UNIVERSIDAD COMPLUTENSE DE MADRID

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DESARROLLO DE PELÍCULAS ACTIVAS DE GELATINA CON INCORPORACIÓN DE LIGNINA Y SU APLICACIÓN ALIMENTARIA

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Ruth Núñez Flores

Bajo la dirección de las doctoras

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TESIS DOCTORAL

Ruth Núñez Flores

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Memoria que presenta Ruth Núñez Flores para optar al grado de Doctor por la Universidad Complutense de Madrid

Bajo la dirección de la Dra. M. Elvira López Caballero y la Dra. M. Carmen Gómez Guillén, actuando como tutora la Dra. Manuela Fernández Álvarez

INSTITUTO DE CIENCIA Y TECNOLOGÍA DE ALIMENTOS Y NUTRICIÓN
(CSIC)

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La DOCTORA MARÍA ELVIRA LÓPEZ CABALLERO, Científico Titular del Instituto de Ciencia y Tecnología de los Alimentos y Nutrición, ICTAN (CSIC), y la DOCTORA MARÍA DEL CARMEN GÓMEZ GUILLÉN, Investigador Científico del Instituto de Ciencia y Tecnología de los Alimentos y Nutrición, ICTAN (CSIC),

CERTIFICAN:

Que la presente Memoria titulada **"Desarrollo de películas activas de gelatina con incorporación de lignina y su aplicación alimentaria"**, presentada por **RUTH NÚÑEZ FLORES** para optar al grado de **Doctor**, ha sido realizada en el Instituto de Ciencia y Tecnología de los Alimentos y Nutrición, ICTAN (CSIC) bajo su dirección, y que, hallándose concluida, autorizan su presentación para que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste a los efectos oportunos, firman la presente certificación en Madrid, a 25 de abril de dos mil trece.

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Índice

LISTADO DE ABREVIATURAS

a.f. ángulo de fase

ABTS: ácido 2,2 azino-bis (3-etilbenzotiazolina-6-sulfónico)

ADN: ácido desoxirribonucleico

AP5: salmón tratado por alta presión (300 MPa, 5 °C, 10 min)

AP40: salmón tratado por alta presión (300 MPa, 40 °C, 10 min)

ATP-asa: enzimas capaces de hidrolizar el adenin trifosfato en adenin difosfato

ATR-FTIR: reflectancia total atenuada- espectro-fotometría de infrarrojo por transformada de Fourier

B: gelatina bovina /bovine gelatin

C: salmón cocinado a 90 °C

Ca: calcio

CE: Comunidad Europea

CECT: colección española de cultivos tipo

CEE: Comunidad Económica Europea

DPPH: 2,2-Difenil-1-picrilhidrazilo

DSC: calorimetría diferencial de barrido

EAR: elongación en la ruptura

EET: encefalopatía espongiforme de transmisión

F: gelatina de peces de aguas frías / cold-water fish gelatin

FRAP: capacidad de reducción del hierro

FT: fuerza de tensión

G: gelatina de peces de aguas cálidas /warm-water fish gelatin

G': módulo elástico

G'': módulo viscoso

Gly: glicina

GRAS: generalmente reconocido como seguro

IC_{50}: concentración requerida para una inhibición del 50 % de un proceso dado

LS: lignosulfonato /lignosulfonate

LSA: lignosulfonato cálcico-magnésico de alto contenido en azúcar

LSb: lignosulfonato cálcico-magnésico de bajo contenido en azúcar

LSc: lignosulfonato sódico de bajo contenido en azúcar

L1000: lignina 1000

L2400: lignina 2400

MAP: envasado en atmósfera modificada

MPa: Megapascales

Mg: magnesio

Na: sodio

NBVT: nitrógeno básico volátil total

-OCH_3: grupo metoxi

Ph-OH: grupo fenólico

PVA: permeabilidad al vapor de agua

PAP5: salmón recubierto con película de G-L1000 y tratado por alta presión (300 MPa, 5 °C, 10 min)

PAP40: salmón recubierto con película de G-L1000 y tratado por alta presión (300 MPa, 40 °C, 10 min)

S: salmón sin tratar

SAP: filetes de sardina tratados con alta (300 MPa, 5 °C, 10 min)

SF: solución filmogénica

SP: filetes de sardina cubiertos con película

SPAP: filetes de sardina cubiertos con película y tratados con alta (300 MPa, 5 °C, 10 min)

SV: filetes de sardina envasados a vacío

TBA: ácido tiobarbitúrico

Tf: temperatura de fusión

Tg: temperatura de transición vítrea

Tgel: temperatura de gelificación

TSE: transmission spongiform encephalopathy

1 Resumen / Abstract

1.1 *Resumen*

1.1.1 Introducción

Los envases juegan un papel importante en la conservación de los alimentos. En la sociedad actual existen envases formados por compuestos sintéticos que generan gran cantidad de residuos que ocasionan desventajas económicas y medioambientales (Kirwan & Strawbridge, 2003). Como alternativa se presenta la utilización de compuestos biodegradables (Martín-Belloso, Rojas-Gräu & Soliva-Fortuny, 2009) principalmente biopolímeros, utilizados normalmente como matriz y soporte, y compuestos naturales con propiedades antioxidantes, antimicrobianas, etc.

La gelatina es un material biopolimérico y biodegradable que se presenta como opción para el envasado de alimentos. La gelatina puede proceder de distintas fuentes. En la literatura se recogen numerosos trabajos en los que se utilizan gelatina bovina debido a sus excelentes propiedades; sin embargo puede ser rechazada en algunas ocasiones por razones socioculturales, religiosas o sanitarias (encefalopatía espongiforme de transimisión, EET), por lo que el estudio de gelatinas de diferentes orígenes, alternativas a la gelatina bovina resulta de gran interés (Gómez-Guillén, Giménez, López-Caballero & Montero, 2011), como es el caso de la gelatina de piel de pescado.

La lignina y el lignosulfonato son residuos industriales susceptibles de utilización para la formulación de materiales filmogénicos por su naturaleza polimérica, así como por su biodegradabilidad, baja citotoxicidad, alta capacidad antioxidante, etc. (Satyanarayana, Arizaga & Wypych, 2009; Ugartondo, Mitjans & Vinardell, 2009). La lignina es un subproducto en la industria del papel pero al mismo tiempo está presente en todos los vegetales y forma parte de la fibra dietética (Elleuch, Bedigian, Roiseux, Besbes, Blecker & Attia, 2011).

En la presente Memoria se han seleccionado como compuestos principales de estudio la gelatina (de distinta procedencia) y la lignina y lignosulfonatos (forma soluble de la lignina obtenida mediante sulfonación (Lehman, 1970)). Estos compuestos presentan la ventaja de ser biodegradables y además son subproductos de la Industria, por lo que su utilización representa una disminución de residuos, un aprovechamiento de los mismos y una mejora medioambiental.

El pescado es un producto altamente perecedero, muy suceptible a sufrir alteración por microorganismos y procesos de enranciamiento, principalmente en especies grasas (Andersen, Bertelsen, Christophersen, Ohlen & Skibsted, 1990). Es por ello que un envasado activo solo o en combinación con tecnologías de conservación resulta de interés para retardar su deterioro y aumentar su vida útil.

1.1.2 Objetivos

El objetivo de la presente Memoria es el desarrollo y estudio de películas compuestas de gelatina de diversos orígenes y lignina o lignosulfonato, y su aplicación para la conservación de pescado. Este objetivo general se puede desglosar en los siguientes objetivos parciales:

- Formulación y caracterización físico-química de las películas obtenidas a partir de diversas gelatinas (gelatina bovina, gelatina de peces de aguas frías y de peces de aguas cálidas) y distintos tipos de lignosulfonato y de lignina.

- Evaluación de la capacidad antioxidante, antimicrobiana y citotoxicidad del lignosulfonato y la lignina para su posible uso combinado con la gelatina en la obtención de películas activas.

- Evaluación de la estabilidad físico-química y funcional de las películas de gelatina y lignosulfonato en sistemas modelo.

- Estudio de conservación en refrigeración de sardina (*Sardina pilchardus*) y de salmón (*Salmo salar*) recubiertos con película de gelatina y lignina o lignosulfonato mediante tratamientos combinados con alta presión.

1.1.3 Resultados

El plan de trabajo se llevó a cabo en dos etapas divididas a su vez en varios pasos:

a) Desarrollo de películas de gelatina bovina (B) y de peces de aguas frías (F) combinadas con lignosulfonatos (LSa, LSb, LSc) y su aplicación a filetes de sardina (Sardina pilchardus).

- Cuando se utiliza un ratio de LSa superior a 80:20 (F:LSa) se dificulta la generación de la tripe hélice de colágeno, la propiedades reológicas empeoran y se produce una hidratación mayor de la proteína. Las películas formadas con un ratio 80:20 (F:LSa) presentan una Tg y una solubilidad menor y una opacidad mayor que las películas que no tienen incorporadas en su composición LSa. Otros parámetros como las propiedades mecánicas o la capacidad gelificante descienden y la permeación al vapor de agua aumenta levemente. El ratio óptimo para el desarrollo de películas de gelatina procedentes de peces de aguas frías y lignosulfonato es 80:20.

- El estudio físico-químico de las películas formadas por F o B junto con LSa u otros LS, (ratio 80:20) revela que B mejora las propiedades de las películas, en especial las formadas por B:LSa en ratio 80:20.

- Los LS presentan una actividad antioxidante importante que resultan muy similares entre sí. La actividad antioxidante efectiva es mucho menor que la concentración citotóxica en cada tipo de LS. Los LS apenas poseen actividad antibacteriana, si bien presentan cierta capacidad antifúngica.

- Las películas B:LSa 80:20 se conservaron en dos tipos de sistemas modelo (al aire y en contacto con aceite de girasol). El contacto con el aceite produce modificaciones en la superficie de las películas, lo que disminuye la solubilidad y refuerza las propiedades mecánicas. Durante el almacenamiento, las películas de gelatina sufren procesos de entrecruzamiento, que se previenen en pre-

sencia del LS. Las películas de B en contacto con agua forman un gel amorfo mientras que las películas de B:LSa mantienen su estructura. La capacidad antioxidante no disminuye con el tiempo, independientemente del modelo de almacenamiento.

- La combinación de la alta presión junto con película de B:LSa aumenta la vida útil de la sardina, ya que durante la conservación se reduce el crecimiento microbiano y los fenómenos de oxidación.

b) Desarrollo de películas de gelatina de peces de aguas cálidas (G) combinadas con lignina 1000 (L1000) o lignina 2400 (L2400) (ratio 85:15) y su aplicación a salmón (Salmo salar).

- Las películas elaboradas con L1000 presentan mayor fuerza de tensión y elongación en la ruptura y menor solubilidad, permeación al vapor de agua y actividad de agua que una película de G o de G:L2400. Por todo ello, L1000 se considera la mejor lignina.

- La lignina provoca un mayor grado de interferencia con G (85:15 G:L1000) que el LS con B (80:20 B:LSa), tanto desde el punto de vista reológico al estudiar las soluciones filmogénicas como desde el punto de vista físico al evaluar las películas.

- La lignina no presenta capacidad antimicrobiana pero es más antioxidante que cualquier lignosulfonato. La lignina es citotóxica a concentraciones inferiores a los LS pero en todos los casos se da a concentraciones muy elevadas y muy superiores a las concentraciones de actividad efectiva antioxidante. Las películas con L1000 son más antioxidantes que las elaboradas con L2400 y generalmente muestran mayor capacidad antioxidante que las películas con LSa (por secuestro de radicales libres).

- El cocinado a 90 °C es la técnica que más modifica el músculo del salmón al compararlo con el salmón crudo, mientras que la alta presión a 5 °C junto con la película de G:L1000 es la técnica que conlleva la menor modificación. Estos resultados destacan especialmente al estudiar la desnaturalización y oxidación proteica y los cambios en el color. La alta presión a 5°C combinada con película prolonga la vida útil del salmón en mayor medida que otros tratamientos.

1.1.4 Conclusiones

PRIMERA. El lignosulfonato puede incorporarse en una proporción óptima de 80 % gelatina – 20 % lignosulfonato para aumentar la resistencia al agua de películas de gelatina de peces de aguas frías, que son extremadamente solubles, y para mejorar sus propiedades de barrera a la luz con el mínimo perjuicio de sus propiedades mecánicas. Dichas películas, sin embargo, son menos resistentes al agua que las de gelatina bovina independientemente del tipo de lignosulfonato.

SEGUNDA. La utilización de gelatina de peces de aguas cálidas adicionada de lignina L1000, en una proporción de 85 % gelatina – 15 % lignina, origina una película de resistencia al agua comparable a la de gelatina bovina y lignosulfonato, pero de mayor resistencia mecánica, mayor barrera a la luz y menor permeabilidad al vapor.

TERCERA. A nivel estructural, tanto el lignosulfonato como la lignina actúan preferentemente a modo de relleno físico de la matriz de gelatina e interfieren, con mayor intensidad en el caso de la lignina, en la interacción entre las cadenas polipeptídicas, favoreciendo una mayor interacción de las mismas con las moléculas de agua.

CUARTA. La capacidad antioxidante de la lignina L1000 es superior al doble de la de cualquier lignosulfonatos estudiados, pero también lo es su citotoxicidad. No obstante, ambos tipos de compuestos producen películas con alta capacidad antioxidante a concentraciones no citotóxicas, por lo que se estiman potencialmente aptas para uso alimentario, a pesar de no presentar actividad antimicrobiana efectiva.

QUINTA. El lignosulfonato dificulta la tendencia natural de la película de gelatina a sufrir procesos de agregación, razón por la cual las propiedades físicas y antioxidantes de las películas complejas no se modifican sustancialmente al cabo de 28 días de conservación.

SEXTA. El tratamiento combinado de alta presión (300 MPa – 5 ^{o}C – 10 min) y recubrimiento con película de gelatina – lignosulfonato resulta más efectivo que el envasado a vacío para prevenir la oxidación lipídica y el deterioro microbiano del músculo de sardina durante la conservación en refrigeración.

SÉPTIMA. El tratamiento combinado de alta presión (300 MPa – 5 ^{o}C – 10 min) y recubrimiento con película de gelatina – lignina previene parcialmente la oxidación y agregación proteica del músculo de salmón y mantiene de forma más eficaz su color característico, en comparación con un tratamiento térmico convencional.

1.1.5 Bibliografía

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1.2 *Abstract*

1.2.1 Introduction

The use of specific packaging plays an important role in food quality and preservation. In today's society, packaging made of synthetic compounds produces a lot of waste, which cause economic and environmental disadvantages (Kirwan & Strawbridge, 2003). Alternatively, biodegradable compounds, mainly biopolymers, could be used as support matrix (Martin- Belloso et al., 2009) in combination with natural compounds with antioxidant, antimicrobial, etc. properties.

Gelatin is a biodegradable biopolymer that could be presented as an option for food packaging. Gelatin comes from different sources. In the literature, many works have been carried out using bovine gelatin because of its excellent properties, but sometimes it can be rejected for cultural, religious or sanitary reasons (transimission spongiform encephalopathy, TSE), so the study of gelatins from different origins, as an alternative to bovine gelatin, is of great interest (Gómez-Guillén et al., 2011), as is the case of fish skin gelatin.

Lignin and lignosulphonate are industrial waste susceptible to be used in the formulation of filmogenic materials due to their polymeric nature, as well as biodegradability, low cytotoxicity, high antioxidant capacity, etc. (Satyanarayana et al., 2009; Ugartondo et al., 2009). Lignin is a byproduct of the paper industry but lignin is also present in all plants and takes part of the dietary fiber (Elleuch et al., 2011).

In the present Report, gelatin (from different sources) and lignin and lignosulphonates (soluble form of lignin obtained by sulfonation (Lehman, 1970)) have been selected as lead compounds. These compounds have the advantage of being biodegradable and they are industrial byproducts, so their use represents an important waste reduction and a profitable solution for environmental improvement.

Fish is a highly perishable product, very susceptible to be spoiled by microorganisms and oxidative processes, especially in fatty species (Andersen, 1990). That is why active packaging alone or in combination with preservation technologies could be of interest to delay spoilage and extend the self- life of fish.

1.2.2 Objectives

The purpose of this Report is the development and study of gelatin composite films of various origins and lignin or lignosulphonate, and their application to fish preservation. This overall objective can be divided into the following sub-objectives:

- Formulation and physicochemical characterization of films obtained from various gelatins (bovine gelatin, cold water fish gelatin and warm water fish gelatin) and different types of lignin and lignosulphonates.

- Evaluation of the antioxidant, antimicrobial and cytotoxicity properties of lignin and lignosulphonate for their possible use in combination with gelatin to obtain active films.

- Evaluation of the physicochemical and functional stability of lignosulphonate gelatin films in model systems.

- Study of cold preservation of sardine (*Sardina pilchardus*) and salmon (*Salmo salar*) coated with gelatin and lignin or lignosulphonate films alone and in combination with high pressure treatments.

1.2.3 Results

The work plan was carried out following two stages divided into several steps:

a) Development of bovine gelatin films (B) and cold-water fish gelatin films (F) combined with lignosulphonates (LSa, LSb, LSc) and its application for sardine (Sardina pilchardus) fillets preservation.

- When a ratio of LSa higher than 80:20 (F: LSa) was used the collagen triple helical structure was difficult to generate. The rheological properties got worse and a greater protein hydration was produced. Films formed with a ratio 80:20 (F: LSa) had lower Tg and water solubility and higher opacity than the films without LSa in their composition. Other parameters such as mechanical properties or the gelling ability descended whereas water vapor permeability increased slightly. The optimum ratio for the development of cold water fish gelatin lignosulphonate films was 80:20.

- Physico-chemical study of films formed by F or B with other LS (ratio 80:20) revealed that the use of B and LSa in ratio 80:20 improved the film properties. All LS had significant antioxidant activities which were very similar among them. The effective antioxidant concentrations were considerably smaller than the cytotoxic ones for each type of LS. The LS did not possess antibacterial activity, while some antifungal activity was detected.

- Films B:Lsa (80:20) were preserved in two model systems (in air and in contact with sunflower oil). The oil induced changes at the surface of films, thereby decreasing the solubility and enhancing the mechanical properties. During storage, gelatin films suffered crosslinking processes, which were prevented in the presence of LS. B films in contact with water formed a gel while B: Lsa films mantained their structure. The antioxidant capacity did not decrease with time, independently of the storage model.

- The combination of high pressure with film B:LSa extended the self-life of sardine fillets during storage since the combined treatment reduced both microbial growth and oxidation phenomena.

b) Development of warm-water fish gelatin films(G) combined with lignin 1000 (L1000) or lignin 2400 (L2400) (ratio 85:15) and their application for salmon (Salmo salar) preservation.

- The films made with L1000 had higher tensile strength and elongation at break, and lower solubility, water vapor permeability and water activity than G or G: L2400 films. Therefore, L1000 lignin was considered the best one.

- From the study of the rheological behavior of film forming solutions and physical properties of films, lignin was found to cause higher interference with G (85:15 G: L1000) than LS with B (80:20 B: LSa). Lignin had no antimicrobial properties but presented higher antioxidant capacity than any ligno-sulphonate. Lignin was cytotoxic at lower concentrations than LS but in all cases cytotoxic concentrations were much higher than effective antioxidant concentrations. Films with L1000 were more antioxidant than those made with L2400 and generally showed higher antioxidant capacity than films with LSa (free radical scavenging capacity).

- Heating at 90 °C was the treatment that greater modified the salmon muscle when compared to the raw salmon, while the high pressure at 5 °C combined with the film G: L1000 was the mildest treatment. These results highlighted especially when studying protein denaturation and oxidation, as well as color changes. The high pressure at 5 °C combined with film extended the self-life of salmon more than other treatments.

1.2.4 Conclusions

FIRST. The lignosulphonate may be incorporated in an optimal ratio of 80% gelatin - 20% lignosulfonate to increase the water resistance of cold water fish gelatin films, which are extremely soluble, and to improve their light barrier properties with the minimum damage in their mechanical properties. Such films, however, were less water resistant than those developed from bovine gelatin, regardless of the lignosulphonate type.

SECOND. The use of warm water fish gelatin and lignin L1000, added in a proportion of 85% gelatin - 15% lignin, led to a film with water resistance comparable to that made of bovine gelatin and lignosulphonate, but with higher strength and lower water vapor permeability.

THIRD. Structurally, both lignin and lignosulphonate acted preferably as a physical filler in the gelatin matrix and interfered, with higher intensity in the case of lignin, in the self-aggregation of the polypeptide chains, favoring greater gelatin-water interactions.

FOURTH. The antioxidant capacity of lignin L1000 was approximately 2.5 times higher than any lignosulphonate studied, but also was its cytotoxicity. However, both types of compounds produced films with high antioxidant capacity at non-cytotoxic concentrations, and therefore they were potentially suitable for food use, despite presenting no effective antimicrobial properties.

FIFTH. The lignosulphonate hampers the natural tendency of the gelatin film to suffer aggregation processes, which explained why the physical and antioxidants properties of the complex films did not change substantially after 28 days of storage.

SIXTH. A combined treatment of high pressure (300 MPa - 5 ^{o}C - 10 min) and covering with gelatin- lignosulphonate film was more effective than vacuum packaging to prevent lipid oxidation and microbial spoilage of sardine muscle during chilled storage.

SEVENTH. A combined treatment of high pressure (300 MPa - 5 ^{o}C - 10 min) and covering with gelatin-lignin film partially prevented protein aggregation and oxidation of salmon muscle, and maintained more effectively its characteristic color in comparison with a conventional heat treatment.

1.2.5 References

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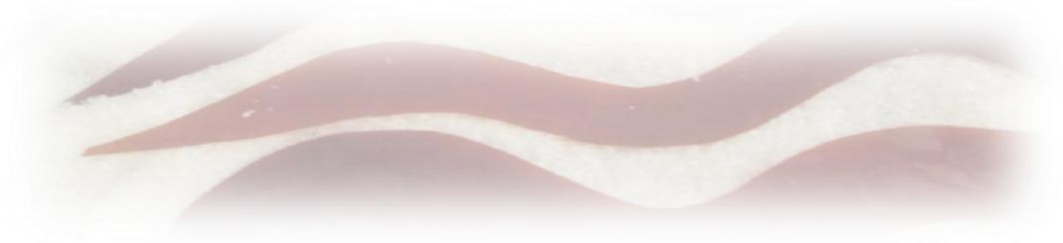
2 Introducción



2.1 *Los envases*

2.1.1 Introducción

Desde la antigüedad, el envasado es una técnica de conservación utilizada para preservar los alimentos. El envasado juega un papel importante en la producción de alimentos seguros y de calidad (Rahman, 1999) y tiene cinco principales funciones como son: contener el producto, preservar y mantener la calidad, buena presentación y comodidad, proteger al alimento y proporcionar información al consumidor.

El envasado de los alimentos es una técnica fundamental para conservar su calidad, reducir al mínimo su deterioro y limitar el uso de aditivos. El envase preserva la forma y la textura del alimento que contiene, evita que pierda sabor o aroma, prolonga el tiempo de almacenamiento y regula el contenido de agua o humedad. Los envases protegen a los alimentos y bebidas de una serie de agentes externos procedentes del ambiente como el calor, la humedad, la luz, microorganismos, suciedad, gases, etc. El envase asimismo permite a los fabricantes ofrecer información sobre las características del producto envasado, su contenido nutricional y su composición (Restuccia, Spizzirri, Parisi, Cirillo, Curcio, Lemma & Picci, 2010) y constituye un buen vehículo para transmitir a los usuarios información sobre el contenido; debe tener una forma y tamaño correctos y una presentación atractiva a los ojos del consumidor.

2.1.2 Problemática con los materiales utilizados actualmente: uso de plásticos

Los materiales más comúnmente utilizados para el envasado de alimentos y bebidas son los plásticos derivados del petróleo, seguidos del papel, fibras, vidrio, acero y aluminio, etc. Los plásticos son muy versátiles en cuanto a la forma y tamaño, además de por sus propiedades como ligereza, resistencia, comodidad, higiene, etc. (García-Díaz & Macías-Matos, 2008) y presentan una serie de ventajas adicionales relacionadas con su dureza y bajo peso. Sin embargo, los plásticos generan una gran cantidad de residuos no biodegradables (Kirwan et al., 2003). La acumulación de este tipo de residuos se debe fundamentalmente al gran nivel de consumo y la incapacidad de la naturaleza de degradar estos productos (Gómez Antón & Gil Bercero, 1997). Además, por problemas de seguridad y medioambientales, el reciclado de plásticos resulta complicado por razones técnicas y económicas (Aguado & Serrano, 1999). De forma adicional a la problemática que se plantea en cuanto a su acúmulo y falta de degradación, los plásticos pueden transportar hacia el alimento o bebida ciertos compuestos no deseables, como por ejemplo plastificantes o aditivos (Nerín De La Puerta, 2009). Por ello resulta de gran interés el desarrollo de alternativas a los plásticos convencionales, considerando la utilización de biopolímeros una buena opción con aplicación en el campo de los envases.

2.1.3 Tecnologías de conservación

La calidad sensorial, nutritiva y organoléptica de los alimentos se puede modificar debido a la actividad metabólica del propio producto y de los microorganismos presentes. Las bajas temperaturas y una correcta manipulación higiénica son factores importantes que afectan a la calidad del alimento.

2.1.3.1 Envasado en atmósferas modificadas

El envasado en atmósferas modificadas (Modified Atmosphere Packaging, MAP) es un método de conservación que minimiza los fenómenos fisiológicos y microbianos adversos de los productos perecederos mediante la implantación de una atmósfera diferente de la composición normal del aire (Church, 1994). El envasado a **vacío** se define como el envasado de un producto en un recipiente hermético en el cual se elimina el aire con el fin de prevenir el crecimiento de microorganismos aerobios, oxidaciones, alteraciones de color (Genigeorgis, 1985). El envasado a vacío es una variación de atmósfera modificada, puesto que eliminar el aire de un envase es en sí mismo una forma de modificar la atmósfera; además, en el interior de estos envases es frecuente la producción de 10-20% de CO_2 debido al metabolismo microbiano (Jensen, Petersen, Roge & Jepsen, 1980; Silliker & Wolfe, 1980; Hintlian & Hotchkiss, 1986). El envasado a vacío favorece la retención de los compuestos volátiles responsables del aroma, lo que resulta muy apreciado en determinados productos como el café. Además impide las quemaduras por frío, la formación de cristales de hielo y la deshidratación de la superficie del alimento gracias a la barrera de humedad de pequeño espesor existente entre el material de envasado y el producto.

2.1.3.2 Alta presión en el envasado de alimentos

La alta presión hidrostática se basa en el principio de Le Chatelier, de acuerdo al cual cualquier reacción, cambio conformacional o transición de fase que se acompaña por un descenso de volumen se verá favorecida a altas presiones, mientras que las reacciones que involucran un aumento de volumen se inhibirán (Ledward, 1995).

La presión es una variable termodinámica importante y puede afectar a un amplio campo de estructuras, reacciones y procesos biológicos (Earnshaw, 1996), y su efecto dependerá del nivel de presión aplicada, tiempo, temperatura de proceso y tipo de alimento.

Una de las principales ventajas de la alta presión es que afecta muy ligeramente la composición del alimento, y como consecuencia los atributos nutricionales permanecen prácticamente intactos. La alta presión conserva micronutrientes como aminoácidos y vitaminas, así como componentes responsables del sabor (Knorr, 2000). Sin embargo, los tratamientos aplicados por encima de 200 MPa pueden producir modificaciones en el color, dando al alimento un aspecto de producto cocinado (Montero & Gómez-Guillén, 2005).

La alta presión modifica las propiedades funcionales en productos cárnicos y pesqueros, induciendo la desnaturalización y la gelificación de la proteína del músculo (Cheftel & Culioli, 1997). Al mismo tiempo, induce una agregación similar a la que se obtiene cuando hay tratamientos térmicos a nivel de interacción de la cabeza de la miosina (Yamamoto, Hayashi & Yasui, 1993). Sin embargo, la naturaleza de tales interacciones difiere ligeramente. Así, las presiones favorecen la formación de enlaces de hidrógeno lábiles al calor mientras que los enlaces disulfuro o interacciones hidrofóbicas juegan un rol importantes en la estabilización de estructuras inducidas por el calor (Angsupanich, Edde & Ledward, 1999).

Aunque está generalmente asumido que la alta presión puede favorecer la oxidación lipídica, este efecto depende en gran parte del nivel de presión, el tipo de alimento y el almacenamiento, así como de la temperatura del proceso a la que se lleva a cabo (Gómez-Estaca, Gómez-Guillén & Montero, 2007)

Sin duda, uno de los principales efectos de la alta presión es el relacionado con la inactivación de los microorganismos. Si bien el mecanismo de acción no está total esclarecido, la membrana constituye un órgano diana (Hoover, Metrick, Papineau, Farkas & Knorr, 1989). También tiene lugar una posible inactivación de las enzimas, como las involucradas en la replicación del ADN y la transcripción (Hoover et al., 1989). Además de la alteración de la membrana, también se produce un descenso del pH en el medio intracelular debido al incremento de la disociación iónica; la presión provoca la separación de las cargas eléctricas ya que éstas organizan las moléculas de agua alrededor de ellas, descendiendo el volumen total del sistema (Cheftel, 1995). También se origina una pérdida de la actividad ATP-asa en la membrana (Smelt, 1995). En resumen, la alta presión puede desnaturalizar enzimas o provocar una alteración en las funciones de membrana, y como consecuencia los microorganismos pueden morir por acidificación interna. Por otro lado, las presiones superiores a 100-200 MPa a menudo provocan disociación de estructuras oligoméricas en subunidades, desdoblamiento parcial y desnaturalización de estructuras monoméricas, agregación de proteínas y gelificación proteica si la concentración de proteínas y la presión son suficientemente altas (Cheftel, 1995).

2.1.3.3 Envasado activo

Desde hace varios años los materiales utilizados en el envasado de alimentos se diseñan con el objetivo de interactuar con los alimentos. En el "envasado activo", a los materiales se les incorporan agentes, por ejemplo antimicrobianos o antioxidantes, con objeto de alargar la vida útil y mantener o incluso aumentar la calidad del alimento (López De Lacey, 2012). Para llevar a cabo este tipo de envases se utilizan materiales no comestibles como papel, plástico, metales, etc. (Dainelli, Gontard, Spyrooulos, Zondervan Van Den Beuken & Tobback, 2008) aunque también se pueden utilizar materiales biodegradables (Martín-Belloso et al., 2009). Los agentes activos se incorporan al material que conforma el envase con el objetivo de que se liberen al alimento por contacto directo o por

proximidad. Se puede distinguir entre agentes activos, ya mencionados y agentes bioactivos, que además tienen una repercusión sobre la salud del consumidor.

La incorporación de un agente activo/bioactivo a un envase ofrece una serie de ventajas a la incorporación directa del agente al alimento, como por ejemplo la liberación controlada del agente, efecto localizado e incluso reducir la dosis del mismo (Martín-Belloso et al., 2009). Además, hay que valorar las posibles interacciones entre el agente activo y los componentes del envase, así como del propio alimento.

2.1.4 Uso de biopolímeros y su aplicación en películas comestibles

La sociedad occidental está experimentando una tendencia creciente hacia el consumismo "verde", un deseo por la menor incorporación de aditivos sintéticos a los alimentos y por los productos que impacten menos al medioambiente (Burt, 2004). Esta tendencia conlleva una investigación en el desarrollo de nuevos materiales biodegradables para el envasado, con el empeo de polímeros de origen natural que constituyan una alternativa parcial a los envases plásticos. Los biopolímeros, considerados como polímeros derivados de recursos renovables (Petersen, Nielsen, Bertelsen, Lawther, Olsen, Nilsson & Mortensen, 1999), pueden utilizarse en la fabricación de envases biodegradables que se transformarán como consecuencia de la acción de microorganismos, foto-degradación o degradación química (Petersen et al., 1999), y que además pueden ser comestibles.

Los biopolímeros se utilizan para la elaboración de dos tipos de envases: películas y recubrimientos (Petersen et al., 1999). Un recubrimiento o cobertura comestible es una capa formada como un revestimiento sobre el alimento, mientras que una película es una capa ya prefabricada que se aplica sobre el producto (Krochta & De Mulder-Johnston, 1997) (Gómez-Estaca, Montero, Giménez & Gómez-Guillén, 2007). La presente Memoria está dirigida hacia la formación y estudio de películas, por lo que es en este tipo de envase en el que se centrará la información.

