each powder sample was determined and compared with the theoretical incorporated amount (**Table 1**). **Table 5** shows the different EE values for each sample. EE was very high (around 94-99%) when using only WP or LE as wall materials. These values were higher than those found by other authors encapsulating eugenol with solid lipid nanoparticles (SLN) (Cortes-Rojas et al., 2014) or β-cyclodextrin grafted chitosan complexes (Phunpee et al., 2016) and similar to those found by Seo, Min & Choi (2010) using β-cyclodextrin. The incorporation of OA into the emulsions slightly decreased the EE values, only being significant in the EOA-WP samples. On the other hand, the use of chitosan remarkably reduced ($p<0.05$) the EE values to 22 and 46% for WP and LE systems, respectively. The presence of free OA containing eugenol on the surface of the dried particles (**Table 5**) could explain the lower EE values, especially in the samples containing CH. To verify this hypothesis, the total and surface lipid contents were analysed, as described in section 2.5, through the analyses of fatty acids present in the whole particles (total lipid content: TLC) and on their surface (surface lipid content: SLC).

Table 4 shows the TLC and SLC, and the specific content of the different fatty acids found in each spray-dried particle. Particles from LE systems contained a higher fat content and different fatty acid profiles (both in TLC and SLC) than those from WP systems, in line with the lecithin composition. As expected, the TLC values were always higher than the SLC, indicating the predominant location of lipids in the internal core of the particles, with a partial retention at surface level. WP based samples without OA had a very low lipid content, coming from the raw WP powder, and about 40 % were on the particle surface. In the rest of the samples, the TLC quantified through the total fatty acids was, as expected, lower than the theoretical lipid load in the powders (OA and/or LE), although in samples containing OA the values were very close, since this component was present at a higher ratio than LE (**Table 1**). However, the percentage of the SLC with respect to TLC greatly differed from powder to powder. Whereas only 4.5 and 3.5 % was present on the particle surfaces of OA loaded WP and LE systems, respectively, powders with CH contained 65 and 54 % of the total lipids on the particle surface, in WP and LE systems, respectively. These results indicate that the majority of the lipids carrying eugenol were entrapped in the internal core of the dried particles, except when CH was incorporated into the emulsions, where a very high ratio of lipids was present on the particle surface. This could be attributed to the greater instability of the flocculated emulsions, which promotes the oil droplet coalescence during the spray drying process, reaching larger sizes than the atomized droplets. In this context, the lipid phase was not efficiently entrapped in the core of the dried particles, but extended/adsorbed on their surface, also carrying eugenol to the particle surface, from which it could easily evaporate. This behavior explains the much lower EE values for eugenol in powders containing CH.

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Figure 5 shows the profile of major fatty acids (individual content with respect to the total content) in the whole particles and on their surface, compared with the typical profile of the raw OA component. Powders with OA (EOA-WP, EOA-LE, EOA-WPCH and EOA-LECH) exhibit a very similar profile at internal and surface levels. This was also very close to that of the raw OA, due to its higher mass fraction in the powder. This suggests that there was no notable amount of the LE lipids present on the particle surface and most of the formed liposomes were entrapped in the particle core, carrying most of the incorporated eugenol. In samples without OA, significant differences were observed in the fatty acid profiles of the particle surfaces and whole particles, according to the specific surface adsorption capacity of the different lipids of raw LE or WP products. This was particularly notable in OA-free LE samples, where LE lipid fractions containing more OA were predominantly adsorbed on the particle surface.

It can be assumed that the eugenol carried by the surface lipids quickly evaporates, mainly during the spray drying process, due to the lack of a true encapsulation, decreasing the total content in the powder or the EE. Taking into account the SLC values and considering that most of the surface lipids come from the incorporated OA component, the loss of eugenol during the drying process was estimated from the E:OA ratio in the emulsions. In this sense, around 4 and 3% of the incorporated eugenol would be present on the particle surface in EOA-WP and EOA-LE samples, respectively, whereas 60 and 50% of the incorporated E would be on the particle surface in EOA-WPCH and EOA-LECH formulations. The sum of the encapsulated and surface eugenol was nearly 100 % of the incorporated E in every case, which verifies the hypothesis that only when lipids carrying E exhibited small droplet size in the initial emulsions, was the EE high and a great amount of the compound could be retained in the powder. Therefore, all of the factors contributing to a reduction in droplet size and emulsion stability will favour the EE in the spray drying processes. The less efficient retention of E in powders containing CH during its thermal release, deduced from the TGA analyses, is also coherent with the higher degree of instability of the emulsions containing CH.
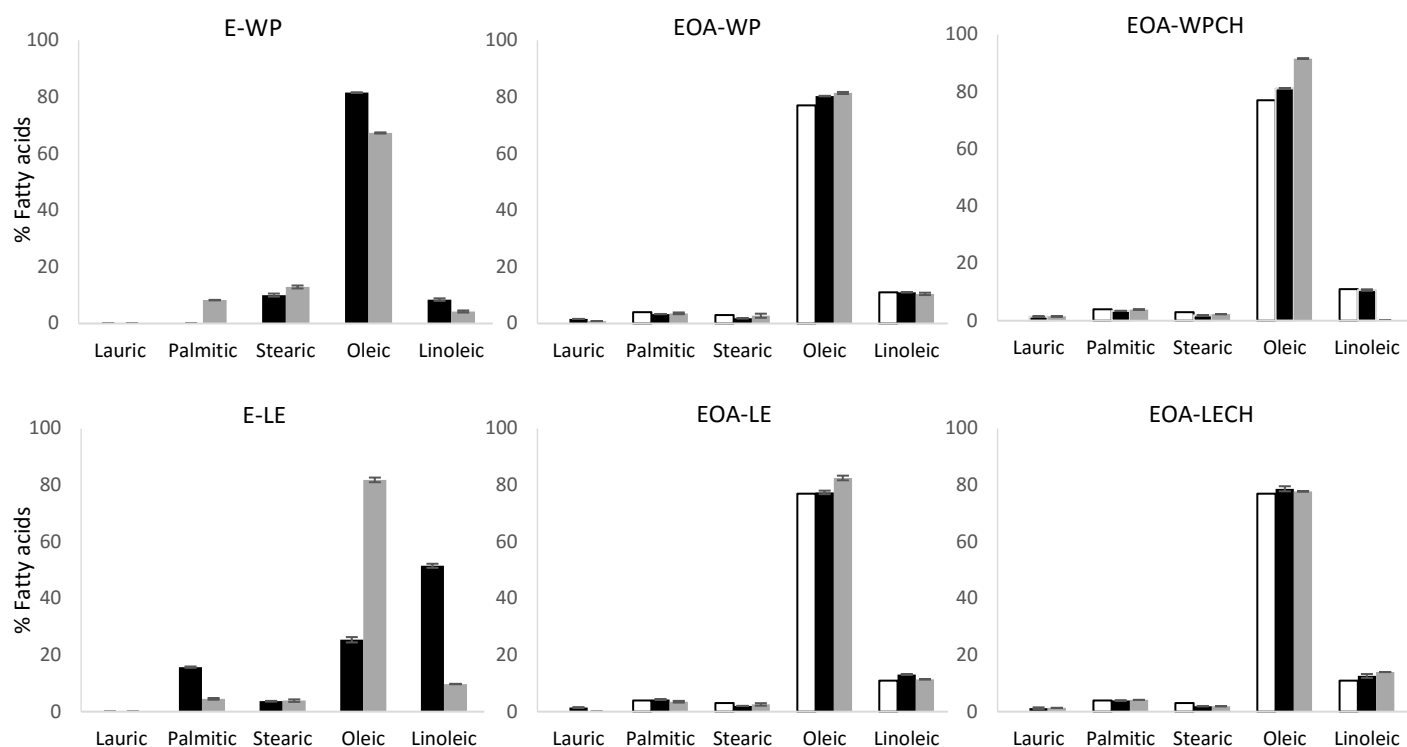
Figure 5. Profile of major fatty acids (individual content with respect to the total content) found in the different sample formulations, in the total extracted lipid fraction (black) and in the lipid extracted from the capsule surface (grey). White bars correspond to the profile of fatty acids in the incorporated oleic acid.

Table 4. Lipid profile of the different formulations expressed as total (TLC) and superficial lipid content (SLC), in mg fatty acid/g powder. Total fatty acids is the sum of the different lipids in TLC and SLC. % SLC is the total amount of fatty acids present on the surface with respect to the total lipid content. Mean values (and standard deviation).

Fatty acids	E-WP TLC	E-WP SLC	EOA-WP TLC	EOA-WP SLC	EOA-WPCH TLC	EOA-WPCH SLC
Lauric C12:0	n.d.*	n.d.	1.95 (0.04)	0.046 (0.014)	1.79 (0.05)	1.18 (0.18)
Miristic C14:0	n.d.	n.d.	0.592 (0.014)	0.013 (0.018)	0.583 (0.095)	0.41 (0.03)
Palmitic C16:0	n.d.	0.0130 (0.0004)	4.03 (0.10)	0.19 (0.04)	4.186 (0.07)	3.096 (0.108)
Margaric C17:0	n.d.	0.0115 (0.0007)	n.d.	0.0099 (0.0002)	n.d.	0.032 (0.002)
Estearic C18:0	0.0386 (0.0007)	0.0202 (0.0004)	2.214 (0.009)	0.1443 (0.0018)	2.290 (0.009)	1.81 (0.04)
Oleic C18:1 (n9)	0.31 (0.02)	0.106 (0.002)	97.14 (1.09)	4.4 (1.2)	98.8 (1.3)	73 (3)
Vaccenic C18:1 (n7)	n.d.	n.d.	0.57 (0.03)	0.031 (0.009)	0.3 (0.5)	0.009 (0.012)
Linoleic C18:2	0.032 (0.004)	0.0067 (0.0007)	13.34 (0.24)	0.57 (0.18)	13.06 (0.14)	0.1171 (0.0004)
Linolenic C18:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arachidic C20:0	n.d.	n.d.	0.258 (0.002)	n.d.	0.12 (0.17)	n.d.
Gondoic C20:1	n.d.	n.d.	0.674 (0.010)	n.d.	0.55 (0.03)	n.d.
Behenic C22:0	n.d.	n.d.	0.108 (0.004)	n.d.	n.d.	n.d.
Lignoceric C24:0	n.d.	n.d.	0.0917 (0.0010)	n.d.	n.d.	n.d.
Total fatty acids (mg/g)	**0.38 (0.03)**	**0.157 (0.003)**	**121.0 (1.5)**	**5.5 (1.5)**	**121.7 (1.5)**	**79 (3)**
% SLC	40.85		4.51		65.10	

Fatty acids	E-LE TLC	E-LE SLC	EOA-LE TLC	EOA-LE SLC	EOA-LECH TLC	EOA-LECH SLC
Lauric C12:0	n.d.	n.d.	1.8 (0.2)	n.d.	1.5 (0.4)	0.869 (0.015)
Miristic C14:0	n.d.	n.d.	0.58 (0.05)	n.d.	0.51 (0.08)	0.304 (0.004)
Palmitic C16:0	1.36 (0.12)	0.051 (0.005)	5.4 (0.5)	0.15 (0.03)	4.7 (0.2)	2.71 (0.03)
Margaric C17:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Estearic C18:0	0.317 (0.013)	0.044 (0.006)	2.56 (0.09)	0.1117 (0.0008)	2.325 (0.007)	1.251 (0.010)
Oleic C18:1 (n9)	2.19 (0.09)	0.927 (0.014)	98 (4)	3.6 (0.6)	95 (3)	50.8 (0.8)
Vaccenic C18:1 (n7)	n.d.	n.d.	0.5 (0.4)	n.d.	0.680 (0.017)	0.079 (0.003)
Linoleic C18:2	4.5 (0.4)	0.111 (0.004)	16.6 (1.0)	0.50 (0.09)	15.2 (1.4)	9.14 (0.16)
Linolenic C18:3	0.32 (0.04)	n.d.	0.28 (0.03)	n.d.	0.24 (0.02)	0.16571 (0.00009)
Arachidic C20:0	n.d.	n.d.	0.257 (0.013)	n.d.	n.d.	n.d.
Gondoic C20:1	n.d.	n.d.	0.530 (0.015)	n.d.	0.54 (0.03)	n.d.
Behenic C22:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lignoceric C24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total fatty acids (mg/g)	**8.6 (0.7)**	**1.13 (0.03)**	**127 (6)**	**4.4 (0.7)**	**121 (5)**	**65.36 (1.05)**
% SLC	13.11		3.45		54.08	

* n.d.: Non-detected.

Table 5. Theoretical and extractable eugenol content (mg/g dried powder), encapsulation efficiency and eugenol content on the particle surface (SLC) of different encapsulates. Antioxidant activity in terms of EC_{50} values of particles encapsulating eugenol was also shown referred per mass unit of powder and mass unit of the encapsulated eugenol. Mean values and (standard deviation).

Formulation	Theoretical eugenol (mg/g)	Extractable eugenol (mg/g)	Encapsulation efficiency (%)	Eugenol in SLC[1] (mg/g powder)	% Eugenol in SLC[2]	EC_{50} (mg powder /mg DPPH)	EC_{50} (mg eugenol/ mg DPPH)
E-WP	65.22	62 (2)	95 (3)[d]	-	-	1.64 (0.05)[a]	0.107 (0.003)[a]
EOA-WP	56.60	49 (3)	87 (5)[c]	2.3 (0.6)[a]	4.13 (1.14)[a]	2.12 (0.15)[a]	0.120 (0.008)[a]
EOA-WPCH	56.43	12.6 (1.7)	22 (3)[a]	34.0 (1.4)[c]	60 (3)[c]	7.8 (0.9)[c]	0.44 (0.05)[c]
E-LE	65.22	64 (4)	98 (6)[d]	-	-	1.78 (0.14)[a]	0.116 (0.009)[a]
EOA-LE	56.60	53 (3)	95 (5)[d]	1.9 (0.5)[a]	3.31 (0.02)[a]	1.709 (0.015)[a]	0.1114 (0.0010)[a]
EOA-LECH	56.43	26.2 (1.9)	46 (3)[b]	28.0 (0.4)[b]	49.6 (0.08)[b]	4.6 (0.3)[b]	0.260 (0.019)[b]
Pure E							0.092 (0.002)[a]

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

(1) Estimated from surface lipid content values (SLC) and nominal E:OA ratio in the powders (3:7).

(2) Percentage of the incorporated eugenol not encapsulated in the samples, deduced from the eugenol in SLC and the theoretical eugenol in the powder.

3.3. Release Kinetics

As concerns the release kinetics of the encapsulated E from the different formulations into food simulants of differing polarity, **Figure 6** shows the amount of released eugenol (expressed as mg eugenol/g powder) as a function of time for LE powders. Very similar behaviour was observed for WP-based formulations (data not shown). The experimental data (points) and curves predicted (lines) by the fitted Peleg model are shown. **Table 6** shows the parameters of the Peleg model, where k_1 is the kinetic constant of the model (min/(mg E/g powder)) related to the mass transfer rate at the beginning of the process and k_2 is related to the asymptotic value of the curve or amount released at equilibrium ($1/k_2 = M_\infty$, mg eugenol/g powder). The maximum release ratio (M_∞/M_0) was estimated with respect to the total methanol extracted eugenol (M_0) in each powder. A good fit of the model was obtained in every case, as reflected by the R^2 values in **Table 6**.
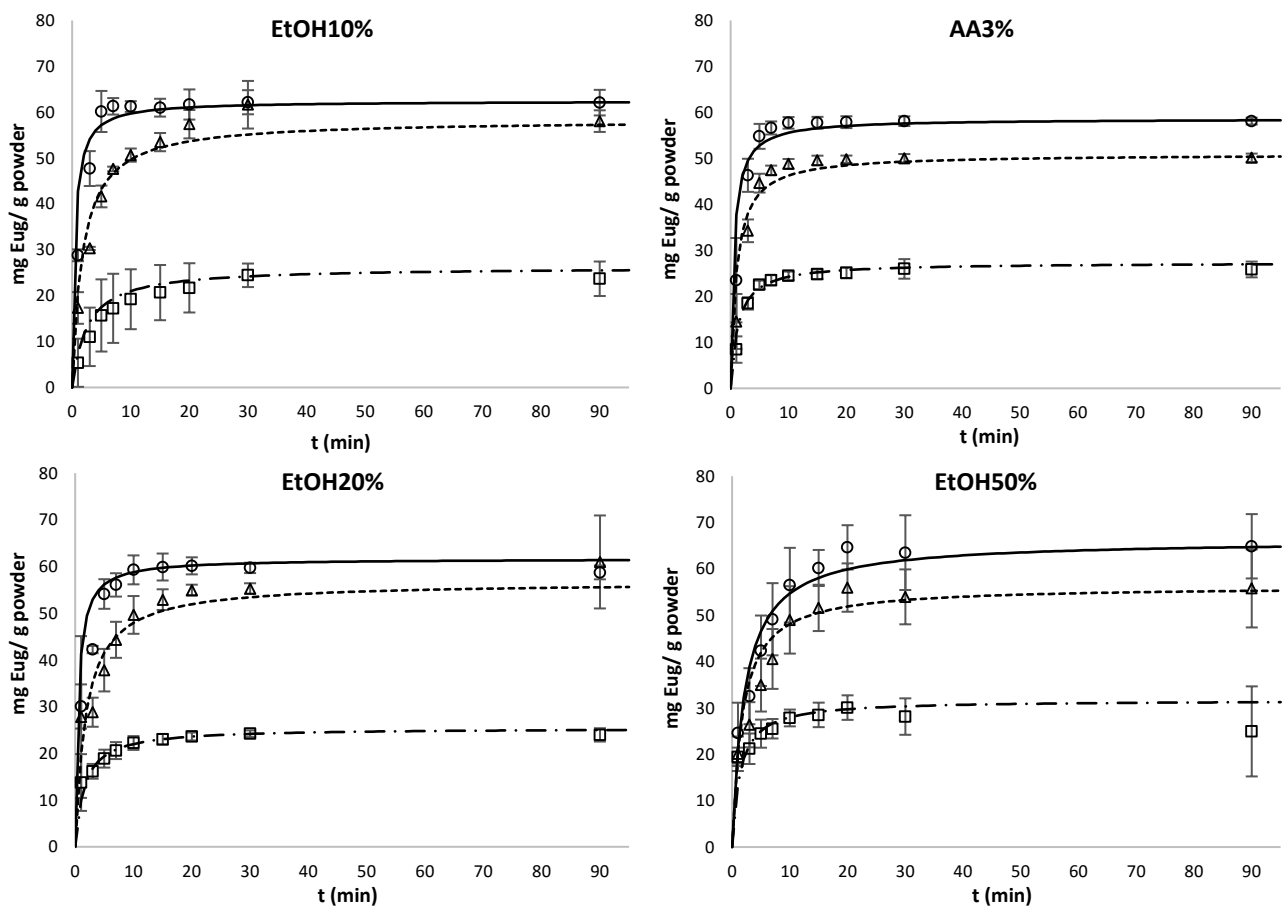
Figure 6. Eugenol release from encapsulated eugenol using lecithin in 4 different aqueous food simulants: 3% acetic acid, 10% ethanol, 20% ethanol and 50% ethanol. Experimental data (O E-LE; Δ EOA-LE; ☐ EOA-LECH) and values predicted by Peleg's model (⎯ E-LE; ‐‐‐ EOA-LE; ⎯·⎯· EOA-LECH).

Table 6. Maximum eugenol release ratio (M_∞/M_0)* and parameters of Peleg´s model for the different encapsulated systems in the different food simulants: inverse of the initial release rate (k_1) (min/(mg eugenol /g powder)) and equilibrium value, M_∞ ($1/k_2$) (mg eugenol/g powder).

Formulation	Parameters	SIMULANTS			
		EtOH10%	EtOH20%	EtOH50%	AA3%
E-WP	k_1	0.0065 (0.0009)[a,1]	0.0053 (0.0010)[a,1]	0.039 (0.009)[a,1]	0.0041 (0.0019)[a,1]
	$M_\infty=1/k_2$	60.3 (0.3)[d,1]	60.2 (0.8)[d,1]	68.5 (1.7)[d,2]	60.22 (1.06)[d,1]
	*M_∞/M_0 (%)	97.1 (1.7)[c,1]	97.1 (1.3)[c,1]	100 (0)[a,1]	97.1 (1.7)[bcd,1]
	R^2	≥ 0.999	≥ 0.999	≥ 0.999	≥ 0.999
EOA-WP	k_1	0.0109 (0.0013)[a,1]	0,012870[a,1]	0.02888 (0.00102)[a,1]	0.018 (0.013)[ab,1]
	$M_\infty=1/k_2$	47.4 (0.7)[c,1]	48.78 (0.15)[c,1]	56.6 (4.4)[c,2]	59 (5.3)[c,2]
	*M_∞/M_0 (%)	89.4 (1.3)[b,1]	92.0 (0.3)[b,1]	100 (0)[a,2]	100 (0)[c,2]
	R^2	≥ 0.998	≥ 0.998	≥ 0.999	≥ 0.998
EOA-WPCH	k_1	0.06 (0.03)[bc,1]	0.09 (0.02)[bc,1]	0.159 (0.006)[b,2]	0.09 (0.03)[c,1]
	$M_\infty=1/k_2$	10.38 (0.02)[a,1]	14.5 (0.3)[a,12]	18.6 (1.8)[a,2]	16.7 (0.3)[a,2]
	*M_∞/M_0 (%)	74.16 (0.14)[a,1]	100 (0)[c,2]	100 (0)[a,2]	100 (0)[cd,2]
	R^2	≥ 0.958	≥ 0.945	≥ 0.925	≥ 0.998
E-LE	k_1	0.007 (0.005)[a,1]	0.0080 (0.0013)[a,1]	0.032 (0.008)[a,1]	0.009 (0.003)[a,1]
	$M_\infty=1/k_2$	62.6 (3.1)[d,12]	61.7 (1.4)[d,12]	66.7 (6.8)[d,2]	58.6 (0.8)[d,1]
	*M_∞/M_0 (%)	99.2 (1.4)[c,1]	97.8 (0.3)[c,1]	98,9 (2.0)[a,1]	96.1 (1.3)[bc,1]
	R^2	≥ 0.999	≥ 0.998	≥ 0.997	≥ 0.998
EOA-LE	k_1	0.030 (0.002)[ab,1]	0.032 (0.008)[ab,1]	0.03 (0.03)[a,1]	0.020 (0.006)[ab,1]
	$M_\infty=1/k_2$	58.4 (0.7)[d,2]	56.7 (0.6)[d,2]	56.5 (4.6)[c,2]	51 (0.7)[c,1]
	*M_∞/M_0 (%)	100 (0)[c,2]	99.4 (0.9)[c,2]	97 (5)[a,2]	89.4 (1.2)[a,1]
	R^2	≥ 0.997	≥ 0.993	≥ 0.958	≥ 0.996
EOA-LECH	k_1	0.095 (0.008)[c,1]	0.061 (0.014)[c,1]	0.042 (0.013)[a,1]	0.047 (0.011)[b,1]
	$M_\infty=1/k_2$	26.3 (2.3)[b,1]	25.4 (0.8)[b,1]	31.8 (2.4)[b,2]	27.4 (1.3)[b,12]
	*M_∞/M_0 (%)	91 (8)[b,12]	88 (3)[a,1]	100 (0)[a,3]	94.4 (4.4)[b,2]
	R^2	≥ 0.986	≥ 0.999	≥ 0.997	≥ 0.994

* Related to the initial eugenol amount determined by methanol extraction.
[abcd] Different letters in the same column indicate significant differences among formulations (p<0.05).
[1234] Different numbers in the same line indicate significant differences among food simulants (p<0.05).

All of the powders released practically their total content of E at equilibrium (M_∞) (M_∞/M_0 ranged between 84-100%) in the tested aqueous simulants, as shown in **Table 6**. This suggests that the release of the active agent was not notably affected by pH or polarity of the food simulant. No significant differences in the M_∞/M_0 values were found (p>0.05) due to the use of different simulants or wall materials. As concerns the eugenol release rate (inverse of K_1), no significant effect of the wall material (WP or LE) (p>0.05) was observed, but the release rate significantly decreased when incorporating OA and

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CH, obtaining the slowest rates in formulations containing chitosan (greatest k_1 values). This CH effect could be attributed to the lower content of encapsulated eugenol in these formulations, which implies a minor driving force for the release. In general, the different simulants were found to have no significant effect on the K_1 values of a determined sample, exhibiting a burst eugenol release throughout the first 20 min. The behaviour observed is coherent with the high water affinity/solubility of the shell material, which favours the fast disruption of the capsules with the subsequent release of the E content.

3.4. Antioxidant and antibacterial activity

All of the samples exhibited antioxidant and antimicrobial activities to some extent, depending on the eugenol content in each powder. The antioxidant activity was evaluated in terms of EC_{50} values. This parameter indicates the amount of sample needed to halve the DPPH radical amount. Thus, the lower the EC_{50} values, the greater the antioxidant activity. In **Table 5**, the EC_{50} values of the different formulations, together with the pure eugenol, are shown. Pure eugenol showed the lowest EC_{50} value, 0.22 mol eugenol/mol DPPH, which was similar to that previously reported by Bortolomeazzi, Verardo, Liessi & Callea (2010) and Brand-Williams et al. (2005). The EC_{50} values of CH-free powders (expressed in terms of moles of eugenol in the powder per mol DPPH) were in the range of the pure component. These results reflected the fact that the antioxidant activity of eugenol was efficiently preserved during the drying process when using lecithin or whey protein as wall materials, with or without OA as carrier agent. However, powders with CH exhibited higher EC_{50} values (lower antioxidant activity), referred to their E content, which could be due to the partial oxidation of the compound retained in the external zone of the particles (surface lipids).

The antimicrobial activity of the samples was evaluated against one Gram – bacteria (*E. coli*) and one Gram + (*L. innocua*). Powders with CH did not exhibit antibacterial effect at any concentration tested, which may be explained by their low eugenol content, which did not exceed the MIC of either bacterium in any case. In **Figure 7**, the bacterial growth inhibition of the CH-free powders as a function of the powder concentration (mg powder/L) can be observed. No significant differences were found between WP and LE systems and, therefore, the mean values for a determined powder concentration are shown in **Figure 7** for powders with and without OA. As expected, the CH-free samples exhibited a dose-dependent antimicrobial activity against both bacteria. Formulations were more effective against *E. coli* than against *L. innocua,* in agreement with that previously reported by Gaysinsky, Davidson, Bruce & Weiss (2005) for eugenol encapsulated in surfactant micelles.

In the case of *E. coli*, OA-free powders exhibited the most marked antibacterial effect, due to their greater eugenol load (**Table 5**). A complete growth inhibition (bactericidal effect) was obtained with 15 mg/mL, which corresponds to 1 g eugenol/L. This value agrees with the MIC found by other authors (Dhara & Tripathi, 2013; Pei, Zhou, Ji & Xu, 2009; Shah, Davidson & Zhong, 2013) for *E. coli* (around 1-1.6 g eugenol/L). The incorporation of OA into formulations significantly decreased the antibacterial action, only provoking nearly a 3 Log CFU reduction when using 30 mg powder/mL. As concerns *L. innocua*, both powders (with and without OA) had a similar antibacterial effect, despite the different eugenol content, provoking a total inhibition at about 25 mg powder/mL (equivalent to about 1.2 or 1.6 g eugenol/L, respectively for powder with and without OA). This could be attributed to the antimicrobial activity reported for some unsaturated fatty acids (such as oleic acid) against Gram positive bacteria (Zheng et al. 2005).
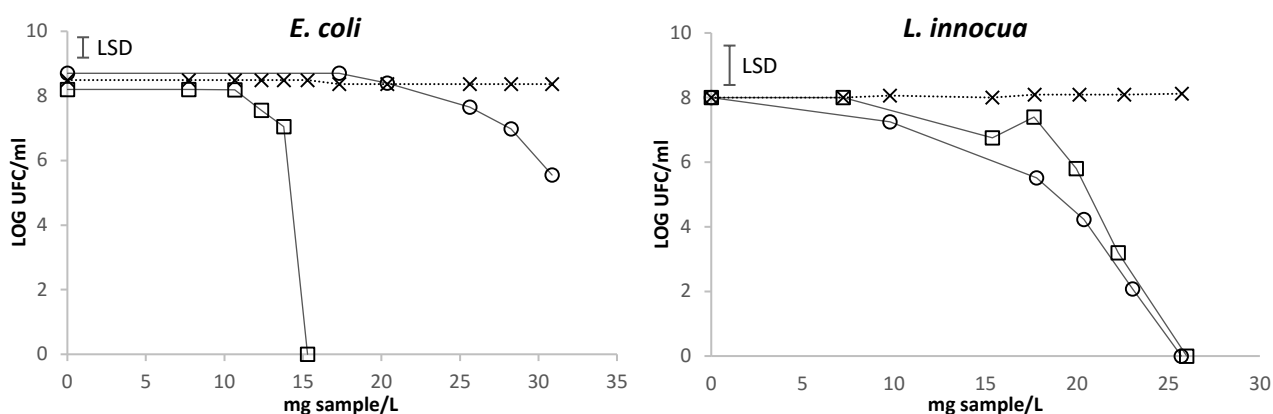
Figure 7. Antimicrobial activity of encapsulated eugenol particles (□ E; ○ EOA; × Control) against *E.Coli* and *L.innocua*. Mean values and 95% LSD intervals.

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4. CONCLUSIONS

The encapsulation efficiency (EE) of eugenol in spray-dried powders containing whey protein or lecithin as wall materials and maltodextrin as drying coadjuvant was very high (95-98 %), while the incorporation of oleic acid (OA) as eugenol carrier or chitosan (CH) to the liquid formulations did not improve EE. CH provoked emulsion destabilization which had a very negative effect on the EE. All of the encapsulating powders exhibited antioxidant activity, coherent with their respective eugenol content, in line with the fast, complete release of eugenol in aqueous systems. The antibacterial effect of the powders against *E. coli* was also coherent with the eugenol content of the powders, but an additional positive effect of OA was detected in the powder's antilisterial action. All of the encapsulating powders presented small particles and a high affinity /solubility in aqueous systems of differing polarity and pH, which allows for a relatively fast, total release of the active compound. The thermal release of eugenol was also inhibited in the powders (mainly in those which were CH-free), which would allow for their use in dry thermal processes, such as the preparation of an active master batch of thermoplastic polymers. Their incorporation as an ingredient or in separate sachets in foodstuffs would permit them to be better preserved against oxidative or microbial decay, thus extending their shelf-life.

Acknowledgements

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Eugenol incorporation into thermoprocessed starch films, using different encapsulating materials

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ABSTRACT

The encapsulation of the volatile active compounds, such as eugenol, before their incorporation into packaging films may limit their losses and modulate their release kinetics. The effect of the incorporation of eugenol (encapsulated and non-encapsulated) on the mechanical, barrier and antioxidant properties of compression moulded corn starch films was studied as well as the release kinetics. Free or spray dried encapsulated eugenol using whey protein or lecithin as wall materials using maltodextrin as drying coadjuvant and oleic acid as a carrying agent, was used. Thermo-processing of the films provoked important eugenol losses (80-65%) which were minimised when microcapsules contained OA. In the films with free eugenol, the compound retention was enhanced through its complexation with starch polymers. Eugenol microcapsules provoked a heterogeneous microstructure in the film matrices, which induced notable changes in the film properties. Films became less resistant and stretchable, with lower water sorption capacity and improved water vapour and oxygen barrier properties. However, a highly variable oxygen permeability was obtained when microcapsules contained oleic due to its very heterogeneous distribution in the matrix. Encapsulates promoted a faster eugenol release and a greater release ratio at equilibrium, especially when they contained more water-soluble components. Acid medium always promoted the release ratio, associated with the progressive partial hydrolysis of the starch matrix.

Key words: optical and mechanical properties, antioxidant activity, controlled release, WVP, OP.

1. INTRODUCTION

Over the last few decades, substantial efforts have focused on the use of natural antioxidants for the development of novel health-promoting ingredients with potential food applications. Eugenol is a natural phenolic compound, present in clove (90%) and cinnamon (60%) essential oils, with antimicrobial and antioxidant properties (Chatterjee & Bhattacharjee, 2013; Devi et al. 2010; Kamatou et al., 2012; Ogata et al., 2000; Woranuch & Yoksan, 2012). It has been widely used in pharmaceuticals, cosmetics and food products and, recently, in active packaging for food preservation. Several authors (Fernandes et al., 2018; Narayanan & Ramana, 2013; Sanla-Ead et al., 2012; Valencia-Sullca et al., 2016) reported the antimicrobial activity of different biopolymer films containing eugenol. This natural compound, incorporated into the film formulation, confers antimicrobial and antioxidant effects to bioactive films for food preservation, thus contributing to the prevention of some deteriorative reactions. However, due to its limited water solubility, volatility and oxidation sensitivity, it is difficult to handle when incorporated into the film master batch (Choi et al., 2009).

The encapsulation of the active compound in different wall-systems may limit its losses during film processing while also helping to modulate the release kinetics of the active from the packaging material (Valencia-Sullca et al., 2016). Microencapsulation by spray-drying is the most commonly used technique at industrial level. This has been used to encapsulate thermosensitive compounds, since it is a fast process in which the temperature reached is relatively low (Barrow et al., 2013). The wall materials in dried powder may also help to improve the retention of volatile compounds in the final encapsulates (Jafari et al., 2007).

The choice of the wall-materials used in the encapsulation of non-polar actives is determinant in the active load of spray-dried microparticles. They greatly affect the stability of the initial aqueous emulsion of the target compound, which is a decisive factor in the encapsulation efficiency during the spray drying process. Likewise, cell wall material may contribute greatly to limiting the subsequent losses of the active from the dried powder (Botrel et al., 2014). Whey protein (WP) is a biopolymer capable of successfully trapping hydrophobic compounds and is widely used to stabilize emulsions (Bae & Lee, 2008). Proteins are often combined with carbohydrates, such as maltodextrins (MD), as they improve drying properties, increasing oxidative stability due to the formation of a dry crust around the droplets during drying (Kagami et al., 2003). The chemical structure of lecithins (LE) allows the formation of liposomes. These have the ability to act effectively as encapsulants of active compounds. Lecithin in combination with MD produced a good encapsulation system stabilizing oil-in-water emulsions (Taylor et al., 2005). On the other hand, several studies demonstrated that the presence of oleic acid (OA) in the initial emulsion enhanced the retention of the active compound due to its carrying effect (Perdones et al., 2014, Pokharkar et al., 2011).

Corn starch is of great potential use for obtaining biodegradable packaging materials, due to its easy availability and low cost. Many studies (Averous et al., 2000; Jiménez et al., 2012a; Muller et al., 2017; Park et al., 2002) have shown the possibility of obtaining starch-based films by thermoprocessing (extrusion, compression moulding, …) under de-structuring and plasticization conditions. Nevertheless, the incorporation of different active compounds, encapsulated or not, into starch films could have an impact on their physical properties which may affect their functionality as a packaging material (Cano et al., 2016; Sánchez-González et al., 2015).

The aim of this study was to evaluate the physical and antioxidant properties of compression moulded corn starch based films containing microencapsulated eugenol, and the release kinetics of this compound into different food simulants, as a function of capsule composition: different wall-materials (whey protein isolate-maltodextrin or lecithin-maltodextrin) and the use of oleic acid as a carrier agent.

2. MATERIALS AND METHODS

2.1. Raw materials

To obtain the microencapsulated eugenol, Lecithin (LE) lipoid S45 (Batch 574510, Lipoid, Germany), Whey Protein Isolate (WP) 90S (Batch 131848) and maltodextrin (MD) Kyrosan E18 (Batch 02157372) from Haarla (Tampere, Finland), Eugenol (E) (Batch STBD6235V, Sigma-Aldrich, Madrid, Spain) and purified Oleic acid (OA) (Batch 15C030505, VWR Chemicals Germany) were used.

Corn starch (S) (Batch RMA12, Roquette Laisa SA, Benifaió, Spain) and glycerol (Panreac Química SLU, Castellar del Vallès, Barcelona, Spain) were used to prepare the films.

2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) and Folin-Ciocalteu, from Sigma-Aldrich (Madrid, Spain) and glacial acetic acid, absolute ethanol, methanol, sodium carbonate (Na_2CO_3), magnesium nitrate ($Mg(NO_3)_2$) and phosphorus pentoxide (P_2O_5), provided by Panreac Química SLU (Barcelona, Spain) were used to carry out the different analyses.

2.2. Preparation of spray-drier microcapsules

Microencapsulated eugenol was obtained by spray-drying. Briefly, 43% (w/w) of WP/LE:MD solids (ratio 1:42) and 3% (w/w) of eugenol with and without 7% (w/w) of OA were dissolved. All dispersions were homogenized with a Rotor Stator (Ultra-Turrax T 25 Basic, IKA Werke GmbH and Co. KG, Germany) at 11.000 rpm for 6 minutes, and after they were three times microfluidized with the high pressure homogenizer (Microfluidics M-110Y, Newton, Massachusetts, USA) at 15000 psi pressure. The different encapsulated eugenol powders (E-WP; EOA-WP; E-LE and EOA-LE powders) were obtained by using a spray-drier (Mobile Minor TM spray-dryer, GEA Niro, GEA Process Engineering A/S, Søborg, Denmark) with a rotary atomiser with an inlet air temperature of 180°C. The outlet temperature was kept at 80 ± 2°C by controlling the feed rate.

2.3. Preparation of the films

Compression moulded films were obtained by pre-mixing the starch, microencapsulates and glycerol using the starch:powder:glycerol mass ratio of 1:0.35:0.3. Each mixture was hot-blended in a two-roll mill (model LRM-M-100, Labtech Engineering, Samutprakarn 10280, Thailand) at 160°C and 12 rpm for 10 min until a homogeneous blend was obtained. The pellets obtained were conditioned for 10 days at 25°C and 53% RH by using oversaturated solution of magnesium nitrate. The films were obtained by compression moulding using a hot-plate press (model LP20, Labtech Engineering). Four grams of the pellet were preheated at 150 °C during 2 min in the press plate and then

pressed at 150°C and 50 bar for 2 min, followed by 120 bar for 6 min. After that, a cooling cycle for 3 min was applied. As controls, pure starch and non-encapsulated eugenol films incorporating the same E concentration as in the encapsulated films were also prepared (non-encapsulated eugenol films). To this aim, pure eugenol was added to the blend starch:glycerol (ratio 1:0.3), hot blended in the two-roll mill and submitted to the same processing conditions as the rest of the films. The final composition of the films and nomenclature is shown in **Table 1**. The films were finally conditioned at 25°C and 53% RH for 5-7 days previous further analysis.

Table 1. Composition of the starch films incorporating or not microencapsulated eugenol (g/100 g dry film).

	Nomenclature	S (%)	Glycerol (%)	WP (%)	LE (%)	MD (%)	Eugenol (%)	OA (%)
Films + encapsulated eugenol	**E-WP-S**	60.606	18.182	0.461		19.368	1.383	
	EOA-WP-S	60.606	18.182	0.400		16.810	1.201	2.802
	E-LE-S	60.606	18.182		0.461	19.368	1.383	
	EOA-LE-S	60.606	18.182		0.400	16.810	1.201	2.802
Control films	**E-S**	75.700	22.710				1.590	
	S	76.923	23.077					

2.4. Characterization of the films

2.4.1. Moisture content, solubility and thickness

The water content of the different films previously conditioned at 53% RH and 25 °C was determined by cutting the films in small pieces and placing them into pre-weighed capsules. In order to accelerate the dehydration process, the samples were placed in a vacuum oven (TEM-T vacuum. J.P. Selecta, S.A., Barcelona, Spain) at 60°C for 48 hours and then stored in a desiccator with phosphorus pentoxide until reaching constant weight. Three replicates were considered per formulation. Moisture content in dry basis (g H_2O/100g dry matter) was calculated from the initial and final sample weights.

Regarding the solubility, pieces of dry samples were transferred to a mesh of known weight and distilled water was added at 1:50 ratio (film:distilled water; w/v). Samples were kept for 7 days at 20°C. The meshes with the samples were placed in an oven (J.P. Selecta, S.A., Barcelona, Spain) at 60°C for 48 hours and subsequently, they were transferred to a desiccator with phosphorus pentoxide until constant weight was reached. The assay was performed in triplicate and the results were expressed as soluble film/100g film.

The thickness of the films was measured in sixteen random points of three samples of all formulations conditioned at 25°C and 53% RH by means of a digital electronic micrometer with an accuracy of 0.001 mm (Palmer model COMECTA, Barcelona).

2.4.2. Barrier properties: water vapour and oxygen permeabilities

Water vapour permeability (WVP) was determined gravimetrically using a modification of the ASTM E96-95 gravimetric method (1995), at 25°C and a RH gradient of 53–100%. Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL,Hermelle/s Argenteau, Belgium) were filled with 5 mL of distilled water (100% RH). Three circular samples of each formulation were prepared and the thickness of each sample was measured in six random points with an electronic digital micrometer (Comecta S.A., Barcelona, Spain). Samples were fixed in the cups and were placed in equilibrated desiccators containing saturated solutions of magnesium nitrate (53% of RH) and with a fan on the top of the cup in order to reduce the resistance to water vapour transport. The cups were weighed periodically using an analytical balance (ME36S, Sartorius, Germany; ±0.00001 g) at intervals of 1.5 h for 24 h after the steady state had been reached. The slope of the weight loss versus time was plotted and the WVP was calculated according to Bonilla, Atarés, Vargas and Chiralt (2013).

The oxygen permeability (OP) was determined by following the ASTM Standard Method D3985-05 (2010). Three 50 cm^2 replicates of each formulation were measured by using the Ox-Tran equipment (Model 1/50, Mocon, Minneapolis, USA) at 25°C and 53% of RH. Oxygen permeability was calculated by dividing the oxygen transmission rate (OTR) by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the film thickness.

2.4.3. Tensile properties

The mechanical behavior of the films was analyzed using a Universal Testing Machine (Stable Micro System TA-XT plus, Haslemere, England) according to ASTM standard method D882 (2001). The thickness of eight pre-conditioned film pieces of 25 mm × 100 mm was measured in six random points by means of an electronic digital micrometer (Comecta S.A., Barcelona, Spain) and samples were mounted in the film's extension grip of the equipment and stretched at a rate of 50 mm/min until breaking. Force-distance curves were obtained and transformed into stress-Hencky strain curves. The mechanical behaviour was analysed in terms of elastic modulus (EM), tensile strength (TS) and percentage of elongation at break (%E).

2.4.4. Optical properties

The optical properties (transparency and psychometric coordinates) were determined in triplicate by measuring the reflection spectrum of the samples from 400 to 700 nm of wavelength using a MINOLTA spectrocolorimeter (model CM-3600d, Minolta CO., Tokyo, Japan). The transparency was measured by means of the internal transmittance (Ti), applying the Kubelka-Munk theory of the multiple dispersion of reflection spectrum (Hutchings, 1999) given the reflection spectra of both black and white backgrounds. The CIEL*a*b* colour coordinates were obtained from the reflectance of an infinitely thick layer of the material by considering illuminant D65 and observer 10°. Psychometric coordinates Chroma (C_{ab}*) and hue (h_{ab}*) were also determined (Talón et al., 2017a).

2.4.5. Microstructural analysis

The microstructure of films' cross sections was observed by Field Emission Scanning Electron Microscope (FESEM) (JEOL, model JSM-5410, Japan). The samples were maintained with phosphorus pentoxide for one week to ensure the total dehydration of the films and cryofractured by immersion in liquid nitrogen in order to observe the transversal zones. The pieces of the film were mounted on the sample holder using the double-sided carbon tape. Samples were coated with platinum and observed using an accelerating voltage of 2kV.

2.4.6. Antioxidant activity

The antioxidant activity (AA) was carried out by using the DPPH method (Brand-Williams et al., 1995), based on the reduction of the DPPH˙ radical in an alcoholic solution by a hydrogen-donor antioxidant. In the radical form, this molecule shows absorbance at 515 nm of wavelength, which disappears after accepting an electron or hydrogen radical from antioxidant compounds (Scherer & Godoy, 2009).

For this purpose, around 2 g of films were dispersed in 100 mL of methanol and kept under stirring for 15 days. After that, samples were homogenised by using the Rotor Stator during 1 min, in order to provoke the total release of the antioxidant compound content.

The determination of the antioxidant capacity of the different films was carried out mixing 0.15 to 1.05 mL (0.15 ml volume increments) of different appropriately diluted samples with a methanol solution of DPPH˙, whose concentration provides an absorbance of 0.70 ± 0.02 (Pastor et al., 2013). Samples were kept in dark and capped to avoid the evaporation of methanol for 4 hours, when reaction stability was achieved (Bortolomeazzi et al., 2007) and after that, were measured in a spectrophotometer (ThermoScientific, Evolution 201 Visible UV Spectrophotometer) with a wavelength of

515 nm. The assay was performed in triplicate and the DPPH· concentration (mM) in the reaction medium was calculated from the calibration curve (**equation 1**) determined by linear regression (R^2 = 0.997). The percentage of remaining DPPH· (%DPPH·$_{rem}$) was calculated following **equation 2**.

$$Abs_{515nm} = 11.79378 \cdot [DPPH^\bullet]$$

<div align="right">**Equation 1**</div>

$$\%[DPPH]_{rem} = \frac{[DPPH^\bullet]_{t=4h}}{[DPPH^\bullet]_{t=0}} \cdot 100$$

<div align="right">**Equation 2**</div>

Where, the DPPH·$_{t=4h}$ is the concentration of DPPH at 4 hours and DPPH·$_{t=0}$ is the concentration at the start of the reaction.

From these values the parameter EC_{50} (Efficient Concentration) was determined, which indicates the antioxidant concentration necessary to reduce 50% of initial DPPH. This parameter was obtained after representing the %DPPH$_{rem}$ *versus* the mass ratio of film (mg film/mg DPPH or mol eugenol/mol DPPH).

2.4.7. Quantification of the eugenol content

The eugenol content was determined by using the Folin-Ciocalteu (Stojanovic et al., 2012) and through the compound extraction and the UV absorbance measurement. To this aim, around 2 g of films were dispersed in 100 mL of methanol and kept under stirring for 15 days. After that, samples were homogenised by using a Rotor Stator homogenizer (Yellow Line DL 25 Basic, IKA, Janke and Kunkel, Germany) for 1 min and filtered before measurements.