Estos biopolímeros se clasifican en cuatro grandes categorías: polisacáridos, proteínas, lípidos y poliésteres (Tharanathahan, 2003) donde en muchos casos estos productos son desechos y/o excedentes de productos de la pesca, agricultura o ganadería (Gómez-Estaca, López De Lacey, López-Caballero, Gómez-Guillén & Montero, 2010).

2.1.4.1 Proteínas

Las proteínas utilizadas en la elaboración de las películas son de origen vegetal o animal y las más representativas son la albúmina, gluten, proteínas lácteas, zeína o gelatina. Las películas elaboradas a partir de proteínas poseen buenas propiedades mecánicas y ópticas pero son altamente sensibles a la humedad y presentan una escasa propiedad de barrera al vapor de agua. Este hecho podría representar un problema a la hora de aplicarlos a productos con alto contenido en humedad,

porque las películas se podrían hinchar, disolver o desintegrar al ponerse en contacto con el agua (Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez & Montero, 2009).

2.1.4.2 Polisacáridos, lípidos y poliésteres

Los polisacáridos se utilizan generalmente como estabilizantes o agentes gelificantes. Tienen varios orígenes: celulosa y derivados, almidones y derivados, pectina y arabinoxilanos, gomas procedentes de algas (alginatos o carragenatos), o gomas procedecentes de microorganismos (pululano, xantano o gelan) (Guilbert & Contard, 2005). Las propiedades de las películas formadas por polisacáridos dependen de la estructura del mismo, ya que el número de enlaces de hidrógeno intermoleculares o el peso molecular afectan a las propiedades finales de las películas. Así por ejemplo, los polisacáridos de alto peso molecular no iónicos forman películas más resistentes mientras que los polisacáridos más ramificados (con carga o no), dan lugar a películas más débiles (Nieto, 2009). Al igual que las películas realizadas a base de proteínas, las películas de polisacáridos poseen buenas propiedades mecánicas y ópticas pero son muy sensibles al agua y al vapor de agua.

Por otro lado, las películas elaboradas a partir de lípidos (ceras, aceites, etc.) y poliésteres (poli-D-β-hidroxibutirato, ácido poliláctico, etc.) tienen buenas propiedades de barrera al vapor de agua pero normalmente son poco flexibles y opacas; además, las películas de lípidos tienden a enranciarse y presentan gran fragilidad (Gontard & Guilbert, 1999) (Gómez-Guillén et al., 2009).

2.1.4.3 Combinación de polímeros

La tendencia actual en el diseño de materiales biodegradables para el envasado de alimentos es combinar diferentes biopolímeros para mejorar así las propiedades finales del envase. Las películas formuladas con hidrocoloides mejorarán las propiedades de barrera al agua o al oxígeno con la incorporación de lípidos a su formulación. A modo de ejemplo, aceite de girasol se ha incorporado a películas de almidón (García, Martino & Zaritzky, 2000) y se han combinado alginatos con mono y diglicéridos de ácido acético (Hambleton, Debeaufort, Bonnotte & Voilley, 2009) donde también se mejoran las propiedades mecánicas. Otra combinación es el uso de gelatina de peces de aguas frías junto con polisacáridos (gelano y *K*-carragenato), donde se observó un aumento en la fuerza de tensión y una mejora en las propiedades de barrera frente al vapor de agua, si bien las películas resultaron ser ligeramente más oscuras (Pranoto, Lee & Park, 2007). También se recogen en la literatura otros ejemplos como películas realizadas a partir de de gelatina y caseinatos (Chambi & Grosso, 2006), gelatina y alginatos (Boanini, Rubini, Panzavolta & Bigi, 2010), gelatina y goma gellan (Lee, Shim & Lee, 2004), almidón y derivados (Ban, Song, Argyropoulos & Lucia, 2006), o celulosa, almidón y lignina (Wu, Wang, Li, Li & Wang, 2009), etc.

2.1.4.4 Películas activas: incorporación de agentes antimicrobianos y antioxidantes

Las películas comestibles contribuyen a mantener la calidad del producto, mejoran las propiedades sensoriales, aumentan su seguridad e incrementan la vida útil de productos listos para comer (Beverlya, Janes, Prinyawiwatkula & No, 2008). Estas películas actúan como barrera al oxígeno y al agua y por tanto disminuyen la velocidad de reacciones de oxidación; también ayudan a mantener la humedad, mejorando así la calidad y alargando la vida útil del alimento (Gennadios, Hanna & Kurth, 1997). Para controlar de forma más eficaz la oxidación de los alimentos y su deterioro debido a la acción de los microorganismos, existe un gran interés en la formulación de películas activas comestibles y/o biodegradables, basadas en la incorporación de un compuesto antioxidante o antimicrobiano en la matriz polimérica (Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez & Montero, 2009; Guilbert, Gontard & Gorris, 1996).

Un antioxidante o una mezcla de ellos puede incorporarse al envase y liberarse al alimento, protegiendo a la superficie de procesos de enranciamiento contribuyendo así al aumento de su vida útil. Una liberación lenta además proporciona una reposición continua de antioxidantes al alimento. Así por ejemplo, existe un gran interés por antioxidantes naturales como la vitamina E, sesamol y ácido carnosínico en sistemas alimentarios (Rooney & Yam, 2004), hidroxitolueno butilado (Butylated-hydroxytoulene, BHT) (Jongjareonrak, Benjakul, Visessanguan & Tanaka, 2008) o bien sobre muchos otros compuestos naturales como ácido ascórbico, ácido cítrico, etc. (Martín-Belloso et al., 2009), especias como orégano y romero (Gómez-Estaca, Bravo, Gómez-Guillén, Alemán & Montero, 2009), etc.

Los compuestos reconocidos como seguros (*Generally Recognized As Safe*, GRAS) se extienden a compuestos antimicrobianos que se emplean para controlar la incidencia o minimizar el número de patógenos transmitidos por los alimentos. En los últimos años aparecen investigaciones basadas en utilizar cultivos iniciadores de bacterias (bioconservanción) y extractos de plantas como barreras antimicrobianas (Zhu, Du, Cordray & Ahn, 2005). Un agente antimicrobiano ha de controlar la microbiota del alimento para aumentar así su vida útil. Son muchos los antimicrobianos utilizados y recogidos en la literatura, entre ellos el quitosano (López-Caballero, Gómez-Guillén, Pérez-Mateos & Montero, 2005), aceites esenciales (Burt, 2004) como el clavo (Gómez-Estaca, López De Lacey, Gómez-Guillén, López-Caballero & Montero, 2009), sangre de drago (Rossi, Guerrini, Maietti, Bruni, Paganetto, Poli, Scalvenzi, Radice, Saro & Sacchetti, 2011), extractos de té (Friedman, 2007), extractos de romero (Del Campo, Amiot & Nguyen-The, 2000), especias (Ceylan & Fung, 2004), etc.

2.2 *Gelatina*

2.2.1 Introducción

La gelatina se obtiene por hidrólisis del colágeno, siendo la principal proteína fibrosa constituyente de los huesos, cartílagos y pieles (Gómez-Guillén et al., 2011). La fuente, edad del animal y el tipo de colágeno son factores intrínsecos que influyen en las propiedades de la gelatina (Johnston-Banks, 1990). Hasta la fecha se conocen más de 27 tipos de colágeno, pero es el denominado colágeno tipo I el que se encuentra en mayor cantidad en el tejido conectivo.

El colágeno intersticial está compuesto por tres cadenas enrolladas en sí mismas formando la denominada triple hélice de colágeno. Esta particular estructura, principalmente estabilizada por puentes de hidrógeno existentes intra- e intercadenas, es el producto de una casi repetitiva secuencia Gly-X-Y, donde Gly es el aminoácido Glicina y X e Y suelen ser prolina e hidroxiprolina, respectivamente (Asghar & Henrickson, 1982).

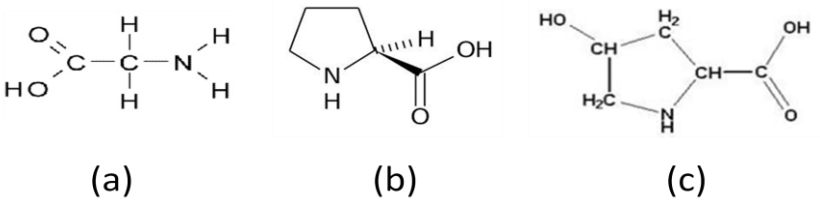


Figura 1. Estructura de los aminoácidos glicina (a), prolina (b) e hidroxiprolina (c).

La región N- y C- terminal, llamada telopéptidos (de entre 15 y 26 aminoácidos), no forma la estructura triple helicoidal y en gran parte está formada por lisina e hidroxilisina, así como por sus aldehídos derivados. Generalmente de cuatro a ocho moléculas de colágeno se encuentran estabilizadas y reforzadas por enlaces covalentes para constituir la unión básica de fibras de colágeno. Por tanto, la dureza y rigidez típica de la piel, tendones y huesos se debe a la estructura básica formada por muchas de estas fibras de colágeno entrecruzadas.

El colágeno nativo es insoluble y debe sufrir un pre-tratamiento antes de convertirse en un producto disponible para posterior extracción, que normalmente se realiza por calentamiento en agua a temperaturas superiores a 45 °C.

Un pre-tratamiento químico rompe los enlaces no covalentes y desorganiza la estructura proteica, produciendo por tanto un hinchamiento y solubilización del colágeno (Stainsby, 1987). Subsecuentes tratamientos por calor romperán los enlaces covalentes, lo que provocará la desestabilización de la triple hélice, la cual se desenrollará y finalmente dará lugar a gelatina soluble (Gómez-Guillén et al., 2011) .

El grado de conversión del colágeno en gelatina está relacionado con la severidad del pre-tratamiento y del procedimiento de extracción en agua caliente, así como del pH, temperatura y tiempo de extracción (Johnston-Banks, 1990). De este modo se obtienen dos tipos de gelatinas denominadas tipo A (con punto isoeléctrico entre pH=8 y pH=9), obtenidas con un pre-tratamiento ácido, y gelatinas tipo B (con punto isoeléctrico entre pH=4 y pH=5), obtenidas con pre-tratamiento alcalino. Debido a la sensibilidad a pH ácido del colágeno inmaduro (por ejemplo, el que está presente en la piel del pescado), un tratamiento ácido moderado es suficiente para conseguir su solubilización (Montero, Borderías, Turnay & Leyzarbe, 1990).

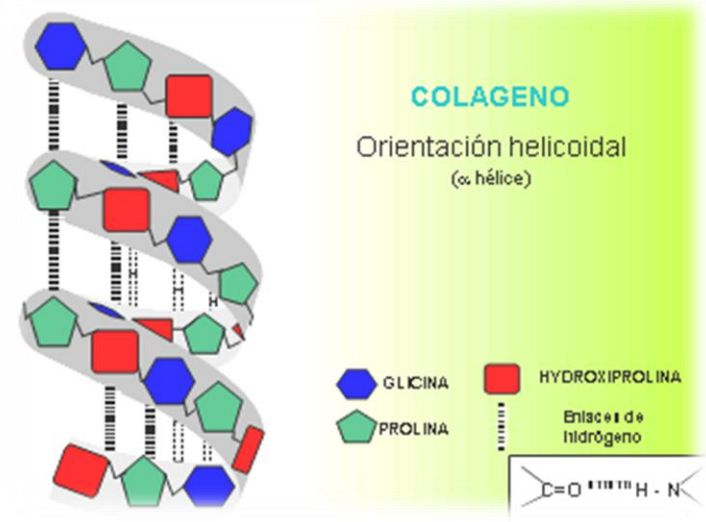


Figura 2. Estructura del colágeno. (Adaptado de la Universidad de Colombia, Dirección de Nacional de Servicios Académicos Virtuales).

La calidad de la gelatina obtenida y las aplicaciones depende en gran medida de sus propiedades reológicas (Stainsby, 1987). Además de las propiedades físico-químicas básicas (parámetros de composición, solubilidad, transparencia, color, olor y gusto), los principales atributos que mejor definen la calidad comercial de una gelatina es su fuerza de gel y su estabilidad térmica (temperatura de gelificación y de fusión). Para estandarizar estos valores, las medidas de fuerza de gel se determinan mediante el llamado *test de Bloom*, que consiste en llevar a cabo un protocolo definido a una concentración de gelatina (6,67 %), temperatura (10 °C) y tiempo de maduración (17 h), permitiendo así normalizar el parámetro (Wainewright, 1977). Ambos, fuerza de gel y termoestabilidad, son altamente dependientes de las propiedades moleculares de la gelatina, en especial con respecto a la composición de aminoácidos y a la distribución de peso molecular (Gómez-Guillén et al., 2011). Los procesos de extracción se pueden modificar para aumentar el rendimiento de la extracción y mejorar las propiedades reológicas. Así por ejemplo se han utilizado diferentes ácidos orgánicos en el pre-tratamiento de las pieles (Giménez, Turnay, Lizarbe, Montero & Gómez-Guillén, 2005), distintas sales

para el lavado (Giménez, Gómez-Guillén & Montero, 2005), tratamientos de alta presión (Gómez-Guillén, Giménez & Montero, 2005), o incorporando enzimas como la pepsina (Nalinanon, Benjakul, Visessanguan & Kishimura, 2008). De esta manera, se pueden obtener gelatinas con óptimas propiedades y de gran aplicación en la industria alimentaria.

Figura 3. Escultura de colágeno (realizada por Julian Voss-Andraea), Stainless steel, "Unraveling Collagen", 2005.

2.2.2 Películas de gelatina

La gelatina se emplea como ingrediente para mejorar la elasticidad, consistencia y estabilidad de los alimentos. Su utilización para encapsular y elaborar películas hace de la gelatina un producto interesante en varios sectores como el fotográfico, el farmacéutico y el alimentario. El uso de películas comestibles ayuda a mantener la calidad de los alimentos, a mejorar las propiedades sensoriales y la seguridad e incrementar la vida útil de varios productos "listos para comer" (Beverlya et al., 2008).

Como norma, las propiedades físicas de las películas de gelatina dependen principalmente de las propiedades de los materiales de partida (diferente según origen) y de las condiciones de obtención. Las propiedades también dependen de los parámetros físicos utilizados en el proceso de fabricación de la película, como la temperatura y el tiempo de secado (Menegalli, Sobral, Roques & Laurent, 1999) y de los ingredientes de la formulación, como la adición de plastificantes (Vanin, Sobral, Menegalli, Carvalho & Habitante, 2005) o agentes entrecruzantes (Bigi, Cojazzi, Panzavolta, Rubini & Roveri, 2001). Los plastificantes son hidrofílicos, tienen bajo peso molecular y se pueden unir a la red proteica fácilmente formando puentes de hidrógeno con los grupos activos o los aminoácidos, reduciendo así las interacciones proteína-proteína; una concentración alta de plastificantes dará lugar a

unas películas menos rígidas y duras y más extensibles (Arvanitoyannis, 2002; Cuq, Gontard, Cuq & Guilbert, 1997).

Las películas de gelatina se forman principalmente mediante dos procesos: por moldeo ("*casting*") o por extrusión (Hernández-Izquierdo & Krochta, 2008). El más utilizado es por "casting", proceso por el que el biopolímerose disuelve junto con resto de ingredientes de la formulación para obtener una solución filmogénica, la cual se vierte sobre una placa y se seca para eliminar el disolvente. El método de extrusión se basa en el comportamiento termoplástico de las proteínas en condiciones de bajo grado de humedad, siguiendo un método de presión en caliente a temperaturas superiores a 80°C. Este método puede afectar a las propiedades, si bien su utilización podría aumentar la comercialización de las películas, pues permite el trabajo en continuo (Gómez-Guillén et al., 2009).

2.2.3 Tipos de gelatina

La gelatina se extrae convencionalmente a partir del colágeno presente en la piel y en los huesos de ciertas especies de mamíferos (principalmente ganado vacuno y porcino), pero debido a las consideraciones socioculturales, religiosas (la religión musulmana y judía no permiten comer productos porcinos mientras que la religión hindú lo hace con los productos bovinos) y de sanitarias (problemas relacionados con la transmisión de encefalopatía espongiforme bovina) se están considerando otras alternativas. Los materiales de desecho de pescado y aves de corral reciben una considerable atención en la actualidad, si bien su producción sigue siendo limitada y por ello son menos competitiva en precio que las gelatinas de mamíferos (Gómez-Guillén et al., 2009).

2.2.3.1 Gelatinas de mamífero

Los materiales de desecho más comúnmente utilizados para la extracción del colágeno y la obtención de la gelatina son las pieles y cueros, huesos, tendones y cartílagos. La piel porcina fue el primer material de desecho utilizado para la manufactura de la gelatina en la década de los '30 y continúa siendo uno de los más importantes materiales en la escala de la producción industrial (Gómez-Guillén et al., 2009), junto con la obtenida del cuero bovino (Pranoto et al., 2007). La temperatura de fusión y gelificación de la gelatina está relacionada con la proporción de los aminoácidos Prolina e Hidroxiprolina en el colágeno original (Veis, 1964), involucradas en la generación de las zonas de nucleación que dan lugar a la formación de las estructuras de triple hélice (Ledward, 1986) y que son responsables de las buenas propiedades reológicas (Gudmundsson & Hafsteinsson, 1997) y mecánicas. El contenido en estos aminoácidos suele ser del 24 % para los mamíferos y del 16-18 % para la mayoría de los peces (Gilsenan & S.B., 2000), por lo que existen grandes diferencias entre estos tipos de gelatinas.

2.2.3.2 Gelatinas de pescado

Sólo el 25-30 % del pescado o de los productos del mar capturados se consumen, el resto se utiliza para alimentación animal o se desechan como residuos, lo que representa un alto coste económico y medioambiental. Con el fin de reducir estas pérdidas, desde hace varios años se fomentan estrategias para desarrollar productos funcionales de alta calidad para su utilización en alimentación humana o incluso compuestos biológicamente activos con beneficios nutricionales o medicinales, lo que incrementaría su valor en el mercado.

Las gelatinas de pescado poseen buenas propiedades formadoras de películas, ya que son transparentes, poco coloreadas y extensibles (Avena-Bustillos, Olsen, Olson, Chiou, Yee & Bechtel, 2006). El colágeno de las pieles de pescado se caracteriza por el bajo grado de entrecruzamiento covalente (a nivel intra e inter catenario) (Montero, 1990). La principal desventaja de las gelatinas de pescado, respecto a las de mamífero, es que forman geles menos estables, en especial las procedentes de peces de aguas frías (bacalao, salmón o abadejo, (Gudmundsson et al., 1997)). Este hecho está relacionado con el menor número de regiones ricas en Prolina e Hidroxipolina en el colágeno comparado con el número existente en los animales de sangre caliente. Además, las películas a las que da lugar son muy solubles en agua (Carvalho, 2008) lo que supone una desventaja a la hora de aplicarlas en los alimentos.

Figura 4. Imágenes de gelatina de peces de aguas frías (a), de peces de aguas cálidas (b) y bovina (c).

Sin embargo, algunos estudios indican que cierto tipo de gelatinas de pescado (atún, tilapia, pez gato, etc.), aunque no son superiores a las gelatinas de mamíferos, sí se pueden comparar por su nivel de calidad, que depende de las condiciones del proceso (Yang, Wang, Zhou & Regenstein, 2008). En este sentido, las especies de aguas cálidas (lenguado, tilapia, carpa herbívora, etc.) se caracterizan por tener un mayor rendimiento y por sus buenas propiedades. Así, las gelatinas extraídas de la piel de la perca del Nilo tienen valores en las propiedades mecánicas similares a los de la gelatina de huesos de bovino (Muyonga, Cole & Duodu, 2004) o las propiedades mecánicas o de barrera al vapor de agua en las gelatinas del pez gato tienen valores comparables a los obtenidos en gelatinas

comerciales de mamíferos (Zhang, Wang, Herring & Oh, 2007). Por otro lado, Avena-Bustillos et al. (2006) demuestran que la permeabilidad al vapor de agua de las películas elaboradas con gelatina procedente de peces de aguas frías es más baja que las elaboradas con gelatinas de peces de aguas cálidas o de mamíferos debido a la diferente composición en aminoácidos. Otras diferencias entre estos tipos de gelatinas se recogen en la literatura (Gómez-Guillén et al., 2011), donde se establece una comparativa entre las temperaturas de gelificación y fusión de las gelatinas de peces de aguas frías (~4-12 °C o <17 °C, respectivamente) o de peces de aguas cálidas (~18-19 °C y ~24-29 °C, respectivamente). También se encuentran diferencias en la fuerza de gel, con valores de 100 g o incluso inferiores en geles procedentes de peces de aguas frías, mientras que en gelatinas de peces de aguas cálidas se obtienen valores superiores a los 200 g.

Por todo lo anteriormente expuesto se puede considerar que las gelatinas de peces de aguas cálidas pueden ser una alternativa importante a las gelatinas de mamíferos.

2.2.4 Incorporación de compuestos a las películas de gelatina

2.2.4.1 Componentes que mejoran las propiedades físicas

Las películas de gelatina destacan por sus buenas propiedades mecánicas, son transparentes y presentan gran versatilidad a la hora de incorporar otros productos si bien presentan ciertas desventajas. Como se mencionó anteriormente, las propiedades físicas de la gelatina dependen del material de origen, así como del proceso de obtención, pues parámetros como temperatura o tiempo de secado influyen en sus propiedades finales (Menegalli, Sobral, Roques & Laurent, 1999). Además de estos factores, las propiedades físicas finales de la gelatina se pueden modificar adicionando ciertas sustancias como por ejemplo los plastificantes (Vanin, Sobral, Menegalli, Carvalho & Habitante, 2005) o agentes entrecruzantes (Cao, Fu & He, 2007) como el ácido ferrúlico o el ácido tanínico, la carboximetilcelulosa (Mu, Guo, Li, Lin & Li, 2012), genipina (Bigi, Cojazzi, Panzavolta, Roveri & Rubini, 2002) o glutaraldehído (Bigi, Cojazzi, Panzavolta, Rubini & Roveri, 2001). El uso de estos agentes no es siempre necesario o deseado; en ocasiones las películas con bajo grado de entrecruzamiento interesan por ejemplo por su mayor elasticidad. Por otro lado, el tiempo y la temperatura también juegan un papel importante a la hora de entrecruzar las películas.

El sorbitol y el glicerol son los plastificantes más comúnmente utilizados en las películas elaboradas a base de gelatina (Gómez-Guillén et al., 2009), pues son compuestos hidrofílicos de bajo peso molecular que se fijan fácilmente a la red de proteínas y forman puentes de hidrógeno con los grupos reactivos, produciéndose por tanto interacciones plastificante-proteína (Cuq et al., 1997). Los plastificantes producen películas menos rígidas y más extensibles; el glicerol da lugar a películas sensibles al agua, débiles y extensibles mientras el sorbitol produce películas menos sensibles al agua, más fuertes y menos extensible, por lo que una mezcla de ambos plastificantes da lugar a pelí-

culas con propiedades mecánicas, viscoelásticas y propiedades de barrera intermedias (Thomazine, Carvalho & Sobral, 2005). Se pueden utilizar otros plastificantes como el polietilenglicol o etilenglicol pero dan lugar a propiedades finales inferiores a las obtenidas cuando se usa el glicerol y el sorbitol (Vanin et al., 2005).

Otros procedimientos que se pueden llevar a cabo para mejorar las propiedades son los tratamientos enzimáticos (Carvalho & Grosso, 2004) y mezclas con otros componentes no polares como ácidos grasos (para disminuir la velocidad de transmisión de vapor de agua (Limpisophon, Tanaka & Osako, 2010)). Hay que destacar también la posibilidad de incorporar otros biopolímeros a las películas de gelatina para mejorar sus propiedades, como por ejemplo caseína (Chambi et al., 2006), pectina (Liu, Liu, Fishman & Hicks, 2007), el quitosano (Gómez-Estaca et al., 2010), etc.

2.2.4.2 Componentes antioxidantes y antimicrobianos

Las películas actúan como barrera frente al oxígeno y al agua y por tanto ayudan a ralentizar las reacciones de oxidación y pérdida de humedad, mejorando así la calidad y aumentando la vida útil de los alimentos (Gennadios et al., 1997). Por ello la formulación de películas comestibles y/o biodegradables basadas en la incorporación de compuestos antimicrobianos y antioxidantes en la matriz es de gran interés.

En la literatura actual se recogen trabajos en los que se diseñan y elaboran películas o recubrimientos donde se incorporan compuestos con propiedades antioxidantes y/o antimicrobianas de muy distinta naturaleza y posterior aplicación a alimentos, por ejemplo películas de quitosano aplicados a bacalao atlántico (Jeon, Kamil & Shahidi, 2002) o quitosano y gelatina en bacalao (López-Caballero et al., 2005), gelatina y extracto de té verde en carpa plateada (Wu, Chen, Ge, Miao, Li & Zhang, 2013), recubrimientos probióticos para el pan (Altamirano-Fortoul, Moreno-Terrazas, Quezada-Gallo & Rosell, 2012), películas de quitosano y aceite esencial de limón en fresas (Perdones, Sánchez-González, Chiralt & Vargas, 2012), películas de alginato cálcico y partículas de plata en zanahorias (Costa, Conte, Buonocore, Lavorgna & Del Nobile, 2012), etc. Las combinaciones en cuanto a matrices, compuestos activos y posterior aplicación son enormes y de gran interés, por lo que continúa siendo un campo de estudio abierto.

2.3 *Lignina*

En la búsqueda de materiales para el diseño de películas y recubrimientos, la lignina es un candidato de gran interés ya que está presente en la naturaleza, forma parte de la fibra dietética y es un material barato por ser subproducto de la industria papelera. Entre sus características están las de poseer propiedades antioxidantes y antimicrobianas, puede actuar como surfactante y presenta citotoxicidad a concentraciones muy elevadas. Por ello se seleccionó como material objeto de estudio.

2.3.1 Introducción

El término lignina fue introducido en 1819 por de Candolle; lignina es una palabra que deriva de *lignum* (en latín) que significa madera (Sjöström, 1981) y que posteriormente evolucionó al castellano al término "leño".

La lignina es un compuesto que se encuentra en las paredes celulares de las plantas junto con la celulosa, hemicelulosa y pectina. Está unida covalentemente a la hemicelulosa y por tanto se entrecruza con distintos polisacáridos de la planta, produciendo rigidez en la pared celular (Chabannes, Ruel, Yoshinaga, Chabbert, Jauneau, Joseleau & Boudet, 2001). Su misión consiste en cementar las fibras celulósicas de la madera, proporcionar rigidez a las mismas y actuar además como barrera contra la degradación enzimática de la pared celular (Sjöström, 1981). La lignina es un producto presente en la naturaleza y constituye el 20-25% de las paredes celulares de las plantas (Demirbas, Balat & Balat, 2009); se trata de un compuesto polimérico natural derivado del resultado de la actuación de la enzima deshidrogenasa que inicia la polimerización de tres precursores primarios: trans-coniferyl, trans-sinapil y trans-coumaril.

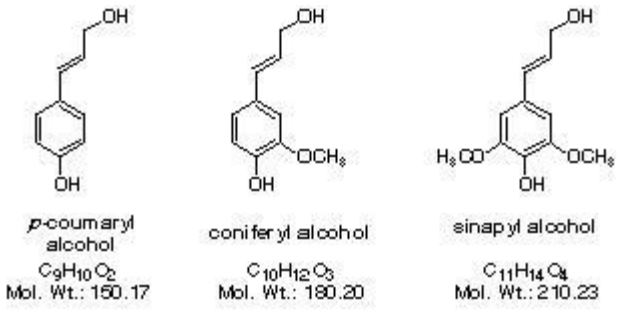


Figura 5. Representación de precursores de lignina (adaptada de Wool y Sun, 2005).

La lignina es un polímero natural de carácter fenólico muy ramificado principalmente constituido por unidades básicas de fenil-propano; tiene enlazado al anillo bencénico un número variable de grupos hidroxílicos y metoxílicos que presentan diferencias estructurales según la especie considerada. Los principales grupos funcionales de la lignina son el hidroxilo, metoxilo, carbonilo y grupos carboxilos en distintas cantidades y proporciones (Gosselink, Snijder, Kranenbarg, Keijsers, De Jong & Stigsson, 2004). Tiene un peso superior a 10.000 Da y está constituida por tres monómeros de monolignol, metoxilados de distintas formas y su porcentaje difiere dependiendo de si la planta en la que está presente es gimnosperma o angiosperma (Boerjan, Ralph & Baucher, 2003). Así, en maderas de gimnospermas predomina el radical guaiacil-propano (metoxi–3-hidroxi-4-fenil-propano), mientras que en las angiosperas predominan los radicales de siringil-propano (dimetoxi-3-5-hidroxi-4-fenil-propano). Su composición también varía en función del método de separación de la misma y de si se

obtiene de árboles coníferas o latifoliadas. Por lo tanto, cuando se habla de lignina no se habla de un compuesto en particular sino de un compendio de varios compuestos.

La lignina es el segundo polímero terrestre más abundante después de la celulosa. Se trata de un compuesto hidrofóbico,lo que posibilita la conducción del agua de modo efectivo a través del sistema vascular de las plantas (Sarkanen & Ludwig, 1971). La lignina se produce en grandes cantidades como material de desecho en la industria del papel y pulpa (el refinado del material para la industria papelera) en todo el mundo. Su contenido en la madera varía del 18 al 25 % si se trata de maderas duras mientras que varía del 25 al 35 % si son maderas blandas (Pool, 2005). Hasta ahora, la lignina se ha tratado como material de desecho debido a la falta de utilización adecuada (Moon, Eoma, Kim, Kim, Lee, Choi & Choi, 2011), ya que por su muy compleja estructura es un polímero amorfo con un uso industrial bastante limitado (Pouteau, Dole, Cathala, Averous. L. & Boquillon, 2003). Sin embargo, los polímeros basados en celulosa presentan dos ventajas: por un lado, hay gran disponibilidad (abundantes toneladas de lignina como deshechos en las industrias del papel) y además la lignina es completamente biodegradable, aunque lo sea de modo lento. Por ello, se puede considerar como un polímero biodegradable "del futuro" (Vengal & Srikumar, 2005). Por otro lado los materiales lignocelulósicos son particularmente atractivos como materiales de partida para la producción de biofuel debido a su bajo coste, gran abundancia y suministro sostenible (Lacerda, De Paula, Zambon & Frollini, 2012).

Figura 6. Aspecto de la lignina.

Es importante destacar la presencia de la lignina en la fibra dietética, entendiéndose como tal la mezcla de polímeros presentes en las plantas, oligosacáridos y polisacáridos, como la celulosa, hemicelulosa, gomas, almidón resistente, inulina (que puede estar asociada con lignina) y otros componentes que no son carbohidratos como polifenoles, ceras o proteínas resistentes (Elleuch et al., 2011). Algunos autores han demostrado que aumentando la ingesta de fibra se obtienen benefi-

cios para la salud (disminuyendo el riesgo de enfermedades coronarias, diabetes, obesidad y algunos tipos de cáncer (Mann & Cummings, 2009).

2.3.2 Obtención de lignina y sus derivados

2.3.2.1 A partir de la madera

La deslignificación es el proceso de extracción de lignina a partir de fuentes vegetales y se realiza por diferentes métodos. El objetivo es la desintegración lignocelulósica dentro de los componentes fibrosos (Marton, 1971).

2.3.2.1.1 Métodos químicos

Son los métodos más convencionales. Se dividen en:

2.3.2.1.1.1 Método Kraft

El método alcalino o proceso Kraft es el proceso dominante y da lugar a la lignina alcalina o tio-lignina. Así, en el método Kraft se forman los licores o lejías negras, residuos originados en la conversión de madera en pulpa de celulosa durante el proceso de separación de la lignina, hemicelulosa y otros componentes de la madera, con la liberación de las fibras de celulosas (Stenius, 2000). El proceso Kraft se utiliza para producir más del 80 % de la pulpa química en todo el mundo (Dang, Bhardwaj, Hoang & Nguyen, 2007). Si bien la mayoría de la lignina obtenida se quema como fuente de fuel, una pequeña fracción se purifica para utilizarse como producto polimérico de bajo coste (Pearl, 1982)

2.3.2.1.1.2 Método de bisulfito

El método de bisulfito da lugar a los ácidos sulfónicos de la lignina, llamados comúnmente lignosulfonatos (Lehman, 1970). Como se mencionó anteriormente, la lignina no es soluble en agua y esa propiedad se puede modificar mediante la sulfonación. Los lignosulfonatos tienen grupos sulfonados y se obtienen con distintos cationes como el sodio, calcio y magnesio (Shen, Zhang & Zhu, 2008). Los lignosulfonatos tienen grupos hidrofílicos (grupos sulfónicos, fenólicos y alcohólico)s pero también grupos hidrófobos como cadenas hidrocarbonadas (Dawy, Shabaka & Nada, 1998) o grupos alifáticos, por lo que los lignosulfonatos pueden actuar como surfactantes (Telysheva, Dizhbite & Paegle, 2001).