In the Folin-Ciocalteu (F-C) method, 100 µL of the previously diluted samples was mixed with 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of Na_2CO_3 and was completed with water until 10 mL. After 2 h of reaction, the absorbance of the samples was measured at 765 nm, in triplicate, by using a spectrophotometer (ThermoScientific, Evolution 201 VisibleUV Spectrophotometer). Pure eugenol was used as a standard and the results were expressed as mg·L^{-1} of Eugenol.

In the UV quantification method, the absorbance of 2 mL samples was measured spectrophotometrically (ThermoScientific, Evolution 201 Visible UV Spectrophotometer) at 282 nm of wavelength, where the eugenol absorbance is maximum (Pramod et al., 2013). The absorbance measurements obtained were related to the corresponding standard calibration curve in order to obtain the concentration of eugenol.

2.5. Kinetics of eugenol release

Four different liquid food simulants were used to perform the release study from the different films according to the Commission regulation (EU) 10/2011 (14 January 2011): ethanol 10% (v/v) (EtOH10%), acetic acid 3% (w/v) (AA3%), ethanol 20% (v/v) (EtOH20%), which are assigned for aqueous foods, aqueous foods with pH values below 4.5 and alcoholic foods up to 20%, respectively and ethanol 50% (v/v) (EtOH50%), which simulates alcoholic food above 20% and oil in water emulsions.

To carry out the release experiments, pieces of film with were weighted, cut in small pieces and introduced into vials containing 100 mL of each simulant. Release studies were carried out in triplicate during 16 days at 22°C under slight stirring conditions. The amount of eugenol released was quantified by the UV method, taking around 2 ml of each simulant solution. This volume was returned to the respective vial after the spectrophotometric measurement.

2.5.1. Mathematical modelling of eugenol release

Peleg's empirical model (Peleg, 1988), described by **equation 3**, was applied to experimental data in order to predict the release kinetics.

$$M_t = M_0 + \frac{t}{k_1 + k_2 t}$$ **Equation 3**

where M_t is eugenol content at time t, M_0 is the initial eugenol content, k_1 is the kinetic constant of the model that is inversely related to the mass transfer rate at the beginning of the process, and k_2 is a constant of the model that is related to the asymptotic value, which can be related to the equilibrium value ($1/M_\infty$, where M_∞ is the amount of active compound released at equilibrium).

2.6. Statistical analysis

Results were submitted to analysis of variance (ANOVA) using Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.). Fisher's least significant difference (LSD) procedure was used at the 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Microstructure

The FESEM images of the cross section of the films are shown in **Figure 1**. This analysis provides information about the internal microstructure, which it is affected by the spatial organization of the different components of the films and by their interactions stablished during the melt blending and thermo-compression processes. Starch (S) films exhibited the typical continuous and homogeneous appearance in agreement with the development of a compact arrangement of polymer chains (Castillo et al., 2013; Ortega-Toro et al., 2015), which indicates that starch granules were totally disrupted during the thermal processing. The micro-cracks observed are probably due to highly dried state of the sample during observation, which make it very brittle and sensitive to the electron impact. Incorporation of non-encapsulated eugenol hardly affected the internal structure of films, although qualitative differences could be appreciated in the matrix. Eugenol became entrapped in the polymer matrix, which exhibits extensive micro-channels, were a part of eugenol could be retained. Likewise, a part of the compound could be complexed in the helical conformation of amylose, as reported by other authors (Eliasson, 1994) for a high number of lipid compounds, leading to lipid-starch complexes.

The effect of the incorporation of encapsulated eugenol on the internal microstructure of the films depended on the composition of the encapsulating formulation. In E-WP formulations, samples exhibited a heterogeneous appearance, showing a coarser cross section than the control films, which suggests that the polymer chain packing was interrupted by the microencapsulating particles. On the contrary, when using E-LE capsules, a smoother and homogenous matrix was obtained. This suggests that a high degree of compatibility between the E-LE particles (lipid nature) and starch has been developed, probably due to the establishment of specific interactions between components during the thermoprocessing. According to Eliasson (1994), starch and lecithin are also able to form lipid-amylose complexes during a thermal process.

When capsules containing OA were used, the structural discontinuities in the films remarkably increased, giving rise to a greater number of thicker micro-channels and cavities. This effect put in evidence the disruption of the micro-particles during the melt blending process, thus causing the OA release to the blend. No oil droplets were formed due to the high viscosity of the melt and the lack of intense shear forces to promote the oil phase disruption in small droplets. Thus, a part of the OA was retained in the starch matrix into micro-channels or cavities, as reflected by the dark zones on the film structure, associated with the initial locust of the lipid, which was partially evaporated under the vacuum conditions in the microscope, as reported by others authors (Jiménez et al., 2012b). These microstructural features were common for all films containing

capsules with OA, regardless the type of wall-material (WP or LE). These discontinuities affected the mechanical resistance of the films, as commented on below.
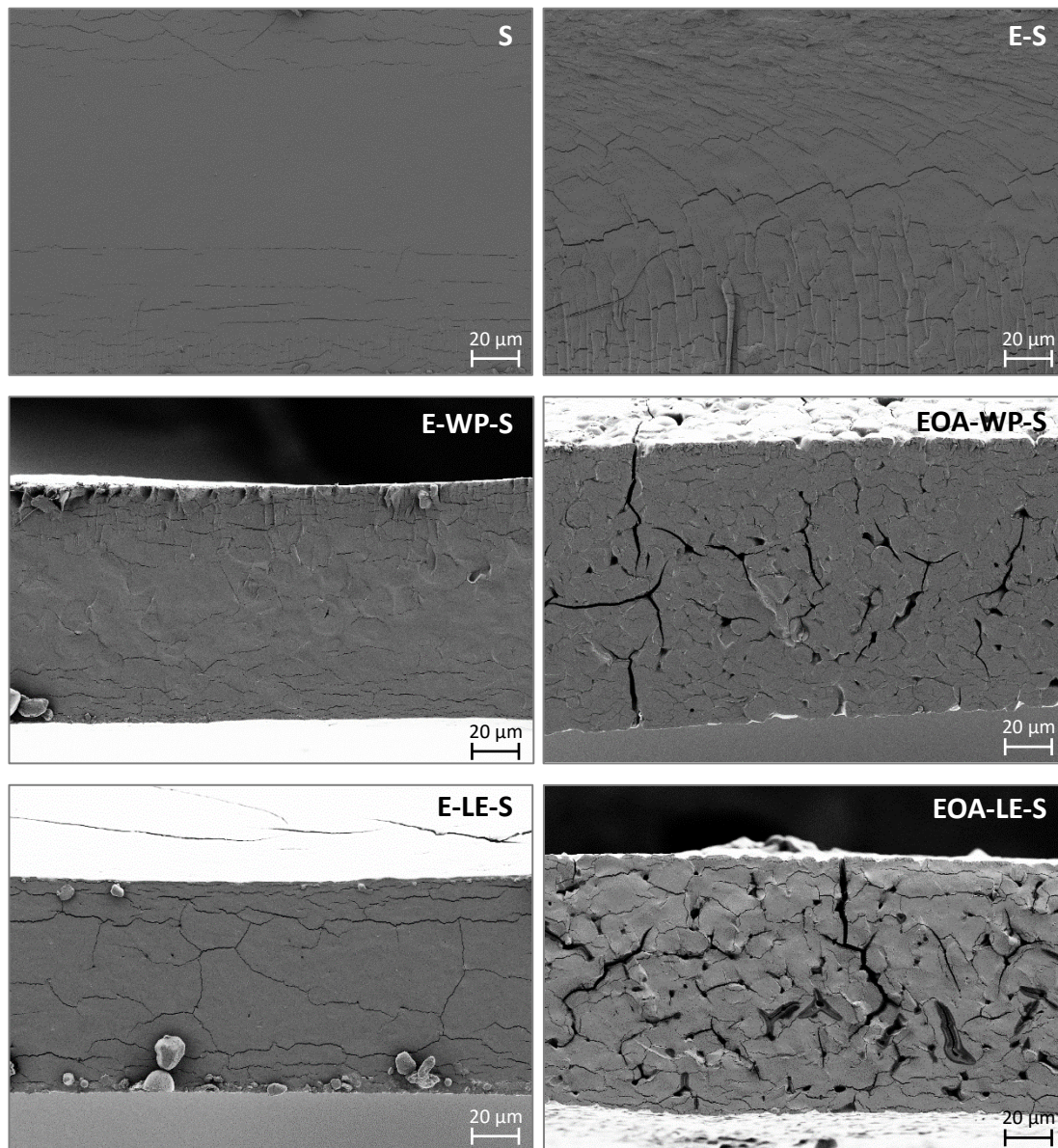
Figure 1. Field emission scanning electron microscope micrographs of the cross-sections of the films.

3.2. Tensile properties

The tensile strength (σ), deformation at break (%E) and elastic modulus (EM) parameters are useful parameters for describing the mechanical behavior of the films, and are closely related to their internal structure. **Figure 2** shows the typical stress–strain curves of the different formulations and the mean values of tensile parameters are shown in **Table 2** for the different films. In **Figure 2,** the effect of the addition of non-encapsulated and microencapsulated eugenol on the mechanical response of the films can be observed.

Starch films showed a similar tensile behaviour to that found by other authors for melt blended and compression moulded starch films (Ortega Toro et al., 2015). Lipid addition generally acted as plasticizers, thus decreasing the EM values and TS and increasing the stretchability (greater %E values, **Table 2**) of the films, since lipids introduce discontinuities in the polymer matrix which contribute to a reduction in the polymer cohesion forces, and thus in the mechanical resistance of the films (Acosta et al., 2015; Jiménez et al., 2012b). Nevertheless, the effect depends on the type of lipid incorporated and its solid state (liquid, solid), particle size and distribution in the polymer matrix (Jiménez et al, 2012b).

The incorporation of non-encapsulated eugenol to the starch matrix caused the reduction of the tensile strength (p<0.05) and EM values, but also the film extensibility (10% reduction), leading to a less resistant, stiff and stretchable films. These results also pointed out to the development of starch-eugenol interactions, mainly through the formation of starch-eugenol complexes, in which the hydrophobic cavity of the helical conformations of amylose and amylopectin chains are involved (Jiménez et al., 2012a; Jiménez et al., 2013; Eliasson, 1994). This effect can promote the amylose crystallization (V-type) enhancing the film brittleness. The formation of these complexes can also be deduced from the microstructural observations (**Figure 1**), where the lipid molecules seemed to be well integrated in the polymer matrix, no exhibiting lipid phase separation.

On the other hand, the incorporation of microencapsulated eugenol to the starch matrix also affected the mechanical behavior of the starch films and it was depended on the capsule formulation. In general, capsule addition turned the film less resistant (lower TS, except in E-LE-S films) and less stretchable (lower %E) due to the presence of discontinuities in the polymer matrix, which contribute to a reduction in the polymer cohesion forces. This behavior was especially noticeable when capsule formulations contained OA, in agreement with the development of a more heterogeneous (less continuous) microstructure as previously commented on. The increase in the TS value for E-LE-S films, in comparison with the control film, also points to the formation of lecithin-starch complexes, thus enhancing the aggregation of the helical lineal segments of the starch polymer chains.

The effect of the capsule incorporation on the film stiffness also depended on the capsule formulation. The EM values increased when using E-WP and E-LE particles but decreased in both cases when they contained OA. This effect was more marked in LE capsules, despite a greater increase in EM was observed for films with OA-free E-LE particles. The high plasticizing effect of the released OA from the capsules (Fabra et al. 2009) provoke a high reduction of the polymer chain interaction forces, while discontinuities introduced in the matrix (**Figure 1**) promote the film breakability.

Table 2. Elasticity modulus (EM), Tensile strength (TS) and percentage of elongation (%E) of films. Mean and (standard deviation).

Formulation	EM	TS (MPa)	%E
S	324 (83)[c]	8.43 (1.07)[d]	30 (9)[d]
E-S	38 (6)[a]	2.32 (0.17)[a]	20 (4)[c]
E-WP-S	398 (36)[d]	7.0 (0.4)[c]	8 (3)[b]
EOA-WP-S	252 (41)[b]	3.6 (0.3)[b]	3.52 (1.14)[a]
E-LE-S	552 (87)[e]	9.7 (0.7)[e]	2.9 (0.7)[a]
EOA-LE-S	87 (53)[a]	1.7 (1.3)[a]	4.6 (1.8)[ab]

[abcde] Different letters in the same column indicate significant difference among formulations (p<0.05).
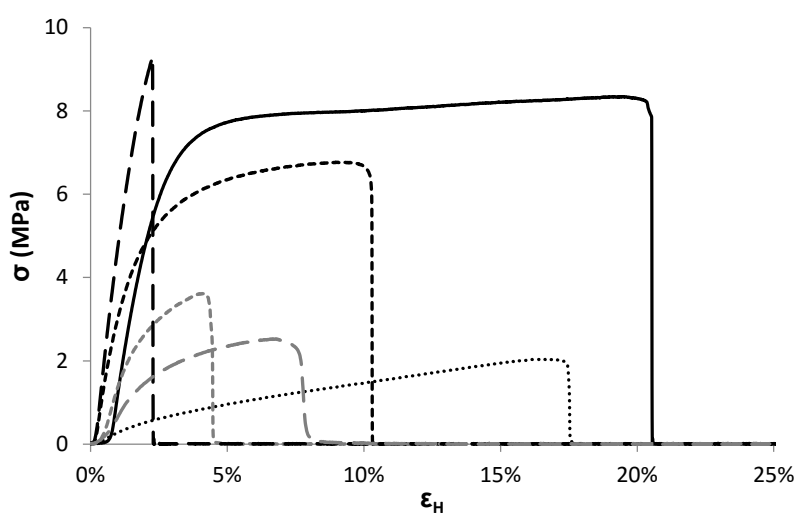
Figure 2. Typical stress-Hencky strain curves of the films: S (—), E-S (···), E-WP-S (- - -), EOA-WP-S (grey - - -), E-LE-S (——) and EOA-LE-S (grey ——).

3.3. Barrier properties, moisture content, solubility and thickness

The oxygen and water vapor permeabilities of the films stored at 53% RH and 25°C is shown in **Table 3**, together with the water content, solubility and thickness of the films. Pure starch films (S) showed similar water and oxygen barrier capacity to those reported by others authors for melt-blended and compression-moulded starch films (Ortega-Toro et al., 2015; Muller et al., 2017). In general, the addition of lipid compounds to hydrophilic film matrices decreases the WVP due to the promotion of hydrophobic nature and increases the OP values due to the higher oxygen solubility in the hydrophobic regions of the matrices (Jiménez et al., 2012b). Films containing non-encapsulated eugenol showed higher values of water content, WVP and OP than pure starch films and films incorporating microencapsulated eugenol. This suggests that the expected effect of the more hydrophobic nature of the compound could be mitigated by its inclusion in the helical formations, thus limiting its active role in the matrix to reduce the matrix water affinity. This implied higher equilibrium water content with the subsequent higher water plasticising effect, which enhanced all diffusion-dependent process, such as water vapour or gas permeation (Dole et al., 2004; Mahieu et al 2015).

When microencapsulated eugenol (E-WP, E-LE, EOA-LE, EOA-WP) was added to the starch films, WVP values and water content decreased in line with the incorporation of more hydrophobic components (Sánchez-González et al., 2011b; Acosta et al., 2016; Ghasemlou et al., 2013). In general, non-significant differences in these values were found among the different formulations. OP values of starch films containing WP or LE based capsules with OA exhibited very high variability, with values ranging from 0.14 to 18.6 and 0.6–23.9 cc·mm·m^{-2}·atm^{-1}·day^{-1}. This can be explained by their very heterogeneous microstructure (**Figure 1**), with large micro-channels filled with oleic acid, where oxygen molecules can easily diffuse. Different orientation and distribution of these channels in each film could lead to very different OP values.

As regards water solubility of the films, the lowest values were obtained for S and E-S films (45 and 64 g soluble film/100g film, respectively). The incorporation of eugenol capsules (E-WP, E-LE, EOA-LE, EOA-WP) resulted in a significant (p <0.05) increase in solubility, coherently with the higher water solubility of the major compound of the capsules (maltodextrins). WP based capsules promote the starch film solubility to a lesser extent than LE based capsules, probably due to the strong interfacial adhesion between proteins and starch, as previously reported by other authors (Azevedo et al. 2017) for corn starch-whey protein blend film obtained by extrusion.

As concerns the film thickness (**Table 3**), net starch films (S) and free-eugenol starch films (E-S) showed the highest values, whereas films containing capsules were thinner, despite the constant conditions applied in the compression molding. This indicates that encapsulates promoted the blend flowability, in line with the plasticizing effect provoked by the low molecular weight compounds (maltodextrin and/or lipids) released

from capsules to the starch matrix. The different flow response of the starch pellets to the thermo-compression gave rise to differences in the final film thickness (Ortega-Toro 2014). In those formulations incorporating more lipid compounds (OA or lecithin), this effect was significantly more marked (p<0.05).

Table 3. Oxygen permeability (OP), water vapor permeability (WVP), moisture content, water solubility and thickness of starch based films equilibrated at 25°C-53% RH. Mean values and (standard deviation).

Formulation	OP (cc·mm/ (m²·atm·day)	WVP (g mm/kPa h m²)	WC (g/100g dry film)	Solubility (g/100g film)	Thickness (μm)
S	0.44 (0.08)[b]	9.9 (0.6)[c]	10.19 (0.19)[c]	45 (9)[a]	204 (28)[d]
E-S	1.05 (0.04)[c]	11.0 (0.2)[d]	11.5 (0.3)[d]	64 (7)[b]	206 (18)[d]
E-WP-S	0.17 (0.06)[a]	6.6 (0.2)[ab]	8.9 (0.6)[a]	85 (8)[c]	166 (15)[c]
EOA-WP-S	0.14 – 18.6	6.2 (0.4)[ab]	8.8 (0.3)[a]	100 (0)[d]	136 (13)[a]
E-LE-S	0.13 (0.02)[a]	6.1 (0.2)[a]	9.0 (0.3)[ab]	94 (5)[cd]	144 (11)[b]
EOA-LE-S	0.6 – 23.9	6.9 (0.5)[b]	9.7 (0.4)[bc]	100 (0)[d]	144 (15)[b]

[abcd] Different letters in the same column indicate significant difference among formulations (p<0.05).

3.4. Optical properties

Optical properties are relevant for polymeric materials useful as a packaging material. Ghanbarzadeh et al. (2010) reported that the film colour can be an important factor in terms of the consumer acceptance of both edible and inedible films. **Table 4** shows the lightness, chrome and hue parameters, together the whiteness index and the internal transmittance of the films at 460 nm wavelength.

The internal transmittance (Ti) of the films, which is related to their transparency, depends on the internal microstructure of the matrix and the distribution of the components that produced more or less light scattering. High values of internal transmittance are associated with highly transparent films. On the contrary, more opaque films correspond with low values of internal transmittance. The Ti values ranged from 0.63 to 0.81 and were dependent on the formulation. Considering these results, all of the films can be considered quite transparent. It is remarkable that the incorporation of encapsulated eugenol promoted reduction in transparency in agreement with the presence of dispersed phases in the matrix (**Figure 1**) which enhanced light scattering, according to the differences in the refractive index of each phase and distribution of their different domains.

When non-encapsulated eugenol was added to pure starch films, the hue values increased due to the yellowness of the eugenol colour while chrome and lightness producing less vivid and dracker colour.

The incorporation of microencapsulated eugenol (E-WP, E-LE, EOA-LE, EOA-WP) in the starch films provoked significant changes ($p < 0.05$) leading to darker (lower L* values) and more saturated reddish colours (lower C* and h* values, respectively) than control films. This was visually perceived as certain brownish colorations. This brownish coloration could be associated with caramelization reactions (in those films with no-proteins) taking place when carbohydrate are heated at high temperature in absence of amino compounds and with the occurrence of Maillard reactions between amino acids derived from proteins and reducing sugars (in the case of E-WP-S films). Both reactions can be produced during the melt blending process and thermocompression of the film material, at high temperatures (Rhim et al., 1998). In fact, this effect was more pronounced in E-WP-S films, as can be deduced from their lowest Whiteness Index (WI) values shown in **Table 4**.

Table 4. Lightness (L*), hue (h_{ab}*), chroma (C_{ab}*), whiteness index (WI) and internal transmittance (T_i) at 460 nm of wavelength values of the film. Average values and standard deviations in brackets.

Formulation	L*	C_{ab}*	h_{ab}*	WI	T_i (460 nm)
S	73.6 (0.9)[e]	16.7 (0.5)[b]	85.6 (0.5)[d]	68.8 (0.9)[d]	0.811 (0.005)[d]
E-S	71.967 (0.115)[d]	12.5 (0.4)[a]	95.9 (0.3)[e]	69.27 (0.06)[d]	0.809 (0.003)[d]
E-WP-S	59.1 (1.3)[a]	25.5 (0.7)[d]	72.68 (1.14)[a]	52.2 (1.7)[a]	0.63 (0.04)[a]
EOA-WP-S	67.1 (0.4)[b]	22.3 (0.4)[c]	77.0 (0.5)[c]	60.1 (0.6)[b]	0.742 (0.013)[c]
E-LE-S	67.50 (0.18)[bc]	21.8 (0.2)[c]	75.97 (0.17)[b]	60.84 (0.15)[bc]	0.745 (0.005)[c]
EOA-LE-S	68.1 (1.2)[c]	21.8 (1.0)[c]	77.6 (0.2)[c]	61.322 (1.105)[c]	0.69 (0.03)[b]

[abcde] Different letters in the same column indicate significant difference among formulations ($p < 0.05$).

3.5. Antioxidant activity

The antioxidant activity (AA) of the films was evaluated though the dissolution of the films in a controlled amount of distilled water, applying the method described by Brand-Williand et al (1995), based on the DPPH free radical. From the absorbance data both at zero reaction time and after achieving the steady state, the EC_{50} parameter was calculated. EC_{50} parameter quantifies the mass of tested sample that causes a 50% decrease in the initial DPPH$^{\cdot}$ concentration. Thus, a higher antioxidant activity is reached in samples exhibiting the lower EC_{50} values.

Table 5 shows the EC_{50} values for pure eugenol and the different films, expressed in terms of moles of eugenol per DPPH˙ moles and in kg films per DPPH mol, by taking into account the initial amount of eugenol in each film sample. Eugenol was found to react slowly with the DPPH˙ (slow kinetic behavior), taking around 4 hours to reach the steady state in agreement with that reported by Bortolomeazzi et al., (2010). The EC_{50} value of pure eugenol was also measured (0.219±0.005 mol eugenol/mol DPPH), being consistent with values obtained by Brand-Williams et al. (1995) (0.27 mol eugenol/mol DPPH).

All formulations incorporating eugenol exhibited antioxidant activity, which depended on the capsule formulation used. The obtained values for EC_{50} expressed in terms of moles of eugenol per mole DPPH˙ significantly increased in the films in comparison with the pure eugenol values, which reflected that significant losses of the expected antioxidant activity occurred in the films. This can be attributed to the eugenol losses or oxidation during the film thermoprocessing or to the formation of eugenol-amylose complexes, that inhibited the free eugenol release to the solvent media. These losses were greater in the non-encapsulated eugenol films and in the encapsulated eugenol films without OA. Thus, the greatest antioxidant activity were found in the films with capsule formulations containing oleic acid, which showed EC_{50} values closer to those of pure eugenol, regardless of the wall material used (WP or LE) of the capsules.

Table 5. Antioxidant activity of films containing non-encapsulated and microencapsulated eugenol, expressed in terms of EC_{50} values.

	EC_{50} parameter of DPPH assay		
Formulation	**kg film/mol DPPH**	**mg eugenol/mg DPPH**	**mol eugenol/mol DPPH**
E-WP-S	35 (3)[b]	1.24 (0.10)[c]	3.0 (0.2)[c]
EOA-WP-S	17.3 (0.3)[a]	0.552 (0.010)[b]	1.33 (0.03)[b]
E-LE-S	52 (4)[d]	1.88 (0.12)[d]	4.5 (0.3)[d]
EOA-LE-S	20 (4)[a]	0.65 (0.13)[b]	1.6 (0.3)[b]
E-S	40.5 (1.7)[c]	1.34 (0.05)[c]	3.21 (0.12)[c]
Pure E		0.091 (0.002)[a]	0.219 (0.005)[a]

[abcd] Different letters in the same column indicate significant difference among formulations ($p < 0.05$).

The films antioxidant activity was related to the amount of eugenol retained in the films after film processing. The eugenol retention efficacy (%) in each film is showed in **Table 6**, which was calculated taking into account the final eugenol content in the films and the nominal eugenol content added (free or encapsulated) before the film processing. To quantity the final eugenol content, two methods were used for comparison purposes, the Folin-Ciocalteu (F-C) and methanol extraction of the films and UV

spectrophotometric quantification. As can be observed, the values obtained by both methods were very similar in all cases, except in the E-S films, being the F-C values three times higher than those obtained by the UV method. This can be explained taking into account that when E-S films were submitted to dissolution, a chemical equilibrium between the free E and E-starch complexes was achieved, for a given temperature. In the F–C assay, polyphenols react with specific redox F-C reagent to form a blue complex that can be quantified by visible-light spectrophotometry (Sanchez-Rangel et al, 2013; Blainski et al., 2013). The formation of such eugenol-F-C complexes would provoke the displacement of the initial equilibrium towards a greater amount of free eugenol, which would explain the higher eugenol content measured in these films by the F-C method. On the contrary, the E methanol extraction from the films could be no effective at releasing E from the starch complexes, which would limit the UV spectrophotometric response, giving rise to a non-total E quantification.

Table 6. Eugenol content and percentage of eugenol retained in the films (g final eugenol content/100 g nominal eugenol), determined by the Folin-Ciocalteu method and eugenol extraction and UV spectrophotometric quantification.

Formulation	Folin-Ciocalteu method		UV (Methanol extract)	
	mg Eug/g film	% Eugenol retention	mg Eug/g film	% Eugenol retention
E-WP-S	2.8 (0.3)[a]	22 (2)[a]	2.83 (0.17)[b]	23.0 (1.2)[b]
EOA-WP-S	4.7 (0.4)[c]	45 (4)[c]	4.16 (0.18)[c]	40.6 (1.9)[c]
E-LE-S	2.55 (0.09)[a]	19.9 (0.7)[a]	2.33 (0.12)[b]	18.2 (0.8)[b]
EOA-LE-S	5.0 (0.2)[c]	45 (2)[c]	4.6 (0.3)[c]	41 (3)[c]
E-S	3.24 (0.18)[b]	26.6 (1.5)[b]	1.54 (0.04)[a]	12.7 (0.4)[a]

[abc] Different letters in the same column indicate significant difference among formulations ($p<0.05$).

By considering the F-C values, encapsulating materials containing OA highly promoted the E retention in the films during thermoprocessing (45%), whereas lower and similar retention (20-26 %) was observed for the incorporated free E or encapsulated in OA-free materials. This increment in the retention yield reflected the effectiveness of OA as a carrier compound, which modulated compound interactions involved in the encapsulation process and decreased the eugenol losses during film thermo-preparation. A similar effect of OA at retaining the essential oil compounds in chitosan films was found by Perdones et al. (2014).

3.6. Kinetics of eugenol release

The kinetic behaviour of the eugenol release from films in four food simulants of different polarity and pH (EtOH10%; AA3%, EtOH20% and EtOH50%) was also studied. **Figure 3** shows the percentage of eugenol released as a function of time in the different food simulants. As can be observed, the eugenol release reached the equilibrium after about 20-25 hours in all simulants and films. The experimental data (points) and curves predicted (lines) by the fitted Peleg model are shown in **Figure 3**. **Table 7** shows the parameters of the Peleg model, where k_1 is the kinetic constant of the model (min/(mg E/g film)) related to the inverse of the mass transfer rate at the beginning of the process and k_2 is related to the asymptotic value of the curve or amount released at equilibrium ($1/k_2 = M_\infty$, mg eugenol/g film). The maximum release ratio (M_∞/M_0) was estimated with respect to the total extracted eugenol (M_0) in each film by UV method, since this method was also used to quantify the released E content into each simulant. A good fit of the model was obtained in every case, as reflected by the R^2 values in **Table 7**.

Table 7. Maximum eugenol release ratio (M_∞/M_0)* and parameters of Peleg´s model for the different systems in the different food simulants: inverse of the initial release rate (k_1) (min/(mg eugenol /g film)) and equilibrium value, M_∞ ($1/k_2$) (mg eugenol/g film). Mean values and (standard deviation).

		E-WP-S	EOA-WP-S	E-LE-S	EOA-LE-S	E-S
EtOH10%	k_1	18 (5)[a,1]	15 (4)[a,12]	83 (40)[b,2]	21 (15)[a,12]	676 (154)[c,2]
	$1/k_2$	2.60 (0.08)[d,1]	1.47 (0.09)[c,1]	1.113 (0.105)[b,1]	1.4 (0.2)[bc,1]	0.29 (0.03)[a,1]
	R^2	≥ 0,9975	≥ 0,9993	≥ 0,9939	≥ 0,9987	≥ 0,9884
	% M_∞/M_0*	92 (3)[d,1]	35 (2)[b,1]	48 (4)[c,1]	30 (5)[b,1]	19.0 (1.8)[a,1]
AA3%	k_1	34.3 (0.3)[a,1]	45 (10)[a,2]	276 (64)[b,3]	58.5 (0.7)[a,2]	6300 (2051)[c,3]
	$1/k_2$	3.1 (0.3)[cd,2]	2.9 (0.2)[c,3]	1.86 (0.16)[b,2]	3.41 (0.07)[d,4]	0.50 (0.16)[a,1]
	R^2	≥ 0,9982	≥ 0,9990	≥ 0,9932	≥ 0,9993	≥ 0,9385
	% M_∞/M_0*	103 (8)[d,2]	69 (6)[b,3]	80 (7)[c3]	74.1 (1.5)[bc,3]	32 (10)[a,2]
EtOH20%	k_1	6 (2)[a,1]	13 (3)[a,12]	59 (16)[b,2]	40 (7)[ab,12]	519 (165)[c,2]
	$1/k_2$	2.4 (0.2)[d,1]	2.01 (0.18)[c,2]	1.61 (0.15)[b,2]	2.9 (0.4)[e,3]	0.2598 (0.0007)[a,1]
	R^2	≥ 0,9993	≥ 0,9991	≥ 0,9969	≥ 0,9935	≥ 0,9905
	% M_∞/M_0*	86 (8)[d,1]	48 (4)[b,2]	69 (6)[c,2]	63 (8)[c,2]	16.8 (0.05)[a,1]
EtOH50%	k_1	4.2 (1.3)[a,1]	2.35 (0.19)[a,1]	7.1 (1.6)[a,1]	14 (7)[a,1]	72 (88)[b,1]
	$1/k_2$	2.47 (0.17)[c,1]	2.5 (0.4)[c,3]	1.789 (0.020)[b,2]	2.5 (0.3)[c,2]	0.49 (0.02)[a,1]
	R^2	≥ 0,9999	≥ 0,9993	≥ 0,9996	≥ 0,9972	≥ 0,9995
	% M_∞/M_0*	87 (6)[c,1]	61 (9)[b,3]	76.7 (0.8)[c,23]	54 (7)[b,2]	32 (1.5)[a,2]

* Maximum eugenol released with respect to the total extracted eugenol (M_0) in the film measured by the UV method (**Table 6**).

abcd Different letters in the same line indicate significant difference among formulations (p<0.05).
1234 Different numbers in the same column indicate significant difference among food simulants(p<0.05).

The total amount of eugenol released (M_∞ values), depended on both the food simulant and the composition of the microencapsulated material present in each film. M_∞ values were around 0.2-0.5 mg eugenol/g film in non-encapsulated films and ranged between 1.1 and 3.4 mg eugenol/g film for those films incorporating encapsulated eugenol. According to several authors (Buonocore et al. 2003; Sánchez-González et al., 2011a; Talon et al., 2017b), the release of an active compound from a polymeric network is dependent on different factors such as the liquid diffusion into the matrix and its subsequent swelling and relaxation, (or even partial polymer solubilization), the diffusion capacity of the active compound through the swelled polymer matrix to the food simulating liquid, and to the relative chemical affinity of the compound to the swelled matrix and liquid phase. Thus, the highest value of total released eugenol was quantified in the acidic solution (AA3%), probably due to the partial acid hydrolysis of the amorphous regions of starch matrix (Wang and Copeland, 2015) leading to a more open structure, which favours the diffusion of eugenol into the simulant. A decrease in the polarity of the medium would favour the eugenol release due to its greater affinity with the liquid phase. However, in general, no notable differences on M_∞ values were found for the different ethanol-water simulants, although the values in 10% ethanol aqueous systems were lower.

The maximum release ratio (M_∞/M_0) ranged between 19% and 100%, depending on the capsule formulation present in the films, following the order: E-WP-S > E-LE-S > EOA-LE-S = EO-AWP-S > E-S. Thus, the lowest ratio was found in the non-encapsulated eugenol film, remaining 17-32% of eugenol in the films at equilibrium, depending on the simulant. The low release ratio of the non-encapsulated E from the films could be explained by the formation of lipid-amylose complexes, previously commented, which limit the free E able to migrate to the solvent medium. In fact some authors report a lipid complexation capacity of amylose in the helical hydrophobic cavity of 10g lipid/100 g amylose (Eliasson, 1994). Considering the amylose ratio in corn starch (20%, Jiménez et al., 2013) and the ratio starch-eugenol in the film, a total complexation could occur, without saturation of the amylose complexation capacity. This effect would be limited in films containing encapsulated E due to the fact that the contact between the lipid compound and amylose was highly restricted when the compound was in the core of the capsules. The greater ratio of eugenol released was found when using E-WP particles, probably due to the higher water solubility of this microencapsulates in the different solvents which enhanced the E release, in comparison with E-LE-S formulations. In OA based formulations, the hydrophobic nature of these microparticles seemed to limit the complete release of the active compound. Nevertheless, this effect significantly decreased when the affinity of the lipid compounds to the medium increased in the 50 % ethanol aqueous simulant.

As regards the release rate, the k_1 value was remarkably high when eugenol was not previously encapsulated (E-S), especially when using acetic acid as food simulant, so at the very beginning of the process, the rate of eugenol release was very slow. Thus, the maximum release ratio of E when using the acidic solvent was notably affected by the hydrolysis of the starch matrix throughout time, which promote the final release of eugenol not only in E-S films but also in the rest of the formulations. Likewise, the low E concentration in the E-S films will also contribute to decrease the driving force of the diffusion process.

In films containing microencapsulated eugenol, the initial release rate was higher in E-WP films probably due to its greater solubility previously commented on. The lowest release rate was found in E-LE films, in which a more gradual release of eugenol was found. No significant differences in k_1 values was found between EOA-WP-S and EOA-LE-S formulations, which exhibited intermediate values. All formulations showed lower k_1 values (higher initial rates) when the polarity of food simulant decreased (50% ethanol), due to the greater affinity of the lipid active compound to the solvent. Similar behaviour were reported by other authors for the release of other essential oil compounds from different polymer matrices (Sánchez-González et al., 2011a; Requena et al., 2017 and Muller et al., 2017c).
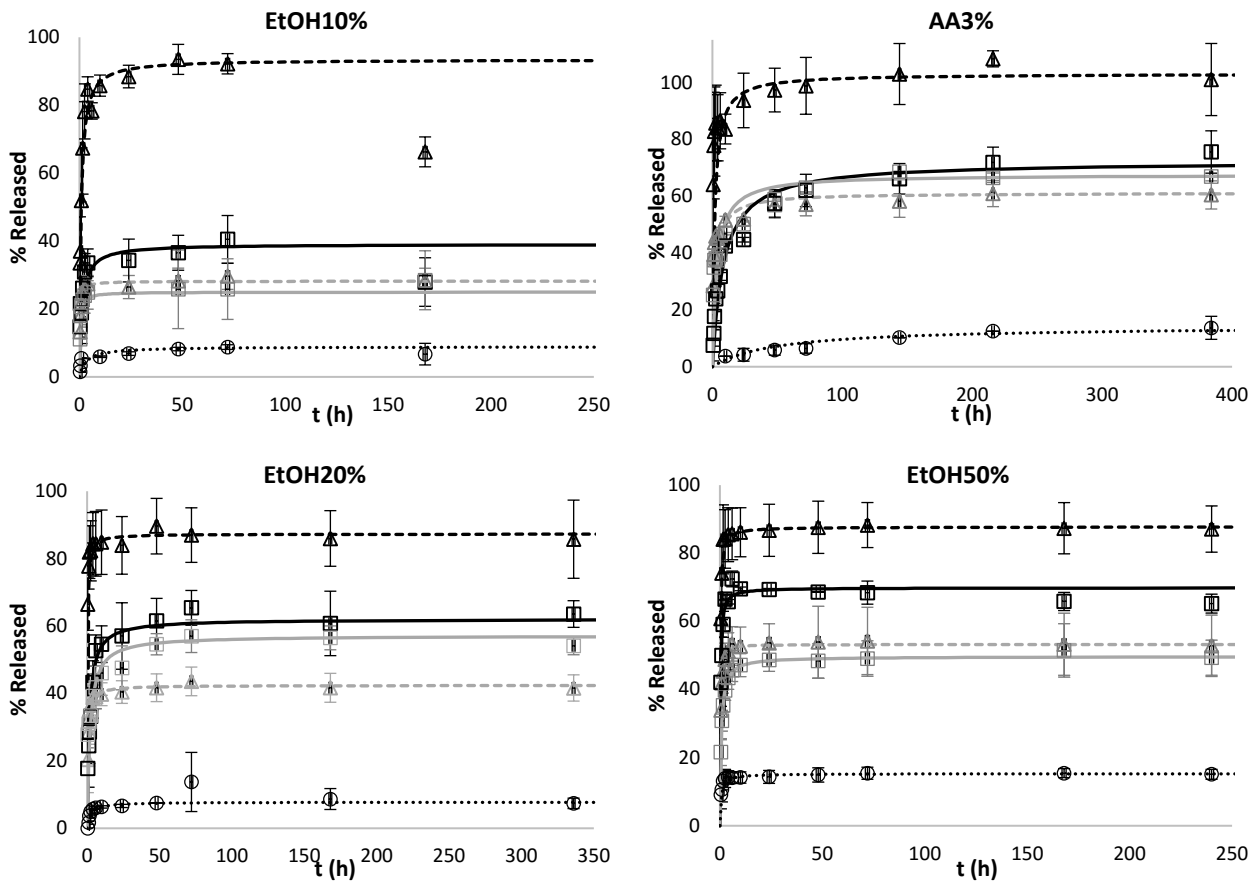
Figure 3. Percentage of total eugenol released at 22°C as a function of time in different solvents (EtOH10%: Ethanol aqueous solution (10% v/v); AA3%: Acetic acid aqueous solution (3%, w/v); EtOH20%: Ethanol aqueous solution (20%, v/v); EtOH50%: Ethanol aqueous solution (50%, v/v)); experimental data (△ E-WP-S; △ EOA-WP-S; □ E-LE-S; □ EOA-LE-S; ○ E-S) and fitted Peleǵs model (- - - E-WP-S; - - - EOA-WP-S; — E-LE-S; — EOA-LE-S; ⋯ E-S).

4. CONCLUSIONS

Incorporation of eugenol (encapsulated or not) into the thermo-compressed starch films significantly affected their physical and antioxidant properties. In non-encapsulated eugenol films, this was attributed to the formation of eugenol-amylose complexes which modify the starch matrix. In films with encapsulated eugenol, the partial disruption of the capsules during the film thermoprocessing release different compounds to the starch matrices provoking different degrees of compatibility among components and starch, which induced heterogeneous film microstructure. The microstructural arrangement of the different components in the matrix caused notable changes in tensile and barrier properties in the starch films. In general, the films with encapsulates turned less stretchable and resistant than pure starch films, with lower water sorption capacity and improved water vapor barrier properties. However, microcapsules containing oleic acid provoked a very heterogeneous distribution of this compound in the matrix, which controlled the film oxygen permeability, giving rise to a high variability in this property.

The protective effect of microencapsulates on the eugenol retention during the film processing was only effective when encapsulates contained oleic acid as lipid carrier, regardless the wall material used (whey protein or lecithin). The loss of the non-encapsulated eugenol from thermo-compressed film was limited probably due to the formation of lipid-starch complexes, which drastically decreased the amount of eugenol released into the different food simulants.

The fastest kinetic and greatest amount of eugenol released was observed in films containing microencapsulated eugenol, especially when the encapsulating materials were more water soluble (E-WP-S) and when the solvent was acid and provoked a partial hydrolysis of the starch matrix, favoring the compound diffusion to the aqueous medium.

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Starch-eugenol based films for sunflower oil preservation: release kinetics and functional properties

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ABSTRACT

Starch films containing eugenol, which was added to the film-forming dispersion in free form or microencapsulated with different wall materials (whey protein or lecithin) were obtained by casting. The physical properties of the films, the release kinetics of eugenol in different food simulants and the antioxidant performance in preventing sunflower oil oxidation during storage were evaluated. The addition of microencapsulated eugenol powder in the film-forming dispersion modified film microstructure, yielding less resistant and less elastic films with reduced moisture content, transparency and oxygen permeability as compared to films formulated with non-encapsulated eugenol. The addition of eugenol microencapsulated with oleic acid promoted the preservation of the antioxidant activity of the films. Films developed with microencapsulated eugenol powder containing lecithin and oleic acid were effective in preventing sunflower oil oxidation during 53 days of storage at 30ºC, maintaining low and almost constant values of peroxide index, conjugated dienes and trienes.

Key words: antioxidant, release, barrier properties, tensile properties, optical properties, microstructure.

1. INTRODUCTION

Sunflower oil is an unsaturated mixture of mainly oleic and linoleic acid group of oils. Regular sunflower oil contains 69% linoleic acid, 20% oleic acid and 11% saturated fatty acids (Khan et al., 2015), although several varieties of sunflower oil are produced containing high linoleic, high stearic (~18%) and high oleic (~70%) acids (Anushree et al., 2017). High reactivity of those unsaturated fatty acids is associated with oxidation (rancidity) that can modify the organoleptic properties of the product causing losses of nutritional value and quality (Kucuk et al., 2005) thus affecting its shelf life.

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) have been used as food additives for sunflower oil preservation. However, there is an increasing interest in food technology area for replacing these conventional additives for natural antioxidant compounds. The physical characteristics of the packaging can directly affect the quality and shelf-life of sunflower oil. Polyethylene (PE), polyvinylchoride (PVC) and polyethylenetetraphthalate (PET) are the main components of commercial plastic bottles used for sunflower oil packaging. These synthetic materials have some drawbacks, such as their relatively limited barrier properties as compared to glass and their impact on the environment (Kucuk et al., 2005).

In this sense, current research efforts have been focused on the use of natural and biodegradable packaging materials, which could also contain active compounds, such as antioxidants and antimicrobial agents that provide them with several functional properties that do not exist in conventional packaging systems (Carrizo et al. 2016).

New emerging tendencies in food packaging are directed towards the development of multilayer packaging materials containing natural active compounds with functional properties. In this sense, Carrizo et al. (2016) developed a new antioxidant multilayer packaging material containing green tea extract, at the industrial level, by using commercial plastics. The new material was used to pack chocolate peanuts and milk chocolate cereals, and the results revealed a good protection of these foods for a long-term period.

Eugenol is a natural phenolic compound abundant in clove (90%) and cinnamon (60%) essential oils with interesting antimicrobial and antioxidant properties (Chatterjee & Bhattacharjee, 2013; Devi et al. 2010; Kamatou et al., 2012; Ogata et al., 2000; Woranuch & Yoksan, 2012). Eugenol has been widely used in pharmaceuticals, cosmetics and food products and, recently, in active packaging for food preservation. Sanla-Ead et al. (2012) studied the antimicrobial activity of cellulose films incorporating cinnamaldehyde and eugenol. This natural compound could be incorporated into the formulation of bioactive films to be used for food preservation, leading to an antioxidant effect on the surface of the product, thus preventing the occurrence of some oxidative

reactions. However, it is difficult to handle due to the limited water solubility and it is susceptible of losing functionality during its handling or processing (Choi et al., 2009).

The encapsulation of the active compound in different wall systems before film preparation can diminish the losses during its development and it can also help to modulate the release kinetics of the active in the product (Valencia-Sullca et al., 2016). In this sense, the microencapsulation by spray-drying technique, a popular and common technique, represents an interesting option. This technique is especially used in the microencapsulation of thermosensitive compounds, since it is a fast process in which the temperatures reached are relatively low (Barrow et al., 2013). This method could also contribute to improve the retention of volatile compounds in the final encapsulated powder (Jafari et al., 2007).

The choice of the wall materials for the encapsulation of active ingredients is critical in the production of microparticles by spray-drying, since they have great influence in the properties of the emulsion before drying and in the retention of the active compound and life of the product in powder after drying (Botrel et al., 2014). Whey protein (WP) and lecithin (LE) are capable of successfully encapsulating active compounds and stabilizing oil-in-water emulsions (Bae & Lee, 2008; Taylor et al., 2005). When developing micro and nanocapsules by means of spray-drying techniques WP and LE are often combined with carbohydrates like maltodextrins, since they increase oxidative stability due to the formation of a dry crust around the droplets during drying (Kagami et al., 2003). The addition of oleic acid can increase the retention and activity of the active compound (Perdones et al., 2014, Pokharkar et al., 2011).

Packaging systems based on petroleum-based plastic materials are gradually being replaced by biodegradable materials that are environmentally friendly (Fadini et al., 2013, Martelli & Laurindo, 2012). The use of biopolymers, such as starch, exhibit a great potential to obtain biodegradable active packaging materials due to its easy availability and low cost. Nevertheless, the incorporation of different active compounds like eugenol to starch films could have an impact on their physical properties, which in turn can affect their functionality as a packaging material (Sánchez-González et al., 2015). Thus, the antioxidant properties of the active compound could be affected by the release kinetics towards the food system. These parameters have been little explored and could depend on several factors such as the food product characteristics and film structure.