2.3.2.1.2 Método *organosolv*

El método *organosolv* es un procedimiento comercial a menor escala y se presenta como alternativa por su menor coste económico y contaminante. En este proceso, a los licores de cocción se

incorpora una determinada cantidad de disolvente orgánico que es de sencilla recuperación y facilita la deslignificación del material de partida (Oliet, Rodríguez, García & Gilarranz, 2001).

2.3.2.2 A partir de restos de alimentos

Los restos lignocelulósicos que se obtienen de la industria alimentaria o de la industria agrícola sirven omo fuente de obtención de lignina. Así, se puede aislar lignina procesando el azúcar de caña o las pieles de naranja y manzana (Chiellini, Cinelli, Imam & Mao, 2001). También se ha aislado lignina de los licores negros del aceite de palma usando como disolventes el 2-metoxietanol y y el dimetilsulfóxido (DMSO). Este estudio refleja que los grupos funcionales de la lignina extraída de los licores negros y de la lignina comercial son muy similares, por tanto esta fuente se puede considerar como una alternativa más para aplicaciones en la industria alimentaria (Bhat, Khalil & Karim, 2009). Otra vía donde se puede encontrar este compuesto es en los licores negros generados durante la producción de bioetanol de la paja de arroz (Minu, Kurian Jiby & Kishore, 2012) o a partir de bambú, donde se separa la lignina de la hemicelulosa y celulosa con líquidos iónicos seguidos de extracción alcalina (Yanga, Zhong, Yuanb, Penga & Suna, 2013).

Por tanto, teniendo en cuenta todas las fuentes de las que se puede obtener lignina y sus derivados y las propiedades que presentan, se concluye el interés de su utilización, aplicación y estudio en distintos campos.

2.3.3 Propiedades de películas con lignina

2.3.3.1 Propiedades físicas

La lignina influye en las propiedades físicas finales de las películas porque da lugar a un descenso de la resistencia a la tracción y de la elongación en la ruptura, así como a un aumento en la absorción de agua. Este hecho se evidenció al utilizar lignina en la elaboración de películas para cápsulas de medicinas con menor coste de fabricación (Vengal et al., 2005). En otro estudio, la lignina procedente del procesado de caña de azúcar se incorporó a películas de gelatina, con lo que se redujo la elongación y la fuerza de tensión, mientras que el módulo de Young se incrementó (Chiellini, Cinelli, Fernandes, Kenawy & Lazzeri, 2001). La lignina también se ha utilizado como agente plastificante en películas de almidón (Wu, Wang, Li, Li & Wang, 2009), aunque cuando se utiliza en *composites* con micropartículas de almidón modificadas con ácido adípico (dentro de una matriz de almidón de maíz), aumenta la fuerza de tensión y disminuye la elongación (Spiridon, Teaca & Bodirlau, 2011).

2.3.3.2 Propiedades químicas

Los productos poliméricos que se obtienen en la industria de la madera tienen muchas aplicaciones basadas en la capacidad dispersante, ligante, emulsificante y secuestrante de la lignina (Northey, 1992). La lignina destaca por sus propiedades antioxidantes al ser un compuesto que evita/reduce la peroxidación lipídica y su poder antioxidante está íntimamente relacionado con el peso molecular. La lignina del bagazo con menor peso molecular presenta mayor poder antioxidante, y el lignosulfonato con el mayor peso molecular posee la menor capacidad antioxidante (Ugartondo et al., 2009). Además, está descrito el importante efecto inhibitorio del lignosulfonato frente la peroxidación lipídica, a pesar de su alto peso molecular comparado con otros tipos de lignina (Ugartondo et al., 2009). La captación de radicales libres de ligninas está influida por las características estructurales del compuesto, tales como la presencia de grupos hidroxilo fenólicos, grupos metoxi, conjugación de π-sistemas así como el peso molecular, la heterogeneidad y la polidispersidad (Dizhbite, Telysheva, Jurkjane & Viesturs, 2004). Así, un estudio reciente en el que se trabaja con lignina extraída del bagazo de la caña de azúcar utilizando etanol o soluciones alcalinas, demuestra que con ambos procedimientos se obtiene lignina con alto poder antioxidante, si bien el procedimiento alcalino da mejores resultados porque se obtiene mayor cantidades de grupos fenólicos (Ph-OH) y metoxi (-OCH_3) (Zhili & Ge, 2012). Por otro lado, al someter al bambú a una extracción con soluciones acuosas de ácidos orgánicos y complementada con microondas se obtiene lignina con alta capacidad antioxidante (Li, Sun, Xu & Sun, 2012) por el alto contenido de grupos hidroxílicos y fenólicos. Otros estudios corroboran la importancia del método de extracción y separación de la lignina, pues influye en la capacidad antioxidante final (García, Toledano, Andrés & Labidi, 2010).

Como componente importante en la fibra dietética, la lignina puede inhibir la actividad de las enzimas relacionadas con la generación de radicales superóxidos y dificultar el crecimiento y viabilidad de células cancerígenas (Lu, Chu & Gau, 1998). La lignina es un compuesto muy antioxidante por su acción sobre los radicales hidroxilos, inhibiendo la acción de la xantin oxidasa y glucosa-6-fosfato deshidrogenasa, e inhibiendo la peroxidación lipídica enzimática y no enzimática (Lu et al., 1998). Además, la lignina purificada (a diferencia de la lignina nativa) no representa una barrera a la digestión en animales monogástricos o rumiantes (Baurhoo, Ruiz-Feria & Zhao, 2008).

2.3.3.3 Propiedades biológicas y citotoxicidad

Las propiedades antimicrobianas de las ligninas se han recogido previamente en la literatura, como por ejemplo en hidrolizados de diferentes materiales lignocelulósicos (extractos de etil acetato) (Cruz, Domínguez, Domínguez & Parajó, 2001), en estructuras lignínicas de extracciones alcalinas (Oh-Hara, Sakagami, Kawazoe, Kaiya, Komatsu & Ohsawa, 1990) y en ligninas Kraft (Dizhbite et al., 2004). El origen de la lignina puede influir en sus propiedades antimicrobianas. Así, la actividad antimicrobiana inducida por las ligninas comerciales es mucho menor que la inducida por fracciones de

extractos de cono de pino obtenidas por sucesivas extracciones alcalinas (Oh-Hara et al., 1990). Comparando los espectros, los mencionados autores encontraron que algunos extractos de cono de pino incluyen dobles enlaces alquénicos y menor número de grupos dimetiléster (-OCH_3) que la lignina alcalina comercial, y que la estructura del ácido cumárico presente en la lignina podría ser responsable de la actividad antimicrobiana. Algunos autores sugieren que la inhibición del crecimiento microbiano por los ácidos fenólicos (dentro de los cuales se incluyen el ácido *p*-cumárico), se incremente con el descenso del pH (Wen, Delaquis, Stanich & Toivonen, 2003). También se ha determinado la actividad antimicrobiana de la lignina extraída de los residuos generados durante la producción del etanol y se han observado resultados distintos en función de las condiciones de extracción; se puede concluir que la lignina presenta actividad antimicrobiana contra bacterias Gram-postivas (*Listeria monocytogenes y Staphylococcus aureus*) y levaduras (*Candida lipolytica*), pero no contra bacterias Gram-negativas (*Escherichia coli O157:H7* y *Salmonella Enteritidis*) o bacteriófago MS2 (Dong, Dong, Lu, Turley, Jin & Wu, 2011).

La lignina es un compuesto no tóxico y biodegradable por lo que se ha incorporado en multitud de materiales (Julinová, Kupec, Alexy, Hoffmann, Sedlarík & Vojtek, 2010). Por otra parte, muchos derivados de lignina, incluyendo los lignosulfonatos, son igual o menos citotóxicos que otros compuestos biológicos como la epicatequina en keratinocito humano HaCat y fibroblasto murina 3 T3 (Ugartondo, Mitjans & Vinardell, 2008). Estos autores encontraron una concentración antioxidante efectiva de lignina concentraciones no citotóxicas, haciendo posible así su utilización en la formulación para el envase activo de alimentos. Tan solo unos pocos estudios describen los efectos citotóxicos de las ligninas. De este modo se ha visto que hay una buena correlación entre citotoxicidad y algunas características como el contenido de carbohidratos y polidispersidad, ya que las ligninas con mayor polidispersidad y menor contenido en carbohidratos son las más citotóxicas (Ugartondo et al., 2008).

Por otro lado se han realizado estudios en intestino de rata en el momento de la administración de fármacos antidiarréicos que contienen 90% de lignina, viendo que no hay modificación de la actividad en la sucrasa ni fosfatasa alcalina y no se observaron modificaciones histológicas (Mitjans, García, Marrero & Vinardell, 2001).

2.3.4 La lignina y otros componentes

La lignina, debido a sus múltiples propiedades beneficiosas, se ha utilizado en mezclas con otros biopolímeros biodegradables, como el aislado de proteína de soja (Huang, Zhang & Chen, 2003) o el almidón (Baumberger, Lapierre, Monties, Lourdin & Colonna, 1997), consiguiendo mejorar la resistencia al agua y las propiedades mecánicas de las películas finales. La sílice ha sido otro compuesto utilizado (Klapiszewski, Nowacka, Milczarek & Jesionowski, 2013) con la lignina con el objetivo de mejorar sus propiedades fisicoquímicas y electrocinéticas. También la lignina se utiliza junto

con el acetato butirato de celulosa (Davé & Glasser, 1997) y con el almidón para favorecer la biodegradación de películas de polivinilalcohol, e hidrolizados de proteína (Julinová, Kupec, Alexy, Hoffmann, Sedlařík, Vojtek, Chromčáková & Bugaj, 2010). Es importante resaltar la utilización de la lignina con polímeros sintéticos debido a su carácter renovable, no tóxico y biodegradable (Ban, Song & Lucia, 2007; Vengal et al., 2005). Los derivados de lignina emplean como agentes de relleno en distintas matrices de polímeros sintéticos para desarrollar materiales con propiedades físicas mejoradas (Cui, Xia, Chen, Wei & Huang, 2007; Mishra, Mishra, Kaushik & Khan, 2007). Otro ejemplo es su utilización con polipropileno, ya que la capacidad antioxidante de la lignina permite que las reacciones oxidativas indeseadas que se originan durante la elaboración de este polímero no se den en tan alto grado (Pouteau et al., 2003). La lignina también se ha utilizado junto con el polivinilalcohol PVA para mejorar su estabilidad térmica y fotoquímica (Fernandes, Winkler Hechenleitner, Job, Radovanocic & Gómez Pineda, 2006) o su biodegradación (Julinová et al., 2010), y el lignosulfonato se adiciona al poliuretano con el objetivo de mejorar sus propiedades mecánicas (Cui et al., 2007). Además, cuando se realizan mezclas de ligninas y compuestos termoplásticos, la adición de plastificantes disminuye el grado de asociación de lignina a la mezcla, previamente estudiado como algo que afecta negativamente a las propiedades mecánicas (Li, Mlynar & Sarkanen, 1997); (Feldman, Banu, Campanelli & Zhu, 2001).

Por otro lado, la lignina *organosolv* se ha incluido en la elaboración de resinas como sustitutos del fenol, y el glutaraldehído como sustituto del formaldehido, obteniéndose resinas con propiedades iguales o mejores que las resinas fenol-formaldehído (Da Silva, Grelierb, Pichavantb, Frollini & Castellan, 2013). La lignina también se utiliza con un procedimento por “grafting” junto con polímeros sintéticos para preparar una novedosa clase de plásticos (G. Sena-Martins, 2008) y se ha aplicado como precursor de fibras de carbono (Otani, Fukuoka, Igarashi & Sasak, 1969). También se utilizan geles químicos de uretano o epoxídicos con derivados de lignina para estudiar su termorrespuesta (Uraki, Imura, Kishimoto & Ubukata, 2004).

Es interesante la utilización de la lignina modificada tras procesos enzimáticos, en los que destaca la copolimerización de la lignina procedente del proceso Kraft con el cresol, usando peroxidasa del rábano “horseradish” como enzima catalítica de polimerización. Los compuestos resultantes se pueden usar como sustitutos de resinas fenólicas (Liu, Eiping & Lo, 1999). También se puede hacer una copolimerización de diferentes ligninas con ácido vanílico, diisocianato y acrilamida catalizada por lacasa (Hüttermann, Mai & Kharazipour, 2001); los productos resultantes ofrecen un alto potencial para nuevos materiales (G. Sena-Martins, 2008). Al mismo tiempo, la lignina se ha aplicado (en distintas formas) junto con formaldehído con el objetivo de elaborar recubrimientos para una liberación prolongada de urea a la hora de usarla como fertilizantes (Muldera, Gosselinka, Vingerhoedsa, Harmsena & Easthamb, 2011).

2.4 *Legislación*

En Europa hay leyes vigentes que regularizan los envases activos:

- Reglamento CE 1935/2004 sobre los materiales y objetos destinados a entrar en contacto con alimentos. En este reglamento se describe: "los materiales y objetos activos en contacto con alimentos están diseñados para incorporar deliberadamente componentes *activos* destinados a pasar a los alimentos o a absorber sustancias de los mismos".

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:338:0004:0004:ES:PDF)

- Reglamento CE 450/2009 sobre los materiales activos e inteligentes y artículos que entran en contacto con alimentos. En este reglamento se describe: "los materiales y objetos activos pueden incorporar deliberadamente sustancias destinadas a ser liberadas en el alimento".

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:135:0003:0003:ES:PDF)

Queda expuesta la legislación pertinente a las películas estudiadas en la presente memoria, pues se clasifican como envasado activo.

Por otro lado, las películas están elaboradas con aditivos alimentarios. En el Reglamento CE 1129/2011 se recoge una lista positiva de aditivos admitidos en la Unión Europea. En esta lista figuran el sorbitol (E-420) y Glicerol (o glicina, E-422).

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:295:0001:0177:ES:PDF)

En el Reglamento CE 853/2004 se establecen normas específicas de higiene de los alimentos de origen animal. Aquí se recogen los requisitos de fabricación, materias primas autorizadas, etc., por ejemplo la gelatina, siendo esta sustancia un componente esencial de las películas estudiadas.

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:139:0055:0205:ES:PDF)

El Real Decreto 1125/1982 recoge la reglamentación técnico-sanitaria para la elaboración, circulación y comercio de materiales poliméricos. Se detalla una lista positiva de sustancias destinadas a la fabricación de compuestos macromoleculares para la elaboración de envases u otros utensilios en contacto con alimentos. En dichas listas figuran los sulfonatos de lignina y sales de calcio, potasio y sodio. La lignina no se recoge en la legislación, pero sí figuran en estas listas las fibras naturales y sintéticas y maderas de árboles varios. (http://www.boe.es/diario_boe/txt.php?id=BOE-A-1982-13244). Este Real Decreto está posteriormente modificado por los Reales Decretos 2814/1983, 1425/1988 y 293/2003, pero no le afecta la incorporación de las sustancias de interés.

(http://www.boe.es/diario_boe/txt.php?id=BOE-A-1983-29113)

(https://www.boe.es/buscar/doc.php?id=BOE-A-1988-27709)

(http://www.boe.es/boe/dias/2003/03/11/pdfs/A09480-09482.pdf)

Por último, el Real Decreto 866/2008 recoge una lista de sustancias permitidas para la fabricación de materiales y objetos plásticos destinados a entrar en contacto con alimentos. (http://www.boe.es/boe/dias/2008/05/30/pdfs/A25070-25120.pdf). Este Real Decreto establece un límite máximo de migración para películas de 10mg/ dm^2 y unas condiciones de ensayo de migración

(Directivas 82/711/EEC u 85/572/EEC, requerimientos para testar la migración de materiales que estén en contacto con alimentos).

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31982L0711:ES:NOT)

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31985L0572:ES:NOT)

3 Hipótesis

Hipótesis

Los envases juegan un papel importante en la conservación de los alimentos. En la sociedad actual existen diversos tipos de envases formados por compuestos sintéticos, los cuales generan gran cantidad de residuos que ocasionan problemas económicos y medioambientales. Como alternativa se presenta la utilización de compuestos biodegradables para el envasado de alimentos donde destacan los biopolímeros, utilizados normalmente como matriz y soporte, y de compuestos naturales con propiedades antioxidantes, antimicrobianas, etc. La combinación de varios de estos compuestos da lugar a envases activos cuya finalidad es alargar la vida útil del alimento originando la menor modificación organoléptica y nutricional posible.

En la presente Memoria se utilizan como compuestos principales la gelatina (de diversos orígenes), la lignina (presente en las células vegetales y componente de la fibra dietética) y lignosulfonatos (forma soluble de la lignina obtenida mediante sulfonación). Estos compuestos presentan la ventaja de ser biodegradables y además son subproductos industriales, por lo que su utilización representa una disminución de residuos, un aprovechamiento de los mismos y una mejora medioambiental. Además, la lignina y derivados son compuestos altamente antioxidantes, antimicrobianos y presentan baja citotoxicidad, por lo que su combinación con la gelatina podría constituir un material de envasado activo de alimentos.

Por otro lado, el pescado es un producto muy perecedero con alta tendencia a deteriorarse, especialmente en el caso del pescado graso, pues sufre enranciamiento con gran facilidad. Es por ello que un envasado activo solo o combinado con otras tecnologías de conservación podría constituir una alternativa para retardar su deterioro y alargar su vida útil.

Por todo ello, el desarrollo de películas de gelatina de varios orígenes junto con lignina y derivados, y su aplicación a pescados grasos puede dar lugar a resultados de interés para el futuro del envasado activo de alimentos.

4 Objetivos

Objetivos

El objetivo de la presente Memoria es el desarrollo y estudio de películas compuestas de gelatina de diversos orígenes y lignina o lignosulfonato, y su aplicación para la conservación de pescado. Este objetivo general se puede desglosar en los siguientes objetivos parciales:

1. Formulación y caracterización físico-química de las películas obtenidas a partir de diversas gelatinas (gelatina bovina, gelatina de peces de aguas frías y de peces de aguas cálidas) y distintos tipos de lignosulfonato y de lignina.

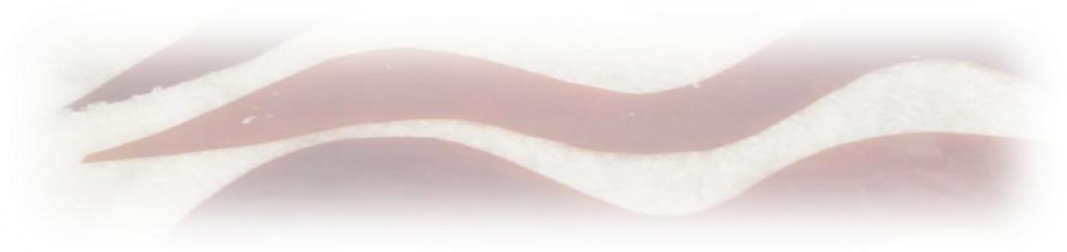
2. Evaluación de la capacidad antioxidante, antimicrobiana y citotoxicidad del lignosulfonato y la lignina para su posible uso combinado con la gelatina en la obtención de películas activas.

3. Evaluación de la estabilidad físico-química y funcional de las películas de gelatina y lignosulfonato en sistemas modelo.

4. Estudio de conservación en refrigeración de sardina (*Sardina pilchardus*) y de salmón (*Salmo salar*) recubiertos con película de gelatina y lignina o lignosulfonato mediante tratamientos combinados con alta presión.

5 Trabajo experimental.

Artículos en prensa científica



5.1 *Artículo 1. Role of lignosulphonate in properties of fish gelatin films*

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Role of lignosulphonate in properties of fish gelatin films

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ABSTRACT

A commercial low-gelling fish skin gelatin was used to prepare edible films by casting with glycerol and sorbitol added as plasticizers. In order to improve the extremely low water resistance of gelatin films, composite films were prepared with increasing concentrations (wt/wt) of lignosulphonate (LS) from eucalyptus wood (100:0, 85:15, 80:20, 75:25, 70:30 and 65:35, gelatin:LS). How gelatin film properties were affected by the different types of gelatin and LS was determined by comparing bovine gelatin and three different LS (Ca^{2+}, Mg^{2+} and Na^{+}) in a mixture ratio of 80:20. Physical properties of films were characterized in terms of tensile strength, elongation at break, water solubility, water vapour permeability and opacity. Dynamic oscillatory tests of film-forming solutions revealed strong LS interference with the cold-renaturation ability of gelatin. LS ratios equal to or higher than the 80:20 blend interfered with intermolecular aggregation of gelatin helices. Supposedly, LS acted as a filler, inducing mostly nonbonding interactions with gelatin, as deduced from Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) and Differential Scanning Calorimetry (DSC) studies. Lignosulphonate significantly reduced the elongation at break of fish gelatin films, water solubility being drastically reduced with a mixture ratio of 80:20 or higher. The water solubility of bovine gelatin–LS composite films was significantly lower than that for fish gelatin, regardless of the type of LS employed. For potential food packaging applications, the three LS were characterized in terms of cytotoxicity, radical scavenging capacity (DPPH assay) and antimicrobial capacity. The effective antioxidant levels (IC_{50}: 83.4–97.5 μg/mL) were noticeably lower than the cytotoxic ones (IC_{50}: 1480–1745 μg/mL), indicating that these compounds could be used as antioxidants at non-cytotoxic concentrations. No relationship between antioxidant and antimicrobial properties could be observed, the only notable antimicrobial finding being some activity against yeasts.

1. Introduction

The increasing tendency to use biodegradable films based on biopolymers from renewable sources is becoming an important factor in reducing the environmental impact of petroleum-derived plastic waste (Tharanathan, 2003). Gelatin has been one of the most studied biopolymers on account of its film-forming ability and its usefulness as an outer film to protect food from drying and exposure to light and oxygen (Arvanitoyannis, 2002). The production of gelatin from sources other than those of the mammalian species is growing in importance as a result of the outbreak of bovine spongiform encephalopathy, and the banning of collagen from pig skin and bone in some regions for religious reasons. Furthermore, the rising interest in putting fish industry by-products to good use is one of the reasons why exploring multifunctional fish gelatins from different species has attracted the attention of researchers in the last decade, as reviewed by Gómez-Guillén, Giménez, López-Caballero, and Montero (2011), Gómez-Guillén et al. (2009) and Karim and Bhat (2009). Fish gelatins exhibit good film-forming properties, yielding transparent, nearly colourless and highly extensible films (Avena-Bustillos et al., 2006; Carvalho et al., 2008; Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007; Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006; Zhang, Wang, Herring, & Oh, 2007). Films based on low-gelling gelatin from cold-water fish species present very poor water resistance, being almost completely soluble in water (Carvalho et al., 2008; Denavi et al., 2009; Pérez-Mateos, Montero, & Gómez-Guillén, 2009). This could be a drawback when they are applied to food products with high moisture content, because the films may swell, partially dissolve or disintegrate upon contact with the wet surface.

The mechanical and barrier properties of fish gelatin films have been enhanced by blending with different types of biopolymers such

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as proteins (Denavi et al., 2009), lipids (Jongjareonrak et al., 2006; Pérez-Mateos et al., 2009) or polysaccharides (Kołodziejska, Piotrowska, Bulge, & Tylingo, 2006; Liu, Liu, Fishman, & Hicks, 2007; Pranoto, Lee, & Park, 2007). Different cross-linking agents, such as glutaraldehyde (Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001), microbial transglutaminase or 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (Kołodziejska & Piotrowska, 2007; Yi, Kim, Bae, Whiteside, & Park, 2006), as well as oxidized phenols from oxygenated seaweed extracts (Rattaya, Benjakul, & Prodpran, 2009) have also been proposed for improving the water resistance of fish gelatin films.

Lignin is a complex polydisperse natural polymer made up of phenylpropane (C6–C3) units that bind cellulose fibres together, thus hardening and strengthening the plant cells. Most commonly derived from wood, lignin is largely thrown off as a waste product in pulp and paper industries. However, on account of its renewable, non-toxic and biodegradable character, lignin has been incorporated as a filler in different polymer matrices to develop lignin-based materials (Cui, Xia, Chen, Wei, & Huang, 2007; Feldman, Lacasse, & Beznaczuk, 1986; Julinová et al., 2010; Kadla & Kubo, 2003; Mishra, Mishra, Kaushik, & Khan, 2007; Wu, Wang, Li, Li, & Wang, 2009). Lignin has been reported to produce a decrease in tensile strength and elongation at break, along with an increase in water absorption, when included in gelatin-based films with the purpose of producing cheaper drug capsules (Vengal & Srikumar, 2005). Similarly, the incorporation of sugar cane bagasse, a lignin cellulosic waste from sugar cane processing, in gelatin films sharply reduced both elongation and tensile strength while increasing Young's modulus (Chiellini, Cinelli, Fernandes, Kenawy, & Lazzeri, 2001). According to these authors, the dark colour of gelatin-sugar cane bagasse composites might turn out to be a good structural attribute for the potential application of these films in soil solarisation and weed control. Recently, a gelatin-lignin film was shown to improve the appearance, protein quality and oxidative stability of salmon fillets subjected to high pressure processing (Ojagh, Nuñez-Flores, López-Caballero, Montero & Gómez-Guillén, 2011).

In the process of cellulose manufacture, lignosulphonates (LS) can be isolated from spent sulphite liquors. Unlike lignin, LS are water-soluble polydisperse polyelectrolytes containing metal ions (Ca^{2+}, Mg^{2+} or Na^{+}), hydrophilic groups such as sulphonic, carboxyl and hydroxyl groups, as well as hydrophobic aromatic and aliphatic groups. The presence of highly polar functional sulphonic groups on a hydrophobic skeleton gives LS important surface-active properties (Ng, Rana, Neale, & Hornof, 2003; Telysheva, Dizhbite, Paegle, Shapatin, & Demidov, 2001). LS have also been blended with other biodegradable biopolymers, such as soy protein isolate (Huang, Zhang, & Chen, 2003) or starch (Baumberger, Lapierre, Monties, Lourdin, & Colonna, 1997) in order to improve the water resistance and mechanical properties of the composite films.

On account of their superoxide and hydroxyl radical scavenging capacity (Lu, Chu, & Gau, 1998), lignins have been shown to be efficient antioxidants, thus opening up the possibility of new potential applications (Dizhbite, Telysheva, Jurkjane, & Viesturs, 2004; Pan, Kadla, Ehara, Gilkes, & Saddler, 2006; Satoh et al., 1999; Ugartondo, Mitjans, & Vinardell, 2008). In this regard, Ugartondo, Mitjans, and Vinardell (2009) reported the significant inhibitory effect of LS against lipid peroxidation despite its higher molecular weight compared to other types of lignin. Moreover, several lignin derivatives, including LS, were shown to be equal or less cytotoxic than (−)-Epicatechin in human keratinocyte HaCaT and murine fibroblast 3T3 cells (Ugartondo et al., 2008). These authors found that the effective antioxidant concentrations of all lignin derivatives were from 5- to 10-fold lower than the cytotoxic ones, indicating that the compounds were effective antioxidants even at non-cytotoxic concentrations, thus furthering their possible use in the formulation of active food packaging biomaterials.

The aim of the present work was to study the formation and physicochemical properties of composite films obtained from a commercial low gelling fish-skin gelatin mixed with different proportions of LS, and also to determine the effect produced by using different gelatins, including bovine, and three different LS (Ca^{2+}, Mg^{2+} and Na^{+}). In order to establish the harmlessness and potential functionality in food packaging applications, the three LS tested were characterized in terms of cytotoxicity, radical scavenging capacity and antimicrobial capacity.

2. Materials and methods

2.1. Materials

Commercial High Molecular Weight Fish Gelatin (F) was supplied by Norland (Cranbury, NJ, USA). As referred by the manufacturer, this type-A gelatin, with non- measurable Bloom value, has been produced from the skin of deep water fish such as cod, haddock and Pollock.

For comparison purposes, a commercial type-A bovine-hide gelatin (Bloom 200/220) (B) from Sancho de Borja S.L. (Zaragoza, Spain) was used. Lignosulphonate (LS) from eucalyptus wood was kindly supplied by LignoTech Ibérica, S.A. (Torrelavega, Spain). According to manufacturer's specifications, three different LS preparations (50% dry matter) were tested: (i) ID081203, calcium-magnesium LS with a molecular weight of 6236 Da and high sugar content (16% reducing sugars) (LSa); (ii) ID081204, calcium-magnesium LS with a molecular weight of 6664 Da and low sugar content (6% reducing sugars) (LSb); (iii) ID081205, sodium LS with a molecular weight of 7085 Da and low sugar content (4% reducing sugars) (LSc). Glycerol and sorbitol were obtained from Panreac (Barcelona, Spain). All other reagents used were of analytical grade. The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical was purchased from Sigma–Aldrich (St. Louis, MO, USA). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) salt was supplied by Promega Biotech Ibérica (Madrid, Spain).

2.2. Preparation of films

The gelatin film-forming solution was prepared by dissolving dry gelatin in distilled water (4% w/v) at 40 °C, adding sorbitol (15 g/100 g gelatin) plus glycerol (15 g/100 g gelatin) as plasticizers. Gelatin–LS films were prepared by mixing the dissolved gelatin with LS at different ratios (calculated on dry matter content) using sorbitol (15 g/100 g dry matter) plus glycerol (15 g/100 g dry matter) as plasticizers. Six groups of films with different ratios of gelatin to LS (100:0, 85:15, 80:20, 75:25, 70:30 and 65:35) were prepared. The mixtures were stirred at 40 °C for 30 min. The films were made by casting an amount of 40 mL over a plate of 12×12 cm^2 and drying at 45 °C in a forced-air oven for 15 h to yield a uniform thickness of 100 ± 10 μm. Films were conditioned over a saturated solution of KBr at 21 °C in desiccators for 4 d.

2.3. Viscoelastic properties of film-forming solutions

Dynamic oscillatory study of the film-forming solutions was carried out on a Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4°, gap 0.15 mm). Cooling and heating from 30 to 2 °C and back to 30 °C took place at a scan rate of 1 °C/min, a frequency of 0.5 Hz, and a target strain of 0.5%. The elastic modulus (G'; Pa), viscous modulus (G''; Pa) and phase angle (°) were plotted as functions of temperature in the heating ramp from 2 to 30 °C. At least two determinations were performed for each sample, with an experimental error of less than 6% in all cases.

2.4. Attenuated Total Reflectance (ATR)-FTIR spectroscopy

Infrared spectra between 4000 and 650 cm^{-1} were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin Elmer Inc, Waltham, MA, USA) equipped with an ATR prism crystal accessory. The spectral resolution was 4 cm^{-1} and 32 scans were averaged for each spectrum. Measurements were performed at room temperature directly on films (previously conditioned during 5 days in desiccators containing silica gel), which were placed on the surface of the ATR crystal, and pressed with a flat-tip plunger until spectra with suitable peaks were obtained. All experiments were performed at least in triplicate and represented as average spectra. Background subtraction and second derivative of amide I band were done using the Spectrum software version 6.3.2 (Perkin Elmer Inc.).

2.5. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of the films were determined by tensile test using a TA.XT.plus Texture analyser (SMS, Surrey, UK). The samples were cut into rectangles with 20 mm width and 50 mm length, fixed on the grips of the device with a gap of 20 mm, and tractioned at a speed of 1 mm/s. Results of TS and EAB were average of five determinations, and were expressed as N/m^2 and %, respectively.

2.6. Water solubility

Film portions of 4 cm^2 were weighed and placed in beakers with 15 mL of distilled water, then sealed and let stand at 22 °C for 15 h. The solution was then filtered through Whatman no. 1 filter paper to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h. Film solubility was calculated by the equation FS (%) = ((Wo − Wf)/Wo)·100, where Wo was the initial weight of the film expressed as dry matter and Wf was the weight of the undissolved desiccated film residue. All tests were carried out in triplicate.

2.7. Water vapour permeability

Water vapour permeability (WVP) was determined following the gravimetric method described by Sobral, Menegalli, Hubinger, and Roques (2001) with slight modification. Films were attached over the openings of cells (permeation area = 15.9 cm^2) containing desiccated silica gel, and the cells were placed in desiccators with distilled water at 22 °C. The cells were weighed every hour for at least 6 h. Water vapour permeability was calculated using the equation $WVP = w \cdot x \cdot t^{-1} \cdot A^{-1} \cdot \Delta P^{-1}$, where w was weight gain (g), x film thickness (mm), t elapsed time for the weight gain (h), and ΔP the partial vapour pressure difference between the dry atmosphere and pure water (2642 Pa at 22 °C). Results have been expressed as g mm h^{-1} cm^{-2} Pa^{-1}. All tests were carried out at least in triplicate.