The aim of this work was to evaluate the physical properties, the release kinetics in different food simulants and the antioxidant performance for sunflower oil preservation of corn starch-based films containing eugenol, which was added to the film-forming dispersions in free form or microencapsulated in powder with different wall-materials (whey protein or lecithin).

2. MATERIALS AND METHODS

2.1. Raw materials

To obtain the microencapsulated eugenol, lecithin (LE) lipoid S45 (Batch 574510, Lipoid, Germany), whey protein isolate (WP) 90S (Batch 131848) and maltodextrin (MD) Kyrosan E18 (Batch 02157372) from Haarla (Tampere, Finland), Eugenol (E) (Batch STBD6235V, Sigma-Aldrich, Madrid, Spain) and purified oleic acid (OA) (Batch 15C030505, VWR Chemicals Germany) were used.

Corn starch (S) (Batch RMA12, Roquette Laisa SA, Benifaió, Spain) and glycerol (Panreac Química SLU, Castellar del Vallès, Barcelona, Spain) were used to prepare the films.

2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) and Folin-Ciocalteu, from Sigma-Aldrich (Madrid, Spain) and glacial acetic acid, absolute ethanol, methanol, sodium carbonate (Na_2CO_3), magnesium nitrate ($Mg(NO_3)_2$) and phosphorus pentoxide (P_2O_5), provided by Panreac Química SLU (Barcelona, Spain) were used to carry out the different analyses.

2.2. Preparation of the microcapsules

Microencapsulated eugenol was obtained by spray-drying. Briefly, 43% (w/w) of WP:MD or LE:MD solids (ratio 1:42) and 3% (w/w) of eugenol (total eugenol amount: 65.22 mg Eu/g powder) with and without 7% (w/w) of OA (total eugenol amount: 56.60 mg E/g powder) were dissolved. All dispersions were homogenized with a rotor-stator (Ultra-Turrax T 25 Basic, IKA Werke GmbH and Co. KG, Germany) at 11.000 rpm for 6 minutes, and after they were three times microfluidized with a high-pressure homogenizer (Microfluidics M-110Y, Newton, Massachusetts, USA) at 103.4 MPa. The different microencapsulated eugenol powders were obtained by using a spray-drier (Mobile Minor TM spray-dryer, GEA Niro, GEA Process Engineering A/S, Søborg, Denmark) with a rotary atomiser with an inlet air temperature of 180°C. The outlet temperature was kept at 80 ± 2°C by controlling the feed rate.

2.3. Preparation of the films

Firstly, an aqueous starch dispersion 3% (w/w) was heated at 95°C for 30 minutes, in order to gelatinize the starch, shaking every 5 minutes to promote a better homogenization of the gelatinization. Thereafter, the dispersion was cooled for 10 minutes in an ice bath and glycerol was added in a 1.5:0.5 starch:glycerol ratio (w/w). The formulations were homogenized with a rotor-stator (Yellow Line DL 25 Basic, IKA, Janke and Kunkel, Germany) at 13500 rpm for 3 minutes and degassed by means of a vacuum pump. Eugenol (E) in free form or microencapsulated eugenol powder were

then added to obtain the film-forming dispersions (FFDs) with a starch eugenol ratio in the film that is shown in **Table 1**. FFDs were poured into Teflon plates by keeping the total amount of starch and glycerol constant (11 mg/cm^2) in the plate. After drying at controlled relative humidity and temperature (53% RH and 25°C) the films were conditioned in a desiccator at 25°C and 53% RH, using an over saturated Mg(NO$_3$)$_2$ solution.

Table 1. Mass fraction (g/g total solids) of each component of the films and theoretical starch:eugenol ratio.

Film	S	Gly	WP	LE	MD	E	OA	S:E
S	0.750	0.250	-	-	-	-	-	
S-E	0.728	0.243	-	-	-	0.029	-	25
S-E-WP	0.500	0.167	0.007	-	0.305	0.021	-	24
S-EOA-WP	0.500	0.167	0.006	-	0.266	0.017	0.044	29
S-E-LE	0.500	0.167	-	0.007	0.305	0.021	-	24
S-EOA-LE	0.500	0.167	-	0.006	0.265	0.018	0.044	28

S: Starch, Gly: Glycerol, E: Eugenol, WP: Whey Protein, OA: Oleic acid, LE: Lecithin

2.4. Characterization of the films

2.4.1. Microstructure, moisture content, solubility and thickness

The microstructure of film cross sections was observed by Field emission scanning electron microscope (FESEM) (JEOL, model JSM-5410, Japan). The samples were maintained with phosphorus pentoxide for one week to ensure the total dehydration of the films and were cryofractured by immersion in liquid nitrogen in order to observe the transversal zones. The pieces of the film were mounted on the sample holder using the double-sided carbon tape. Samples were coated with platinum and observed using an accelerating voltage of 2kV.

The water content of the different films previously conditioned at 53% RH and 25°C was determined. Films were cut in small pieces and filled into pre-weighed capsules. In order to accelerate the dehydration process, the samples were placed in a vacuum oven (TEM-T vacuum. J.P. Selecta, S.A., Barcelona, Spain) at 60°C for 48 hours and then stored in a desiccator with phosphorus pentoxide until reaching constant weight. Three replicates were considered per formulation. Moisture content in dry basis (g H$_2$O/100g dry matter) was calculated from the initial and final sample weights.

For the solubility test, pieces of dry samples were transferred to a mesh of known weight and distilled water was added at 1:50 ratio (film:distilled water; w/v). Samples were kept

for 7 days at 20°C. The meshes with the samples were placed in an oven (J.P. Selecta, S.A., Barcelona, Spain) at 60°C for 48 hours and subsequently were transferred to a desiccator with phosphorus pentoxide until constant weight was reached. The assay was performed in triplicate and the results were expressed as soluble film/100g film.

Thickness was measured in sixteen random points of three samples of all formulations conditioned at 25°C and 53% RH by means of a digital electronic micrometer with an accuracy of 0.001 mm (Palmer model COMECTA, Barcelona).

2.4.2. Barrier, tensile and optical properties

Water vapour permeability (WVP) was determined gravimetrically using a modification of the ASTM E96-95 gravimetric method (1995), at 25°C and a RH gradient of 53–100%. Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 mL of distilled water (100% RH). Three circular samples of each formulation were prepared and the thickness of each sample was measured in six random points with an electronic digital micrometer (Comecta S.A., Barcelona, Spain). Samples were fixed in the cups and were placed in equilibrated desiccators containing saturated solutions of magnesium nitrate (53% of RH) and with a fan on the top of the cup in order to reduce the resistance to water vapour transport. The side of the films in contact to air during film drying was exposed to the atmosphere at the lowest RH (53%). The cups were weighed periodically using an analytical balance (ME36S, Sartorius, Germany; ±0.00001 g) at intervals of 1.5 h for 24 h after the steady state had been reached. The slope of the weight loss versus time was plotted and the WVP was calculated according to Bonilla et al. (2013).

Oxygen permeability (OP) was determined by following the ASTM Standard Method D3985-05 (2010). Three 50 cm^2 replicates of each formulation were measured by using the Ox-Tran equipment (Model 1/50, Mocon, Minneapolis, USA) at 25°C and 53% of RH. Oxygen permeability was calculated by dividing the oxygen transmission rate (OTR) by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the film thickness.

The mechanical behavior of the films was analyzed using a Universal Testing Machine (Stable Micro System TA-XT plus, Haslemere, England) according to ASTM standard method D882 (2001). The thickness of eight pre-conditioned film pieces of 25 mm × 100 mm was measured in six random points by means of an electronic digital micrometer (Comecta S.A., Barcelona, Spain) and samples were mounted in the film's extension grip of the equipment and stretched at a rate of 50 mm·min^{-1} until breaking. Force-distance curves were obtained and transformed into stress-strain curves. The tensile properties were analysed in terms of elastic modulus (EM), tensile strength (TS) and percentage of elongation at break (%E).

Optical properties were determined in triplicate by measuring the reflection spectrum of the samples from 400 to 700 nm of wavelength using a MINOLTA spectrocolorimeter (model CM-3600d, Minolta CO., Tokyo, Japan). The transparency was measured by means of the internal transmittance (Ti), applying the Kubelka-Munk theory of the multiple dispersion of reflection spectrum (Hutchings, 1999) given the reflection spectra of both black and white backgrounds. Measurements were taken on the side of film which was in contact with air during film drying. CIEL*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 illuminant D65 and observer 10°.

2.4.3. Antioxidant activity

The antioxidant activity of the films was evaluated by means of the 2,2-diphenyl-1-pikryl-hydrazyl (DPPH) method and by the Folin-Ciocalteu method. DPPH method is based on the reduction of the DPPH$^\cdot$ radical in an alcoholic solution by a hydrogen-donor antioxidant (Brand-Williams et al., 1995). In the radical form, this molecule shows absorbance at 515 nm of wavelength, which disappears after accepting an electron or hydrogen radical from antioxidant compounds. The Folin-Ciocalteu method is based on the phenolic compounds redox reaction with the Folin-Ciocalteu reagent, in a basic pH, giving rise to a blue coloration susceptible to be determined spectrophotometrically at 765 nm (Stojanovic et al., 2012). Since eugenol is a phenolic compound, it is also possible to relate its antioxidant power to the total amount of the compound.

To perform the antioxidant activity measurements, film samples (1.5 g) were dispersed in 100 mL of methanol and were keep under stirring for 15 days. After that, samples were homogenised by using the rotor-stator (Yellow Line DL 25 Basic, IKA, Janke and Kunkel, Germany).

The determination of the antioxidant capacity by means of DPPH was carried out mixing 0.15 to 1.05 mL (0.15 ml volume increments) of different appropriately diluted samples with a methanol solution of DPPH$^\cdot$ (with an absorbance of 0.70 ± 0.02 (λ = 515 nm), as described by Pastor et al. (2013). Samples were kept in dark in covered cuvettes to prevent the evaporation of methanol for 4 hours, when reaction stability was achieved (Bortolomeazzi et al., 2007). Absorbance measurements (λ = 515 nm) were carried out in a spectrophotometer (Evolution 201 VisibleUV, ThermoScientific, Germany). The assay was performed in triplicate and the DPPH$^\cdot$ concentration (mM) in the reaction medium was calculated from the calibration curve determined by linear regression. The percentage of the remaining DPPH$^\cdot$ (%DPPH$^\cdot_{rem}$) was calculated following **equation 1.**

$$\%[DPPH^\bullet]_{rem} = \frac{[DPPH^\bullet]_{t=4h}}{[DPPH^\bullet]_{t=0}} \cdot 100 \qquad \textbf{Equation 1}$$

Where, the $DPPH^{\cdot}_{t=4h}$ is the concentration of DPPH at 4 hours and $DPPH^{\cdot}_{t=0}$ is the concentration at the start of the reaction.

From these values the parameter EC_{50} (Efficient Concentration) was determined, which indicates the antioxidant concentration necessary to reduce 50% of initial DPPH. This parameter was obtained after representing the $\%DPPH_{rem}$ *versus* the mass ratio of film (mg film/mg DPPH or mol eugenol/mol DPPH).

To perform the Folin-Ciocalteu analyses 100 µm of appropriately diluted samples was mixed with 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of Na_2CO_3 and was completed with distilled water until 10 mL. After 2 h of reaction, the absorbance of the samples was measured at 765 nm, in triplicate, by means of a spectrophotometer (Evolution 201 VisibleUV, ThermoScientific, Germany). Gallic acid was used as a standard and the results were expressed as mg gallic acid $\cdot L^{-1}$.

2.5. Kinetics of eugenol release

Four different food simulants were used to perform the release rate of eugenol from the different films according to the Commission regulation (EU) 10/2011 (14 January 2011): A (ethanol 10% (v/v)) (ET10%), B (Acetic acid 3% (w/v) (AA3%), and C (ethanol 20% (v/v)) (ET20%) food simulants, which are assigned for aqueous foods, aqueous foods with pH values below 4.5 and alcoholic foods up to 20%, respectively and D1 (ethanol 50% (v/v)) (ET50%), which simulates alcoholic food above 20% and oil in water emulsions. Pieces of films from each formulation with controlled weight were cut in small parts and placed into vials containing 100 mL of each food simulant. Release studies were carried out in triplicate during 16 days at 22°C under stirring conditions. Successively, the samples were taken at different film-solvent contact times and the absorbance was measured at 282 nm where eugenol absorbance shows a maximum (Pramod et al., 2013) by means of a spectrophotometer (Evolution 201 VisibleUV, ThermoScientific, Germany). Absorbance measurements were related to the corresponding standard calibration curve to obtain the concentration of eugenol released in each case, expressed as amount of eugenol per gram of film.

Fick's second law was considered to study the diffusion process of the eugenol from the films to the different food simulants. Film samples were considered as infinite plane sheets with the half thickness as a characteristic dimension, where the active compound diffuses only in an axial direction (Requena et al., 2017). The values of the diffusion coefficient (D) of eugenol into the different solvents were obtained by fitting experimental values to the diffusional long-time equation for an infinite plane sheet (equation 5) with ten terms (Crank 1979). The Solver tool (Microsoft Excel 2013®) was

used to optimize the D values, by minimizing the Sum of Squared Errors (SSE), and considering the following boundary conditions:

$$t = 0; \quad 0 < x < L; \quad c = c_0$$

$$t > 0; \quad x = L; \quad c = 0 \qquad \qquad \textbf{Equation 2}$$

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

Where, M_t is the mass of eugenol released at time t, M_∞ is the mass of eugenol released at equilibrium and L is half thickness of film.

To predict the release kinetics, Peleg's model (Peleg, 1988), described by **equation 3,** was applied to experimental data.

$$M_t = M_0 + \frac{t}{k_1 + k_2 t} \qquad \qquad \textbf{Equation 3}$$

where M_t is eugenol content at time t, M_0 is the initial eugenol content, k_1 is the kinetic constant of the model that is inversely related to the mass transfer rate at the beginning of the process, and k_2 is a constant of the model that is related to the asymptotic value, which can be related to the equilibrium value ($1/M_\infty$, where M_∞ is the amount of active compound released at equilibrium).

2.6. Antioxidant performance of the films on sunflower oil

The films that showed the highest antioxidant activity and adequate mechanical behavior were considered as potential packaging materials for sunflower oil. To evaluate the antioxidant performance, film samples (area of 11 cm × 6 cm) were thermosealed with a vacuum packing machine (SAECO Vacio Press Elite, Barcelona, Spain) to form bags, as described by Galarza et al. (2017). Then, 10 mL of commercial sunflower oil were poured to each bag, which was thermos-sealed. The protective effect of films in delaying sunflower oil oxidation was evaluated as compared to low density polyethylene (PE) and pure corn starch films (S). As control, an open glass Petri dish containing 10 mL of sunflower oil was considered. All samples were stored at 30ºC and 53% RH and exposed to fluorescent light at intensity of 1000-1500 lux (measured by using a digital Luxometer; model RS Pro ILM1332A, RS Components, Madrid, Spain). The oxidative stability of sunflower oil was measured in terms of the peroxide value (PV) and diene

and triene content at 0, 4, 7, 11, 14 and 18 days of storage. In order to determine the PV of the samples, the titrimetric method was employed (IUPAC, 1987) by using an automatic titrator (Titrando, Metrohm Ion Analysis, Switzerland). With this aim, 1 g of oil was dissolved in 10 mL of solvent (glacial acetic acid:1-decanol volume ratio of 3:2, containing 10-15 mg·L^{-1} of iodine) and mixed with 200 μL of an oversaturated KI solution. The mixture was thoroughly sacked and kept in a dark place for 1 min. Then, 50 mL of distilled water was added, and the solution was titrated with 0.01M or 0.001M Na$_2$S$_2$O$_3$, depending on the PV predicted. A blank control sample, without sunflower oil, was also prepared by following the same procedure described. All the analyses were performed in triplicate.

The conjugated dienes and conjugated trienes were determined by a spectrophotometric method, according to the European Regulation EC2568/91. The absorbance of the appropriately diluted samples in isooctane was measured at 232 nm and 268 nm of wavelength to determine the conjugated dienes and trienes, respectively (spectrophotometer Evolution 201 VisibleUV, ThermoScientific, Germany).

2.7. Statistical analysis

Results were submitted to analysis of variance (ANOVA) using Statgraphics Centurion XVI software (Manugistics Corp., Rockville, Md.). Fisher's least significant difference (LSD) procedure was used at the 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Microstructure, moisture content, solubility, thickness, barrier mechanical and optical properties

The microstructure of the films was studied to observe the organization of the different components and the effects on the film structure when the active compound is added, and for a better understanding of physical properties of the film (Vargas et al., 2011). The microstructure of the cross-section of the films is shown in **Figure 1.** The presence of eugenol did not notably affect the microstructure of the starch matrix. This could be explained by high losses of eugenol during the drying process due to water vapor distillation and the good integration of the remaining eugenol within the films.

When eugenol was incorporated microencapsulated in powder, film thickness was increased, as shown in **Table 2**, and film microstructure was modified, especially in S-E-LE where a certain level of phase separation and creaming was observed. This shows that the incorporation of microencapsulated eugenol powder modified film microstructure, which is consistent with the mechanical properties of the films (**Table 2**). The most significant differences in terms of film thickness were obtained in films containing OA, which showed structural discontinuities in accordance with the lack of miscibility of the components (Fabra et al., 2009). In these films, small cavities produced by OA droplets that evaporated under vacuum conditions in the FESEM equipment were observed. This coincides with the observations of Jimenez et al., (2012a) in wheat starch films containing fatty acids. In films containing LE, the effect of OA addition was less marked, since lecithin retained the OA.

The equilibrium moisture content (EMC) of the films stored under conditions of 53% RH and 25°C is also presented in **Table 2**, together with film solubility in water, barrier and tensile properties. The highest EMC was observed for S and S-E films due to their higher starch and glycerol content (**Table 1**). Glycerol contains hydroxyl groups capable of interacting with water by means of hydrogen bonds, providing greater affinity for water in the films (Farahnaky et al., 2013).
The incorporation of microencapsulated eugenol significantly decreased the EMC of the films, possibly due to the reduced starch and glycerol content of these films (**Table 1**). As expected, EMC was reduced significantly in S-EOA-WP and S-EOA-LE ($p < 0.05$) since OA shows a lower water adsorption capacity (Sánchez-González et al., 2011; Fabra et al., 2010).

As concerns water solubility, the lowest values were obtained in S-E and S formulations. The incorporation of encapsulated eugenol produced a significant ($p < 0.05$) increase in solubility, consistent with the soluble character of the wall materials, especially MD. S-EOA-WP and S-EOA-LE films showed intermediate values due to the presence of OA, which implied an increase in the lipid fraction that is not soluble in water.
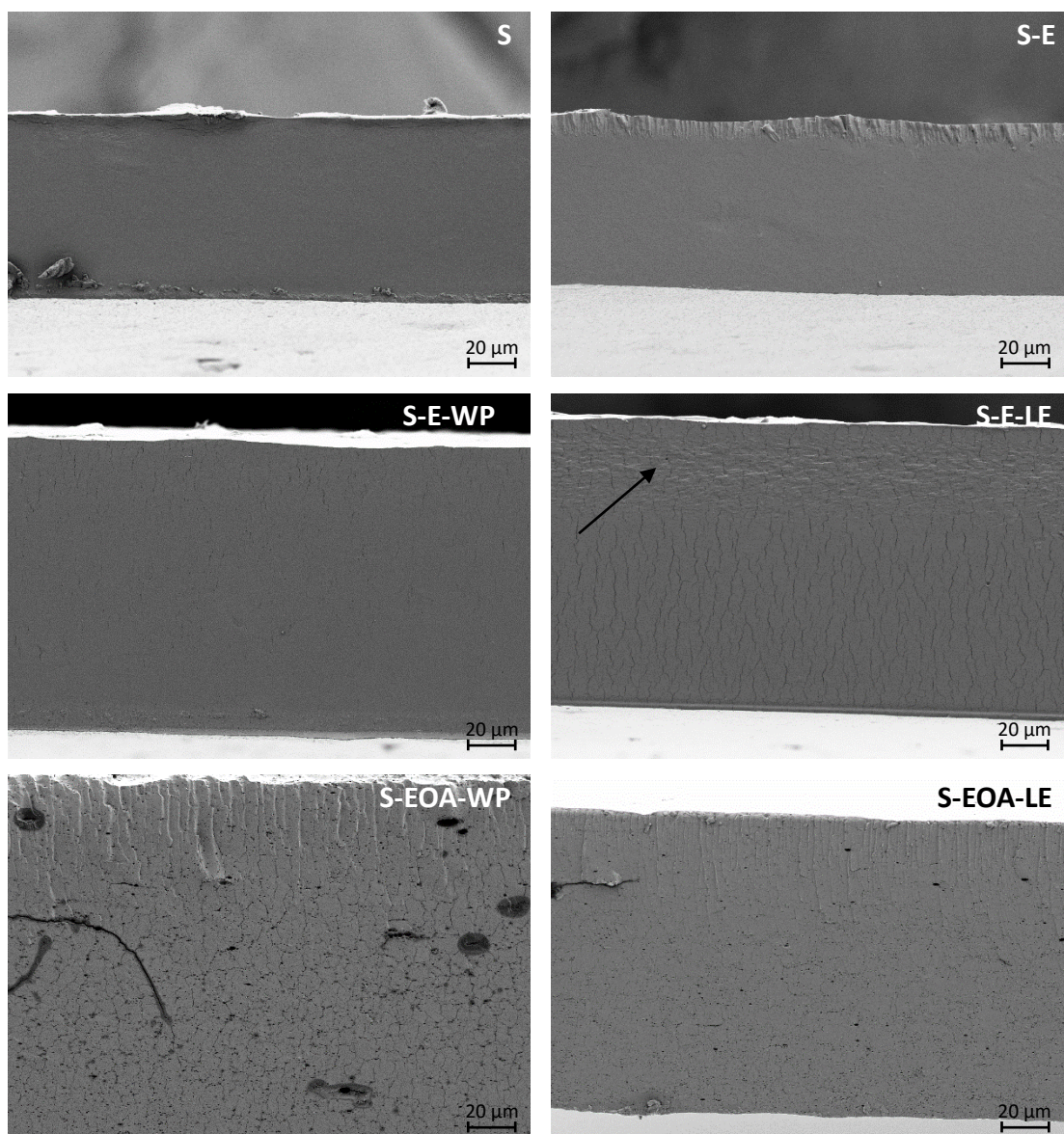
Figure 1. Field emission scanning electron microscope micrographs of the cross-sections of the films.

Table 2 shows the OP of the films. Eugenol exhibited oxygen scavenging and significantly reduced the OP of the films, especially when eugenol was incorporated in S-E-WP films, in accordance with the lowest amount of lipids of this film (**Table 1**). Bonilla et al. (2013b) obtained similar results when adding lipids with antioxidant properties into hydrocolloid-based films. When the lipid shows antioxidant activity, a chemical oxygen blocking effect produced by the lipid could be responsible for the reported improvement in the oxygen barrier properties. In the same way, Jimenez et al., (2013) observed a similar reduction in the OP when incorporating tocopherol into starch-sodium caseinate films.

The addition of essential oils into hydrophilic films usually causes a decrease in the WVP, as the hydrophobicity and tortuosity of the film matrix is increased (Acosta et al., 2016). In the present study, no changes in WVP values were registered when eugenol was incorporated in the starch matrix in free form (**Table 2**). Nevertheless, a significant increase in the WVP was obtained when eugenol was added in WP-based microcapsules. The water vapor transfer process in films also depends on the hydrophilic/hydrophobic ratio of the film constituents. Besides, interactions of oil components with hydrophilic proteins such as WPI could promote the decrease in the hydrophobic character of film matrix, thus could increase the WVP (Ahmad et al., 2012). On the contrary, when eugenol was added encapsulated into LE-based powder, the negative effect on WVP was mitigated, probably due to the lipid nature of LE, which conferred a more hydrophobic character to the matrix. However, when OA was added WVP of S-EOA-LE films significantly increased, which indicates that there is a limit for addition of hydrophobic compounds into hydrophilic films (Kechichian et al., 2010).

The tensile strength (σ) and the elongation at break (%E) are the maximum stress and elongation that a film can with stand before it breaks up, respectively (Parris & Coffin, 1997). The elastic modulus (EM) is related to the stiffness of the material at low strains. These three mechanical parameters are useful for describing the mechanical properties of the films, and are closely related to its structure (McHugh & Krochta, 1994). The values of the elastic modulus (EM), the maximum tensile strength (σ) and elongation (%E) at break are shown in **Table 2**. Pure starch films showed a mechanical behavior that was similar to that found in previous studies (Jiménez et al., 2012b; Talón et al., 2017). The incorporation of eugenol had variable effects on the mechanical parameters, depending on film composition. When eugenol was added to starch films in free form, films became more extensible with a lower EM. Lipids introduced discontinuities in the polymer chain organization that contributed to a reduction in the polymer cohesion forces and favoured the sliding of the chains during film stretching thus reducing EM and increasing %E (Bonilla et al., 2012). This plastic behaviour was more pronounced with the increase in the lipid content of the films. S-E-WP films showed the opposite effect in terms of mechanical behaviour, due to the non-lipid nature of WP and its possible incompatibility with starch, which could promote interruptions in the film matrix.

Table 2. Oxygen permeability (OP, $cm^3 \cdot mm \cdot m^{-2} \cdot atm^{-1} \cdot day^{-1}$), water vapor permeability (WVP, $g \cdot mm \cdot KPa^{-1} \cdot h^{-1} \cdot m^{-2}$), equilibrium moisture content (EMC, g $H_2O \cdot 100^{-1}$g dry film), water solubility (WS, g soluble film/100^{-1}g film), thickness (μm), elastic modulus (EM, MPa), tensile strength (σ, MPa) and percentage of elongation (%E) at break of films equilibrated at 25°C-53% RH. Mean values and standard deviation, in brackets.

Formulation	OP (cc·mm/ (m²·atm·day)	WVP (g·mm/KPa·h·m²)	EMC (g H_2O/100g dry film)	Solubility (g soluble film/100g film)
S	0.537 (0.019)[c]	5.33 (0.08)[a]	14.2 (0.3)[d]	24 (4)[a]
S-E	0.49 (0.02)[bc]	5.31 (0.19)[a]	13.3 (0.3)[c]	22 (8)[a]
S-E-WP	0.13 (0.03)[a]	5.79 (0.11)[b]	10.80 (1.02)[b]	45 (5)[b]
S-EOA-WP	0.324 (0.016)[ab]	5.94 (0.06)[bc]	9.7 (0.2)[a]	37 (2)[b]
S-E-LE	0.26 (0.14)[ab]	5.4 (0.3)[a]	10.18 (0.19)[ab]	46 (4)[b]
S-EOA-LE	0.6 (0.3)[c]	6.12 (0.13)[c]	9.5 (0.2)[a]	37 (5)[b]

Formulation	Thickness (µm)	EM (MPa)	σ (MPa)	%E
S	82 (5)[a]	238 (38)[b]	6.5 (0.7)[b]	15 (5)[b]
S-E	85 (6)[b]	167 (94)[a]	6.3 (1.6)[b]	30 (9)[c]
S-E-WP	121 (7)[c]	342 (90)[c]	6.23 (1.15)[b]	4.0 (1.7)[a]
S-EOA-WP	133 (7)[d]	158 (70)[a]	4.3 (0.8)[a]	33 (9)[cd]
S-E-LE	132 (5)[d]	109 (25)[a]	4.4 (1.0)[a]	40 (8)[de]
S-EOA-LE	136 (6)[e]	144 (27)[a]	4.9 (0.9)[a]	45 (11)[e]

[abcde] Different letters in the same column indicate significant difference among formulations (p<0.05).

3.1.1. Optical properties

Table 3 shows the lightness, chroma and hue of the films. Starch films containing free eugenol became more yellow (higher h*ab values) with respect to pure starch films. The incorporation of microencapsulated eugenol significantly reduced the lightness and hue and increased the chroma (towards a more saturated colour), especially in films containing LE. The addition of microencapsulated eugenol to films reduced the T_i values and especially in those films containing oleic acid. Jimenez et al. (2014) observed a similar behaviour when adding nanoliposomes in corn starch and sodium caseinate films, which showed reduced Ti values mainly at low wavelength.

Table 3. Lightness (L*), hue (h_{ab}*), chroma (C_{ab}*) and internal transmittance (T_i) at 460 nm of wavelength values of the film. Average values and standard deviations, in brackets.

Formulation	L*	C_{ab}*	h_{ab}*	Ti (460 nm)
S	82.21 (1.05)[d]	3.3 (0.3)[a]	102.1 (1.4)[f]	0.857 (0.003)[d]
S-E	82.7 (0.3)[d]	6.367 (0.103)[b]	93.5 (0.5)[e]	0.86 (0.00)[d]
S-E-WP	79.2 (0.4)[b]	11.9 (0.3)[c]	87.13 (0.15)[c]	0.836 (0.003)[c]
S-EOA-WP	79.9 (0.7)[c]	13.2 (0.5)[d]	91.6 (0.4)[d]	0.806 (0.004)[b]
S-E-LE	78.0 (0.7)[a]	14.0 (0.3)[e]	78.6 (0.3)[a]	0.830 (0.003)[c]
S-EOA-LE	77.36 (0.10)[a]	18.2 (0.4)[f]	83.6 (9.3)[b]	0.751 (0.013)[a]

[abcdef] Different letters in the same column indicate significant difference among formulations (p<0.05).

3.2. Antioxidant activity

The EC_{50} and total eugenol content values of the films, according to Folin-Ciocalteu method are shown in **Table 4**. EC_{50} is defined as the concentration of antioxidant compound that causes a 50% decrease in the $DPPH^{\cdot}$ radical absorbance. Thus, the lower the EC_{50} values, the greater the antioxidant activity of the tested sample. The reaction took place for 4 hours of reaction, based on previous studies (Talón et al. 2017). A similar reaction time was found by Bortolomeazzi et al., (2010) in studies carried out with eugenol and other polyphenols from wood smoke and smoke flavourings used by the food industry.

All formulations exhibited antioxidant activity and there were significant differences among the films ($p <0.05$). EC_{50} value of pure eugenol is in accordance with the results obtained by Brand-Williams et al. (1995).

The lowest antioxidant activity was found in films containing non-encapsulated eugenol (the greatest EC_{50} value), which is consistent with the lowest total eugenol content of this films detected by the Folin-Ciocalteu method (5.6 g eugenol/ 100 g initial eugenol). The loss of eugenol during film drying is in the range of the losses reported by Sanchez-González et al. (2011a) during the formation of chitosan films containing essential oils.

When eugenol was previously microencapsulated, the total eugenol content detected in the films significantly increased, and thus the antioxidant activity was also improved (lower EC_{50}). The lowest EC_{50} values were found in S-EOA-LE films (5.6 mg GAE/100 g, 31 g eugenol/100 g initial eugenol). Thus, the addition of oleic acid and LE as carriers improved the preservation of the antioxidant activity of eugenol during film formation. This can be explained by the affinity of eugenol to OA, which contribute to the binding of the compound. Monedero et al. (2010) observed a similar behaviour when studying the release of n-hexanal from soy protein films with blends of beeswax and OA. Pokharkar et al. (2011) also observed a positive effect of OA on the retention of eugenol in nanostructured gels with mixtures of stearic acid and OA.

Table 4. Antioxidant activity of films containing free and microencapsulated eugenol, expressed in terms of EC$_{50}$ values, and their total eugenol content calculated by the Folin-Ciocalteu method and by UV-method.

Sample	EC$_{50}$			Folin-Ciocalteu		UV-method
	mg film/mg DPPH	mg E/mg DPPH	mol E/mol DPPH	mg GAE/g film	g extracted E/100 g initial E	mg E/g film
S-E	117 (12)[d]	3.8 (0.4)[d]	9.09 (1.04)[d]	1.82 (0.14)[a]	5.6 (0.4)[a]	1.7 (0.2)[a]
S-E-WP	87 (4)[c]	2.07 (0.10)[c]	5.0 (0.2)[c]	2.3 (0.5)[ab]	11 (2)[ab]	1.6 (0.3)[a]
S-EOA-WP	40.9 (1.3)[a]	0.84 (0.03)[b]	2.03 (0.06)[b]	4.5 (0.3)[c]	27 (2)[c]	3.9 (0.2)[c]
S-E-LE	74 (2)[b]	1.81 (0.06)[c]	4.36 (0.15)[c]	3.0 (0.8)[b]	13 (4)[b]	2.41 (0.18)[b]
S-EOA-LE	34 (4)[a]	0.69 (0.08)[b]	1.65 (0.19)[b]	5.6 (0.6)[d]	31 (3)[d]	5.2 (0.6)[d]
Eugenol	-	0.091 (0.002)[a]	0.219 (0.005)[a]			

[abcd] Different letters in the same column indicate significant difference among samples (p<0.05).

3.3. Kinetics of eugenol release

The ratio of the active compound released in each food simulant, with respect to that of the equilibrium value, as a function of contact time and fitted Fick's model is shown in **Figure 2a**, and apparent diffusivity (D) values are shown in **Table 5**. Diffusivity values of eugenol were significantly affected by the type of simulant and by film composition (p<0.05). Diffusion of eugenol is the result of swelling, the affinity of eugenol with both the film matrix and the food simulant. The slower release (high D) obtained in films containing pure eugenol as compared with the films containing Le or OA is explained by the reported complex formation between amylose from starch and lipids (Eliasson, 1994). In this way, in films containing L and OA, starch forms complexes with these lipids and eugenol is released at a higher rate. In general, films containing lecithin showed lower D than those containing WP, since lecithin can retain eugenol in aqueous solution. Moreover, WP can interact with S and promote eugenol release. The lowest D was obtained in ET50% where the swelling is significantly reduced.

Table 5 shows the Peleg's model kinetic constants (k$_1$, k$_2$ and M$_\infty$) for the four food simulants, and the corresponding regression coefficients. In all cases, a good fitting of the model was obtained (R^2 ≥ 0.98) as seen in **Figure 2b**. Constant k$_1$ is related to the release rate at the very beginning of the process, thus the lower value of k$_1$, the higher initial rate. k$_2$ is related to the asymptotic value which can be related to the equilibrium value. In all food simulants, the addition of OA resulted in an increase in the initial eugenol release rate (lower values of k$_1$) possibly due to the more open structure of these films (**Figure 1**).

Films with pure eugenol (S-E) showed the lowest values of M$_\infty$ (total eugenol concentration in equilibrium) in all simulants. However, the amount of eugenol released with respect to the total eugenol amount of these films was significantly higher in ET50% simulant (the less polar solvent), due to the highest affinity of the eugenol with this

simulant. When eugenol was microencapsulated prior to its incorporation into the films, the released amount of eugenol was higher, especially when lecithin was used as the encapsulating material. In all cases, S-EOA-LE films showed the highest M_∞ values regardless the simulant.

Films containing oleic acid (S-EOA-WP and S-EOA-LE) showed higher equilibrium eugenol concentrations as compared to S-E-WP and S-E-LE in all simulants. Moreover, the ratio M_∞/M_0 in these films decreased significantly with the decrease in the polarity of the simulants. The addition of OA could promote the retention of eugenol during film processing, being this effect more significant in the less polar solvent (ET50%). Perdones et al., (2014) observed that OA promoted the retention of cinnamon leaf essential oil in chitosan-based matrices during film drying.

Table 5. Diffusivity (D, $m^2 \cdot s^{-1}$), parameters of Peleg´s model: k_1 (min·g film·mg^{-1} eugenol); k_2 (g film·mg^{-1} eugenol); M_∞ ($1/k_2$, mg E released at equilibrium·g^{-1} film), M_∞/M_0 (g eugenol released at equilibrium/g eugenol in the film). Mean values and standard deviation, in brackets.

Simulants	Parameters	S-E	S-E-WP	S-EOA-WP	S-E-LE	S-EOA-LE
AA3%	$D \times 10^{13}$	0.77 (0.06)[a,1]	1.7 (0.3)[ab,1]	3.2 (0.4)[c,2]	2.6 (0.2)[bc,1]	2.615 (0.012)[bc,1]
	k_1	90 (26)[c,2]	99 (32)[c,2]	12.11 (0.18)[a,1]	52.8 (1.5)[b,2]	13.7 (0.9)[a,1]
	k_2	0.99 (0.07)[d,2]	0.63 (0.06)[c,1]	0.470 (0.019)[b,1]	0.62 (0.07)[c,12]	0.3150 (0.0016)[a,1]
	M_∞	1.02 (0.07)[a,12]	1.59 (0.15)[b,2]	2.13 (0.09)[c,2]	1.63 (0.19)[b,12]	3.175 (0.017)[d,23]
	M_∞/M_0*	0.61 (0.04)[ab,1]	0.96 (0.09)[c,3]	0.54 (0.02)[a,2]	0.68 (0.08)[b,12]	0.61 (0.03)[ab,1]
	R^2	≥ 0.9958	≥ 0.9847	≥ 0.9996	≥ 0.9951	≥ 0.9994
ET10%	$D \times 10^{13}$	4.9 (0.3)[b,3]	11.2 (0.4)[d,3]	3.3 (0.4)[a,2]	8.8 (1.6)[c,3]	8.2 (0.8)[c,3]
	k_1	45 (27)[b,1]	11 (3)[a,1]	10.0 (1.3)[a,1]	15 (8)[a,1]	8.6 (0.8)[a,1]
	k_2	1.09 (0.12)[e,3]	0.767 (0.017)[d,23]	0.42 (0.03)[b,1]	0.66 (0.03)[c,2]	0.309 (0.015)[a,1]
	M_∞	0.9274 (0.1114)[a,1]	1.30 (0.03)[b,1]	2.38 (0.17)[c,3]	1.51 (0.07)[b,1]	3.24 (0.16)[d,3]
	M_∞/M_0*	0.55 (0.07)[a,1]	0.790 (0.017)[b,12]	0.60 (0.04)[a,2]	0.61 (0.03)[a,1]	0.62 (0.03)[a,1]
	R^2	≥ 0.9976	≥ 0.9987	≥ 0.9979	≥ 0.9994	≥ 0.9922
ET20%	$D \times 10^{13}$	2.43 (0.02)[a,2]	6.4 (0.4)[bc,2]	9.10 (0.15)[d,3]	5.38 (0.15)[b,2]	7.2 (1.6)[c,2]
	k_1	66 (19)[b,12]	29 (4)[a,1]	8 (2)[a,1]	30 (10)[a,12]	10.4 (1.2)[a,1]
	k_2	1.025 (0.018)[d,23]	0.76 (0.05)[c,2]	0.48 (0.03)[b,1]	0.56 (0.02)[b,1]	0.339 (0.016)[a,1]
	M_∞	0.976 (0.017)[a,1]	1.31 (0.08)[b,1]	2.09 (0.13)[d,2]	1.80 (0.06)[c,2]	2.96 (0.14)[e,12]
	M_∞/M_0*	0.58 (0.09)[a,1]	0.80 (0.05)[b,2]	0.53 (0.03)[a,2]	0.75 (0.03)[b,2]	0.57 (0.03)[a,1]
	R^2	≥ 0.9981	≥ 0.9988	≥ 0.9997	≥ 0.9954	≥ 0.9932
ET50%	$D \times 10^{13}$	0.21 (0.14)[a,1]	1.54 (0.05)[b,1]	2.1 (0.5)[b,1]	2.3 (0.5)[b,1]	2.5 (0.4)[b,1]
	k_1	333 (22)[d,3]	124 (28)[c,2]	88 (24)[b,2]	82 (4)[b,3]	33 (23)[a,1]
	k_2	0.82 (0.09)[c,1]	0.86 (0.04)[c,3]	0.62 (0.08)[b,2]	0.62 (0.05)[b,12]	0.36 (0.05)[a,1]
	M_∞	1.24 (0.14)[a,2]	1.17 (0.06)[a,1]	1.6 (0.2)[b,1]	1.62 (0.13)[b,12]	2.8 (0.4)[c,1]
	M_∞/M_0*	0.74 (0.08)[c,2]	0.71 (0.04)[c,1]	0.42 (0.06)[a,1]	0.67 (0.05)[c,12]	0.54 (0.07)[b,1]
	R^2	≥ 0.9817	≥ 0.9978	≥ 0.9915	≥ 0.9824	≥ 0.9972

* Calculated from the total initial eugenol content in the film obtained by methanol extraction UV (**Table 4**).

[abcd] Different letters in the same line indicate significant difference among formulations (p<0.05).

[1234] Different numbers in the same column indicate significant difference among food simulants (p<0.05).
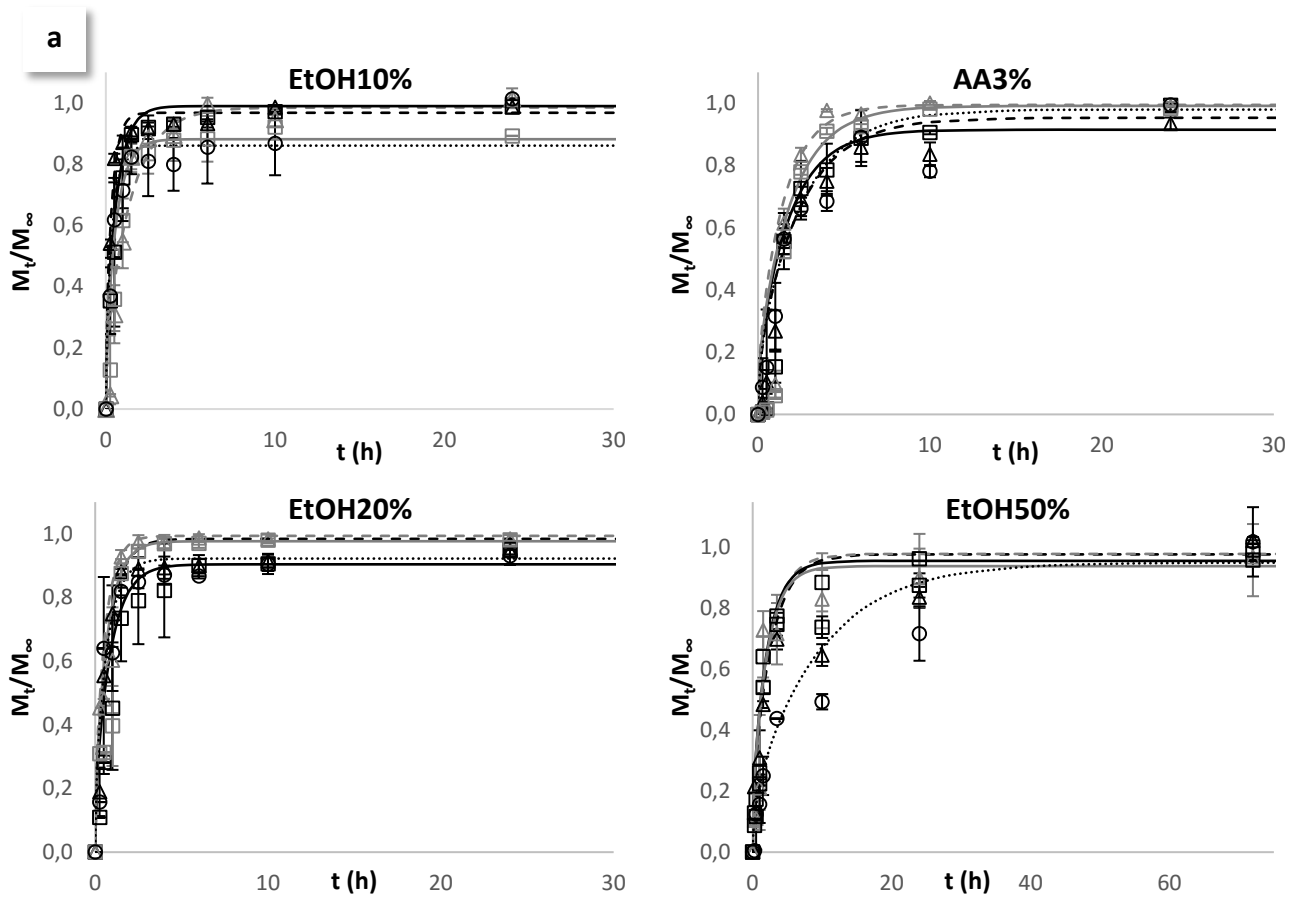
Figure 2. a) Ratio of the active compound released in each food simulant, with respect to that of the equilibrium value, as a function of contact time (points) and fitted Fick's model (lines): experimental data (Δ S-E-WP; △ S-EOA-WP; □ S-E-LE; □ S-EOA-LE; O E-S) and fitted Fick's model (- - - S-E-WP - - - S-EOA-WP; —— S-E-LE; —— S-EOA-LE; ··· S-E).
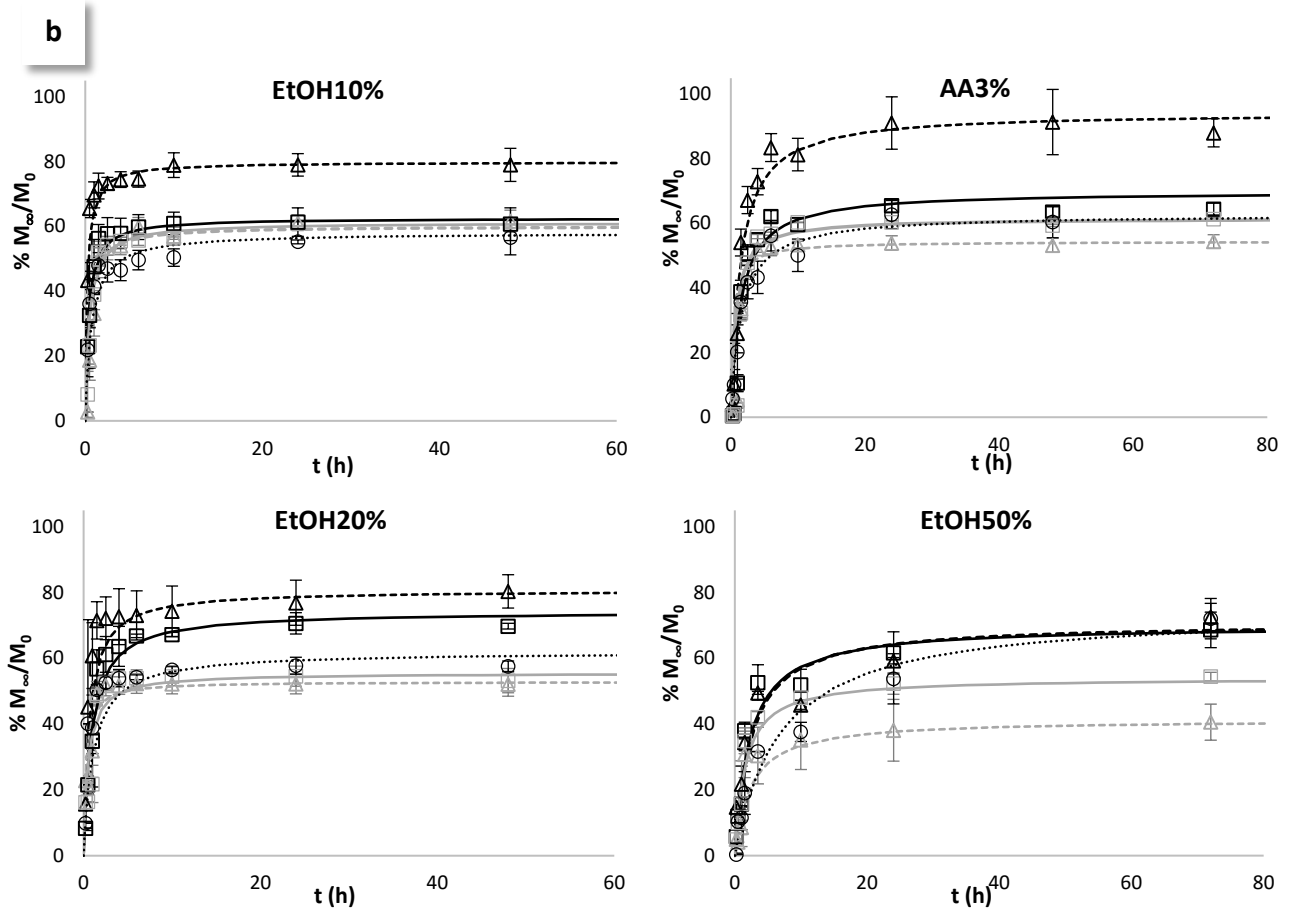
Figure 2. b) % Released (M_∞/M_0) with respect to the total eugenol content (**Table 4**) as a function of contact time (points) and fitted Peleg's model (lines), in different solvents: experimental data (△ S-E-WP; △ S-EOA-WP; □ S-E-LE; □ S-EOA-LE; O E-S) and fitted Peleg's model (- - - S-E-WP - - - S-EOA-WP; —— S-E-LE; —— S-EOA-LE; ••• S-E).