2.8. Opacity

The films were cut into a rectangle piece and directly placed against one side of the spectrophotometer test cell, using an empty test cell as the reference. The opacity of the films was calculated by the equation $O = Abs_{600}/x$, where Abs_{600} is the value of absorbance at 600 nm and x is the film thickness in mm. Measurements were done at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan).

2.9. Thermal properties

Calorimetric analysis was performed using a model TA-Q1000 differential scanning calorimeter (DSC) (TA Instruments, New Castle, DE, USA) previously calibrated by running high purity indium (melting point 156.4 °C; enthalpy of melting 28.44 J/g). Samples of approximately 10 mg (±0.002 mg) were weighed out using a model ME235S electronic balance (Sartorious, Goettingen, Germany) and were tightly encapsulated in aluminium pans and scanned under dry nitrogen purge (50 mL/min). Freshly conditioned films were rapidly cooled to 0 °C and scanned between 0 and 90 °C at a heating rate of 10 °C/min. Glass transition temperatures, Tg (°C), were determined only on the first heating scans, in order to characterize the same material used in the rest of analyses (values obtained on the second scans being deemed not relevant because of the practical impossibility of reproducing the original film conditioning). The glass transition temperature was estimated as the midpoint of the line drawn between the temperature at the intersection of the initial tangent with the tangent through the inflection point of the trace and the temperature of the intersection of the tangent through the inflection point with the final tangent. Tg data were reported as mean values of at least duplicate samples for each film, usually within ±1–2 °C.

2.10. Antioxidant activity of LS

The antioxidant activity of the LS was determined based on the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability. The assay was carried out as described by Brand-Williams, Cuvelier, and Berset (1995) with some modifications (Fukumoto & Mazza, 2000). The LS were analyzed by triplicate testing at least five different concentrations ranging from 50 to 800 μg/mL. The radical scavenging activity was expressed as IC_{50} value, the concentration necessary to quench 50% of initial DPPH radical. The percentage of scavenged DPPH was plotted versus the concentration of LS, and that required to quench 50% of initial DPPH radical was obtained from the graph by linear regression. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analogue, was used as reference compound. The DPPH radical scavenging capacity of Trolox, expressed as IC_{50} value, was determined by testing concentrations ranging from 3 to 50 μg/mL.

2.11. Cytotoxic effect of LS

2.11.1. Culture of cell lines

The mouse fibroblast cell line 3T3-L1 was grown in DMEM medium (4.5 g/L glucose) supplemented with 10% foetal bovine serum (FBS), at 37 °C under a humidified 5% CO_2 atmosphere. When the cells were approximately 70% confluent, they were split by mild trypsinization and seeded into 24-well plates at a density of 1×10^4 cells/well. The 24-well plates were incubated at 37 °C and 5% CO_2 for 24 h. Runs were performed in triplicate with different passage cells.

2.11.2. Experimental treatments

After 1 day of incubation, cultures were exposed to increasing concentrations of LS (from 2.5 to 1.75 mg/mL) sterilized by filtration and diluted in DMEM medium supplemented with 10% FBS. Controls (containing only the culture medium) were included in each plate. The plates were incubated at 37 °C with 5% CO_2 for 24 h.

2.11.3. MTS assay

The viability of the 3T3-L1 cells treated with increasing concentrations of LS for 24 h was determined by the MTS assay, composed of the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent (PMS, phenazine methosulfate). This assay is based on the conversion of the tetrazolium

compound into a coloured, aqueous soluble formazan product by mitochondrial activity of viable cells at 37 °C. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in culture.

MTS and PMS were combined in a ratio of 20:1 and the mixture was added to the culture medium in a ratio of 1:5 (reagent mixture:culture medium). Cells were washed with PBS and the medium containing MTS/PMS was added (500 µl per well). After 1 h of further incubation, the absorbance was measured in a microplate reader at 485 nm (Appliskan, Thermo Scientific, Madrid, Spain).

2.12. Antimicrobial capacity of LS

The antimicrobial activity of the lignosulphonates was determined by the disk diffusion method in agar against 26 microbial strains as previously described (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010). The strains, selected because of its importance in health (such as probiotics or pathogens) or for being responsible for food spoilage, were obtained from the Spanish Type Culture Collection (CECT): *Lactobacillus acidophilus* CETC 903, *Salmonella cholerasuis* CECT 4300, *L. acidophilus* CECT 903, *Listeria innocua* CECT 910, *Citrobacter freundii* CECT 401, *Escherichia coli* CECT 515, *Shigella sonnei* CECT 4887, *Pseudomonas aeruginosa* CECT 110, *Yersinia enterocolitica* CECT 4315, *Brochothrix thermosphacta* CECT 847, *Staphylococcus aureus* CECT 240, *Bacillus cereus* CECT 148, *Listeria monocytogenes* CECT 4032, *Clostridium perfringens* CECT 486, *Aeromonas hydrophila* CECT 839T, *Photobacterium phosphoreum* CECT 4192, *Shewanella putrefaciens* CECT 5346T, *Pseudomonas fluorescens* CECT 4898, *Vibrio parahaemolyticus* CECT 511T, *Bacillus coagulans* CECT 56, *Bifidobacterium animalis* subespecie *lactis* DSMZ 10140, *Bifidobacterium bifidum* DSMZ 20215, *Enterococcus faecium* DSM 20477, *Lactobacillus helveticus* DSM 20075, *Debaryomyces hansenii* CECT 11364, *Aspergilus niger* CECT 2088, *Penicilium expansum* DSMZ 62841. After incubation, the inhibition area -considered the antimicrobial activity- was measured with Acrobat Reader Professional software ver. 6. Results were expressed as percentage of growth inhibition respecting to the total plate surface. Each determination was performed in duplicate.

2.13. Statistical analysis

Statistical tests were performed using the SPSS computer program (SPSS Statistical Software Inc., Chicago, IL, USA). One-way analysis of variance was carried out. Differences between pairs of means were assessed on the basis of confidence intervals using the Duncan test. The level of significance was $p \leq 0.05$.

3. Results and discussion

3.1. Viscoelastic properties of film-forming solutions

To what extent lignosulphonate (LSa) contributes to gelatin renaturation into the triple helical structure has been evaluated by studying the viscoelastic properties of the film-forming solutions obtained by mixing different proportions of both biopolymers in the presence of sorbitol plus glycerol as plasticizers. Fig. 1 shows the viscoelastic behaviour upon heating from 2 °C to 30 °C of previously cold-gelled film-forming solutions. Importantly, we have used a cold-water fish gelatin with very low gel forming capacity, deducible from the low value of the elastic modulus (G′, 0.02 Pa), achieved by cooling the film-forming solution (4% gelatin, w/v) to 2 °C at a rate of 1 °C/min. The lower content of both proline and especially hydroxyproline residues in cold-water fish gelatins, as compared to gelatins from mammals or fish from tropical waters,

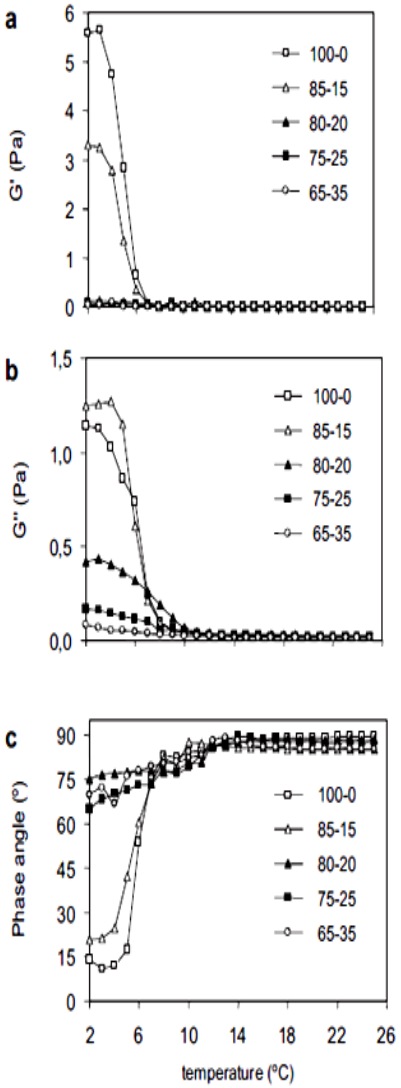

Fig. 1. Viscoelastic properties upon heating from 2 °C to 25 °C of film-forming solutions based on mixtures of fish gelatin (F) and lignosulphonate (LSa) at different ratios. a) Modulus of elasticity (G′, Pa); b) modulus of viscosity (G″, Pa); c) phase angle (°).

has been argued as the main reason for their lower thermostability and poorer rheological properties, due to a reduced hydrogen-bonding ability needed to induce the refolding of gelatin chains into the typical collagen-like triple helical structures (Gilsenan & Ross-Murphy, 2000; Ledward, 1986). Nevertheless, after a brief period of cold maturation (5 min at 2 °C), the gelatin solution without LS (100–0) resulted in a weak gel (G′ = 4.39 Pa, G″ = 1.10 Pa, $\delta = 14.22°$) which began to melt from 6 °C upwards, as can be deduced from the sharp increase in the phase angle curve (Fig. 1c). The replacement of 15% gelatin by LSa (blend 85:15) reduced the initial value of G′ (at 2 °C) by nearly 50%, while higher LS concentrations mostly hindered the refolding ability of gelatin chains, as is evident from the low G′ and G″ values and the virtual absence of thermal melting transition in the phase angle curve (blend 70:30 not shown). The changes in the viscoelastic behaviour of the different film-forming solutions suggested a considerable interference of LS in the formation of junction zones (short triple helical regions) in the gelatin matrix, increasing as the added quantity rose.

3.2. ATR-FTIR spectroscopy of films

To assess possible interactions between gelatin and LS (LSa) in the films resulting from the different mixture concentrations, a structural analysis by FTIR was conducted. All spectra showed main IR absorption bands at characteristic peak frequency values, corresponding to the amide A (~3278 cm^{-1}), amide I (~1634 cm^{-1}), amide II (~1533 cm^{-1}) and amide III (~1239 cm^{-1}), as befits the predominantly protein nature of the different films (Fig. 2). Replacing gelatin by increasing concentrations of LS produced a progressive decrease in the intensity and amplitude of the amide I, amide II and amide III bands, with the spectra of mixtures 80–20 and 75–25 reaching very similar intermediate values among all tested formulations (blend 70:30 not shown). According to Muyonga, Cole, and Duodu (2004), a decrease in the intensity of the peaks associated with amide bands I, II and III would indicate protein denaturation, however, a gelatin "dilution effect" cannot be excluded given the increased relative concentration of LS in the mixture. This effect was clearly visible through the progressive increase in the intensity of IR absorption in the range of frequencies between 1200 and 1000 cm^{-1}. This coincided with the region of asymmetric extension of the C–O–C bonds of LS, largely associated with the significant carbohydrate content, together with symmetric extension of the sulphone and sulphonate groups (Kadla & Kubo, 2003; Shen, Zhang, & Zhua, 2008). Moreover, the ability of the C–O glycerol group, added as plasticizer to increase IR absorbance in this frequency range, should also be taken into account (Bergo & Sobral, 2007).

The degree of interaction between two biopolymers is measured by the eventual changes in frequency at which main absorption peaks appear. Changes in the amplitude and intensity of the amide A band (associated with the stretching vibration of the NH bond) of gelatin produced by the presence of LS were barely noticeable, with only a slight widening of this band in mixture 65–35. This was attributed to the high content of –OH groups of the more concentrated lignin derivative, whose absorption peak has been described as around 3422 cm^{-1} (Kadla & Kubo, 2003). However, in contrast to the work reported by Kadla and Kubo (2003), the absence of any band shift suggests a possible interaction through hydrogen bonds at this level to be negligible.

The amide I band, related mainly to stretching vibrations of the protein C|O groups, after modification by hydrogen bonding and coupling with neighbouring carbonyl groups, peaked at ~1635 cm^{-1} in the gelatin film without LS and in mixtures up to 75–25. The addition of LS did not produce an apparent shift of wavenumber to lower values, so a possible interaction LS-gelatin by hydrogen bonding was again not evidenced. On the contrary, the highest LS concentration might have led to some interference in the hydrogen-bonding ability of gelatin polypeptides, as denoted by the up-shift to 1639 cm^{-1} in the 65:35 blend. Although IR absorption of carbonyl groups present in lignin has been described at higher frequencies (~1740–1700 cm^{-1}) (Fernandes, Winkler, Job, Radovanocic, & Pineda, 2006; Mishra et al., 2007; Pereira et al., 2007) it was hardly detectable in the spectra of the different film mixtures.

The amide I band is most often used to study in depth changes in the protein secondary structure (Surewicz & Mantsch, 1988). In particular, the amide I band of collagen/gelatin has previously been described as the result of overlapping of several components that appear at frequencies between ~1630 and 1695 cm^{-1} (Payne & Veis, 1988; Prystupa & Donald, 1996). To improve the spectral resolution and identify the main components that make up the amide I band of gelatin–LS films, the second derivative was applied

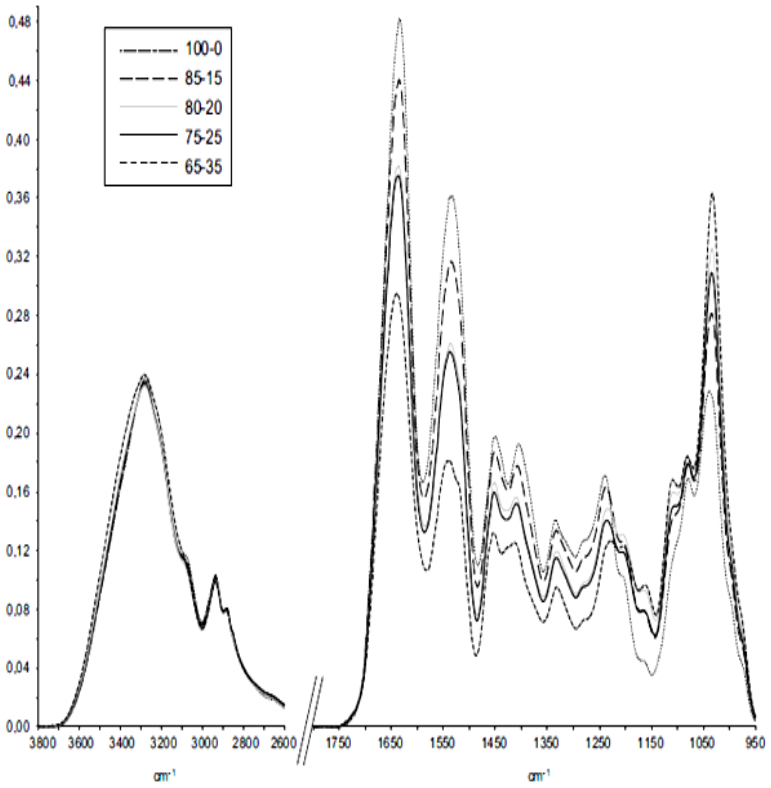

Fig. 2. ATR-FTIR spectra of films based on mixtures of fish gelatin (F) and lignosulphonate (LSa) at different ratios.

and the spectra are shown in Fig. 3. LS addition caused conformational changes in gelatin polypeptide chains resulting in a decrease in the presence of single α-helices, random coils and disordered structures, as evidenced by peak decreases at ~1651 cm^{-1} and ~1645–1635 cm^{-1} (Payne & Veis, 1988; Prystupa & Donald, 1996). This effect was most pronounced in the 65:35 mixture, reaching intermediate values in mixtures 80:20 and 75:25. The component associated mostly with the presence of collagen triple helix (~1660 cm^{-1}) was very poorly defined in the spectrum for the film without LS, consistent with the low-gelling capacity of this cold-water fish gelatin revealed in the study of its viscoelastic properties. Therefore, the effects of LS on the gelatin triple helix structure were hardly visible, with only mixture 65:35 once again presenting the most noticeable changes. On the other hand, LS ratios equal to or higher than the 80:20 blend produced significant reductions in bands at 1695 and 1675 cm^{-1}, indicating that LS interfered with the intermolecular aggregation of gelatin helices and β-turns (Doyle, Blout, & Bendit, 1975; Prystupa & Donald, 1996; Muyonga et al., 2004). According to Prystupa and Donald (1996), β-turns could serve as nucleation sites for the formation of helical segments under appropriate conditions. Therefore, as already observed in the study of viscoelastic properties, LS hinders the formation of primary nucleation sites necessary for triple helical structure development. However, the possible contribution of hydrogen-bonding interactions is difficult to establish, given the absence of significant down-shifts in the frequency of occurrence of the different band centres. Therefore, the marked effect of LS on the conformation of gelatin polypeptide chains may be attributable principally to nonbonding interactions, as reported by Kadla and Kubo (2003) in mixtures of polyethylene oxide and lignin. These non-specific interactions are considered to be due to the strong polar character of LS sulphonic acid groups, which together with the predominantly hydrophobic phenylpropane units, confer high-surface active properties to LS (Telysheva et al., 2001). Huang et al. (2003) reported an improvement in water resistance in soy protein isolate-lignosulphonate composite films attributable mainly to a beneficial microphase separation and the formation of a physical cross-linked network between the two biopolymers. In this regard, LS has also been reported to tend to aggregate as supramolecular complexes inducing the formation of physical interactions in composite matrices with waterborne polyurethane (Cui et al., 2007).

In the case of the amide II, which is associated with IR absorption due to bending vibrations of the NH group and stretching vibrations of the CN group, the centre tended to shift gradually from 1533 cm^{-1} in the sample without LS to 1538 cm^{-1} in the 65:35 mixture. The amide II band is less sensitive to changes in protein secondary structures than amide I, however, it is strongly influenced by the hydration level. According to Yakimets et al. (2005), the observed decrease in band intensity and its shift to higher wavenumbers would indicate an increase in the level of protein hydration, which would be accompanied by a decrease in polypeptide chain interactions (Jakobsen, Brown, Hutson, Fink, & Veis, 1983), possibly favoured by the abundant hydroxyl groups of lignosulphonate.

3.3. Thermal properties of films

DSC traces of gelatin-LS films are shown in Fig. 4a for a wide range of composition. Large glass transition processes, taking place along a wide temperature interval with high heat capacity change (Tg zones indicated by solid arrows), are usually observed for the higher gelatin contents; it may be mainly due to the fact that gelatin is a wholly amorphous polypeptides mixture (result not shown). The blends appeared chiefly miscible since a single Tg is essentially observed; however, minor (even subtle) Tg events (Tg zones indicated by dotted arrows) were visible, particularly in the 85:15 and 75:25 films where both glass transition processes ocurred with overlapping. This kind of phenomenon may likely be related to a gelatin "microphase separation", as discussed latter on.

Glass transition temperatures (Tg) of the several blended films are depicted as a function of composition in Fig. 4b. In the average, despite a somewhat big data scattering (local inhomogeneities in the complex systems may be a matter), a decreasing tendency (illustrated by a dashed line) may be presumed for the main Tg values of G-LS films containing moderate amounts of LS (LSa), as in

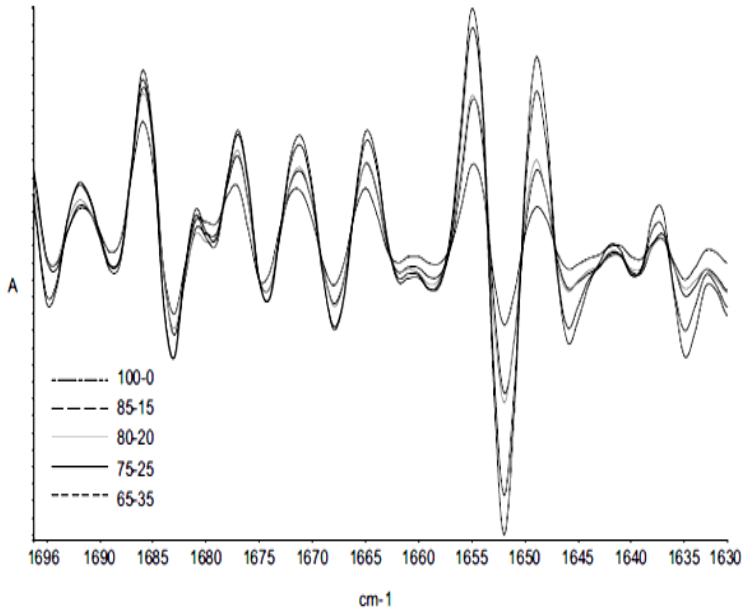

Fig. 3. Second derivative of amide A band from FTIR spectra of films based on mixtures of fish gelatin (F) and lignosulphonate (LSa) at different ratios.

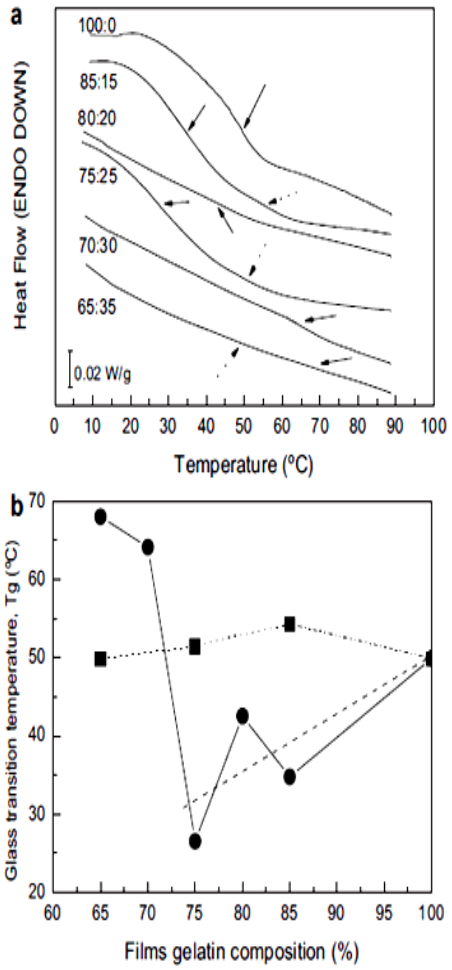

Fig. 4. a) DSC traces of heat flow (W/g) vs. temperature (°C) for several films based on fish gelatin (F) and LS blends with different composition (numerical G:LS legends nearby respective curves). Bar indicates the ordinate scale (0.02 W/g). Arrows indicate glass transition zones: Solid, Mayor effects; Dotted, Minor effects. b) Glass transition temperature (Tg, °C) of G-LS films as a function of the G content (%). Solid circles: Mayor effects; Solid squares, Minor effects.

the 85:15, 80:20 and 75:25 compositions. This kind of LS action appeared to be compatible with the (glycerol plus sorbitol) plasticizing effect on G, i.e., some reduction in the glass transition temperature of gelatin in the films from 75% and upwards in G. Contrarily, at higher LS concentrations such as in the 70:30 and 65:35 blends, lignosulphonate seemed to play an opposite role since the corresponding films exhibited Tg values clearly higher to that of non-blended G film, i.e., some antiplasticizing induced effect when LS content was in the 30–35% concentration. Worthy to note is that, as will be commented below, these last two films did not allow proper handling (sticky problems) for their mechanical properties determination but did however allow sampling for their thermal analysis. Huang et al. (2003) reported that low to moderate levels of LS produced a physical cross-linked complex with soy protein which induced plasticization effects in soy protein plastics. In contrast, higher LS additions in the above mentioned soy protein films, like the 60:40 and 40:60 blends reported by those authors, exhibited glass transition temperatures that clearly showed an inversion in the previous plasticization trend, suggesting the effect of LS as a filler dispersed in the film matrix.

According to Fredheim and Christensen (2003) the cross-linking of its phenylpropanoid monomers yields an LS spherical structure of a compact hydrophobic hydrocarbon core negatively charged because of its sulphonic and carboxylic groups, which are located on the core surface. It seems that interference with the intermolecular aggregation of gelatin G helices by LS interaction would take place mainly on this surface. Since this structure may become large (LS fractions may have molecular weights of several kDa), it could introduce considerable steric hindrance in the system which might impair an even distribution of LS throughout the biopolymer matrix. At low or moderate concentrations, LS undergo a certain "microphase separation" (Huang et al., 2003) thus permitting the free volume to increase in the system and produce a decreasing tendency in Tg values. In the 70:30 and 65:35 blended films, however, the number and size of the LS domains led to greater steric hindrance which considerably restricted the biopolymer molecular motion. The mobility restriction could have been highly intensified by the fact that LS has been shown to form supramolecular complexes by inter- and intramolecular hydrogen bonding of its polar groups (Li & Sarkanen, 2002). To this effect, LS intramolecular ionic bonding was elicited by Hatakeyama, Asano, and Hatakeyama (2003) to be responsible for glass transition not being clearly observed in LS fractions. According to Lemes, Soto-Oviedo, Waldman, Innocentini-Mei, and Durán (2010) the LS domains increased with LS concentration and interacted poorly with a poly(3-hydroxybutirate-co-3-hydroxyvalerate) (PHBV) matrix, as seems to be the case with gelatin G. It is worthy to note that the minor Tg events in the G-LS films seemed to be mainly related to the G component, as illustrated by the dotted line in Fig. 4b, thus being likely associated with current "microphase separation" processes.

3.4. Physical properties of films

At the macroscopic level, the addition of LS (LSa) to the gelatin matrix produced transparent, light brown and visually homogeneous films in all cases (Fig. 5). Except for the 70:30 and 65:35 blends, which were extremely sticky, the other films were easy to unmold and handle. The physical properties of gelatin films with increasing proportions of LS are presented in Table 1.

The gelatin film without LS was characterized by a tensile strength substantially lower than that reported by Chambi and Grosso (2006) for bovine gelatin films or Pranoto et al. (2007) for tilapia (a warm-water species) gelatin films. However, this film presents extraordinary high elongation at break, as reported earlier in gelatin films from cold-water fish such as cod (Pérez-Mateos et al., 2009) or halibut (Carvalho et al., 2008). The addition of LS did not induce significant changes in the tensile strength of the

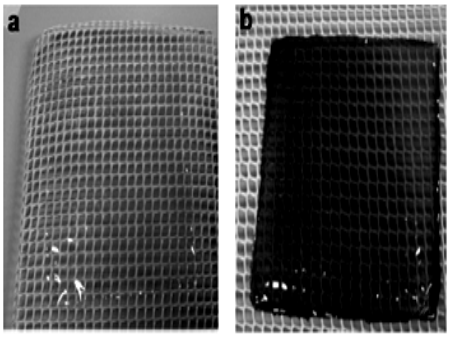

Fig. 5. Visual appearance of films. a) fish gelatin film; b) Fish gelatin-lignosulphonate (LSa) film, at the ratio 80:20.

Table 1
Physical properties of films based on mixtures of fish gelatin (F) and lignosulphonate (LSa) at different ratios. Different letters a,b,c in the same column indicate significant ($p < 0.05$) differences.

F–LSa	TS × 10^6 (N/m^2)	EAB (%)	Solubility (%)	WVP × 10^{-8} (g mm/h Pa cm^2)	Opacity (ABS_{600}/mm)
100–0	5.39 ± 0.42^a	546 ± 7^a	92.50 ± 1.55^a	2.60 ± 0.14^a	0.39 ± 0.02^a
85–15	4.28 ± 0.45^a	375 ± 8^b	93.53 ± 0.95^a	2.21 ± 0.31^a	0.72 ± 0.07^a
80–20	4.76 ± 0.58^a	426 ± 32^c	54.54 ± 2.26^b	5.54 ± 0.89^b	1.93 ± 0.30^b
75–25	4.23 ± 0.74^a	379 ± 9^{bc}	54.90 ± 5.70^b	5.56 ± 0.41^b	1.98 ± 0.42^b
70–30	*nd*	*nd*	*nd*	*nd*	*nd*
65–35	*nd*	*nd*	*nd*	*nd*	*nd*

nd: not determined.

corresponding composite films, but significantly ($p \leq 0.05$) decreased the elongation at break, which agrees with previous work reported by Vengal and Srikumar (2005) on polymeric gelatin-lignin films. The poorer film physical properties of G:LS films, particularly in relation to their mechanical properties, was consistent with an LS filler effect interfering with the aggregation of gelatin helices, as discussed above, becoming more pronounced as the proportion of LS increased in the formulation. Contrary to what was observed in gelatin, LS as such, or in the form of lignin, has been referred to as a plasticizing agent in composite films with starch (Baumberger et al., 1997; Wu et al., 2009), or as a reinforcing agent with cellulose (Rohella, Sahoo, Paul, Choudhury, & Chakravortty, 1996) or polyethylene oxide (Kadla & Kubo, 2003), but in all cases providing adequate miscibility with the polymer. Cui et al. (2007) observed a decrease of strength and modulus as well as an increase of elongation of waterborne polyurethane loaded with high proportions of lignosulphonate.

As previously reported, in soy protein isolate-lignosulphonate composite films (Huang et al., 2003), the addition of LS also contributed greatly to improve the water resistance of fish gelatin films, which showed a water solubility above 90% in the absence of LS. Such high solubility was in accordance with the values reported by Pérez-Mateos et al. (2009) for cod gelatin films, being far superior to those of gelatin films from bovine or from warmer climate fish (Bertan, Tanada-Palmu, Siani, & Grosso, 2005; Gómez-Estaca et al., 2009). Despite the highly polar nature of LS due to the abundance of acid and alcohol groups that give it a highly water-soluble nature (Baumberger et al., 1997), the solubility of gelatin films was drastically reduced in the mixtures 80:20 and 75:25, with no significant differences between them. The decrease of water solubility in these two formulations was accompanied by a significant increase in water vapour permeability (WVP), whose values nearly doubled in these samples, unlike the 85:15 mixture. The increase in WVP could be the result of certain microphase separation, as discussed previously. The pronounced changes produced from the 80:20 ratio onwards, which were consistent with those observed in the study of viscoelastic properties, DSC and FTIR, indicate an LS proportion of 20% to be the threshold for producing substantial physical interference in the entanglement of gelatin polypeptide chains within the film matrix. Such interference, which might have led to a significant blockade of gelatin ability to interact with water molecules, would be a main responsible for reducing the water solubility in the composite films. Moreover, the lower solubility could be also attributed to a possible interaction between LS sugars and the gelatin, by means of a glycation reaction between aldehyde sugar groups and protein amines, principally lysine ε-amine. This amino acid is found mainly in the terminal telopeptide zones of gelatin, and is highly reactive in glycation reactions (Delgado-Andrade, Seiquer, Nieto, & Navarro, 2004). Furthermore, some authors have observed that the pentoses and especially the xyloses are extremely reactive in the Maillard reaction (Kwak & Lim, 2004), and these sugars are present in the composition of the LS. These interactions were not clearly visible in the FTIR spectra; however they might be also responsible to some extent for the observed reduction of nucleation points in the gelatin–LS blends.

The addition of LS tends to reduce the transparency of the films, due in large part to the chromophore nature of lignin, which is highly capable of protecting against UV radiation (Ban, Song, & Lucia, 2007). The marked increase in the opacity level for mixtures 80:20 and 75:25, which are clearly disproportionate with respect to the mixture 85:15, confirmed that the loss of film transparency was due not only to an increased LS concentration, but also to the gelatin-LS non-specific interaction, causing conformational changes in the film protein matrix.

The addition of 20% LS in the formulation of fish gelatin films produced a significant improvement in water resistance, with the least damage in mechanical properties. For this reason, the 100:0 and 80:20 films were selected for a comparative study of the kinetics of solubilisation in water after a conditioning period of 2 and 9 days at 21 °C and 58% air humidity (Fig. 6). Films without LS (100:0) reached maximum values of water solubility within the first 4 h, whereas in the 80:20 film, apart from significantly reducing the solubility from the beginning, the soluble fraction continued to increase up to 9 h, indicating that the film was dissolved more slowly. Unlike the film without LS, after a conditioning period of 9 days the rate of water solubility of the 80:20 film was significantly reduced during the first 9 h in water, but after 16 h it reached values similar to the film conditioned for 2 days, indicating that additional interactions attributed to the ageing effect were also water-labile, which strongly suggests their non-covalent nature.

3.4.1. *Effect of type of gelatin and lignosulphonate*

To determine the effect of the type of gelatin and lignosulphonate, new films were made in the 80:20 ratio with bovine gelatin (B), fish gelatin (F) and three types of LS (calcium-magnesium, high

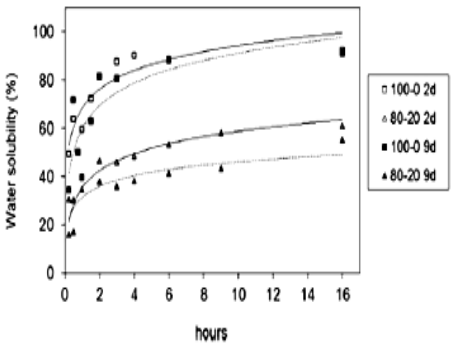

Fig. 6. Water solubility of fish gelatin films (100–0) and fish gelatin-lignosulphonate (LSa) film (80–20) after conditioning for 2 days (2d) and 9 days (9d) at 21 °C.

Table 2
Physical properties of films based on mixtures of fish gelatin (F) or bovine gelatin (B) and three types of lignosulphonates (LSa, LSb, LSc), at the ratio (80:20, gelatin:LS). Different letters a,b,c in the same column indicate significant ($p < 0.05$) differences.

Gelatin–lignosulfonate	TS × 10^6 (N/m^2)	EAB (%)	Solubility (%)	WVP × 10^{-8} (g mm/h Pa cm^2)	Opacity (ABS_{600}/mm)
F–LSa	4.76 ± 0.58^{a}	426 ± 32^{a}	54.54 ± 2.26^{a}	5.54 ± 0.89^{abc}	1.93 ± 0.30^{a}
F–LSb	4.12 ± 0.43^{a}	511 ± 12.8^{b}	58.94 ± 5.61^{a}	6.37 ± 0.34^{bc}	1.98 ± 0.23^{a}
F–LSc	3.78 ± 0.56^{a}	474 ± 46.7^{ab}	62.08 ± 5.73^{a}	5.92 ± 0.44^{abc}	1.92 ± 0.20^{a}
B–LSa	5.28 ± 0.05^{a}	341 ± 39.6^{c}	23.96 ± 1.50^{b}	5.15 ± 0.87^{ab}	2.57 ± 0.33^{a}
B–LSb	7.13 ± 0.71^{b}	362 ± 31.0^{bc}	28.77 ± 3.90^{b}	4.60 ± 0.35^{a}	2.25 ± 0.20^{a}
B–LSc	7.09 ± 0.88^{b}	232 ± 28.6^{d}	25.58 ± 1.61^{b}	7.04 ± 0.51^{c}	2.44 ± 0.55^{a}

sugar content, LSa; calcium-magnesium, low sugar content, LSb; and sodium, low sugar content, LSc) (Table 2). In general, the bovine gelatin–LS composite films present higher tensile strength than the fish gelatin blends, with the exception of the mixture with LSa (with higher sugar content), where differences in this parameter due to type of gelatin were not significant ($p > 0.05$). Intrinsic differences in the gelatin attributes may affect considerable the physical properties of films. To this regard, the molecular weight distribution and amino acid composition, which are the main factors influencing the physical and structural properties of gelatin, are also believed to play a key role in the mechanical and barrier properties of the resulting films. In a previous study, weaker and much more deformable films were obtained using tuna skin gelatin as compared to bovine-hide gelatin (Gómez-Estaca et al., 2009). The lower Pro + Hyp residues in the fish gelatin was suggested as a main factor for the increased deformability of bovine gelatin films, since the pyrrolidine rings of the imino acids may impose conformational constraints, imparting a certain degree of molecular rigidity. As expected, bovine gelatin produced less stretchable films than fish gelatin, with the bovine gelatin–LSc film (B–LSc) (sodium, low-sugar LS) registering the lowest EAB values among all the cases studied and coinciding with the highest TS. As far as the fish gelatin films were concerned, no significant differences ($p > 0.05$) in WVP associated with the type of LS were found, whereas in bovine gelatin films, B-LSc presented WVP significantly higher than with the other types of LS, the main difference being the higher molecular weight and the presence of Na^+ of LS, which may have produced greater aggregation in the protein matrix.

Regardless of the type of LS employed, the water solubility of bovine gelatin–LS composite films was significantly lower than with fish gelatin, probably due to the intrinsic differences in the parent gelatins used. The higher cross-linking degree in the bovine gelatin by the presumptive higher imino acid content would favour the film water resistance. On the other contrary, films from low-gelling cold-water fish gelatin, with low Hyp content, tend to be completely soluble in water (Carvalho et al., 2008; Denavi et al., 2009; Pérez-Mateos et al., 2009). The type of LS did not induce significant differences in the opacity of the films which was very similar in all cases.

Table 3 summarizes the viscoelastic properties of the 80:20 filmogenic solutions with different gelatins and lignosulphonates, after cooling to 2 °C and then heated to 30 °C. Unlike fish gelatin, all mixtures based on bovine gelatin produced a cold-matured gel as indicated by the significantly higher G′ and G″ values at 2 °C together with an apparent decline in phase angle. In addition, bovine gelatin gels showed a melting temperature significantly higher than fish gelatin (26 °C *vs.* 7 °C). This confirmed the greater ability of bovine gelatin to form nucleation sites for development and growth of triple helices (Gilsenan & Ross-Murphy, 2000; Ledward, 1986), which was manifested in the greater tensile strength and lower elongation of the resulting films (Table 2). Regardless of the type of gelatin, the LSa (Ca–Mg, high sugar content) interfered more with the cold-renaturation ability of polypeptide chains, as can be deduced from the lower G′ and higher phase angle. The greatest interference occurred with the bovine gelatin (B–LSa), resulting in a significant decrease ($p \leq 0.05$) in TS (Table 2). In contrast, the LSc (Na, low sugar content) favoured significantly the gel elastic component of both gelatins, possibly due to less hindrance from both the low sugar content and the monovalent nature of the cation Na^+. In this respect, under certain conditions salts can destabilize the structure of the gelatin (Sarabia, Gómez-Guillén, & Montero, 2000; Slade & Levine, 1987), possibly due to protein and salt ions competing for water (Elysée-Collen & Lencki, 1996). Therefore, in the case of bovine gelatin, the LSc permitted more gelatin chain inter- and intramolecular interactions, resulting in a stronger but less stretchable film, with increased WVP (B-LSc) (Table 2). None of the gelatins lost the condition of thermo-reversibility in the presence of any type of LS, as revealed by the viscoelastic parameters (low G″ and high δ) after heating at 30 °C. Complete gel melting, irrespective of the type of gelatin or LS, confirmed the predominance of non-covalent interactions between gelatin and LS.

3.5. Antioxidant activity of LS

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable molecule. This radical is commonly used as a substrate to evaluate the antioxidant activity

Table 3
Viscoelastic properties of film-forming solutions based on mixtures of fish gelatin (F) or bovine gelatin (B) and three types of lignosulphonates (LSa, LSb, LSc), at the ratio (80:20, gelatin:LS). Different letters a,b,c,d in the same column indicate significant ($p < 0.05$) differences.

Gelatin–LS	$G'_{2\,°C}$ (Pa)	$G''_{2\,°C}$ (Pa)	$\delta_{2\,°C}$ (°)	Tm (°C)	$G''_{30\,°C}$ (Pa)	$\delta_{30\,°C}$ (°)
F–LSa	0.092 ± 0.00^{a}	0.407 ± 0.02^{a}	77.27 ± 4.64^{a}	*ng*	0.022 ± 0.001^{a}	88.52 ± 5.31^{a}
F–LSb	0.571 ± 0.03^{a}	0.919 ± 0.05^{a}	58.49 ± 3.51^{b}	7	0.020 ± 0.001^{a}	82.71 ± 4.96^{a}
F–LSc	2.967 ± 0.18^{a}	1.254 ± 0.07^{a}	23.08 ± 1.38^{c}	7	0.023 ± 0.003^{a}	85.14 ± 5.11^{a}
B–LSa	236.7 ± 14.2^{b}	14.36 ± 0.86^{b}	3.48 ± 0.21^{d}	26	0.022 ± 0.001^{a}	86.10 ± 5.17^{a}
B–LSb	518.9 ± 31.1^{c}	15.01 ± 0.90^{b}	1.66 ± 0.10^{d}	26	0.024 ± 0.002^{a}	88.94 ± 5.34^{a}
B–LSc	1040 ± 62.4^{d}	34.80 ± 2.09^{c}	1.91 ± 0.11^{d}	26	0.023 ± 0.001^{a}	88.72 ± 5.32^{a}

Tm: melting temperature.
ng: no gel.

of different compounds. The DPPH assay had previously been proposed to assess the radical scavenging activity of lignins (Dizhbite et al., 2004; Telysheva, Dizhbite, Tirzite, & Jurkjane, 2000). The DPPH radical scavenging capacity of the LS and Trolox, expressed as IC_{50} values, are shown in Table 4. All the LS tested in this study showed a similar ability to quench DPPH radicals, as can be deduced from the IC_{50} values obtained, ranging from around 83 to 97 μg/mL. When compared with Trolox, all the LS showed significantly lower antioxidant activity, with IC_{50} values ranging from 6.5- to 7.5-fold higher than those obtained for Trolox. The antioxidant activity of lignins is influenced by structural features, mainly the presence of several functional groups such as the phenolic hydroxyl groups as well as the molecular weight (Pan et al., 2006; Ugartondo et al., 2008). It has been reported that high molecular weight may be one of the main factors decreasing the radical scavenging activity of lignins (Pan et al., 2006; Ugartondo et al., 2009). The three LS evaluated in this study showed similar molecular weights, which may help to explain the similarity found in antioxidant activity. Furthermore, the carbohydrate content has been reported to influence the antioxidant activity of lignins. Thus, high carbohydrate content has been correlated with a decrease in antioxidant activity, since the polar groups of the carbohydrates may form hydrogen bonds with lignin phenolic groups (Ugartondo et al., 2008). As described above, the LSa was high in carbohydrates, whereas the LSb and LSc had low levels. However, despite the differences in the carbohydrate content, no noticeable differences were observed in antioxidant activity among the three LS.

3.6. Cytotoxic effect of LS

Given the potential application of lignosulphonates as active packaging materials in contact with food, it is of great interest to study their possible cytotoxic effects. Cell cytotoxicity assays are among the most common *in vitro* bioassays to predict the toxicity of a wide range of compounds (Sánchez, Mitjans, Infante, & Vinardell, 2004). The MTS assay has been used in this study to assess the *in vitro* cytotoxic effect of the three LS. This cytotoxicity assay has been reported to be a convenient method for assessing cell viability in a number of studies (Malich, Markovic, & Winder, 1997).

The cytotoxic effects of the LS on fibroblast 3T3 cells are shown in Table 5. The IC_{50} values obtained for all the LS reveal that these compounds have similar cytotoxic effects, but only at very high concentrations. Only a few studies have reported the cytotoxic effects of lignins. Ugartondo et al. (2008) reported that lignins and especially lignosulphonate showed low toxicity against 3T3 cell lines, with IC_{50} values around 1200 μg/mL after 24 h of exposure. In our study, the three LS showed IC_{50} values even higher than those reported by Ugartondo et al. (2008), ranging from around 1500 to 1750 μg/mL. When the cytotoxic potential of the LS was related to their antioxidant activity, it could be observed that the effective antioxidant concentrations were noticeably smaller than the cytotoxic ones (from 15- to 20-fold lower), so these compounds are antioxidants at non-cytotoxic concentrations.

Table 4
Radical scavenging efficacy of lignosulphonates. Different letters a,b,c in the same column indicate significant ($p < 0.05$) differences.

	IC_{50} (μg/ml)
LSa	92.6 ± 8.1^b
LSb	83.4 ± 2.5^b
LSc	97.5 ± 6.4^b
Trolox	12.9 ± 0.2^a

Table 5
Cytotoxicity of lignosulphonates in 3T3 mouse fibroblasts. Different letters a,b,c in the same column indicate significant ($p < 0.05$) differences.

	IC_{50} (μg/ml)
LSa	1480 ± 114^a
LSb	1696 ± 55^a
LSc	1745 ± 16^a

3.7. Antimicrobial properties of LS

In general, lignosulphonates showed no antimicrobial activity against most of the microorganisms studied but with some exceptions. Some activity was detected against *D. hansenii* CECT 11364, with an inhibition percentage of 9.3, 9.8 and 9.9% for LSa, LSb and LSc, respectively. To a lesser extent, there was also some evidence of inhibition against the Gram-positives *S. aureus* CECT 240 and *B. thermosphacta* CECT 847. Cruz, Domínguez, Domínguez, and Parajó (2001) found that the acid ethyl acetate extracts from hydrolysates of several lignocellulosic materials showed activity against bacteria and yeasts, with eucalyptus wood extract being the most effective and *S. aureus* the most sensitive bacteria.

The antibacterial action of a kraft-lignin was reported to be due to the activity of its methanol soluble fraction, suggesting that the suppression of phytopathogenic microorganism growth could be connected, in part, with the inhibition of the radical process by mobile lignin fractions (Dizhbite et al., 2004). The relationship between antioxidant and antimicrobial properties, however, was not observed in our work; despite studying the antioxidant properties of the lignosulphonates (Table 4) antimicrobial activity was barely detected. The lignosulphonates are described as powerful surfactants and hence their possible antimicrobial action. However, it was reported that both wood-pulp and straw-pulp lignosulphonates could accelerate bacteria reproduction in re-circulated cooling water because of the presence of sugars (Lou, Qiu, Yang & Pang, 2004). In our study, the inhibition areas against the yeast were similar, regardless of composition (sugar content or mineral) of the lignosulphonates tested.

4. Conclusions

Lignosulphonate represents an important waste material which could be successfully employed as a filler agent for improving the water resistance of biopolymer films with extremely high water solubility, as is the case of cold-water fish skin gelatin films. In this case, the blend 80:20 (gelatin:lignosulphonate) was the most adequate to produce a significant improvement in water resistance, with the least damage in mechanical properties. Moreover, the proved antioxidant capacity and low cytotoxicity of lignosulphonates make them suitable candidates to be used for potential food packaging applications.

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5.2 *Artículo 2. Functional stability of gelatin-lignosulphonate films and their feasibility to preserve sardine fillets during chilled storage in combination with high pressure treatment*

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Functional stability of gelatin–lignosulphonate films and their feasibility to preserve sardine fillets during chilled storage in combination with high pressure treatment

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ABSTRACT

The physico-chemical and functional stability of gelatin (G) and gelatin–lignosulphonate (GLS) films stored during 4 weeks at 21 °C, (i) in container or (ii) in contact with oil, was examined. Addition of lignosulphonate dramatically increased ABTS radical scavenging and ferric ion reducing capacities, which remained practically unaltered after the storage period. GLS films exhibited reduced elongation at break, irrespective of storage medium, and retained their water resistance. The feasibility of using GLS film to improve the quality of sardine fillets during chilled storage, alone or in combination with high pressure treatment (300 MPa/10 min/7 °C), was evaluated. The combined use of GLS film with high pressure reduced microbial growth, total volatile basic compounds (TVB) and thiobarbituric acid reactive substances (TBARS) during chilled storage. No noteworthy high pressure-induced colour changes were observed in the sardine muscle using this treatment alone, although an increase in yellowness due to the combined treatment was detected.

Industrial Relevance: Addition of lignosulphonate dramatically increased antioxidant properties (ABTS radical scavenging and ferric ion reducing) of gelatin films, which remained practically unaltered during 4 weeks of storage at room temperature. Application of those films confers stability during storage of chilled sardine, especially in combination with high pressure treatment. These novel packaging was promising for fish preservation.

1. Introduction

Most polymers currently used for food packaging are derived from petroleum, contributing greatly to environmental and recycling problems. For these reasons there is a continuous search for other materials that are non-toxic, biodegradable and obtained from natural, renewable sources. Gelatin is an animal protein obtained by controlled hydrolysis of the fibrous insoluble collagen present in bones and skin generated as waste during animal slaughtering and processing (Patil, Mark, Apostolov, Vassileva, & Fakirov, 2000). Gelatin has been one of the most studied biopolymers on account of its film-forming ability and its usefulness as an outer film to protect food from drying and exposure to light and oxygen (Arvanitoyannis, 2002), but many of the potential applications of gelatin films require improvements in some of their properties, such as mechanical and especially water barrier properties. Also, the thermal stability of gelatin itself limits its application as packaging material (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002). As reviewed by Gómez-Guillén et al. (2009), gelatin has been combined with various biopolymers (proteins, polysaccharides, lipids or chitosan) as well as cross-linking agents to overcome the limited food application of the derived films. It was recently proposed that gelatin should be combined with plant or algal phenolic compounds to improve its physical properties (Cao, Fu, & He, 2007; Haroun & El Tourny, 2010; Rattaya, Benjakul, & Prodpran, 2009; Zhang et al., 2010) or to induce active properties in the resulting composite films (Gómez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009; Gómez-Estaca, Giménez, Montero, & Gómez-Guillén, 2009). In this connection, lignin and lignin derivatives with antioxidant properties (Dizhbite, Telysheva, Jurkjane, & Viesturs, 2004; Ugartondo, Mitjans, & Vinardell, 2009) have been shown to form suitable composites with gelatin, providing films with antioxidant capacity (Núñez-Flores et al., 2013).

Lignin is a complex phenolic compound found abundantly in the cell walls of plants in association with cellulose and hemicellulose. As a major component in dietary fibre, lignin can inhibit the activity of enzymes related to the generation of superoxide anion radicals and obstruct the growth and viability of cancer cells (Lu, Chu, & Gau, 1998). Lignin is an abundantly available material because tons of lignin is thrown away as a waste product in the pulp and paper industries. Furthermore, lignin is completely biodegradable, though slowly, and therefore it could be considered for the design of prospective biodegradable polymers (Vengal & Sreekumar, 2005). Lignosulphonate (LS) is a soluble form of lignin containing metal ions (Ca^{2+}, Mg^{2+}, and Na^{+}), hydrophilic groups such as sulphonic, carboxyl and hydroxyl groups, and hydrophobic aromatic and aliphatic groups. In the process of cellulose manufacture, LS can be isolated from spent sulphite liquors (Núñez-Flores et al., 2012).

Biopolymer films are thermodynamically unstable; therefore they are subject to time-dependent changes, producing the so-called ageing, which may considerably affect their physical properties. In the

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particular case of partially crystalline gelatin films, a positive correlation of storage modulus with time (0–72 h, at 25 °C) was reported, associated with an enthalpy relaxation phenomenon occurring in the amorphous regions (Badii, Martinet, Mitchell, & Farhat, 2006). In a high-term temperature accelerated ageing period (from 2 to 30 days at 75 °C), noticeable gelatin insolubilization was found, especially from day 8 onwards, which was related to the formation of huge gelatin aggregates growing in both size and density (Rbii, Violleau, Guedj, & Durel, 2009). Early work by Marks, Tourtellotte, and Andux (1968) suggested that the increased insolubility of gelatin in an accelerated ageing process was probably the result of polymerization and aggregation of gelatin molecules involving cross-linking and hydrogen bonds. Although these reactions would occur relatively slowly in the absence of a catalyst, as would be expected in gelatin films stored at room temperature, a certain degree of gelatin cross-linking would be a desirable consequence of film ageing, increasing the mechanical and water barrier properties and making the films more resistant in contact with the wet surface of many foodstuffs, such as fish fillets.

Fish is one of the most highly perishable food products and the shelf life of such products is limited in the presence of normal air by the chemical effects of atmospheric oxygen and the growth of aerobic spoilage microorganisms (Özogul, Polat, & Özogul, 2004). Several techniques to preserve fish have been studied, such as the application of high pressure and vacuum packaging, together with refrigeration. High hydrostatic pressure has been widely applied as a minimal processing technique to prolong the shelf life of additive-free foods, its major advantage being the preservation of micronutrients such as amino acids and vitamins, as well as flavour compounds (Knorr, 2000), but it is well recognized that pressurizing above 200 MPa leads to changes in colour and texture, giving fish muscle a cooked-like appearance (Montero & Gómez-Guillén, 2005). The combined use of high pressure processing and composite edible films based on gelatin and active plant extracts in cold-smoked sardine has already been shown to be a very promising technology for fish preservation (Gómez-Estaca, Montero, Giménez, & Gómez-Guillén, 2007). In a previous work, a gelatin–lignin film alone and combined with high pressure was shown to improve the appearance and overall quality of salmon fillets for use in ready-to-eat or semi-prepared dishes (Ojagh, Núñez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011). In the present study, raw sardine was the species of choice, given its high oxidation-susceptible fat content and quick spoilage tendency under chilled conditions, making it interesting for it to be preserved with an active packaging to prolong its eating quality (Mohan, Ravishankar, Lalitha, & Srinivasa Gopal, 2012).

In fatty species, such as salmon or sardine, besides the fillet surface humidity, the presence of fat is another important compositional factor that may influence the functionality of the film covering. Recently, lipid oxidation products (oxidized linoleic acid) have been shown to interact with gelatin, causing changes in protein surface hydrophobicity, protein oxidation by increasing the amount of carbonyl groups, and inter- and intramolecular cross-linking of gelatin (Aewsiri, Benjakul, Visessanguan, Wierenga, & Gruppen, 2011).

The objectives of the present work were to study (i) the physico-chemical and functional stability of an antioxidant gelatin–lignosulphonate film during 4 weeks of storage at room temperature (21 °C) under two different conditions, i.e., exposed to air inside a container, and in contact with sunflower oil; and (ii) the feasibility of using this film to improve the quality of sardine fillets during chilled storage, alone or in combination with high pressure treatment.

2. Materials and methods

2.1. Film properties

2.1.1. Materials

A commercial type-A bovine-hide gelatin (Bloom 200/220) from Sancho de Borja S.L. (Zaragoza, Spain) was used. A calcium–magnesium lignosulphonate (LS) from eucalyptus wood was kindly supplied by LignoTech Ibérica, S.A. (ID081203, Torrelavega, Spain). According to the manufacturer's specifications, this LS had 50% dry matter, a molecular weight of 6236 Da and 16% reducing sugars. Glycerol and sorbitol were obtained from Panreac (Barcelona, Spain). All other reagents used were of analytical grade and purchased from Sigma-Aldrich.

2.1.2. Preparation of films

The gelatin film-forming solution was prepared by dissolving dry gelatin in distilled water (4% w/v) at 40 °C, adding sorbitol (15 g/100 g gelatin) plus glycerol (15 g/100 g gelatin) as plasticizers. Gelatin–LS films were prepared by mixing the dissolved gelatin with LS at a ratio of 80–20 (calculated on dry matter content). The mixtures were stirred at 40 °C for 30 min. The films were made by casting an amount of 40 mL over a plate measuring (12 × 12) cm^2 and drying at 45 °C in a forced-air oven for 15 h to yield a uniform thickness of 100 mm ± 10 mm. Prior to the initial analyses, the films were conditioned over a saturated solution of KBr at 21 °C in desiccators for 2 days.

2.1.3. Stability of films

To study the stability of the gelatin (G) and gelatin–lignosulphonate (GLS) films, they were stored in containers over a saturated solution of KBr at 21 °C for 28 days. Additionally, films were also placed on vessels in contact with sunflower oil, and allowed to stand for 28 days at 21 °C, in order to simulate their performance in contact with a very fatty food (GO and GLSO batches). Prior to analysis, the films were removed from the oil and excess oil was cleaned with absorbent filter paper.

2.1.4. Solubility

Film water solubility was determined as previously described by Núñez-Flores et al. (2012). Film portions measuring 16 cm^2 were weighed and placed in beakers with 15 mL of distilled water, then sealed and allowed to stand at 22 °C for 2 h (early solubility) and 16 h (late solubility). The solutions were then filtered through Whatman No. 1 filter paper to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h. Film solubility was calculated by the equation FS (%) = ((Wo − Wf)/Wo) · 100, where Wo was the initial weight of the film expressed as dry matter and Wf was the weight of the undissolved desiccated film residue. All tests were carried out in triplicate.

2.1.5. Opacity

Film opacity was determined as previously described by Núñez-Flores et al. (2012). The films were cut into a rectangular piece and placed directly against one side of the spectrophotometer test cell, using an empty test cell as the reference. The opacity of the films was calculated by the equation $O = Abs_{600}/x$, where Abs600 is the value of absorbance at 600 nm and x is the film thickness in mm. Measurements were done at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan).

2.1.6. Water vapour permeability

Water vapour permeability (WVP) was determined following the gravimetric method described by Sobral, Menegalli, Hubinger, and Roques (2001), with slight modification. Films were attached over the openings of cells (permeation area = 15.9 cm^2) containing desiccated silica gel, and the cells were placed in desiccators with distilled water at 22 °C. The cells were weighed every hour for at least 6 h. Water vapour permeability was calculated using the equation $WVP = w \cdot x \cdot t^{-1} \cdot A^{-1} \cdot \Delta P^{-1}$, where w was weight gain (g), x film thickness (mm), t elapsed time for the weight gain (h), and ΔP the partial vapour pressure difference between the dry atmosphere and pure water (2642 Pa at 22 °C). Results were expressed as g mm h^{-1} cm^{-2} Pa^{-1}. All tests were carried out at least in triplicate.

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2.1.7. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of the films were determined by tensile tests using a TA.XT plus Texture Analyser (SMS, Surrey, UK). The samples were cut into rectangles 20 mm wide and 50 mm long, fixed on the grips of the device with a gap of 20 mm, and tractioned at a speed of 1 mm/s. The TS and EAB results were the average of five determinations, and were expressed as N/m^2 and %, respectively.

2.1.8. Antioxidant properties

Ferric ion reducing capacity (FRAP assay) was determined according to the method described by Pulido, Bravo, and Saura-Calixto (2000). The samples were dissolved in distilled water. A 30 mL dissolved sample was incubated (37 °C) with 90 mL of distilled water and 900 mL of FRAP reagent (containing tripyridyltriazine (TPTZ, Sigma-Aldrich) and FeCl3). Absorbance values were read at 595 nm after 30 min. Results were expressed as mmol Fe^{2+} equivalents/g of sample, based on a standard curve of FeSO4·7H2O which relates the concentration of FeSO4·7H2O (mM) to the absorbance at 595 nm.

The ABTS radical (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) scavenging capacity of samples was evaluated according to the method of Re et al. (1999). The stock solution of ABTS radical (7 mM ABTS in 2.45 mM potassium persulphate) was kept in the dark at room temperature for 12–16 h. An aliquot of stock solution was diluted with distilled water to prepare the working solution of ABTS radical with an absorbance of 0.70 ± 0.02 at 734 nm. A 20 mL aliquot of sample was mixed with 980 mL of ABTS reagent. The mixture was then left to stand at 30 °C for 10 min and absorbance values were read at 734 nm. Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC) per g of sample based on a standard curve of vitamin C (Sigma-Aldrich), which relates the concentration of vitamin C to the amount of absorbance reduction caused by vitamin C. All determinations were performed at least in triplicate.

2.2. Preservation of sardine fillets

2.2.1. Sample preparation and high pressure processing (HPP)

For the preservation trial, sardine (*Sardina pilchardus*) fillets were acquired at a local market in summer. The fillets (about 25 g each, 14 cm length, 0.5 cm thick) were divided randomly to prepare the following batches: fillets covered with a gelatin–lignin film (SF), vacuum-packed fillets (SV), pressurized fillets (SHP) and fillets covered with a gelatin–lignin film and subsequently pressurized (SFHP). The gelatin–lignin films used were obtained as described previously and were first conditioned over a saturated solution of KBr at 21 °C in desiccators for 2 days.

The batches were vacuum-packed (except for SF) into flexible plastic bags using a Multivac packaging machine (A-NG, 85021, GS, Germany). The pressurized batches were treated in a pilot high pressure unit (ACB 665, GEC Alsthom, Nantes, France), the temperature of the immersion medium (distilled water) being regulated by a thermocouple connected to a programmed temperature control unit (model IA/2230AC, INMASA, Barcelona, Spain). High pressure treatment was carried out for 10 min at 300 MPa and at 5 °C. Pressure was increased by 2.5 MPa/s and after the high pressure treatment was completed the time for the pressure to drop back to atmospheric pressure was around 3 s. In the covered samples, when applied on the surface of the sardine fillets, the films retained their structural integrity and were not altered by the high pressure treatment, and could be easily separated from the muscle for further analyses. All batches were stored at 7 °C and periodically sampled.

2.2.2. pH

Approximately 5–10 g of muscle was homogenized with distilled water (1:2, w/v). After 5 min at ambient temperature, the pH was determined with a pHm93 pH-meter and a combined pH electrode (Radiometer, Copenhagen, Denmark). The experiments were performed at least in quintuplicate.

2.2.3. TVB-N

Total volatile basic nitrogen (TVB-N) determinations were carried out over the storage period using the method of Antonacopoulos and Vyncke (1989). Samples of sardine fillets (10 g) were homogenized with 90 mL of perchloric acid (6%) in an Osterizer (at 5000 rpm for 1 min) to precipitate proteins. The mixture obtained was filtered through Whatman No. 1 paper, washed with 5 mL of perchloric acid, and adjusted to 100 mL. The filtrate was distilled in a Tecator AB device (model 1002, Kjeltec Systems, Sweden). The distillate was collected on boric acid (0.3% w/v) and was titrated with 0.05 mol/L HCl. Analyses were performed at least in triplicate, and results were expressed as mg TVB-N/100 g muscle.

2.2.4. Microbiological assays

The microbiological analyses were as follows: a total amount of 10 g of muscle, from at least 3 different packages, was collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 mL of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min in a stomacher blender (model Colworth 400, Seward, London, UK), appropriate dilutions were prepared for the following microorganism determinations: (i) total bacterial counts (TBC) on spread plates of Iron Agar (Scharlab, Barcelona, Spain), 1% NaCl incubated at 15 °C for 3 days; (ii) H2S-producing organisms, as black colonies, on pour plates of Iron Agar incubated at 15°C for 3 days; (iii) luminescent bacteria on spread plates of Iron Agar 1% NaCl incubated at 15 °C for 5 days; (iv) total aerobic mesophiles on pour plates of Plate Count Agar, PCA (Oxoid) incubated at 30 °C for 72 h; (v) *Pseudomonas* on spread plates of Pseudomonas Agar Base (Oxoid) with added CFC (Cetrimide, Fucidine, Cephalosporine) supplement for *Pseudomonas* spp. (Oxoid) incubated at 25 °C for 48 h; (vi) *Enterobacteriaceae* on double-layered plates of Violet Red Bile Glucose Agar (VRBG, Oxoid) incubated at 30 °C for 48 h [after first adding 5 mL of Tryptone Soy Agar (Merck, Darmstadt, Germany) and incubated at room temperature for 1 h]; and (vii) lactic acid bacteria on double-layered plates of MRS Agar (Oxoid) incubated at 30 °C for 72 h. All microbiological counts are expressed as the log of the colony-forming units per gram (log cfu/g) of sample. All analyses were performed in triplicate.

2.2.5. Thiobarbituric acid reactive substances (TBARS)

The determination of TBARS was performed as described by Vyncke (1970). Samples of sardine fillets (15 g) were homogenized with 30 mL of 7.5% w/v trichloroacetic acid (TCA; Panreac Química S.A.U., Barcelona, Spain) in an Osterizer device (Oster©, Sunbeam Products, Inc., USA, at 5000 rpm for 1 min). The mixture was left to stand for 30 min and was subsequently filtered through Whatman No. 1 paper. The filtrate (or appropriate dilutions in TCA) was subjected to the colorimetric reaction with thiobarbituric acid (TBA; Sigma-Aldrich Chemical Co., St Louis, USA). The reaction was performed at 90 °C for 40 min, and the absorbance at 538 nm was immediately read in a spectrophotometer (Shimadzu model UPS-240, Japan). A calibration curve was prepared using 1,1,3,3-tetraethoxypropane (TEP; Sigma-Aldrich Chemical Co., St Louis, USA) as the standard. Results were expressed as μmol malondialdehyde (MDA) per kg of muscle. Determinations were performed at least in triplicate.

2.2.6. Colour measurements

The colour parameters lightness (L*), redness (a*) and yellowness (b*) were measured using a Konica Minolta CM-3500d colorimeter (Osaka, Japan). Measurements were taken at a number of locations in different muscle portions and each point is the mean of at least 10 measurements.

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2.3. Statistical analyses

Statistical tests were performed using the SPSS computer programme (v. 18, SPSS Statistical Software, Inc., Chicago, IL.) One-way analysis of variance was carried out. The difference of means between pairs was resolved by means of confidence intervals using Bonferroni and Tamhane's T2 tests at a level of significance of $p < 0.05$.