3.4. Antioxidant performance of the films in preventing sunflower oil oxidation

The antioxidant power of S-EOA-LE films in protecting sunflower oil was evaluated through the analysis of the evolution of peroxide index (PV) for 18 days, at 30°C and 53% RH, and with the presence of light to promote oil oxidation. S-EOA-LE film was selected as a potential food packaging material due to its interesting mechanical properties together with its higher eugenol content and greater antioxidant capacity (**Table 4**). The antioxidant effect of this film was compared to that of commercial low density polyethylene packaging (PE), to that of pure starch film (S, without eugenol) and to an open glass Petri dish (Open Control). The PV is associated with the presence of peroxides derived from the polyunsaturated fatty acids present in the sample. Hydroperoxides are produced as primary oxidation products that could be derived into secondary products. Thus, the PV shows the initial oxidation stage (Sadeghi et al., 2017; Kiralan et al., 2017).

Figure 3 shows the evolution of PV and conjugated dienes and trienes during storage. The initial PV of sunflower oil was 1.34 mEq O_2/kg. Similar results were obtained by Mohdaly et al. (2010). A continuous increase in PV was observed with the increase in storage period for all controls (S, PE, and Open Control), reaching values of 9.4 mEq/kg, 135.8 mEq/kg and 115.8 mEq/kg, respectively at 18 days of storage. As observed, different range of PV were reached and significant differences between samples were detected (p<0.05). The lowest increase in PV was registered for samples packaged in S and S-EOA-LE films. This could be explained by the low OP of this films, with no significant differences in terms of oxygen barrier properties (**Table 2**). In samples packaged with S-EOA-LE films, PV was kept at an average of 3 mEq O_2/kg during all storage period, thus indicating the great antioxidant effect of eugenol in this film. These results are in accordance with previous studies on the antioxidant effect of essential oils. In this sense, Reis et al. (2015) observed a reduction in the oxidation ratio in terms of PV of palm oil packaged in cassava starch films containing mango pulp and yerba mate extract. Galarza et al. (2017) reported the prevention of oxidation in sunflower oil that was packaged in red rice flour films.

In the present study, PE samples led to PV higher than those obtained in the Open Control during storage. This could be explained by the degradation of low density PE due to the presence of oxygen, which leads to formation of peroxide and hydroperoxide products by the reaction of oxygen with an alkyl site (Chabira et al., 2008; Chabira et al., 2011).

In order to demonstrate the long-term effectiveness of S-EOA-LE film packaging in preventing oil oxidation as compared to S films, PV was also measured after 53 days of storage. Samples packaged with S films reached a PV value of 10.69 ± 1.09 mEq/kg, which was above the peroxide limit established by the Codex Alimentarius for refined oils (10 mEq/kg). However, samples packaged in S-EOA-LE films showed a PV value

around 4.0 ± 0.4 mEq/kg, thus indicating that the antioxidant effect of eugenol remained for a long time.

Conjugated dienes and conjugated trienes were also measured and the results are shown in **Figure 3B** and **3C**. These compounds are formed by the rearrangement of the hydroperoxides double bonds during oxidation (Dobarganes et al., 2002; Mohdaly et al., 2010). Thus, conjugated dienes represent the primary degradation products of oil and could be used to confirm the PV content while conjugated trienes are related with the secondary products of the oxidation. The initial contents of conjugated dienes and trienes were 3.81 g/100g and 2.85 g/100g, respectively. Samples packaged with PE and the Open Control samples showed an increase in conjugated dienes, which was proportional to the increase in PV. However, both S-EOA-LE and S films maintained low values of conjugated dienes during all storage, which indicates that these films prevent the occurrence of primary degradation products. Conjugated trienes remained constant during the whole storage period for all samples, which shows that sunflower oil did not reach the second stage of degradation (Galarza et al., 2017).
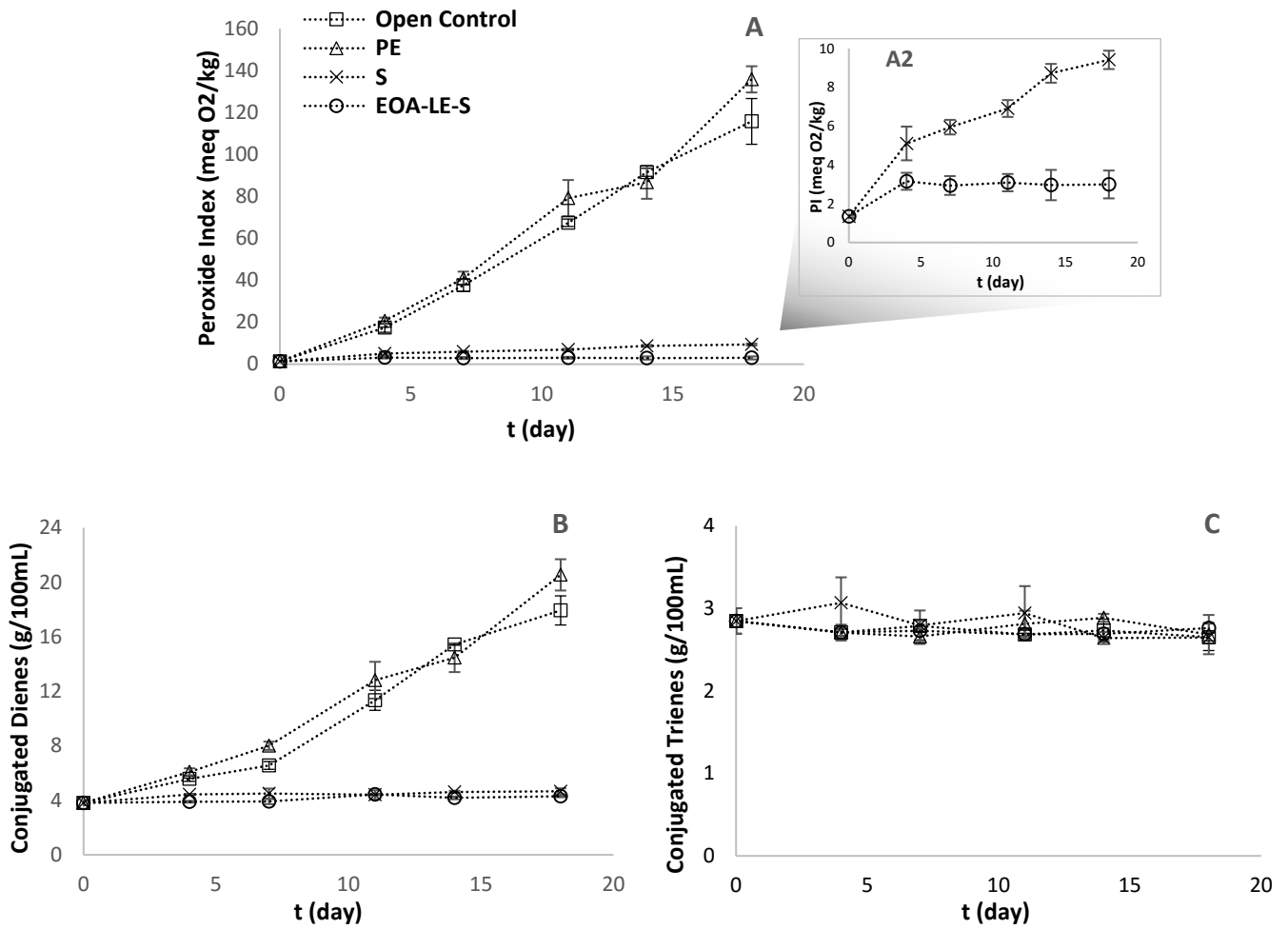
Figure 3. A) Peroxide Index (meq O_2/kg), B) conjugated dienes and C) conjugated trienes (in g/100g) of sunflower oil packaged with S-EOA-LE (O), S (×) and PE (Δ) films and in a glass Petri dish (□ open control).

4. CONCLUSIONS

The addition of eugenol encapsulated with lecithin or whey protein in starch-based films modified film microstructure and yielded less resistant and less elastic films with reduced moisture content, transparency and oxygen permeability as compared to films prepared with pure eugenol. The release kinetics of eugenol in different food simulants and their antioxidant effect were affected by the wall-material used for the encapsulation and by the polarity of the solvent. The addition of oleic acid and lecithin promoted the release of eugenol and the preservation of the antioxidant activity of the films. Films containing eugenol with lecithin and oleic acid were effective in preventing sunflower oil oxidation in accelerated storage conditions.

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GENERAL DISCUSSION

Currently, in the field of food packaging, there is great interest in the development of biodegradable materials, such as starch and chitosan, in order to contribute both to a reduction in the use of synthetic plastic wastes and to a longer shelf-life of the food products by means of the incorporation of active substances. These materials could eventually replace conventional plastic polymers partially or entirely, since they are low cost, renewable and environmentally friendly. However, the use of these biopolymers as packaging materials is often limited by their high hydrophilicity and their poor mechanical properties when compared with conventional plastic materials.

As commented on above, edible and biodegradable films show a high potential when containing active ingredients such as antioxidant and antimicrobials that can help to extend the shelf life of the food, and their development has been particularly prominent in recent years due to its potential applications in food preservation.

In the present Doctoral Thesis, different strategies to improve the incorporation of different active compounds into starch based films are evaluated. More specifically, the direct inclusion of polyphenols in starch and starch-chitosan based films, with tannic acid as a cross-linking agent, and the incorporation of microencapsulated eugenol in starch matrices, produced both by casting and thermo-compression methods are studied.

The first strategy studied, which correspond with the **Chapter 1**, was to incorporate polyphenols from thyme extract in pea starch, chitosan and starch-chitosan blend films, and to evaluate the effect of tannic acid as a cross-linking agent.

Polyphenols are one of the most numerous and universal groups of active compounds with great antioxidant capacity. The use of polyphenols from aqueous thyme extract could be especially desirable because their acceptable flavor and limited aroma (if any), and thus, their impact on the food sensory attributes will be negligible. According to their structure, polyphenols can easily react with free radicals to exert their antioxidant activity. However, their incorporation into films could limit their potential action due to some losses during film development (i.e, oxidation). In this sense, the strategies followed to incorporate efficiently the polyphenols into films are on the way to enhance the antioxidant capacity of the films and to confer them proper mechanical properties.

Previous works have shown that the partial replacement of starch with chitosan in starch-based films can improve the mechanical properties of starch-based films. Moreover, the physical properties of chitosan based-films can be also improved by the addition of tannic acid (TA), a natural polyphenol with antioxidant and crosslinking capacity, due to its multiple phenolic groups that can interact with positively charged macromolecules such as chitosan (Rivero et al., 2010). TA crosslinking properties as regards chitosan have been related with the establishment of electrostatic interactions (ionic complexations in acidic conditions), ester linkages and hydrogen bonds.

The results obtained in Chapter 1 revealed that the establishment of interactions among film components (active and polymer matrix) markedly determines the film physical properties and the releases kinetic of the active component. Thus, the effective cross-linking effect of TA in CH and CH:S blend films led to a more rigid and compact matrix. Polyphenols from thyme extract also strongly interacted with chitosan chains in both pure chitosan and chitosan-starch blend films, leading to a similar mechanical response than when using TA. This response was no observed when blending polyphenols with pure starch films due to the lack of interactions between them, which gave rise to fast delivery rates and high delivery ratio of polyphenols and so, to a low antioxidant capacity, in accordance with the high solubility of starch matrix and the promotion of the polyphenols oxidation, respectively. On the other hand, TE provided the CH based films with remarkable antioxidant activity, despite the lower polyphenol release obtained due to the presence of polyphenols-chitosan interactions. So, we can concluded that the polyphenols from thyme extract were efficiently incorporated in S and CH-S films and their antioxidant activity depended on the amount of polyphenols delivered from the matrix, which was also affected by the interactions established with the polymer matrix and the polarity and pH of the solvent.

In the **second chapter** of this work, the challenge was to incorporate efficiently active volatile compounds into films and to evaluate how the different film processing methods affected the active retention and functionality. Eugenol was selected as active compound and starch, as a polymer matrix due to its thermoplastic properties.

Eugenol is a natural phenolic substance found as a major compound in different plant essential oils, such as clove, nutmeg, cinnamon or basil with interesting antioxidant and antimicrobial activities. The use of essential oils and other volatile compounds in active films for food preservation remained limited mainly due to their intense aroma, which could affect the sensory attributes of the food products, losses during the film processing and toxicity problems. Besides, many essential oils contains chemically labile components, which can suffer from oxidation processes, chemical interactions or volatilization. In this sense, the strategy followed in order to incorporate efficiently the active compound in starch films was to pre-encapsulate the eugenol in different wall-systems to reduce its losses as well as its sensory impact once incorporated into the film.

In the food industry, the main advantages of having an active compound encapsulated are the ease of handling, the protection during storage and transport and their better control in its release towards foods. In this work, eugenol was successfully microencapsulated by spray-drying using whey protein isolate:maltodextrin and lecithin:maltodextrin wall-systems. The effect of using oleic acid (OA) as eugenol carrier, did not show relevant advantages in the encapsulation efficiency of the microparticles incorporating eugenol but, once the microparticles were incorporated into the films (processed by both the casting and the thermo-compression methods), the OA exerted a positive protection effect, thus reducing the eugenol losses.

The use of these microparticles as an ingredient would allow the foodstuffs to be better preserved against oxidative or microbial decay, thus contributing to extend their shelf-life. Besides, the encapsulation process using whey proteins or lecithins as wall materials protected eugenol against evaporation, thus enhancing its thermal stability and leading to suitable eugenol-based microparticles to be used in dry thermal processes, such as the preparation of an active master batch for the development of thermoformed materials. These microencapsulates could be also used to develop multi-layered films (as most of the conventional plastic materials available at the market), where the combination of different layers of polymers (biodegradable or not) with biopolymers (such as starch) containing active compounds (such as eugenol) could enhance the properties of the packaging.

In order to better analyse the feasibility of all the film materials developed in this thesis to be used in food packaging, a general discussion about influence of the different active compounds incorporation on the physical properties (barrier and mechanical properties) of films as well as on their antioxidant activity and release kinetics has been carried out, as it is commented on below.

It is well known that the main drawbacks of the use of starch as film packaging material are the poor mechanical and water barrier properties, when compared with the conventional plastic materials. Different strategies i.e. blending with other polymers such as chitosan could improve these inconveniences. Furthermore, the incorporation of active agents into starch films can directly affect to their functional properties.

The following figures summarize the main physical properties of all films obtained in this doctoral thesis, for better understanding the main results. In order to compare the effect of the film processing and storage conditions, and the effect of the active compounds incorporation on the matrices, the results have been bring together taking into account the different chapters of the thesis, as described in **Table 1**.

Table 1. Composition and nomenclature of all films developed in this doctoral thesis, organized by chapters.

CHAPTER	DESCRIPTION	FILM COMPOSITION (g/g total solids)										FILM PROCESS	NOMENCLATURE
		S	Glycerol	CH	TA	TE	WP	LE	MD	Eugenol	OA		
I and II	Films based on pea starch and chitosan-starch blends containing polyphenols from thyme extracts and TA as crosslinking-agent	0.833	0.167	-	-	-	-	-	-	-	-	Casting	*S
		0.667	0.167	0.167	-	-	-	-	-	-	-		CH:S
		0.645	0.161	0.161	0.032	-	-	-	-	-	-		CH:S:TA
		0.741	0.148	-	-	0.111	-	-	-	-	-		S:TE
		0.593	0.148	0.148	-	0.111	-	-	-	-	-		CH:S:TE
		0.576	0.144	0.144	0.029	0.108	-	-	-	-	-		CH:S:TA:TE
		-	0.167	0.833	-	-	-	-	-	-	-		CH
		-	0.161	0.806	0.032	-	-	-	-	-	-		CH:TA
		-	0.148	0.741	-	0.111	-	-	-	-	-		CH:TE
		-	0.144	0.719	0.029	0.108	-	-	-	-	-		CH:TA:TE
IV	Corn starch films containing microencapulated eugenol in different wall-systems, processed by compression moulding	0.606	0.182	-	-	-	0.005	-	0.194	0.013	-	Thermo-compression moulding	E-WP-S
		0.606	0.182	-	-	-	0.004	-	0.169	0.011	0.028		EOA-WP-S
		0.606	0.182	-	-	-	-	0.005	0.194	0.014	-		E-LE-S
		0.606	0.182	-	-	-	-	0.004	0.169	0.011	0.028		EOA-LE-S
		0.757	0.227	-	-	-	-	-	-	0.016	-		E-S
		0.769	0.231	-	-	-	-	-	-	-	-		*St
V	Corn starch films containing eugenol, obtained by casting	0.500	0.167	-	-	-	0.007	-	0.305	0.021	-	Casting	S-E-WP
		0.500	0.167	-	-	-	0.006	-	0.266	0.017	0.044		S-EOA-WP
		0.500	0.167	-	-	-	-	0.007	0.305	0.021	-		S-E-LE
		0.500	0.167	-	-	-	-	0.006	0.265	0.018	0.044		S-EOA-LE
		0.728	0.243	-	-	-	-	-	-	0.029	-		S-E
		0.750	0.250	-	-	-	-	-	-	-	-		*Sc

* S is a pure pea starch film obtained by casting, S_c and S_t are pure corn starch films produced by casting and by thermo-compression, respectively.
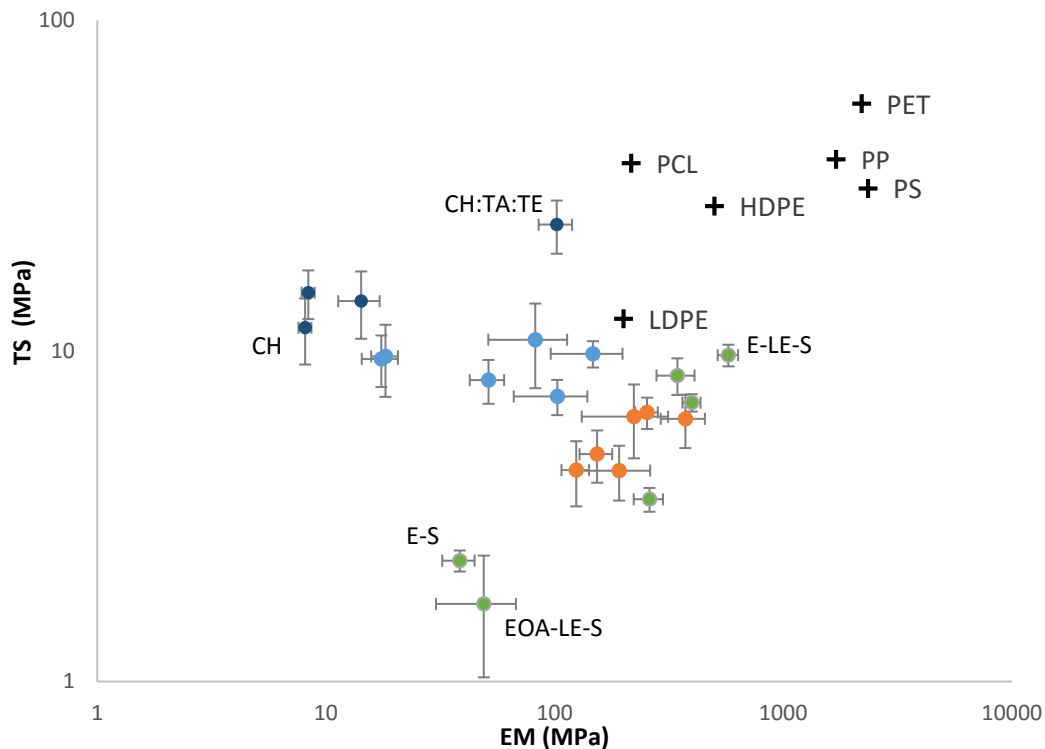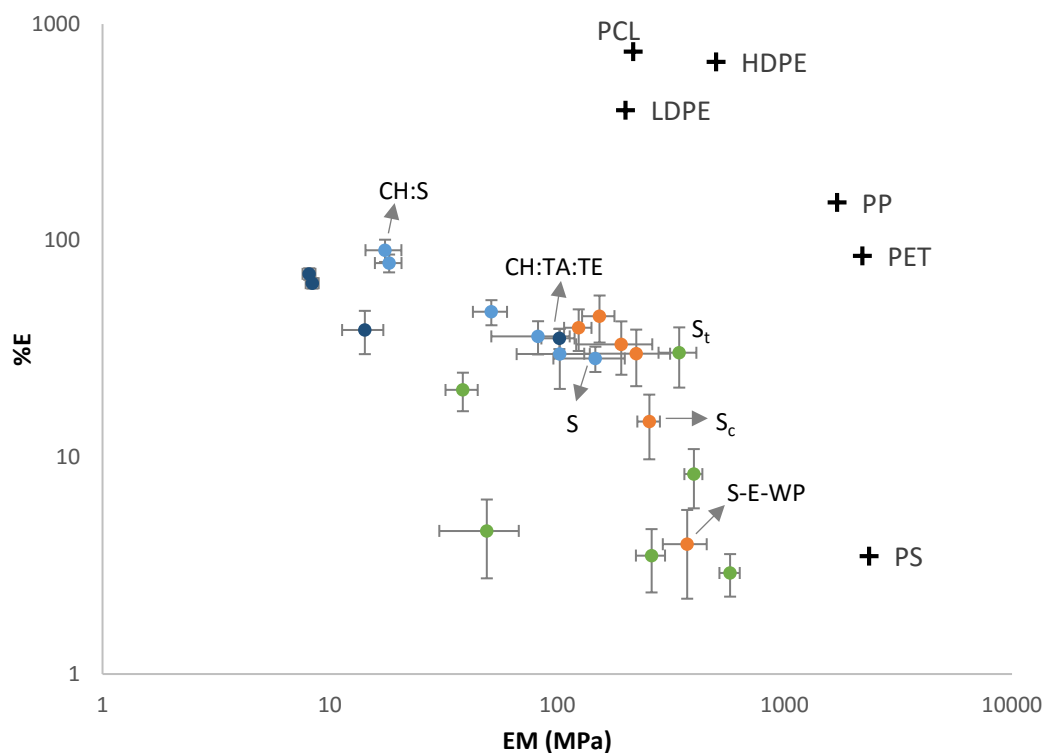
Figure 1. Map of mechanical properties (Tensile Strength *vs.* Elastic Modulus) showing the location of the different film formulations and some synthetic plastics commonly used in food packaging (HDPE, LDPE, PET, PP, PS, PCL) (Cho et al., 1998; Shudesh et al., 2000; Plackett, 2012). ● Chapter 1.I and 1.II (dark blue for CH-based films), ● Chapter 2.IV, ● Chapter 2.V.