3. Results and discussion

3.1. Stability of films

3.1.1. Physical properties

The addition of LS produced a light brownish coloration in the resulting gelatin–lignosulphonate film, which kept a visual homogeneity and transparency, as reported earlier by Núñez-Flores et al. (2012). The physical properties of the gelatin and gelatin–lignosulphonate films, both stored for 28 days in container (G, GLS) or in contact with sunflower oil (GO, GLSO), are presented in Table 1.

The replacement of 20% gelatin by LS did not induce any significant change in tensile strength (TS) values of newly made films (at day 2), in accordance with what has been reported previously concerning the formulation of composite fish gelatin–LS films (Núñez-Flores et al., 2012). With other biopolymer matrices, however, the addition of lignin derivatives has been shown to alter the mechanical properties of the resulting blend films significantly (Kosíková, Mikdesová, & Demianova, 1993).

In the present study, it is worth noting the pronounced increase ($p < 0.05$) in TS exhibited by the gelatin film after being held in oil for 2 days (GO), which was not observed in GLSO films (Table 1). A possible explanation lies in the extremely hydrophobic nature of oil in contact with the predominantly hydrophilic exposed gelatin polypeptide chains, which might greatly affect the surface properties of the film, inducing significant mechanical reinforcement. Such reinforcement might also be supported by presumptive chemical interactions between gelatin and lipid oxidation products as well as lipid-induced gelatin aggregation (Aewsiri et al., 2011). In contrast, the high surface-active properties of LS, which were previously shown to cause gelatin conformational changes and to prevent intermolecular gelatin aggregation in the composite film matrix (Núñez-Flores et al., 2012), were presumed to counteract the oil-induced surface changes.

A significant ($p < 0.05$) increase in TS was registered in G films during the 28-day storage period, which could be attributed to a minor age-induced gelatin aggregation, producing slight film reinforcement. In the case of GLS films, despite a small fluctuation at day 14, possibly due to some conformational rearrangement of both gelatin and LS macromolecules, TS values after one month of storage did not differ significantly from those registered in the newly made films. In both oil-stored films (GO and GLSO), TS did not change substantially during the 28-day storage period.

The elongation at break (EAB) of G film was slightly increased ($p < 0.05$) by the addition of LS in the newly made films (Table 1), as reported in other types of films, such as LS–starch or LS–polyurethane composite films (Wu, Wang, Li, Li, & Wang, 2009). An eventual reduction in EAB occurred in both G and GLS films at day 14 of storage; however, whereas the EAB in G films returned to initial values after 28 days, the reduced EAB in GLS films did not change significantly ($p > 0.05$).

In the case of films stored in contact with oil, the EAB tended to decrease significantly ($p < 0.05$) in both GO and GLSO films during storage, suggesting that oil-induced changes in the surface active properties of the films led to an anti-plasticizing effect. Although not exactly the same case, since the oil in the present study was in the storage medium, incorporated oil has been reported to reduce elongation or puncture deformation in films made of either arabinoxylans (Phan The et al., 2002) or cod skin gelatin (Pérez-Mateos, Montero, & Gómez-Guillén, 2009), in the latter case especially observable after one month of storage. It is possible that the reduced EAB of GO and GLSO films during storage might be the result of an increase in the degree of gelatin cross-linking produced by increasing interactions between gelatin and accumulated lipid oxidation products (Aewsiri et al., 2011).

The addition of LS led to slightly lower water vapour permeability (WVP) values in the newly made films, which were significant ($p < 0.05$) only in the case of films stored in oil (Table 1). In contrast, LS was reported to produce a significant increase in the WVP of similar composite films made with a cold water fish species gelatin (Núñez-Flores et al., 2012). Despite a noticeable decrease in WVP after two weeks of storage in all the films studied, which coincided with a reduction in the EAB, the WVP at day 28 returned to values close to the value registered at day 2, or slightly higher ($p < 0.05$) in the case of GLSO.

The presence of LS produced a marked increase ($p < 0.05$) in the opacity of the composite films (Table 1), as reported previously with fish gelatin–LS films (Núñez-Flores et al., 2012). This increase was attributed to the chromophoric nature of lignin and lignin derivatives, which have a high capacity to protect against UV radiation (Ban, Song, & Lucia, 2007). The storage period slightly increased ($p < 0.05$) the opacity of G films, while GLS remained practically unaltered. The opacity of newly-made oil-stored gelatin film (GO) was slightly higher ($p < 0.05$) than in its G counterpart. Although possible traces of non-removed oil might remain on the film surface, decreasing film transparency, the remarkable increase in TS exhibited by the GO film suggests that the increased opacity could be largely due to gelatin aggregation induced by the oil-induced surface changes. Nevertheless, regarding the storage period, no significant ($p < 0.05$) differences were found in GO films after 28 days. The opacity of GLSO films did not vary substantially as compared to GLS films, with the only exception at day 28, where GLSO registered a higher ($p < 0.05$) value.

Table 1
Physical properties of films based on gelatin and on gelatin–lignosulphonate mixtures, stored under controlled air conditions (G, GLS) or in contact with oil (GO, GLSO) for 28 days.

	Days	G	GLS	GO	GLSO
TS · 10^6 (N/m^2)	2	$8.91 \pm 0.68^{a,x}$	$9.35 \pm 0.51^{a,x}$	$15.91 \pm 2.12^{b,x}$	$9.14 \pm 2.31^{a,x}$
	14	$14.73 \pm 0.17^{a,y}$	$16.01 \pm 0.98^{a,y}$	$16.58 \pm 0.30^{a,x}$	$9.34 \pm 2.61^{b,x}$
	28	$13.78 \pm 0.18^{a,x}$	$6.90 \pm 1.82^{b,z}$	$10.79 \pm 1.91^{a,y}$	$8.48 \pm 0.19^{b,x}$
EAB (%)	2	$207 \pm 11^{a,x}$	$279 \pm 23^{b,x}$	$236 \pm 4^{a,x}$	$240 \pm 11^{a,b,x}$
	14	$160 \pm 18^{a,y}$	$164 \pm 11^{a,y}$	$149 \pm 23^{a,y}$	$126 \pm 18^{a,y}$
	28	$218 \pm 17^{a,x}$	$179 \pm 16^{a,b,y}$	$156 \pm 20^{b,y}$	$169 \pm 18^{a,b,z}$
WVP · 10^{-8} ($g \cdot mmh^{-1} \cdot Pa \cdot cm^2$)	2	$4.97 \pm 0.65^{a,x}$	$3.89 \pm 0.48^{a,b,x}$	$4.75 \pm 0.74^{a,x}$	$3.14 \pm 0.58^{b,x}$
	14	$2.46 \pm 0.30^{a,b,y}$	$2.14 \pm 0.38^{a,b,y}$	$1.64 \pm 0.34^{b,y}$	$2.81 \pm 0.56^{a,x}$
	28	$4.69 \pm 0.62^{a,x}$	$5.20 \pm 0.77^{a,x}$	$5.37 \pm 0.06^{a,x}$	$5.11 \pm 0.21^{a,y}$
Opacity (ABS_{600}/mm)	2	$0.70 \pm 0.01^{a,x}$	$3.12 \pm 0.26^{b,x}$	$0.98 \pm 0.07^{a,x}$	$2.79 \pm 0.13^{b,x}$
	14	$0.80 \pm 0.03^{a,y}$	$3.11 \pm 0.28^{b,x}$	$1.25 \pm 0.22^{a,x}$	$2.94 \pm 0.12^{b,x}$
	28	$0.92 \pm 0.02^{a,z}$	$3.33 \pm 0.10^{b,x}$	$0.88 \pm 0.19^{a,x}$	$5.03 \pm 0.01^{c,y}$

TS: tensile strength; EAB: elongation at break; WVP: water vapour permeability.
Different letters (a,b,c) in the same day mean significant differences ($p \geq 0.05$) as a function of sample. Different letters (x,y) for the same sample mean significant differences ($p \geq 0.05$) as a function of time.

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3.1.2. Water solubility

The early and late solubility in water (after 2 h and 16 h, respectively) of gelatin films (G) and gelatin–LS films (GLS) during the storage period are shown in Fig. 1a. Firstly, it is interesting to note that, although the GLS values were slightly higher than the G values in most cases, the former maintained their structural film integrity visually after either 2 h or 16 h in water, in contrast to the G films, which disintegrated and generated an amorphous water-binding gel. The film solubility after 16 h was significantly higher ($p < 0.05$) than during the first 2 h of water exposure, suggesting some kind of film water resistance in all cases.

After 14 days of film storage, GLS showed some instability, as deduced from the significant ($p < 0.05$) rise in water solubility measured after 2 h of water exposure, attributed to possible conformational changes induced by the presence of LS in the film matrix, also coinciding with some fluctuation in mechanical properties. In contrast, G film showed a clear tendency to reduce early solubility (2 h) with storage time, suggesting some improvement in film water resistance associated with age-induced gelatin cross-linking (Rbii et al., 2009). However, the late film water solubility (16 h) of G and GLS films after 28 days of storage did not differ significantly ($p > 0.05$) with respect to the values registered at day 2, indicating that 4 weeks might be a suitable storage period to overcome possible fluctuations in the gelatin–lignosulphonate film properties.

When films were stored in contact with sunflower oil (Fig. 1b), the early solubility of the newly made GO film (12.79%) was noticeably lower than that of its G counterpart (28.29%), unlike the case of the GLSO films, whose values remained very similar to those of the GLS films. In general, films stored in oil tended to exhibit reduced early water solubility when compared with the films stored under air conditions during the whole storage period, this being especially evident at day 28. At this stage, GO registered the lowest solubility after both 2 h and 16 h of water exposure. In general, when considering the late solubility values (16 h), the effect of oil contact with film during storage was almost negligible, with the sole exception of GO film at day 28, which registered a noticeable decrease, possibly due to

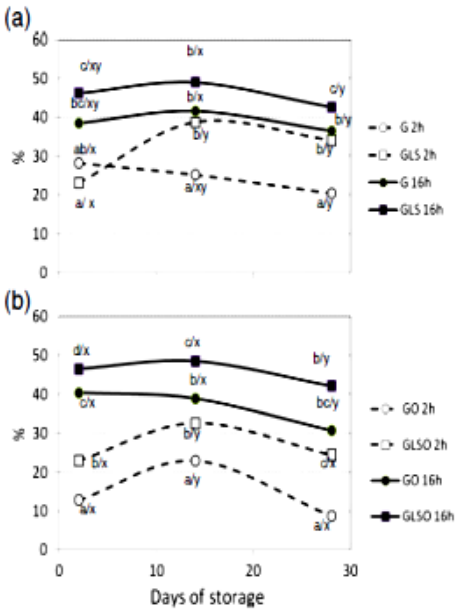

Fig. 1. Early (measured after 2 h) and late (measured after 16 h) water solubility of films based on gelatin and on gelatin–lignosulphonate mixtures, stored for 28 days (a) under controlled air conditions (G, GLS) or (b) in contact with oil (GO, GLSO).

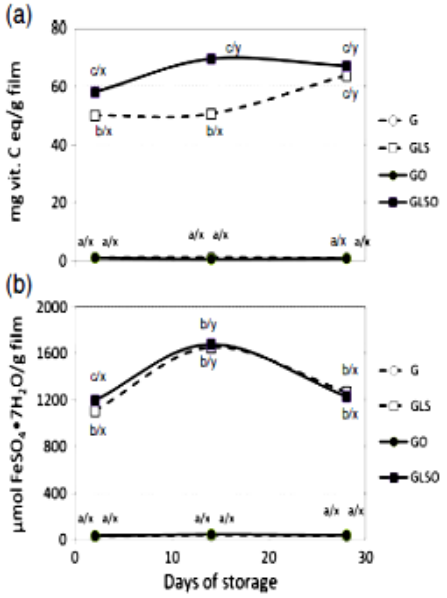

Fig. 2. Antioxidant properties, measured by (a) ABTS assay and (b) FRAP assay, of films based on gelatin and on gelatin–lignosulphonate mixtures stored for 28 days under controlled air conditions (G, GLS) or in contact with oil (GO, GLSO).

covalent interaction of gelatin with cumulative lipid oxidation products (Aewsiri et al., 2011). All these findings suggest that the presence of LS had a negative effect on the presumed gelatin aggregating effect exerted by oil contact, possibly because LS by itself induced gelatin aggregation and the two effects were not additive. In any case, the oil-induced gelatin aggregation in gelatin films was probably mostly of a water-labile nature, except on day 28 of storage, when the decreased late solubility suggested a certain degree of covalent cross-linking in the case of GO films.

3.1.3. Antioxidant properties

The antioxidant capacity of the films should be strongly related to the portion of film that can dissolve in water, and consequently to the release of active compounds, which in the case of gelatin–LS composites would correspond mostly to phenolic compounds and, possibly, also to reducing sugars naturally present in the lignosulphonate preparation. Therefore, the soluble fraction of films after 16 h of water exposure, was considered in order to measure their free radical scavenging (ABTS) (Fig. 2a) and ferric ion reducing (FRAP) capacities (Fig. 2b).

The antioxidant properties (ABTS and FRAP values) increased dramatically in the LS-containing films, irrespective of the storage conditions (GLS and GLSO). Lignin derivatives have been reported to be effective radical scavengers (Dizhbite et al., 2004). Moreover, the same commercial calcium–magnesium lignosulphonate as used in the present study, permitted for use as animal feed, was found to be a potent radical scavenger at non-cytotoxic concentrations in a previous work (Núñez-Flores et al., 2012).

The slightly higher ABTS values exhibited by GLSO films in comparison to GLS was not related with a greater release of LS active compounds, since no appreciable differences in film water solubility between GLS and GLSO were found. In addition, no significant ($p > 0.05$) differences in FRAP values were observed. Therefore, possible traces of active compounds coming from sunflower oil might have contributed to raise the radical scavenging capacity (Schwartz, Ollilainen, Piironen, & Lampi, 2008).

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During the storage time, the films did not lose their antioxidant properties. On the contrary, there was a small gain in free radical scavenging capacity, attributed to possible macromolecular reorganizations within the film matrix, which did not coincide with an increase in water solubility. These findings strongly suggest that in LS-containing gelatin films the effects of gelatin aggregation, induced either by time-ageing or by contact with oil, were slight and did not hinder the ability to release antioxidant compounds from the film matrix.

3.2. Chilled storage of sardine fillets

3.2.1. pH

Initially the pH of the sardines was 6.6, rising to 6.8 by the end of the storage period ($p > 0.05$). With initial values of 6.2, Stamakis and Arkoudelos (2007) reported that the pH in vacuum-packaged sardine at 3 °C remains practically constant. In the present work, the application of gelatin and gelatin–lignosulphonate films and high pressure treatment did not affect the pH during storage ($p > 0.05$).

3.2.2. Total volatile basic nitrogen (TVB-N)

TVB-N is related with the growth of microorganisms and the formation of basic compounds from their metabolism that usually brings an increase in pH, which helps to determine the quality of the product. The initial concentration of TVB-N in fresh sardine was 13.5 mg ± 0.28 mg/100 g, values in the range found by Erkan, Özden, Alakavuk, Yildirim, and Inugur (2006) in iced sardine. The high pressure treatment did not alter the initial value of volatile basic compounds ($p > 0.05$), giving initial values of 14.11 mg ± 0.50 mg/100 g in the pressurized sardine. These results agree with Erkan et al. (2011), who reported that the trimethylamine content (volatile amine) in horse mackerel (*Trachurus trachurus*) was not affected by the HP application.

Table 2 shows the TVB-N for sardine fillets under different treatments during chilled storage. The GLS film alone did not prevent formation of basic compounds, whereas the combined treatment (SFHP batch) effectively reduced their production, recording the lowest values (22.6 mg TVB/100 g) at 11 days ($p \leq 0.05$). A gelatin–lignin film applied in salmon helped to reduce basic compound production when pressurized at 5 °C (Ojagh et al., 2011). In the present work, although the storage study was set to 11 days, an additional analysis was performed at 17 days to determine the extent of the treatments. Except for SF, all batches recorded below 25 mg and 35 mg of TVB-N/100 g of muscle, the acceptability limits set by the European Union for fish (Commission, 1995) and, in particular, for sardine (Ababouch et al., 1996), respectively. In the present study, the SF and SV batches behaved similarly up to day 8, suggesting that the GLS film efficiency for preserving sardine biochemical quality might be similar to conventional vacuum packaging. These values were close to those obtained by Özogul et al. (2004) for sardine stored in air and in vacuum packaging at 4 °C for 12 days.

3.2.3. Microbial growth

Initially the total mesophilic counts in sardine were 4.9 log cfu/g. These values are slightly higher than those found in *Sardinella pilchardus* (Özogul et al., 2010) and *Sardinella aurita* (Özogul et al., 2011). The number of microorganisms was reduced by the effect of the high pressure treatment ($p \leq 0.05$). These reductions ranged from 2.5 logarithmic cycles for total aerobic mesophiles to just under 1 log cycle for lactic acid bacteria. Presumptive *Shewanella putrefaciens*, as black colonies (López-Caballero, Martinez-Alvarez, Gómez-Guillén, & Montero, 2007, was sensitive to high pressure (López-Caballero, Pérez-Mateos, Montero, & Borderías, 2000; Ojagh et al., 2011), although the inhibition obtained in the present work was not very pronounced ($p \leq 0.05$). *Pseudomonas* spp. and *Enterobacteriaceae* were below the detection limit after high pressure treatment. Gómez-Estaca et al. (2007) reported reductions in total bacteria counts (15 °C) in cold smoked sardine pressurized at 300 MPa/20 °C/15 min. The microbial growth rate (pseudomonads, *S. putrefaciens* and lactic acid bacteria) was significantly reduced during storage of salmon following pressure treatment of 150 MPa for 30 and 60 min or 200 MPa at 5 °C (Amanatidou et al., 2000).

The antimicrobial properties of lignosulphonates have been described in the literature. Thus, Dizhbite et al. (2004) reported that the antibacterial action of a kraft lignin was due to the activity of its methanol soluble fraction. In this connection, Núñez-Flores et al. (2012) detected some activity against *D. hansenii*. The application of GLS films in sardine (SF batch) was not as effective as vacuum packaging in preventing the growth of the microorganisms studied during storage ($p \leq 0.05$) (Table 3). In terms of microbiological assessment and according to the results found in the present study, the total viable counts (20 °C) in refrigerated sardine were higher than 7 log cfu/g between days 6 and 9 of chilled storage (Özogul et al., 2004; Stamakis & Arkoudelos, 2007), whereas the shelf life of vacuum-packed sardine was approximately 8–9 days (Özogul et al., 2010).

The smaller counts corresponded to pressurized batches. Both batches (SHP and SFHP) evolved similarly, even though the combined application of high pressure and film showed some additive effects ($p \leq 0.05$). Although the effect of high pressure treatment on microorganisms has been reviewed for decades (Cheftel, 1992), the combined treatments of high pressure and films have been studied in recent years. In this connection, inhibition of microbial growth in fish has been found in high pressure combined with edible films incorporating oregano (Gómez-Estaca et al., 2007), clove (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010) and green tea (López de Lacey, 2012). The effect of gelatin–lignin film applied on salmon on the growth of microorganisms has not been very noticeable, since the counts are similar for batches with or without film, the main differences being attributed to the high pressure treatment (Ojagh et al., 2011).

At the end of the storage period, the H_2S-producing microorganisms and *Pseudomonas* spp. were the largest group in the SF batch (Table 3). Moreover, the H_2S-producing microorganisms but not the aerobic *Pseudomonas* were predominant in the SV batch. Gram and Huss (1996) stated that two species were predominant during storage of vacuum-packed fish (*S. putrefaciens* and *Photobacterium phosphoreum*), with the one whose number was greater initially prevailing at the end of storage. Luminescent colonies, considered as presumptive *P. phosphoreum* (López-Caballero, Álvarez-Torres, Sánchez-Fernández, & Moral, 2002), were not detected in this study, which supports the prevalence of *S.*

Table 2
Total volatile basic nitrogen (mg TVB-N/100 g muscle) in sardine stored at 7 °C for 17 days.

Days of storage					
Batch	2	4	8	11	17
SF	$16.52 \pm 0.49^{a,x}$	$13.05 \pm 0.32^{a,x}$	$22.79 \pm 2.99^{a,y}$	$33.08 \pm 0.42^{a,y}$	$68.53 \pm 11.21^{a,z}$
SHP	$16.82 \pm 0.31^{b,w,x}$	$14.90 \pm 1.60^{b,w,w}$	$20.66 \pm 0.79^{b,w,y}$	$26.61 \pm 0.91^{b,y}$	$24.93 \pm 3.69^{b,w,y}$
SFHP	$15.45 \pm 0.42^{a,x}$	$15.70 \pm 0.02^{b,x}$	$23.85 \pm 1.93^{a,y}$	$22.66 \pm 0.96^{b,y}$	$22.25 \pm 0.75^{b,y}$
SV	$17.05 \pm 0.69^{b,w}$	$15.86 \pm 0.80^{b,w,w}$	$20.18 \pm 2.43^{b,w}$	$25.15 \pm 0.55^{b,y}$	$30.43 \pm 0.84^{b,z}$

SF: sardine with film; SHP: sardine treated with high pressure; SFHP: sardine treated with film and high pressure; SV: sardine under vacuum packaging.
Different letters (a,b,c) in the same day mean significant differences ($p \geq 0.05$) as a function of sample. Different letters (x,y) for the same sample mean significant differences ($p \geq 0.05$) as a function of time.

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Table 3
Microbial counts (log CFU/g) in sardine subjected to different processing treatments and stored at 7 °C.

Treatment	Days of chilled storage				
	2	4	8	11	17
Total viable count					
SV	$4.58^{a,y}$	$5.00^{a,w}$	$6.45^{a,x}$	$10.95^{a,y}$	$9.43^{a,z}$
SF	$4.87^{b,y}$	$6.38^{b,w}$	$9.60^{b,x}$	$10.11^{b,y}$	$11.81^{b,z}$
SHP	$3.62^{c,y}$	$3.84^{c,w}$	$4.00^{c,x}$	$4.15^{c,y}$	$6.82^{c,z}$
SFHP	$3.56^{c,y}$	$3.58^{c,w}$	$3.00^{c,x}$	$4.32^{c,y}$	$5.46^{c,z}$
H 2 S-producing microorganisms					
SV	$4.09^{a,w}$	$4.95^{a,w}$	$6.11^{a,x}$	$10.94^{a,y}$	$8.60^{a,z}$
SF	$4.85^{b,y}$	$6.15^{b,w}$	$7.00^{b,x}$	$10.08^{b,y}$	$9.80^{b,z}$
SHP	$3.30^{c,y}$	$3.60^{c,y}$	$4.45^{c,w}$	$3.95^{c,y}$	$5.00^{c,z}$
SFHP	$3.18^{c,w}$	$3.41^{c,x}$	$3.48^{c,x}$	$4.04^{c,y}$	$5.00^{c,z}$
Total mesophilic count					
SV	$4.27^{a,x}$	$4.46^{a,x}$	$6.75^{a,y}$	$6.81^{a,y}$	$7.44^{a,z}$
SF	$4.84^{b,w}$	$5.82^{b,x}$	$8.90^{b,y}$	$9.96^{b,z}$	$10.89^{b,z}$
SHP	$3.23^{c,w}$	$3.69^{c,x}$	$3.71^{c,x}$	$4.01^{c,y}$	$6.45^{a,b,z}$
SFHP	$3.49^{c,x}$	$3.21^{c,w}$	$3.53^{c,x}$	$4.10^{c,y}$	$6.30^{b,z}$
Pseudomonas spp.					
SV	$3.85^{a,x,w}$	$4.00^{a,w}$	$5.41^{a,x}$	$7.45^{a,y}$	$7.79^{a,z}$
SF	$4.78^{b,y}$	$5.78^{b,w}$	$8.86^{b,x}$	$9.71^{b,y}$	$10.78^{b,z}$
SHP	$2.00^{c,y}$	$2.00^{c,y}$	$2.00^{c,y}$	$3.04^{c,z}$	$3.00^{c,z}$
SFHP	$2.89^{c,x}$	$2.00^{c,y}$	$2.00^{c,y}$	$2.48^{c,z}$	$2.00^{c,y}$
Enterobacteriaceae					
SV	$3.02^{a,w}$	$3.46^{a,w}$	$4.72^{a,x}$	$5.14^{a,y}$	$6.00^{a,z}$
SF	$3.36^{b,x}$	$5.40^{b,y}$	$5.00^{b,y}$	$5.65^{b,y}$	$4.90^{b,z}$
SHP	$1.00^{c,y}$	$1.48^{c,w}$	$1.30^{c,x}$	$2.20^{c,y}$	$2.41^{c,z}$
SFHP	$1.60^{c,w}$	$<1^{a,x}$	$1.00^{c,y}$	$<1^{a,x}$	$3.69^{c,z}$
Lactic acid bacteria					
SV	$3.51^{a,w}$	$3.45^{a,w}$	$5.54^{a,x}$	$7.43^{a,y}$	$7.04^{a,y}$
SF	$3.48^{b,w}$	$3.64^{b,w}$	$5.04^{b,x}$	$5.60^{b,y}$	$7.73^{b,y}$
SHP	$2.34^{b,y}$	$2.00^{c,w}$	$3.00^{c,x}$	$3.56^{b,y}$	$6.37^{c,z}$
SFHP	$1.95^{c,w}$	$1.78^{c,w}$	$2.67^{c,x}$	$3.93^{c,y}$	$6.33^{c,z}$

SF: sardine with film; SHP: sardine treated with high pressure; SFHP: sardine treated with film and high pressure; SV: sardine under vacuum packaging.
Different letters (a,b,c) in the same day mean significant differences ($p \geq 0.05$) as a function of sample. Different letters (x,y) for the same sample mean significant differences ($p \geq 0.05$) as a function of time.

putrefaciens. For the SHP and SFHP batches, H_2S-producing microorganisms and lactic acid bacteria counts were in a similar range, but the TVB production (Table 1), although modest, might indicate the prevalence of H_2S producing microorganisms at the end of storage. In these pressurized batches, *Enterobacteriaceae* were below 3 log cfu/g at day 11 ($p \leq 0.05$). The counts in PCA (30 °C), especially in non-pressurized batches, were lower than in Iron Agar incubated at 15 °C. Differences in total microorganism counts depending on medium and incubation temperature have been reported (López-Caballero et al., 2000; Ojagh et al., 2011), sometimes related to the nature of the psychrotrophic microbiota predominant in fish.

3.2.4. Lipid oxidation

The TBARS method was used to determine changes in secondary lipid oxidation products of sardine processed using different treatments during chilled storage (Table 4). Initially, the TBARS content of fresh sardine was 8.63 mg ± 0.65 mg MDA/kg sardine muscle. Reported TBARS values in fresh sardine range from 2.8 mg MDA equivalents/kg (Erkan & Özden, 2008) to 17.2 mg/kg (Chaijan, Benjakul, Visessanguan, & Faustman, 2005b). High pressure treatment significantly reduced the TBARS content ($p \leq 0.05$) to 6.51 mg ± 0.62 mg MDA/kg, in accordance with Gómez-Estaca, López de Lacey, Gómez-Guillén, López-Caballero, and Montero (2009), who found a significant decrease in TBARS after pressurizing salmon carpaccio at 300 MPa/7 °C/15 min. However, Erkan et al. (2011) reported that the differences in TBA-index between untreated and HP-treated (220, 250, and 330 MPa, 7 and 15 °C, 5 and 10 min) horse mackerel samples were insignificant ($p > 0.05$), except for one sample (220 MPa, 15 °C, 10 min).

Despite the noticeably high in vitro antioxidative potential of the GLS film, covering the sardine fillets with this film (batch SF) did not prevent lipid oxidation during chilled storage, in comparison with the vacuum-packed (batch SV) or pressurized sardine (batches SHP and SFHP). The apparent inefficacy of the GLS film to prevent lipid oxidation in sardine fillets could be due to a limited release of active compounds to the muscle, as compared to the release in water. TBARS levels of pressurized sardine remained very low during the whole 17-day storage period, with the film-covered sample (batch SFHP) registering significantly ($p \leq 0.05$) lower values than the uncovered one up to 8 days of chilled storage. Ojagh et al. (2011) reported that TBARS development in pressurized salmon (300 MPa, 10 min, 5 °C) was significantly minimized by covering the fillets with a gelatin–lignin film, suggesting a possible lignin-induced inhibitory effect against lipid oxidation. According to previous reports, lignin derivatives, including lignosulphonate, have a great potential as antioxidants because they can inhibit non-enzymatic and enzymatic oxidative reactions (Núñez-Flores et al., 2013; Ugartondo et al., 2009). Núñez-Flores et al. (2012) showed that lignosulphonate (the same commercial product as used in the present work) was an effective antioxidant at non-cytotoxic concentrations and was suitable for potential food packaging applications. In the present work, although the GLS film alone did not prevent lipid oxidation in sardine muscle more efficiently than conventional vacuum packaging, a noticeable positive effect was found when it was used in combination with a moderate high pressure treatment.

3.2.5. Colour measurement

The sardine preservation study showed that the use of the GLS film did not prolong shelf life during chilled storage; however, either alone or especially in combination with high pressure treatment, it improved quality parameters during the first 8 days of storage. Colour parameters in terms of L* (lightness), a* (redness) and b* (yellowness) of treated samples after 2 and 8 days of storage are shown in Table 5. The L*, a* and b* values of the fresh sardine fillets were 51.19 ± 2.04, 3.87 ± 0.20 and 4.97 ± 0.52, respectively. Both the L* and the a* values were very similar to those registered in sardine patties during storage at 4 °C (Kilinc, Cakli, & Tolasa, 2008). However, b* values reported by Kilinc et al. (2008) were 3-fold higher than the value reported in the present study, owing to the presence of specific ingredients used in the patty formulation. No significant ($p > 0.05$) changes in sardine muscle colour parameters associated with the high pressure treatment were found, since the L*, a* and b* values in the newly pressurized sardine were 55.23 ± 1.70, 3.39 ± 0.18 and 5.35 ± 0.31, respectively.

Table 4
TBARS (mg of MDA/kg muscle) in sardine stored at 7 °C for 17 days.

Batch	Days of storage				
	2	4	8	11	17
SF	$11.71 \pm 1.39^{a,w}$	$16.38 \pm 3.28^{b,x}$	$11.81 \pm 1.43^{c,w}$	$27.61 \pm 0.76^{a,y}$	$22.78 \pm 1.86^{b,z}$
SHP	$6.51 \pm 0.62^{b,x}$	$5.22 \pm 0.73^{a,y}$	$4.83 \pm 0.21^{a,y}$	$2.52 \pm 0.04^{a,z}$	$3.31 \pm 0.43^{a,z}$
SFHP	$4.66 \pm 0.01^{a,x}$	$2.83 \pm 0.01^{a,w}$	$2.04 \pm 0.66^{a,z}$	$2.26 \pm 0.20^{a,z}$	$3.53 \pm 0.22^{a,y}$
SV	$11.71 \pm 0.37^{b,w}$	$6.13 \pm 0.57^{a,x}$	$3.22 \pm 0.30^{a,b,y}$	$5.11 \pm 0.13^{a,z}$	$6.54 \pm 0.29^{a,x}$

SF: sardine with film; SHP: sardine treated with high pressure; SFHP: sardine treated with film and high pressure; SV: sardine under vacuum packaging.
Different letters (a,b,c) in the same day mean significant differences ($p \geq 0.05$) as a function of sample. Different letters (x,y) for the same sample mean significant differences ($p \geq 0.05$) as a function of time.

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Table 5
Colour parameters (L*, a*, and b*) of sardine stored at 7 °C for 8 days.

Batch	L*		a*		b*	
	2 days	8 days	2 days	8 days	2 days	8 days
SF	47.48 ± 0.48^{a,x}	36.80 ± 1.75^{a,y}	4.13 ± 0.96^{a,x}	4.82 ± 1.35^{a,x}	5.45 ± 0.25^{a,x}	5.49 ± 0.51^{a,x}
SHP	55.98 ± 0.31^{b,x}	53.06 ± 2.03^{b,x}	3.71 ± 0.70^{a,x}	8.33 ± 0.64^{b,y}	6.67 ± 0.25^{b,x}	4.63 ± 0.28^{a,y}
SFHP	54.21 ± 0.81^{c,x}	51.86 ± 1.01^{b,x}	4.37 ± 0.34^{a,x}	8.68 ± 1.03^{b,y}	6.52 ± 0.47^{b,x}	13.61 ± 1.35^{b,y}
SV	51.05 ± 0.08^{d,x}	43.48 ± 0.12^{c,y}	2.11 ± 0.12^{b,x}	7.15 ± 0.64^{b,y}	4.61 ± 0.26^{a,x}	6.00 ± 0.62^{a,x}

SF: sardine with film; SHP: sardine treated with high pressure; SFHP: sardine treated with film and high pressure; SV: sardine under vacuum packaging.
Different letters (a,b,c) in the same day mean significant differences ($p \geq 0.05$) as a function of sample. Different letters (x,y) for the same sample mean significant differences ($p \geq 0.05$) as a function of time.

Fish muscle treated with high pressure has frequently been reported to become opaque with a cooked-like appearance (Oshima, Ushio, & Koizumi, 1993). Indeed, noticeable high pressure-induced increases in L* values and decreases in a* values were reported in various fish species, such as salmon, horse mackerel and turbot (Chevalier, Le Bail, & Ghoul, 2001; Erkan et al., 2011; Ojagh et al., 2011). The slight changes found in sardine muscle denote that high pressure-induced colour modifications are largely species-dependent and sardine may be a good candidate for preservation by high pressure.

After 8 days of storage, a marked reduction in muscle lightness (L* value) occurred in both SF and SV batches, as compared to the fresh sardine, being slightly more intense in sardine covered with GLS film (SF). Meat darkening in sardine stored in ice (*Sardinella gibbosa*) was reported to be the result of a slight increase in metmyoglobin content caused by myoglobin oxidation (Chaijan, Benjakul, Visessanguan, & Faustman, 2005a), which is normally positively correlated with lipid oxidation (Chan, Faustman, Yin, & Decker, 1997). Accordingly, in the present study the level of lipid oxidation after 8 days of storage was significantly higher in the SF batch. With regard to high pressure treatment, no significant ($p > 0.05$) changes in lightness were observed between the SHP and SFHP batches during the 8-day storage period, nor in comparison with the newly pressurized sardine.

SF did not suffer significant ($p > 0.05$) changes in a* or b* values during storage as compared to the fresh sardine, unlike the vacuum-packed (SV) batch, which had a significantly higher a* value at day 8 (Table 5). A noticeable increase in redness was also observed in the pressurized batches (SHP and SFHP). Yellowness (b* values) was considerably ($p \leq 0.05$) higher in the SFHP batch, probably as a result of the transfer of some light colour from the film, favoured by the high pressure treatment, since this behaviour was not evident in the SF batch ($p \leq 0.05$). In a previous work (Ojagh et al., 2011), covering salmon fillets with a gelatin–lignin film significantly affected L* and a* values, in contrast to the present findings in sardine, obviously due to the great differences in the muscle colour of the two species. In the work by Ojagh et al. (2011), the film had some preservative effect on the colour of the pressurized salmon muscle; however, given that the sardine muscle colour was not affected by high pressure, the GLS film produced an evident colour-protective effect during storage only when applied alone.

4. Conclusions

Lignosulphonate has been shown to provide gelatin films with in vitro antioxidative properties. A mid-term film-storage period of 4 weeks at room temperature maintained mechanical and water resistance of the gelatin–lignosulphonate film without any detrimental effect on its antioxidant capacity. Gelatin films in contact with oil underwent some kind of anti-plasticizing effect and a noticeable reduction in water solubility, which was overcome when lignosulphonate was included. Although the composite films did not prevent lipid oxidation when applied for preserving sardine fillets during chilled storage, the combined use of the film with high pressure treatment reduced lipid oxidation and microbial spoilage more efficiently than conventional vacuum packaging. Despite the low cytotoxicity level of lignosulphonate, as previously described, and the suitable film functionality in combination with high pressure, further studies should be necessary to guarantee the safety of its use as a packaging material for human consumption.

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5.3 *Artículo 3. Physical and functional characterization of active fish gelatin films incorporated with lignin*

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Physical and functional characterization of active fish gelatin films incorporated with lignin

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ABSTRACT

In order to provide gelatin films with antioxidant capacity, two sulphur-free water-insoluble lignin powders (L_{1000} and L_{2400}) were blended with a commercial fish-skin gelatin from warm water species at a rate of 85% gelatin: 15% lignin (w/w) ($G–L_{1000}$ and $G–L_{2400}$), using a mixture of glycerol and sorbitol as plasticizers. The water soluble fractions of $G–L_{1000}$ and $G–L_{2400}$ films were 39.38 ± 1.73% and 46.52 ± 1.66% respectively, rendering radical scavenging capacity (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS assay)) of 27.82 ± 2.19 and 15.31 ± 0.88 mg vitamin C equivalents/g film, and ferric ion reducing ability (FRAP assay) of 258.97 ± 8.83 and 180.20 ± 5.71 μmol Fe^{2+} equivalents/g film, respectively. Dynamic oscillatory test on film-forming solutions and Attenuated Total Reflectance (ATR)-FTIR spectroscopy study on films revealed strong lignin-induced protein conformational changes, producing a noticeable plasticizing effect on composite films, as deduced from the study of mechanical (traction and puncture tests) and thermal properties (Differential Scanning Calorimetry, DSC). The gelatin films lose their typical transparent and colourless appearance by blending with lignin; however, the resulting composite films gained in light barrier properties, which could be of interest in certain food applications for preventing ultraviolet-induced lipid oxidation. Lignin proved to be an efficient antioxidant at non-cytotoxic concentrations, however, no remarkable antimicrobial capacity was found.

1. Introduction

Gelatin has been one of the most studied biopolymers on account of its film-forming ability and its usefulness as an outer film to protect food from drying and exposure to light and oxygen (Arvanitoyannis, 2002). Fish gelatins exhibit good film-forming properties, yielding transparent, nearly colourless and highly extensible films (Avena-Bustillos et al., 2006; Carvalho et al., 2008; Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006; Zhang, Wang, Herring, & Oh, 2007). Furthermore, enriching gelatin films with antioxidants and/or antimicrobial substances will extend the functional properties of these biodegradable films and provide an active packaging biomaterial. Because of "clean labelling" concerns, there is growing interest in using natural compounds, such as polyphenolic plant extracts (Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007) or α-tocopherol (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2008) in the formulation of active fish gelatin films.

Lignin, most commonly derived from wood, is largely thrown off as a waste product in pulp and paper industries. It is a complex polydisperse natural polymer made up of phenyl-propane (C6–C3) units that bind cellulose fibres together, thus hardening and strengthening the plant cells. Lignin derivatives have been incorporated as fillers in different synthetic polymer matrices to develop lignin-based materials with improved physical properties (Cui, Xia, Chen, Wei, & Huang, 2007; Feldman, Lacasse, & Beznaczuk, 1986; Kadla & Kubo, 2003; Mishra, Mishra, Kaushik, & Khan, 2007). During the last decade, a great deal of research was devoted to the development of lignin-containing biopolymeric materials, on account of its renewable, non-toxic and biodegradable character (Ban, Song, & Lucia, 2007; Baumberger, Lapierre, Monties, Lourdin, & Colonna, 1997; Chiellini, Cinelli, Fernandes, Kenawy, & Lazzeri, 2001; Julinová et al., 2010; Li & Sarkanen, 2002; Vengal & Srikumar, 2005; Wu, Wang, Li, Li, & Wang, 2009). Lignin has been referred to as a plasticizing agent in composite films with starch (Wu et al., 2009). However, in composites prepared using adipic acid-modified starch microparticles within a corn-starch matrix, addition of lignin produced higher tensile strength and lower elongation capacity (Spiridon, Teaca, & Bodirlau, 2011). Similarly, lignin acted as a reinforcing agent with cellulose (Rohella, Sahoo, Paul, Choudhury, & Chakravortty, 1996) or polyethylene oxide

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(Kadla & Kubo, 2003), in all cases providing adequate miscibility with the polymer. In addition, the incorporation of small amounts of lignin into polypropylene films has been shown to stabilize the composite material against photo-oxidation (Kosikova, Demianova, & Kakurakova, 1993). The hydrophobic nature of lignin has been also shown to produce strong reduction of water absorbency and transparency in starch-based films (Ban et al., 2007).

Due to their complex polyphenolic nature, lignins can exert antioxidant (radical scavenging capacities) (Dizhbite, Telysheva, Jurkjane, & Viesturs, 2004; Lu, Chu, & Gau, 1998; Pan, Kadla, Ehara, Gilkes, & Saddler, 2006; Satoh et al., 1999; Ugartondo, Mitjans, & Vinardell, 2008) and antimicrobial properties (Dong et al., 2011), thus opening up the possibility of new potential applications. As a result of the molecular complexity of lignins, it becomes difficult to assign the antioxidant efficacy to specific structural components, compared to the activities of chemically defined tannins and flavonoids (Sakagami et al., 2005). The radical scavenging activity of lignins is influenced by structural features, such as the presence of phenolic hydroxyl groups, methoxy groups, π-conjugation systems as well as the molecular weight, heterogeneity and polydispersity (Dizhbite et al., 2004). Only a few studies have reported the cytotoxic effects of lignins. A good correlation between cytotoxicity and some features such as carbohydrate content and polydispersity has been reported; the lignins with higher polydispersity and lower carbohydrate content are the most cytotoxic (Ugartondo et al., 2008). Lignin derivatives have been shown to be effective antioxidants at concentrations that are not harmful to normal human cells, thus furthering their possible use in the formulation of active food packaging biomaterials (Núñez-Flores et al., 2012; Ugartondo et al., 2008). In this sense, the appearance, protein quality and oxidative stability of salmon fillets subjected to high pressure processing were enhanced by the combined use of a gelatin–lignin film similar to the one characterized in the present study (Ojagh, Núñez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011).

The antimicrobial properties of lignins have been reported previously in the literature, both in model system and in experimental animals, for example, from hydrolysates of several lignocellulosic materials (ethyl acetate extracts) (Cruz, Domínguez, Domínguez, & Parajó, 2001), lignin-related structures from alkaline extractions (Oh-Hara et al., 1990), kraft-lignins (Dizhbite et al., 2004) and to a lesser extent from lignosulphonates (Núñez-Flores et al., 2012). The origin of lignin might influence their antimicrobial properties. Thus, Oh-Hara et al. (1990) reported that the antimicrobial activity induced by commercial lignins was much lower than that induced by fractions of pine cone extracts obtained by successive alkaline extractions (and then recovered as acid precipitates at pH 5). By comparing the spectra, these authors found that some pine cone extract include more alkenic double bonds and fewer OCH3 than commercial alkali-lignin, while a coumaryl type of lignin structure could be responsible for the antimicrobial activity.

The aim of the present work was to produce antioxidant fish gelatin films by mixing gelatin with two types of lignin, and to characterize structural, mechanical, optical, and thermal properties of the composite active material. In order to establish the harmlessness and potential functionality in food packaging applications, cytotoxicity, radical scavenging capacity and antimicrobial capacity of lignin were also tested.

2. Materials & methods

2.1. Materials

Commercial type A warm-water fish gelatin was supplied by Rousselot S.A.S. (Puteaux, France). For comparison purposes, two sulphur-free water-insoluble commercial lignin powders were used: Protobind 2400 (L_{2400}) and Protobind 1000 (L_{1000}) (Granit Recherche & Developpement SA, Lausanne, Switzerland). According to manufacturer's specifications, both lignins aqueous suspensions presented pH ~ 4, number average molecular weight ~ 1000 Da and particle size <210 micron, differing in bulk density (~0.55 kg/l in L_{2400} *vs* ~0.30 kg/l in L_{1000}) and in softening temperature (~130 °C in L_{2400} *vs* ~200 °C in L_{1000}). Glycerol and sorbitol were obtained from Panreac (Barcelona, Spain). All other reagents used were of analytical grade. The 2,4,6-tripyridyl-s-triazine, the 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, Vitamin C and the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical were purchased from Sigma–Aldrich (St. Louis, MO, USA). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) salt was supplied by Promega Biotech Ibérica (Madrid, Spain).

2.2. Preparation of films

The gelatin–lignin film forming solution (FFS) was prepared by dissolving the fish gelatin in distilled water (3.4% w/v) at 40 °C, adding sorbitol (15 g/100 g gelatin) and glycerol (15 g/100 g gelatin) as plasticizers. The lignin powder was added to a final concentration of 0.6% w/v in the FFS. This concentration was selected according to previous experiments. The mixture was stirred at 40 °C for 15 min and was alkalinized to ~pH = 11 to obtain a good blend with total solubility. The films were made by casting an amount of 40 ml over a plate of 12 × 12 cm^2 and drying at 45 °C in a forced-air oven for 15 h to yield a uniform thickness of ~100 ± 10 μm. Films were conditioned over a saturated solution of KBr in desiccators for 4 d.

2.3. Viscoelastic properties of film forming solutions

Dynamic oscillatory study of the film-forming solutions was carried out on a Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4°, gap 0.15 mm). Cooling and heating from 30 to 2 °C and back to 30 °C took place at a scan rate of 1 °C/min, a frequency of 0.5 Hz, and a target strain of 0.5%. The elastic modulus (G'; Pa), viscous modulus (G''; Pa) and phase angle (°) were plotted as functions of temperature in the heating ramp from 2 to 30 °C. At least two determinations were performed for each sample. The experimental error was less than 6% in all cases.

2.4. Film thickness

Film thickness was measured using a digital micrometer (Mitutoyo, model MDC-25M, Kanagawa, Japan), averaging nine different locations.

2.5. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of the films were determined using a TA.XT.plus Texture analyser (SMS, Surrey, UK). The samples were cut into rectangles (20 mm width and 50 mm length), fixed on the grips of the device with a gap of 20 mm, and tractioned at a speed of 1 mm/s. Results of TS and EAB were average of five determinations, and expressed as N/m^2 and %, respectively.

A puncture test was performed to determine the breaking force and the breaking deformation of the films. Films were placed in a cell 5.6 cm in diameter and punched to the breaking point using the same texture analyser, with a round-ended stainless-steel plunger 3 mm in diameter at a cross-head speed of 60 mm/min. Breaking force was expressed in N and breaking deformation in %, according to Sobral, Menegalli, Hubinger & Roques (2001). All determinations are the means of at least five measurements.

2.6. Water solubility

Water solubility was measured using the same methodology as described by Núñez-Flores et al. (2012). Film solubility was calculated by the equation FS (%) = ((Wo − Wf)/Wo)·100, where Wo was the initial weight of the film expressed as dry matter and Wf was the weight of the undissolved desiccated film residue. All tests were carried out in triplicate.

2.7. Water activity (A_w)

Water activity (A_w) of films was measured using a portable LabMaster-a_w (Novasina, Lachen, Switzerland) instrument. Values were recorded at equilibrium when A_w of any two readings were <0.001 at constant temperature (25 °C). Measurements were done at least in triplicate.

2.8. Water vapour permeability

Water vapour permeability (WVP) was determined following a gravimetric method as described below. Films were attached over the openings of cells (permeation area = 15.9 cm^2) containing desiccated silica gel, and the cells were placed in desiccators with distilled water at 22 °C. The cells were weighed every hour for at least 6 h. Water vapour permeability was calculated using the equation $WVP = w \cdot x \cdot t^{-1} \cdot A^{-1} \cdot \Delta P^{-1}$, where w was weight gain (g), x film thickness (mm), t elapsed time for the weight gain (h), and ΔP the partial vapour pressure difference between the dry atmosphere and pure water (2642 Pa at 22 °C). Results have been expressed as g mm h^{-1} cm^{-2} Pa^{-1}. All tests were carried out at least in triplicate.

2.9. Light barrier properties

The films were cut into a rectangle piece and directly placed against one side of a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) test cell, using an empty test cell as the reference. The light barrier properties of films were measured by exposing the films to light absorption at wavelengths ranging from 690 nm to 200 nm. The opacity of the films was calculated by the equation $O = Abs_{600}/x$, where Abs_{600} is the value of absorbance at 600 nm and x is the film thickness in mm. Measurements were done at least in triplicate.

2.10. Colour measurements

The colour parameters Lightness, Redness and Yellowness were measured in the L^*, a^*, b^* mode of CIE scale using a Konica Minolta 3500-D colorimeter (Osaka, Japan). Results are average of at least 10 replicates. Hue angle (h^*_{ab}) and Chroma (C^*_{ab}) values were calculated using the following equations:

$$h^*_{ab} = \tan^{-1}\left(b^*/a^*\right), \text{ when } \left[+a^*, +b^*\right]$$

$$h^*_{ab} = 180 + \tan^{-1}\left(b^*/a^*\right), \text{ when } \left[-a^*, -b^*\right]$$

$$h^*_{ab} = 360 + \tan^{-1}\left(b^*/a^*\right), \text{ when } \left[+a^*, -b^*\right]$$

$$C^*_{ab} = \left(a^{*2} + b^{*2}\right)^{1/2}$$

2.11. Attenuated Total Reflectance (ATR)-FTIR spectroscopy

Infrared spectra between 4000 and 650 cm^{-1} were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin Elmer Inc., Waltham, MA, USA) equipped with an ATR prism crystal accessory, as reported previously (Núñez-Flores et al., 2012). All experiments were performed at least in triplicate and represented as average spectra.

2.12. Differential Scanning Calorimetry (DSC)

Calorimetric analysis was performed using a model TA-Q1000 differential scanning calorimeter (TA Instruments, New Castle, DE, USA) previously calibrated by running high purity indium. Samples of approximately 10 mg (±0.002 mg) were weighed out using a model ME235S electronic balance (Sartorious, Goettingen, Germany) and were tightly encapsulated in hermetic aluminium pans and scanned under dry nitrogen purge (50 ml/min). An empty hermetic aluminium pan was used as reference. Freshly conditioned films were cooled to −80 °C, or 0 °C, at 10 °C/min and scanned up to 90 °C at a heating rate of 10 °C/min. After cooling at the same rate down to the corresponding initial temperature, a second heating scan was run. Glass transition temperatures, Tg (°C), were calculated by the inflection-midpoint method and usually reported on the first heating scans, in order to thermally characterize the same material used in the rest of analyses. Tg as well as melting transition data (Temperature, Tm; Enthalpy change, ΔH) were reported as mean values with their standard deviations of at least triplicate samples for each film. The energetic parameter was normalized to dry matter content of the corresponding film sample, ΔH (J/g_{dm}), which needed from the desiccation (105 °C, pin hole in the lid) of each individual capsule content.

2.13. Cryoscanning electron microscopy (Cryo-SEM)

Cryoscanning electron microscopy (Cryo-SEM) was used to examine microstructural representative cross sections of films. Samples were mounted with OCT compound (Gurr) and mechanically fixed onto the specimen holder using the Oxford CT1500 Cryosample Preparation Unit (Oxford Instruments, Oxford, England). Samples were frozen in subcooled liquid nitrogen for 2 min and then transferred to the preparation unit. After ice sublimation, the surfaces were gold sputter coated, and subsequently transferred into the cold stage of the SEM chamber. Specimens were observed with a DSM960 Zeiss SEM microscope (Zeiss, Oberkochen, Germany) at −135 °C under a 15 kV acceleration potential.

2.14. Antioxidant properties of films

The ferric ion reducing capacity (FRAP) and the radical scavenging ability (ABTS) assays were used to measure the antioxidant activity of the films. An aliquot of the filtrate obtained from the determination of the film water solubility was employed as the sample. The method used for the FRAP and ABTS assays was previously described by Gómez-Estaca, Giménez, et al. (2009) and Gómez-Estaca, Montero et al. (2009). The results were expressed as μmol Fe^{2+} equivalents per g of film for FRAP and mg of Vitamin C Equivalent Antioxidant Capacity (VCEAC) per g of film for ABTS, based on standard curves of $FeSO_4 \cdot 7H_2O$ and vitamin C, respectively.

2.15. Antioxidant activity of lignin

The antioxidant activity of lignin (L_{1000}) was determined based on the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability. The assay was carried out as described by Brand-Williams, Cuvelier, and Berset (1995) with some modifications (Fukumoto &

Mazza, 2000). L_{1000} was analyzed by triplicate testing at least five different concentrations ranging from 7 to 250 μg/ml. The radical scavenging activity was expressed as IC_{50} value, the concentration necessary to quench 50% of initial DPPH radical. The percentage of scavenged DPPH was plotted *vs* the concentration of L_{1000}, and that required to quench 50% of initial DPPH radical was obtained from the graph by linear regression. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analogue, was used as reference compound. The DPPH radical scavenging capacity of Trolox, expressed as IC_{50} value, was determined by testing concentrations ranging from 3 to 50 μg/ml.

2.16. Cytotoxic effect of lignin

2.16.1. Culture of cell lines

The mouse fibroblast cell line 3T3-L1 was grown in DMEM medium (4.5 g/l glucose) supplemented with 10% foetal bovine serum (FBS), at 37 °C under a humidified 5% CO_2 atmosphere. When the cells were approximately 70% confluent, they were split by mild trypsinization and seeded into 24-well plates at a density of 1×10^4 cells/well. The 24-well plates were incubated at 37 °C and 5% CO_2 for 24 h. Runs were performed in triplicate with different passage cells.

2.16.2. Experimental treatments

After 1 day of incubation, cultures were exposed to increasing concentrations of lignin (L_{1000}) sterilized by filtration and diluted in DMEM medium supplemented with 10% FBS. Controls (containing only the culture medium) were included in each plate. The plates were incubated at 37 °C with 5% CO_2 for 24 h.

2.16.3. MTS assay

The viability of the 3T3-L1 cells treated with increasing concentrations of LS for 24 h was determined by the MTS assay, composed of the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent (PMS, phenazine methosulfate). This assay is based on the conversion of the tetrazolium compound into a coloured, aqueous soluble formazan product by mitochondrial activity of viable cells at 37 °C. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in culture. MTS and PMS were combined in a ratio of 20:1 and the mixture was added to the culture medium in a ratio of 1:5 (reagent mixture:culture medium). Cells were washed with PBS and the medium containing MTS/PMS was added (500 ml per well). After 1 h of further incubation, the absorbance was measured in a microplate reader at 485 nm (Appliskan, Thermo Scientific, Madrid, Spain).

2.17. Antimicrobial capacity of lignin

The antimicrobial activity of lignin was determined by the disk diffusion method in agar against 26 microbial strains (including Gram-positive and Gram-negative bacteria, yeast and molds) as previously described (Núñez-Flores et al., 2012). Each determination was performed in triplicate.

2.18. Statistical analysis

Statistical tests were performed using the SPSS computer program (SPSS Statistical Software Inc., Chicago, IL, USA). One-way analysis of variance was carried out. Differences between pairs of means were assessed on the basis of confidence intervals using the Duncan test. The level of significance was $p < 0.05$.

3. Results & discussion

3.1. Physical properties of films

Table 1 shows the physical properties of gelatin and gelatin–lignin (G–L) composite films. The addition of lignin produced an evident plasticizing effect, as deduced from significant decreases in both tensile strength (TS) and breaking force (BF) in the composite films, together with a marked increase in elongation at break (EAB) as well as in breaking deformation (BD). The composite films were highly stretchable, with percent elongation values similar to those reported previously by Vengal and Srikumar (2005) in gelatin–lignin films, and comparable to the highly extensible gelatin films obtained from the skin of cold water fish species (Carvalho et al., 2008; Pérez-Mateos, Montero, & Gómez-Guillén, 2009). The films containing lignin L_{2400}, which was characterized by a higher bulk density and a lower softening point than lignin L_{1000}, were characterized by significantly ($p < 0.05$) lower TS values than G–L_{1000} films. The reduced TS in the gelatin–lignin composite films was related to an increased water activity, which was more pronounced in G–L_{2400} film. Thus, the apparent plasticizing effect observed in composite films could be attributed not only to a direct effect of lignin but also to the presence of water free molecules, causing a reduction in intermolecular attractive forces between polymer chains (Cuq, Gontard, Cuq, & Guilbert, 1997). Lignin has provided adequate miscibility with different polymers. It has been reported as a plasticizing agent in composite films with starch (Baumberger et al., 1997; Wu et al., 2009) or with soy protein isolate (SPI) (Huang, Zhang, & Chen, 2003), but in the latter case only when incorporated at moderate concentrations (from 10 to 20 parts). In contrast, lignin acts as a reinforcing agent with cellulose (Rohella et al., 1996) or polyethylene oxide (Kadla & Kubo, 2003). A significant increase in TS has been also reported for SPI-lignin blends (Huang et al., 2003). However, it should be noted that SPI films were considerably less resistant than the gelatin films obtained in this study, in accordance to a previous work performed by Cao, Fu, and He (2007).

Regarding film water barrier properties, the water solubility was slightly but significantly ($p < 0.05$) reduced only in the G–L_{1000} film. To this respect, it should be taken into account that the solubility of the control gelatin film (~44%) was relatively low, especially when compared to extremely high water-soluble films prepared with gelatins from cold-water species, such as cod (Pérez-

Table 1
Physical properties of films based on mixtures of fish gelatin (G) and lignin (L_{1000} and L_{2400}) at the ratio 85% gelatin: 15% lignin (w/w).

Gelatin–lignin	Thickness (mm)	Aw	TS × 10^6 (N/m^2)	EAB (%)	BF (N)	BD (%)	Water solubility (%)	WVP × 10^{-8} (g mm/h Pa cm^2)
G	0.096 ± 0.01^a	0.375^a	16.44 ± 0.18^a	136.6 ± 39.8^a	26.08 ± 0.25^a	15.85 ± 4.33^a	44.29 ± 0.97^a	2.06 ± 0.10^a
G–L_{1000}	0.124 ± 0.02^a	0.390^b	12.13 ± 0.48^b	316.48 ± 19.7^b	18.95 ± 1.43^b	54.90 ± 0.13^b	39.38 ± 1.73^b	2.17 ± 0.54^a
G–L_{2400}	0.100 ± 0.02^a	0.467^c	7.51 ± 0.05^c	362.83 ± 39.6^b	16.38 ± 2.05^b	65.70 ± 1.16^c	46.52 ± 1.66^a	4.58 ± 0.53^b

Aw: water activity; TS: tensile strength; EAB: elongation at break; BF: breaking force; BD: breaking deformation; WVP: water vapour permeability.
Different letters a, b, c in the same column indicate significant differences ($p < 0.05$) among samples.

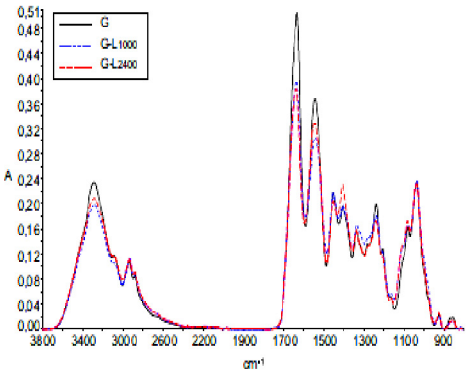

Fig. 2. ATR-FTIR spectra of films based on mixtures of fish gelatin (G) and lignin (L_{1000} and L_{2400}) at the ratio 85% gelatin: 15% lignin (w/w). Single gelatin film, G; compound films with lignin, G–L_{1000} and G–L_{2400}.

lignin-induced protein conformational changes, especially attending to differences in amide I band (Surewicz & Mantsch, 1988). The slight frequency up-shift of amide I peaks in the composite films (G: 1631 cm^{-1}; G–L_{1000}: 1635 cm^{-1}; G–L_{2400}: 1635 cm^{-1}), would indicate an eventual disruption of hydrogen bonding at the C=O groups of gelatin polypeptides by the lignin interference, regardless the lignin type. In this connection, addition of lignin has been also shown to prevent interaction among SPI molecules in SPI-lignin blend films (Huang et al., 2003). Infrared absorption of lignin carbonyl groups, which has been described at higher wavenumbers (~1740–1700 cm^{-1}) (Fernandes, Winkler, Job, Radovanocic, & Pineda, 2006; Mishra et al., 2007; Pereira et al., 2007) were not detectable in the spectra of the gelatin–lignin films. On the other hand, FTIR spectra confirmed the higher protein hydration level in the G–L_{2400} film, in comparison to G–L_{1000}, deduced from the slightly higher intensity of amide A band and the frequency up-shift of amide II peak (1544 cm^{-1} *vs* 1539 cm^{-1}) in those films (Yakimets et al., 2005).

The prominent band at ~1035 cm^{-1} in the gelatin films could be largely attributed to the interactions arising between plasticizers (C–O stretch of glycerol and sorbitol) and film structure (Bergo & Sobral, 2007; Hoque, Benjakul, & Prodpran, 2011). Despite the film plasticizing effect caused by lignin, no evident changes at this level were observed in the gelatin–lignin films, discarding thus remarkable interactions between lignin and plasticizers.

The second derivative of the amide I band, depicted in Fig. 3, revealed noticeable changes induced by the presence of lignin in the composite films, as denoted by the reduced intensity of peaks at ~1660 cm^{-1}, 1652 cm^{-1} and ~1630 cm^{-1}, which had been related, respectively, to the presence of triple helix, single α-helix, and disordered coil structures in gelatin matrices (Payne & Veis, 1988; Prystupa & Donald, 1996). The most pronounced changes in composite films within the range of ~1635–1625 cm^{-1} were indicative of the strong interference caused by both lignins in the hydrogen bonding between water and imide residues (Payne & Veis, 1988). Such an interference, which was also evidenced in the triple helical component at ~1660 cm^{-1}, was consistent with the reduced capacity in triple helix development of lignin-containing film forming solutions.

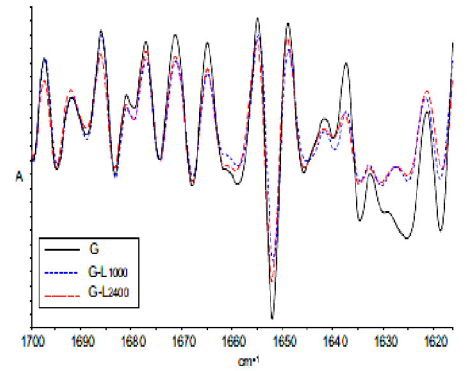

Fig. 3. Second derivative of amide A band from FTIR spectra of films based on mixtures of fish gelatin (G) and lignin (L_{1000} and L_{2400}) at the ratio 85% gelatin: 15% lignin (w/w). Single gelatin film, G; compound films with lignin, G–L_{1000} and G–L_{2400}.

3.5. Thermal properties

Typical DSC behaviour of films from fish gelatin alone (G) or blended with lignin (G–L) are shown in Fig. 4. Films G exhibited thermal characteristics resembling those reported in a previous publication (Gómez-Estaca, Giménez, et al., 2009; Gómez-Estaca, Montero, et al., 2009) concerning a type A tuna-skin gelatin prepared at the laboratory. Essentially, the thermal behaviour of gelatin single films consisted (Trace G, solid line) of a glass transition process with a Tg ~ 18.5 ± 0.5 °C, followed by an endothermic event involving a bimodal melting with maximum temperatures Tm at around 61.3 ± 0.5 °C and 79.5 ± 0.5 °C. These thermal data were something higher than corresponding ones of previous work, particularly Tg, which notwithstanding indicated a close similitude between that tuna-skin gelatin and current commercial warm-water fish gelatin. Melting enthalpy ΔH was 11.3 ± 0.41 J/g_{dm}. This kind of complex endothermic melting in G films has also been reported by Gómez-Estaca, Giménez, et al. (2009) and Gómez-Estaca, Montero, et al. (2009) and Rahmann, Al-Saidi, and Guizani (2008) by additionally using MDSC (Modulated DSC) and tentatively attributed to a normal melting overlapped with a crystal perfection process. Second heating scans (Trace G, dash line) displayed a slightly lower (~3 °C) glass transition temperature but associated with a higher (~1/3) heat capacity change in the system,

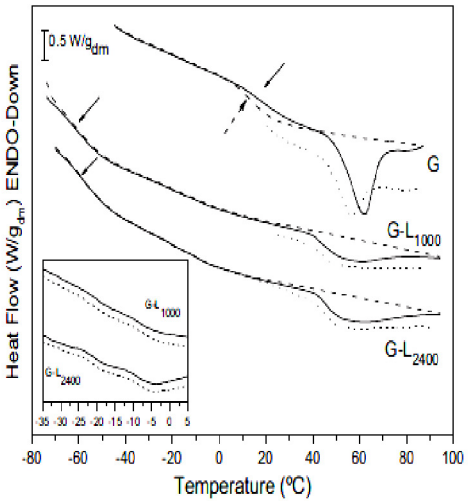

Fig. 4. Normalized typical DSC traces (heat flow, W/g_{dm}, *vs* temperature, °C) of films based on mixtures of fish gelatin (G) and lignin (L_{1000} and L_{2400}) at the ratio 85% gelatin: 15% lignin (w/w). Single gelatin film, G; compound films with lignin, G–L_{1000} and G–L_{2400}. First scans in conditioned films at −80 °C, solid lines; second scans, dash lines. First scans in conditioned films at 0 °C, dot lines. Arrows roughly indicate Tg locations: Inset shows DSC traces of blended films magnified in the temperature zone of the complex glass transition processes. Solid, first scans; dash, second scans. Bar indicates the ordinate scale (0.5 W/g_{dm}).

at the time that no sign of remnant melting was detected. This may likely be due to a different thermal history of a wholly amorphous and bigger (total) G amount participating in the glass transition process.