Figure 1 shows the correlation between EM (Elastic Modulus, MPa) and TS (Tensile Strength, MPa) of all the films. As can be observed, CH-based films (dark blue dots) produced in the first study (**chapter 1**) showed the highest rigidity and resistance to break, especially when incorporating polyphenols (TE and TA), close to some conventional plastic and with better resistance than some LDPE materials. This behaviour is due to the cross-linking effect between polyphenols and chitosan (Rivero et al., 2010). The opposite effect was observed when TE was incorporated into pure starch films, as they became less rigid (lower EM) and less resistant to fracture (lower TS).

The mechanical response of films obtained by casting and compression moulding incorporating eugenol (orange and green dots) in terms of TS and EM values was quite similar, except for those films obtained by thermo-compression incorporating OA and LE and non-encapsulated E, which showed poor mechanical responses. In general, a better resistance to break was obtained in the thermo-pressed films, thus suggesting the development of a different network organization favoured by the high temperature conditions of the thermo-pressing process. Moreover, the stretchability of the films

obtained by thermocompression moulding (shown in **Figure 2**) was lower than those obtained by casting. In this case, the greater isotropy of the material obtained by casting method seems to play also an important role. Mangavel et al. (2004) also found higher stress and lower strain values of wheat gluten thermo-pressed films compared to cast films due to the establishment of a more densely cross-linked network favoured by the thermal process.

On the other hand, pea starch-based films obtained by casting and thermo-processed starch-based films showed similar TS and EM values than some commercial LDPE plastic, as can be observed in **Figure 1.**

Figure 2. Map of mechanical properties (stretchability *vs.* Elastic Modulus) showing the location of the different film formulations and some synthetic plastics commonly used in food packaging (HDPE, LDPE, PET, PP, PS, PCL) (Cho et al., 1998; Shudesh et al., 2000; Plackett, 2012). ● Chapter 1.I and 1.II (dark blue for CH-based films), ● Chapter 2.IV, ● Chapter 2.V.

As commented on above, **Figure 2** shows the dispersion graph for percentage elongation (%E) *versus* EM. In films produced by casting (**Chapter 1** and **2,** blue and orange dots) a negative correlation between %E and EM parameters was observed, as the more stretchable films exhibited the lower rigidity values. This behaviour was more

pronounced in films conditioned at the higher relative humidity (**chapter 1,** blue dots) in coherence with the water plasticizing effect.

The CH:S blend films exhibited a better mechanical response than pure starch (light blue dots), becoming more stretchable. The addition of polyphenols (TA and TE) in CH-based films (dark blue) gave rise to less stretchable due to the cross-linking effect.

In the films of the **second chapter** produced by casting (orange dots), the incorporation of a lipid component (i.e eugenol) in the starch matrix led to a films significantly more stretchable as it contributed to reduce the polymer cohesion forces and favoured the sliding of the chains during film stretching. S-E-WP films showed the lowest %E values, due to the non-lipid nature of WP and its possible incompatibility with starch, which could promote interruptions in the film matrix. When films were obtained by thermo-compression (green dots), the incorporation of microencapsulated eugenol turned the film less stretchable due also to the presence of discontinuities in the polymer matrix.

As can be observed, some of the developed biodegradable films showed %E values in the range of some commercial plastics, such as PP, PET and PS, although the rigidity of these commercial plastics was very high compared with the films of this work.

In **Figure 3**, the barrier properties of the studied film formulations can be observed, in terms of the oxygen permeability and water vapour rates (OP *vs.* WVTR map), together with the values of some commercial plastics. The transmission rate, described as the quantity of permeant passing through a film per unit of area and time, at steady state, which is related to the permeability values, has been calculated for comparison purposes.

As **Figure 3** shows, the OP values of films from **chapter 1.I** and **II** (blue dots) were significantly higher and the WVTR values were remarkably lower in comparison with the values found for the rest of the films. These effects can be explained by the greater equilibrium moisture content of these films (conditioned at 75% RH), which gave rise to a more plasticized and open matrix.

CH based films (dark blue dots) showed the highest OP values, probably due to the greater water uptake capacity of chitosan versus starch. In general, chitosan based films presented greater WVTR and OP values than the starch films, and blend CH:S films exhibited an intermediate behaviour.

As expected, the conventional plastic presented better water barrier capacity and worse oxygen barrier properties than our biodegradable films equilibrated at intermediate moisture content (53% HR). Thus, a two-layer film consisting of a monolayer of conventional plastic material and monolayer of our starch-based films could offer significant advantages for some food applications.

The incorporation of eugenol in a pre-encapsulated form exhibited a different effect on the WVTR values, depending on the film processing conditions used. Thus, when microencapsulated eugenol was added to the thermoprocessed starch films, WVTR decreased (independently of the microparticles used) due to the partial hydrophobic nature of the components and the higher degree of compactation of the matrix promoted by the blend flowability (leading to thinner films). In the casted starch films, the water barrier effect was influenced by the hydrophilic/hydrophobic ratio of the film (and microparticles) constituents.
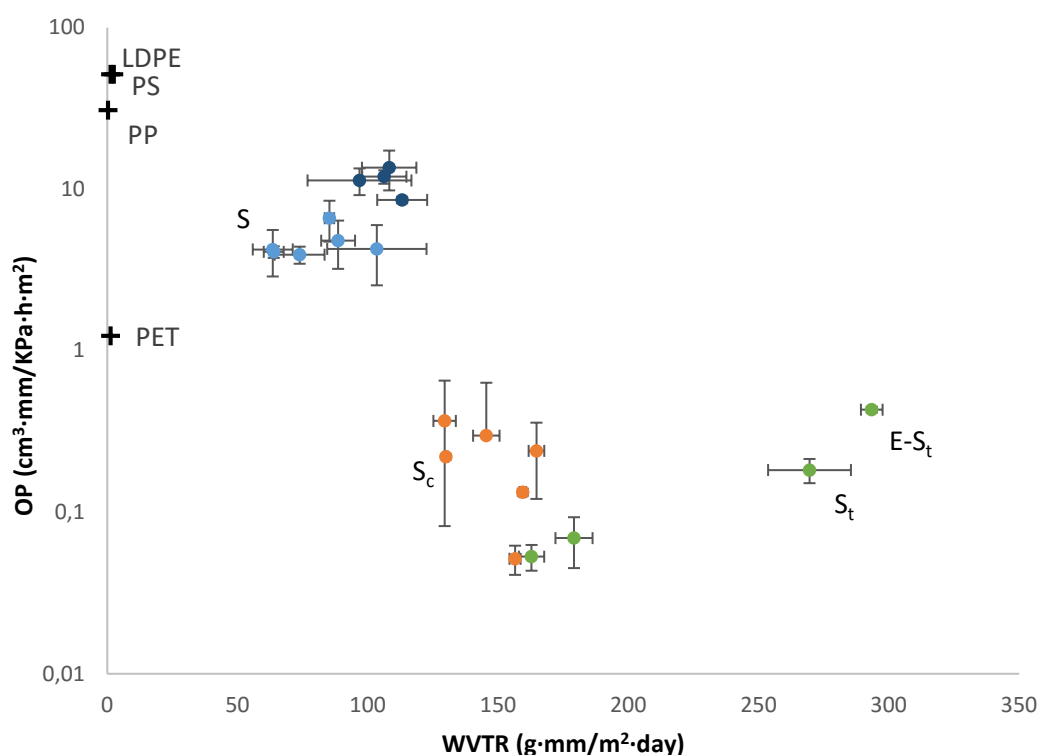
Figure 3. Map of barrier properties showing the location of the different film formulations and some synthetic plastics commonly used in food packaging (HDPE, PET, PP, PS) (Plackett, 2012). 🔵 Chapter 1.I and 1.II (dark blue for CH-based films), 🟠 Chapter 2.V, 🟢 Chapter 2.IV.

Figure 4 shows the maximum percentage of released active ($M_\infty/M_{theoretical}$) from the films, with respect to the initial active compound added to the films ($M_{theoretical}$), which takes into account both the preservation of the active after the film processing and the amount effectively released in different food simulants. In order to compare the films obtained in the different chapters, only two food simulant with different polarity (AA3% and Et20%) have been selected as they showed the more relevant differences.

As can be observed, the ratio depended on the type of active used, polarity and pH of the solvent, the swelling capacity and relaxation of the polymer matrix on each solvent,

the presence of interactions among components and the method used to prepare the films, among other factors.

In both simulants, films obtained in **chapter 1** (casting, blue points) showed release ratio between 10-50%, being the lowest values related to the interactions developed among polyphenols (TA and from TE) and CH. The highest ratio (50 %) was reached by the pure starch films (S-TE) in both simulants. Nevertheless, this can not be seen as an advantage since these films showed the poorest antioxidant activity, as it has been commented on above.

Films obtained by thermo-compression showed the greater M_∞/M_{th} values, as in these films, the eugenol retention during the film processing was promoted in comparison with the cast films. It is recommended to use encapsulation process to incorporate eugenol in the films, as the lowest ratios were found in films using free eugenol (E-S films), due to the losses during film processing and the formation of eugenol-amylose complexes. Other factor affecting the M_∞/M_{th} values of films incorporating eugenol microencapsulated obtained by casting or by thermo-compression were the type of solvent and the composition of the encapsulates. Thus, films containing pre-encapsulated eugenol using OA, obtained by both casting and thermo-compression, obtained the greatest values of the M_∞/M_{th} ratio in the acid medium due to acid hydrolysis of the amorphous regions of starch matrix (Wang and Copeland, 2015), which favoured the complete release of the active compound and to the protective effect of OA against eugenol losses.
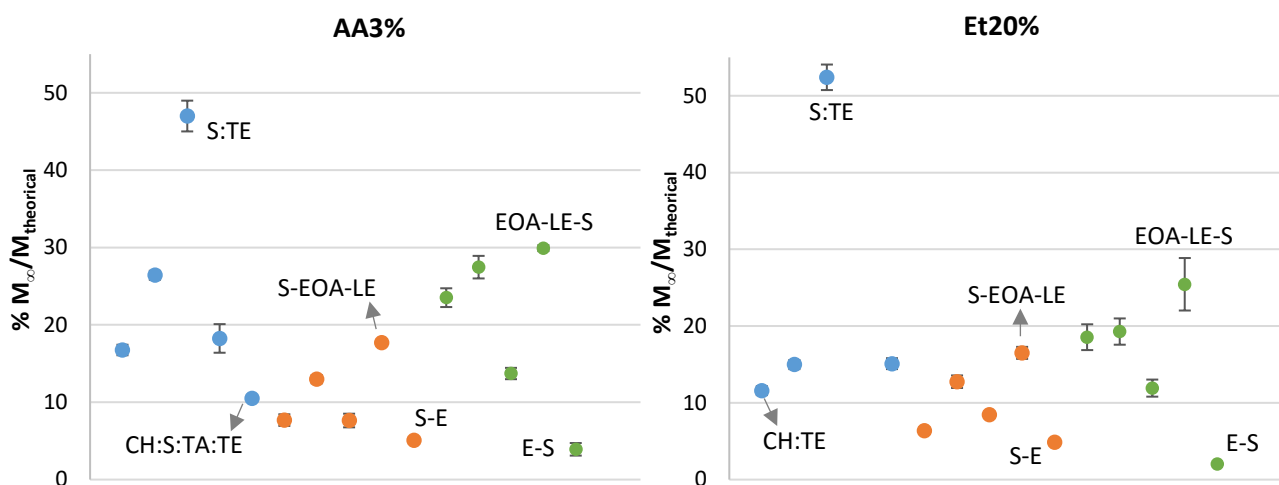

Figure 4. Map of $M_\infty/M_{theoretical}$ (%) ratio of all films obtained from the release studies in acetic acid solution (3%, w/v) (AA3%) and 20% ethanol solution (v/v) (Et20%). M_∞ is the maximum active compound release at equilibrium obtained by fitted Peleg model and $M_{tehoretical}$ is the initial amount of active compound incorporated in the film. ● Chapter 1.II, ● Chapter 2.IV, ● Chapter 2.V.

Finally, **Figure 5** shows the antioxidant activity (AA) of all produced films of this work. For reasons of clarity, the AA was expressed in terms of the antiradical power (ARP), calculated as the inverse of the EC_{50} parameter. Thus, the larger the ARP, the greater the antioxidant activity. Dotted lines indicate the ARP of the pure component incorporated in the different films. As **figure 5** shows, tannic acid was the active compound with the highest AA, followed by eugenol and polyphenols from thyme extract.

The greatest AA was found in the films obtained in the first work containing TA (**chapter 1,** blue dots), in coherence with the greater antioxidant powder of TA. CH and CH blends films were preferred as they prevented the oxidation of polyphenols that occurred during the film formation.

On the other hand, high losses on the AA of starch films incorporating eugenol (**chapter 2.IV** and **2.V**) were observed, especially in films containing eugenol in a free form. This effect was due to eugenol losses, oxidation and the formation of eugenol-amylose complexes. However, when OA was used as eugenol carrier in the microencapsulates, the ARP values significantly increased, thus highlighting certain protective role of OA against eugenol oxidation. Similar results were observed when microencapsulated eugenol was incorporated in thermo-processed starch films.
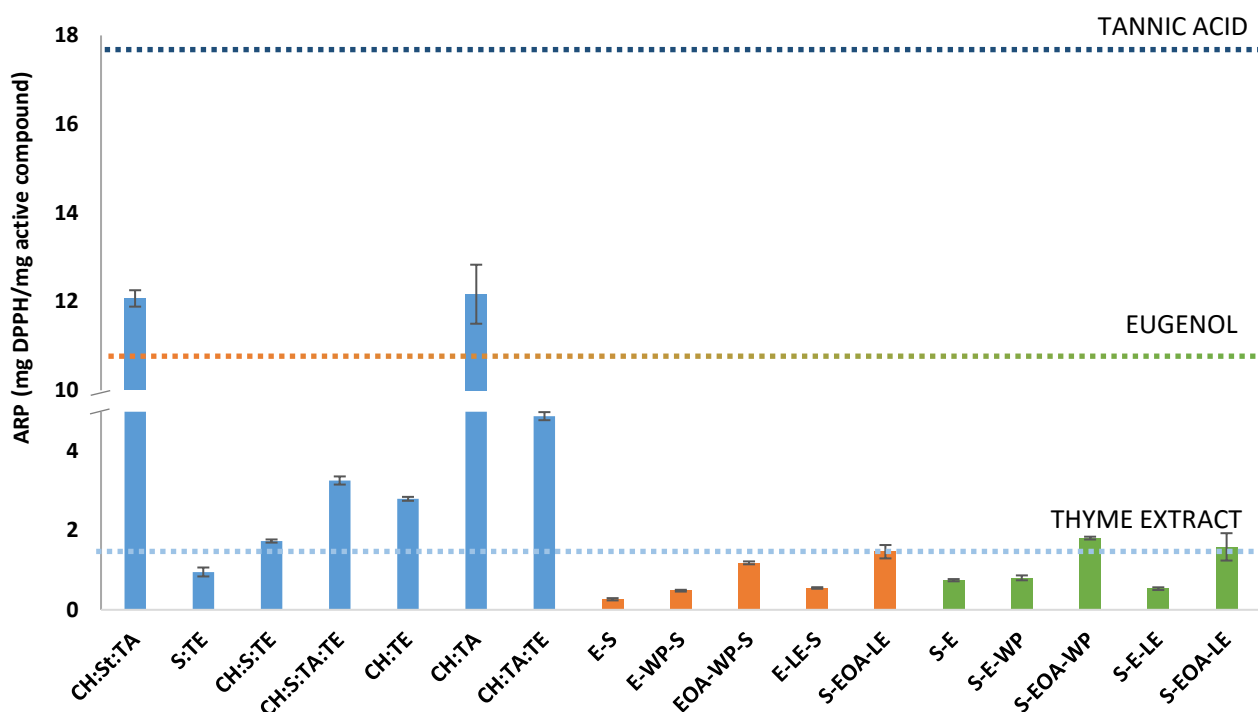
Figure 5. Antiradical power (ARP) of all films (coloured bars) and pure compounds incorporated in the different films (dotted lines). ● Chapter 1.I, ● Chapter 2.IV, ● Chapter 2.V.

Although all films incorporating active compounds showed interesting antioxidant capacity, some losses on the AA occurred due to different factors such as oxidations, establishment of strong interactions with the polymer matrix or formation of complexes that limited the final antioxidant activity of the films. Thus, all these factors play an important role in the final functionality of the films and they have to be taken into account when designing active films for food preservation.

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CONCLUSIONS

1. The use of thyme extract (TE) was analysed as a source of natural polyphenols for the purposes of the development of antioxidant films for food preservation. This extract was efficiently incorporated into starch and chitosan film matrices. The films presented some changes in their microstructural and physical properties due to the incorporation of TE, which depended on the type of matrix. The best results were obtained when thyme extract was added to chitosan based films, due to the cross-linking effect that occurs among the polyphenols and the chitosan, promoting a better tensile response (greater resistance at break and higher degree of stiffness). Nevertheless, films became less stretchable and more opaque due to the structural changes provoked by polyphenols in the matrix. TE improved tensile properties of the films in the same way as TA and the ratio polyphenol-chitosan seems to play an important role in this response. Although the antioxidant activity of TA was higher than of TE, the obtained films also exhibited remarkable antioxidant activity after film formation and conditioning. The results also highlighted the possibility of using these antioxidant films for coating purposes in order to promote the shelf life of the products sensitive to oxidative processes.

2. Polyphenols from thyme extract strongly interact with chitosan chains in both pure chitosan matrix and chitosan-starch blend films. These interactions led to a reduction of polyphenols release rate in aqueous media, even at low pH where, despite the increase in the total delivered amount, this was much lower than that occurred in starch films. Incorporation of tannic acid to the chitosan films provoked the matrix crosslinking, to a greater extent in pure chitosan films, which greatly reduced the ratio and rate of polyphenol release, although in the acid medium, the increase in the chitosan solubility enhanced the total polyphenol delivery. However, in chitosan:starch blends, the cross-linking effect of tannic acid inhibited the release promotion of the acid medium, while both release rate and ratio were enhanced in ethanol solutions, thus indicating the role of chitosan-starch interaction in the blend matrix rearrangement. In fact, the total delivery of polyphenols in CH:S blend films was nearer to that occurred in chitosan films rather than to the delivery in starch films, in spite of the greater starch ratio in the blend.

3. The encapsulation efficiency (EE) of eugenol in spray-dried powders containing whey protein or lecithin as wall materials and maltodextrin as drying coadjuvant was very high (95-98 %), while the incorporation of oleic acid (OA) as eugenol carrier or chitosan (CH) to the liquid formulations did not improve EE. CH provoked emulsion destabilization which had a very negative effect on the EE. All of the encapsulating powders exhibited antioxidant activity, coherent with their respective eugenol content, in line with the fast, complete release of eugenol in aqueous systems. The antibacterial effect of the powders against *E. coli* was also coherent with the eugenol content of the powders, but an additional positive effect of OA was detected in the powder's antilisterial action. All of the encapsulating powders presented small particles and a high affinity /solubility in aqueous systems of differing polarity and pH, which allows for a relatively fast, total release of the active compound. The thermal release of eugenol was also inhibited in

the powders (mainly in those which were CH-free), which would allow for their use in dry thermal processes, such as the preparation of an active master batch of thermoplastic polymers. Their incorporation as an ingredient or in separate sachets in foodstuffs would permit them to be better preserved against oxidative or microbial decay, thus extending their shelf-life.

4. Incorporation of eugenol (encapsulated or not) into the thermo-compressed starch films significantly affected their physical and antioxidant properties. In non-encapsulated eugenol films, this was attributed to the formation of eugenol-amylose complexes which modify the starch matrix. In films with encapsulated eugenol, the partial disruption of the capsules during the film thermoprocessing release different compounds to the starch matrices provoking different degrees of compatibility among components and starch, which induced heterogeneous film microstructure. The microstructural arrangement of the different components in the matrix caused notable changes in tensile and barrier properties in the starch films. In general, the films with encapsulates turned less stretchable and resistant than pure starch films, with lower water sorption capacity and improved water vapor barrier properties. However, microcapsules containing oleic acid provoked a very heterogeneous distribution of this compound in the matrix, which controlled the film oxygen permeability, giving rise to a high variability in this property. The protective effect of microencapsulates on the eugenol retention during the film processing was only effective when encapsulates contained oleic acid as lipid carrier, regardless the wall material used (whey protein or lecithin). The loss of the non-encapsulated eugenol from thermo-compressed film was limited probably due to the formation of lipid-starch complexes, which drastically decreased the amount of eugenol released into the different food simulants. The fastest kinetic and greatest amount of eugenol released was observed in films containing microencapsulated eugenol, especially when the encapsulating materials were more water soluble (E-WP-S) and when the solvent was acid and provoked a partial hydrolysis of the starch matrix, favoring the compound diffusion to the aqueous medium.

5. The addition of eugenol encapsulated with lecithin or whey protein in starch-based films modified film microstructure and yielded less resistant and less elastic films with reduced moisture content, transparency and oxygen permeability as compared to films prepared with pure eugenol. The release kinetics of eugenol in different food simulants and their antioxidant effect were affected by the wall-material used for the encapsulation and by the polarity of the solvent. The addition of oleic acid and lecithin promoted the release of eugenol and the preservation of the antioxidant activity of the films. Films containing eugenol with lecithin and oleic acid were effective in preventing sunflower oil oxidation in accelerated storage conditions.