When the film was conditioned at 0 °C, corresponding glass transition seemed to be only visible by MDSC as reported in a previous work (Gómez-Estaca, Giménez, et al., 2009; Gómez-Estaca, Montero, et al., 2009), probably due to the small of the temperature gap. The melting endotherm (Trace G, dot line) evolved less sharply (rounded peaks) and the main melting temperature was considerably and significantly lower (53.2 ± 2.5 °C) than that at −80 °C, while the second peak remained almost unaltered (80.8 ± 0.3 °C). The melting enthalpy value was 9.57 ± 0.21 J/g_{dm}, also significantly lower (~15%) than in the low temperature conditioning. These differences can likely be due to the different thermal crystallization conditioning which yielded a lower amount and a different crystal material. These thermal data were much more similar to those from Gómez-Estaca, Giménez, et al. (2009) and Gómez-Estaca, Montero, et al. (2009) than previous ones due to much more similar thermal histories between samples. In general, current results were not able for comparison with those from others (Badii & Howell, 2006; Rahmann et al., 2008) on fish gelatin films, because disparity appeared not only among gelatins used but also among the different plasticizers present in the films.

Regarding G–L_{1000} and G–L_{2400} films, a new glass transition process arose at the lowest temperature region (Traces G–L_{1000} and G–L_{2400}, solid lines), with Tg values of − 63.4 ± 0.8 °C and − 54.9 ± 0.6 °C, respectively (Fig. 4). Tg data were significantly lower in G–L_{1000} than in G–L_{2400}, this suggesting a differential plasticization capacity between both lignin types (L_{1000} > L_{2400}). This glass transition process was followed by a second one at the significantly different Tg values of − 23.1 ± 0.4 °C and − 20.6 ± 0.6 °C respectively. Both Tg values were significantly lower than that of G, indicating the plasticization role of lignin (L_{1000} > L_{2400}), as well as an (initially) total miscibility between both types of L and G at the proportions used in the blended films. Corresponding melting endotherms were considerably smaller (with a flatter trace) than in G alone films, with main melting temperatures no significantly different for both blended films at around 55.8 °C and 55.4 °C respectively. Second scans (Traces G–L_{1000} and G–L_{2400}, dash lines) showed little variations in the corresponding lowest Tg data; the second glass transition process underwent however some complexity in the sense that two Tg values emerged, i.e., one at a lower and the other at a higher (more subtly) temperature than in the first scan: −28.3 ± 0.7 °C plus − 11.9 ± 0.9 °C for G–L_{1000}, and −20.7 ± 1.2 °C plus − 8.9 ± 0.9 °C for G–L_{2400}. The two sets of values for both blended G–L films were significantly different in their lower temperature components, but not significantly different in their higher temperature components. It is worth-noting that the first glass transition process at the lowest temperature range (Tg's at roughly −63 °C for G–L_{1000} and −55 °C for G–L_{2400}) seemed similar to that observed in a previous work (Gómez-Estaca, Gómez-Guillén, Fernández-Martín, & Montero, 2011) dealing with several composite films from fish gelatin plus chitosan plasticized with the same glycerol plus sorbitol addition. We reported an average Tg of −32.9 °C as a representative value for all the composition range studied (Gómez-Estaca et al., 2011). Huang et al. (2003) have also reported by DSC similar low temperature Tg values (compositionally varying from −70 to −75 °C) in SPI-lignin films and, by complementarily using dynamic mechanical thermal analysis (DMTA) (Tg values from −66 to −74 °C), ascribed this phenomenon to the α-relaxation process of the SPI side segments in the system. It was followed by a second glass transition process which showed some complexity in the sense that it consisted of two successive transitions (Inset: Traces G–L_{1000} and G–L_{2400}, solid lines) at the significantly different Tg values of −23.1 ± 0.4 °C plus − 11.9 ± 0.9 °C for G–L_{1000}, and −20.7 ± 1.2 °C plus − 8.9 ± 0.9 °C for G–L_{2400}. Both Tg couple values were significantly lower than that single of G, indicating the plasticization role of lignin (L_{1000} > L_{2400}), as well as an (initially) total miscibility between both types of L and G at the proportions used in the blended films. Corresponding melting endotherms were considerably and significantly smaller (with a flatter trace: not significantly different values of 5.23 ± 0.25 J/g_{dm} for G–L_{1000} and 6.14 ± 0.16 J/g_{dm} for G–L_{2400}) than in G alone films, with main melting temperatures no significantly different for both blended films at around 55.8 °C and 55.4 °C respectively. The two sets of values for both blended G–L films were significantly different in their lower temperature components, but not significantly different in their higher temperature components. The Tg doublet presence could likely be due to the appearance of a certain immiscibility between hydrophobic L and hydrophilic G leading to a microphase separation, which provide more space for the G mobility. This phenomenon has been reported by Huang et al. (2003) to occur in the SPI-lignin films, and by Núñez-Flores et al. (2012) in (chemically near) fish gelatin plus lignosulphonate films. The lower temperature component values of the respective Tg doublets suggested that the above discussed immiscibility (microphase separation) enhanced the initial plasticization effect of L_{1000} with respect to L_{2400}. The higher temperature components were not significantly different but presented significant differences to the Tg value of G, suggesting that immiscibility produced the segregation of a L-rich and a L-pour phase respectively. Lignin is the common name of a family of wholly amorphous aromatic polymers derived by oxidative condensation of phenolic precursors, very branchy configured and containing several structural units which may yield very complex structures. According to Guigo, Mija, Vincent, and Sbirrazzouli (2009), glass transition temperatures reported for isolated lignin in the literature cover a wide temperature range, nearly between 80 and 200 °C, mainly depending on differences in molecular weight, delignification processes and vegetal origin of the product. It has been speculated (Kubo & Kadla, 2005) that these high Tg temperatures are mostly related to the low mobility of lignin chains highly constituted by aromatic units with several polar groups, which facilitates the presence of different bond types, hydrogen-bonding contributing significantly to that lignin segments mobility. Second scans showed little variations in the glass transition processes (Inset: Traces G–L_{1000} and G–L_{2400}, dash lines), practically restricted to a down shifting of around 2–3 °C in corresponding Tg data. No remnant melting processes were detected.

When crystallization was carried out at 0 °C, film melting temperatures were recorded at 52.8 ± 0.3 °C for G–L_{1000} and 51.0 ± 1.2 °C for G–L_{2400}, which were not significantly different, nor to the G alone film. Corresponding melting enthalpies were not significantly different, 4.78 ± 0.25 for G–L_{1000} and 5.14 ± 0.25 J/g_{dm} for G–L_{2400}, although somewhat smaller (no significant) than those at subzero temperature conditioning, and significantly lower than in the G alone film. The presence of lignin in the films seems to restrict G from melting in a similar way irrespective of the lignin type in the blend. This suggested that G apparently underwent a strong intermolecular interaction of similar kind and extent with both types of lignin L_{1000} and L_{2400}, everything in main agreement with above FTIR results. Second scans (not shown) yielded flat DSC traces indicating that G underwent complete melting in the previous runs. On the other hand, it is interesting to point out that the G–L films so conditioned (even more at room temperature) are well above their corresponding glass transition temperatures, i.e., they are located at the rubbery state domain of the phase diagram. This could mean that the above commented plasticization role of current lignin types may be not prevalent any more, and water may likely play instead a relevant contribution to the system plasticization and,

consequently to the physical properties of these composite films. Thus, as assessed by water activity and FTIR results, main differences in mechanical properties and WVP between G–L_{1000} and G–L_{2400} films could be largely attributed to a different film hydration level (G–L_{1000} < G–L_{2400}, with subsequently paralleled plasticizing effects in opposition to those previously discussed at the glassy state) rather than to a distinct effect of each lignin on protein conformation, especially concerning triple helical structure. Comparison of current results was not possible due to the absence of precedent literature data on G–L systems.

3.6. Microstructure

The freeze-fractured transversal section microstructure of gelatin and gelatin–lignin films is shown in Fig. 5. The addition of lignin provoked a strong disruption of the smooth and homogeneous structure of the parent gelatin film, inducing a partial laminar-like appearance with decreased density, which tended to be more abrupt in the G–L_{1000} film. This special feature did not apply to the whole section, but only to the side which has been subjected to the water evaporation during drying process. Despite the compatibility of lignin and gelatin denoted by the homogeneous appearance of the corresponding film forming solutions, the bimodal microstructure in the composite films would indicate some phase segregation of the two components, in accordance to the above mentioned gelatin structural changes induced by lignin. Certain degree of immiscibility between hydrophobic groups of lignin and hydrophilic groups in SPI has been shown to prevent interaction among SPI molecules causing microphase separation in the corresponding blends (Huang et al., 2003).

3.7. Antioxidant properties

The water soluble fraction of gelatin and gelatin–lignin films was studied for its antioxidant capacity. Table 4 shows the radical scavenging capacity (ABTS assay) and the ferric ion reducing power (FRAP assay) of gelatin and gelatin–lignin films, which had been solubilized in water for 16 h. The presence of lignin increased significantly ($p < 0.05$) the antioxidant properties of the composite films, especially in the case of the G–L_{1000} film, despite its decreased water solubility (Table 1). The gelatin–lignin films registered ABTS and FRAP values comparable to that achieved in fish gelatin films enriched with an antioxidant borage extract (Gómez-Estaca, Giménez et al., 2009). This property could be useful for preservation of certain types of food in which oxidation process may represent a limiting factor determining its self-life. To this regard, a similar fish gelatin–lignin (G–L_{1000}) film was reported to reduce the levels of protein carbonyl groups formed in Atlantic salmon muscle immediately after high-pressure processing, and prevented lipid oxidation from taking place at advanced stages of chilled storage (Ojagh et al., 2011).

In the present study, composite gelatin films with lignin L_{1000} were found to present considerably higher radical scavenging and Fe reducing capacities than those with lignin L_{2400}. Moreover, the mechanical and water resistance of gelatin–lignin blend films were also higher with L_{1000} than with L_{2400}. For this reason L_{1000} was subjected to a study of functional properties, concerning antioxidant, antimicrobial and cytotoxic properties, in order to determine its potential in food packaging applications.

3.8. Functional properties of lignin

The noticeable antioxidant capacity of gelatin–lignin films is strongly related to the high antioxidant properties of lignin. The DPPH radical scavenging capacity of L_{1000} and Trolox, expressed as

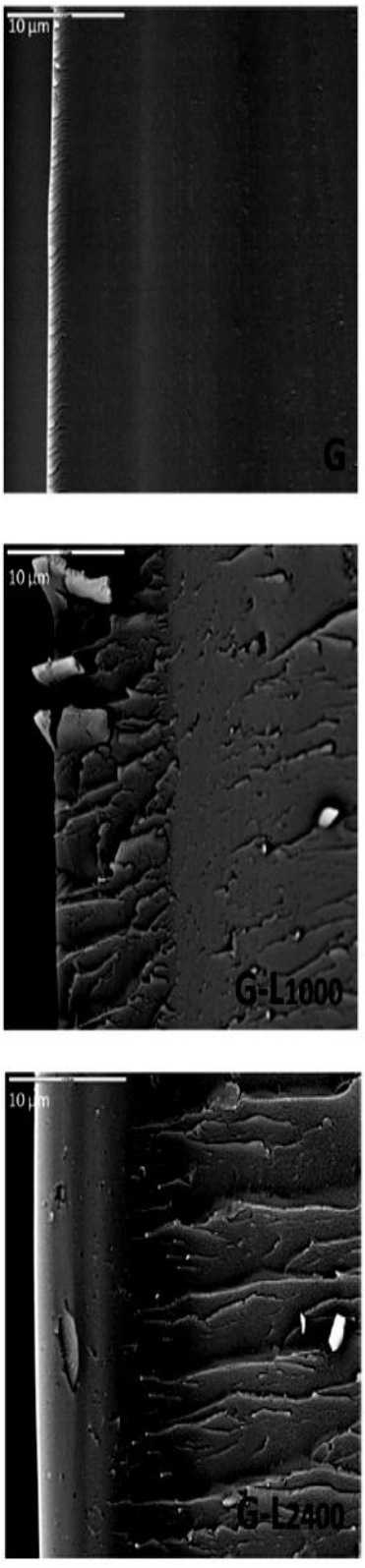

Fig. 5. Scanning electron microscopy at low temperature (Cryo-scanning) of films based on mixtures of fish gelatin (G) and lignin (L_{1000} and L_{2400}) at the ratio 85% gelatin: 15% lignin (w/w). Single gelatin film, G; compound films with lignin, G–L_{1000} and G–L_{2400}.

Table 4
Radical scavenging and Fe ion reducing capacities of films based on mixtures of fish gelatin (G) and lignin (L_{1000} and L_{2400}) at the ratio 85% gelatin: 15% lignin (w/w).

Gelatin–lignin	ABTS (mg vit C/g film)	FRAP (μmol Fe^{2+}/g film)
G	6.54 ± 0.13^a	8.60 ± 0.60^a
G–L_{1000}	27.82 ± 2.19^b	258.97 ± 8.83^b
G–L_{2400}	15.31 ± 0.88^c	180.20 ± 5.71^c

Different letters a, b, c in the same column indicate significant differences ($p < 0.05$) among samples.

Table 5
Radical scavenging efficacy and cytotoxic effect of L_{1000}.

	DPPH IC_{50} (μg/ml)	Cytotoxicity IC_{50} (μg/ml)
L_{1000}	36.5 ± 5.5^b	631 ± 92
Trolox	15.7 ± 0.2^a	

Different letters a, b in the same column indicate significant differences ($p < 0.05$) among samples.

IC_{50} values, are shown in Table 5. When compared with Trolox, L_{1000} showed significantly lower antioxidant activity, with IC_{50} values approximately 2-fold higher than those obtained for Trolox. The radical scavenging activity of L_{1000}, however, was about 3-fold higher than the activity of several lignosulphonates reported by Nuñez-Flores et al. (2012).

Given the potential application of lignin as active packaging material in contact with food, it is of great interest to study its possible cytotoxic effects. The cytotoxic effect of L_{1000} on fibroblast 3T3 cells is shown in Table 5. The IC_{50} value obtained for L_{1000} (631 ± 92 μg/ml) reveals that this compound has cytotoxic effects, but only at very high concentrations. In our study, L_{1000} showed IC_{50} values similar to those reported for Curan 100 (Lignotech) or Bagase (Granit) lignins (600–650 μg/ml) on 3T3 cell line after 24 h of exposure (Ugartondo et al., 2008), but lower to those reported for several lignosulphonates (IC_{50} ~1200 μg/ml, Ugartondo et al., 2008; IC_{50} 1200–1700 μg/ml, Nuñez-Flores et al., 2012). When the cytotoxic potential of L_{1000} was related to its antioxidant activity, it could be observed that the effective antioxidant concentration was noticeably smaller than the cytotoxic one (about 17-fold lower), so this compound could be considered antioxidant at non-cytotoxic concentrations.

The lignin L_{1000} showed no antimicrobial activity against the 26 microbial strains studied (data not shown). The pH of lignin (in our assay lignin was tested at neutral and basic pH) could be partly responsible for this lack of activity, although the antimicrobial effectiveness of polyphenolic compounds from other sources (green tea) was similar in a pH range of 4.0–7.0 (von Staszewski, Pilosof, & Jagus, 2011). Some authors suggested that the inhibition of microbial growth by phenolic acids, among which includes the p-coumaric, increased with decreasing pH (Wen, Delaquis, Stanich, & Toivonen, 2003). In our work no relationship was found between antioxidant and antimicrobial properties of lignin, in contrast to the work of Dizhbite et al. (2004), who assumed the connection between the antibacterial effect and the scavenging activity of the kraft lignin soluble fraction.

4. Conclusions

Lignin represents an important waste material which could be successfully employed as an active food packaging agent for providing fish gelatin films with antioxidant capacity at non-cytotoxic concentrations. At the macroscopic level, lignin provided adequate miscibility with gelatin, reducing the typical transparent appearance and conferring a dark brownish colour to gelatin films, without losing their visual homogeneity. The structural analyses, however, revealed that lignin prevented the interaction among gelatin molecules producing a certain microphase separation between both components. Although the composite films were considerably more plasticized than the single gelatin films, the water solubility was scarcely affected, largely because the gelatin used (from warm water fish species) produced films with reasonable low solubility.

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5.4 *Artículo 4. Lessening of high-pressure-induced changes in Atlantic salmon muscle by the combined use of a fish gelatin–lignin film*

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Lessening of high-pressure-induced changes in Atlantic salmon muscle by the combined use of a fish gelatin–lignin film

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ABSTRACT

Salmon muscle is considerably affected by cooking with the resulting loss of its appealing red colour. The combined use of high pressure with fish gelatin–lignin film is proposed as an alternative to the more aggressive thermal processing procedures, with the aim of improving the appearance and overall quality of salmon fillets in ready-to-eat or semi-prepared dishes. The effects of high pressure processing (300 MPa, 10 min, 5 °C or 40 °C) and conventional heating (90 °C, 10 min) were evaluated in terms of colour changes, protein denaturation, as well as protein and lipid oxidation, by comparison with raw muscle. The stability of the processed products was assessed by monitoring changes in microbial growth and total volatile basic nitrogen and thiobarbituric acid reactive substances during 23 days of chilled storage. Fourier transform infrared spectroscopy (FTIR), apparent viscosity and dynamic oscillatory studies revealed notable differences in the overall degree and nature of protein aggregation between high pressure and heating treatments, especially when performed at 5 °C instead of 40 °C. SDS–PAGE of the protein fraction solubilised in 0.8 M NaCl showed MHC and α-actinin to be the main myofibrillar proteins denatured by high pressure processing at 40 °C, while actin was more denatured when pressurised at 5 °C. The film attenuated colour changes associated with high pressure treatment, especially at 5 °C, where redness was more preserved without jeopardising the appearance of a ready-to-eat product. High pressure processing at 5 °C in combination with gelatin–lignin film was found to improve protein quality of salmon fillets. The film reduced the levels of carbonyl groups formed immediately after processing, and prevented lipid oxidation from taking place at advanced stages of chilled storage. However, the effect on microbial growth was negligible, since total counts were similar for muscle with or without the film.

1. Introduction

High hydrostatic pressure has been widely applied as a minimal processing technique to prolong the shelf-life of additive-free foods, with its major advantage being the conservation of micronutrients such as amino acids and vitamins, as well as flavour compounds (Knorr, 2000, chap. 2). Although it is claimed that high pressure processing has less impact on colour compared to thermal processing treatments (Ohshima, Ushio, & Koizumi, 1993), it is well recognised that pressurising above 200 MPa leads to changes in colour and texture, giving fish muscle a cooked-like appearance (Montero & Gómez-Guillén, 2005). With this idea in mind, high pressure processing could be considered as an alternative to conventional heating treatments for the preparation of ready-to-eat or semi-prepared dishes, with a minimum loss of micronutrients and flavour compounds.

High pressure induces dramatic changes in the colour of Atlantic salmon muscle as this fish species is probably one of the most sensitive in this respect (Amanatidou et al., 2000; Yagiz et al., 2009). Besides haem proteins, carotenoids (mainly astaxanthin and canthaxanthin) are mainly responsible for the typical red colour of Atlantic salmon muscle (Andersen, Bertelsen, Christophersen, Ohlen, & Skibsted, 1990). In particular, the reduction in redness in pressurised smoked salmon has recently been linked with astaxanthin oxidative degradation, coupled with the formation of deoxymyoglobin or deoxy-haemoglobin (Tintchev et al., 2009). Though early studies reported that carotenoids were bound to actomyosin in salmon muscle (Henmi, Hata, & Hata, 1989), α-actinin was later identified as the target myofibrillar protein for binding with astaxanthin (Matthews, Ross, Lall, & Gill, 2006). Thus, the degree of protein denaturation in salmon muscle is relevant, not only affecting textural properties, but also influencing the strong colour changes. High pressure has been shown to modify the palatability and functional properties of meat and fish products by inducing denaturation and muscle protein gelation (more specifically myosin and actin) (Cheftel & Culioli 1997). Yamamoto, Hayashi and Yasui

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(1993) reported that pressure-induced aggregation was similar to that induced by thermal treatment at the level of myosin head interaction. However, the nature of such interactions differs somewhat, since pressure induces the formation of heat labile hydrogen-bonded structures while the contribution of disulphide bonds and hydrophobic interactions plays a key role in the stabilization of heat-induced structures (Angsupanich, Edde, & Ledward, 1999). Fourier transform infrared spectroscopy (FTIR) provides information about the chemical composition and conformational structure of food components, and in particular, it is a useful tool for monitoring changes in secondary protein structures (Jackson & Mantsch, 1995; Willard, Merritt, Dean, & Settle, 1981). Recently, FTIR has been successfully applied in the study of both salted and salted smoked salmon muscle denaturation (Böcker, Kohler, Aursand, & Ofstad, 2008; Carton, Bocker, Ofstad, Sørheim, & Kohler, 2009), however, to our knowledge, the effect of high pressure treatment on fish muscle proteins has not yet been studied by FTIR.

Haem proteins, which are key catalysts of lipid oxidation in fish muscle (Hultin, 1994), can also be denatured by either heat or high pressure treatment, and become pro-oxidative. The oxidation occurs mainly by the release of the haem pigment from the haem-proteins, since free haem pigment is known to be a very potent pro-oxidant (Everse & Hsia, 1997). Although it is generally assumed that high pressure may induce some lipid oxidation, this effect would largely depend on factors such as pressure level, fish species and subsequent storage. In salmon muscle, the presence of asthaxantin, which has been described as a potent antioxidant (Shimidzu, Goto, & Miki, 1996) may provide considerable protection from lipid oxidation. Yagiz et al. (2009) reported that a high pressure treatment of 300 MPa and ~25 °C average temperature reduced the oxidation susceptibility of Atlantic salmon muscle, probably as a result of pressure-induced changes in muscle structure, making astaxanthin more available to protect lipids from oxidation. However, when pressurised at 300 MPa and ~7 °C, a significant increase in TBARs was found for salmon carpaccio (Gómez-Estaca, López-Caballero, Gómez-Guillén, López de Lacey, & Montero, 2009). Thus, the temperature at which high pressure processing is carried out may also be a key factor affecting the oxidative stability of pressurised products.

The use of edible films helps to maintain product quality, enhance sensory properties, improve product safety, and increase the shelf life of various ready-to-eat food products (Beverlya, Janes, Prinyawiwatkula, & No, 2008). These films act as oxygen and water barriers thereby slowing oxidation reactions and retaining moisture, thus enhancing quality and extending storage life (Gennadios, Hanna, & Kurth, 1997). To control lipid oxidation in foodstuffs, there is a growing interest in the formulation of edible and/or biodegradable active films, which are based on the inclusion of an antioxidant or antimicrobial compound in the polymeric matrix. Although there is increasing literature available regarding the development of active biodegradable films (Guilbert, Gontard, & Gorris, 1996; Gómez-Guillén et al., 2009; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004), limited work has been done in relation to their application (in the form of films or coatings) to extend the shelf-life of fish products (Jeon, Kamil, & Shahidi, 2002; López-Caballero, Gómez-Guillén, Pérez-Mateos, & Montero, 2005; Ojagh, Rezaei, Razavi, & Hosseini, 2010; Ouattara, Sabato, & Lacroix, 2001; Sathivel, Liu, Huang, & Prinyawiwatkul, 2007). The application of active plant extract-gelatin composite edible films in combination with high pressure processing in cold-smoked sardine has already been shown to be a very promising technology for fish preservation (Gómez-Estaca, Montero, Giménez, & Gómez-Guillén, 2007). However, more work is necessary in this field to assess the composition and suitability of films, and thereby ensure their resistance to high pressure processing while at the same time taking into account the wet nature of the fish surface.

Lignin is found in plant cell walls in association with cellulose and hemi-cellulose, and is an abundant waste product in the pulp and paper industry. In the present study, and based on preliminary work carried out in our laboratory, a mixture of gelatin and lignin was used to prepare the protective film, which was found to be flexible, transparent, with a relatively strong brownish-yellow colouration and having reasonably high water resistance (unpublished data). Lignin and derivatives have the right chemistry to be used in making coatings and composites given their small particle size, hydrophobicity and their ability to form stable mixtures (Park, Doherty, & Halley, 2008). Lignin has already been successfully used in the preparation of gelatin–lignin films with high swelling capacity (Vengal & Srikumar, 2005). Moreover, lignins have been shown to have efficient antibacterial and antioxidative properties (Ugartondo, Mitjans, & Vinardell, 2009).

The objective of the present work was to evaluate the effects on Atlantic salmon fillets of two high pressure treatments at different temperatures (300 MPa at 5 °C and 40 °C), in combination with gelatin–lignin biodegradable film, compared with conventional heating treatment in an oven at 90 °C. The degree of muscle alteration as a result of the different processing treatments was assessed by comparing the extent of colour changes, protein denaturation, as well as protein and lipid oxidation with raw muscle, The stability of the processed products was evaluated by monitoring changes in microbial growth and total volatile basic nitrogen and thiobarbituric acid reactive substances during 23 days of chilled storage.

2. Materials and methods

2.1. Preparation of film

The gelatin–lignin film forming solution (FFS) was prepared by dissolving a commercial warm-water fish gelatin (Rousselot S.A.S., Puteaux, France) in distilled water (3.4% w/v) at 40 °C, with sorbitol (15 g/100 g gelatin) and glycerol (15 g/100 g gelatin) as plasticizers. The commercial lignin powder (Protobind 1000, Granit R&D SA, Lausanne, Switzerland) was added to a final concentration of 0.6% w/v in the FFS. This concentration was selected according to previous experiments. The mixture was stirred at 40 °C for 15 min and was alkalinized to ~pH 11 to obtain a good blend with total solubility. The films were made by casting an amount of 40 ml over a plate of 12 × 12 cm^2 and drying at 45 °C in a forced-air oven for 15 h to yield a uniform thickness of 100 μm. Prior to covering the fish, films were conditioned over a saturated solution of KBr in desiccators for 5 d. The film retained its structural integrity when applied on the surface of the fillet and was not altered by the high pressure treatment, allowing to be easily separated from the muscle for further analyses.

2.2. Fish sample preparation and High Pressure Processing (HPP)

Salmon fillets were acquired at a local market. The fillets were cut into 85–100 g portions (~5–6 cm thickness) and divided randomly to prepare six lots: (i) untreated raw muscle, R; (ii) cooked muscle, C; (iii) muscle pressurised at 5 °C, HP5; (iv) muscle covered with a gelatin–lignin film and subsequently pressurised at 5 °C, HPF5; (v) muscle pressurised at 40 °C, HP40 and (vi) muscle covered with a gelatin–lignin film and subsequently pressurised at 40 °C, HPF40. In the covered samples, each portion was covered individually with the gelatin–lignin film.

For HPP, all lots were vacuum-packaged into flexible plastic bags by using a Multivac packaging machine (A-NG, 85021, GS, Germany). Vacuum packed samples were treated in an pilot high-pressure unit (ACB 665, GEC Alsthom, Nantes, France), the temperature of the immersion medium (distilled water) being reg-

ulated by a thermocouple connected to a programmed temperature control equipment (model IA/2230AC, INMASA, Barcelona, Spain). HPP was carried out for 10 min at 300 MPa and at two temperature levels (5 °C and 40 °C). Pressure was increased by 2.5 MPa/s and after high pressure treatment was completed, the time for pressure dropping back to atmospheric was around 3 s.

The C lot was prepared by cooking the vacuum packed muscle portions at 90 °C for 10 min in a Rational oven (mod. Combi-Master CM6, Suffolk, UK). All lots were stabilized at −80 °C for further analysis, except for microbiological, TVBN (total volatile basic nitrogen) and TBARs (thiobarbituric reactive substances) analyses, where a study of 23 d of chilled storage at 7 °C was performed.

2.3. Colour measurements

The colour parameters lightness (L^*), redness (a^*), and yellowness (b^*) were measured using a Konica Minolta CM-3500d colorimeter (Osaka, Japan). Measurements were taken at a number of locations in different muscle portions and each point is the mean of at least 10 measurements.

2.4. Protein solubility

One gram of muscle (without connective tissue) was homogenised with 40 ml of 0.8 M NaCl in an Omni-Mixer homogenizer model 17106 (Omni International, Waterbury, CT) for 1 min at setting 6. The resulting homogenates were stirred at ~4 °C for 30 min and then centrifuged for 30 min at 4000g in a Multifuge 3 L-R Heraeus (Hanau, Germany). The protein concentration in the supernatant was determined by using the BCA kit (Meridian RD., Rockford, IL, 61101 USA). Soluble protein was expressed as the percent protein solubilised with respect to total muscle protein, which was determined according to AOAC (Association of Official Analytical Chemists) (1984).

2.5. Apparent viscosity

Apparent viscosity of muscle was determined according to Borderías, Jiménez-Colmenero, & Tejada (1985). Previously thawed samples (50 g) were homogenised with 150 mL of 5% NaCl (w/v) in 0.2 M sodium phosphate buffer (pH 7.0). Apparent viscosity of the resulting homogenate was measured by taking 3 readings at 1-min intervals at a speed of 12 rpm using a Brookfield-LV viscometer (Brookfield Engineering NABS, Stoughton, MA, USA). Alternatively, the homogenate was filtered through gauze and centrifuged for 10 min at 345g in a Multifuge 3 L-R Heraeus (Hanau, Germany). All the procedures were carried out at ~4 °C. The values obtained were expressed in centipoises (cP), and the results presented are averages of at least 3 replicates.

2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Salt-soluble protein was analysed by SDS–PAGE in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA) at 20 mA/gel, using 12% polyacrylamide gels. Samples were treated according to Hames (1985) (2.5% SDS, 5% mercaptoethanol, 10 mM Tris–HCl, 1 mM EDTA and 0.002% bromophenol blue) and then heated for 5 min in a boiling water bath. The protein concentration was adjusted to 1 mg/ml, except in the cooked sample (due to lower protein concentration in the supernatant), and the loading volume was 20 μl in all lanes, except for cooked sample, where 30 μl were loaded. Protein bands were stained with Coomassie brilliant blue R-250. As reference for molecular weights, two standards were used: SDS–PAGE Standard High Range (Cat. Num. 161-0309) (std 1) and SDS–PAGE Standard Low Range (Cat. Num. 161-0305) (std 2), (Bio-Rad, CA, USA).

2.7. FTIR-Attenuated Total Reflectance (ATR) spectroscopy

Infrared spectra between 4000 and 650 cm^{-1} were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc, Waltham, MA, USA) equipped with an ATR prism crystal accessory. For each spectrum 32 scans of interferograms were averaged and the spectral resolution was 4 cm^{-1}. Background was subtracted using the Spectrum software version 6.3.2 (Perkin–Elmer Inc.). Measurements were performed at room temperature using extremely thin layers of semi-thawed muscle, which were placed on the surface of the ATR crystal, and pressed with a flat-tip plunger until spectra with suitable and stable peaks were obtained. All experiments were performed at least in duplicate.

2.8. Dynamic oscillatory studies

Salmon muscle batches of 100 g were homogenised with 2% NaCl in a domestic Braun Minipimer homogenizer at ~4 °C for 3 min. Dynamic viscoelastic studies were performed on a Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4°, gap = 0.15 mm). A dynamic frequency sweep was done at 10 °C by applying oscillation amplitude within the linear region ($\gamma = 0.005$) over the frequency range 0.1–10 Hz. The Elastic modulus (G'; Pa), Viscous modulus (G''; Pa) and Complex modulus (G^*, Pa) were plotted as a function of frequency. The dynamic temperature sweep was done by heating from 10 °C to 90 °C at a scan rate of 1 °C/min, frequency 0.1 Hz, and target strain $\gamma = 0.005$. G' (Pa), G'' (Pa) and phase angle (δ; °) were plotted as a function of temperature. Results were averages of at least two determinations.

2.9. Protein carbonyl groups

Protein carbonyls were assayed as hydrazone derivatives by reacting proteins with 2,4-dinitrophenylhydrazine(DNPH) (Sigma Chemical Co., St. Louis, MO, USA) as described by Levine et al. (1990). Results of carbonyl groups content were expressed in nm/mg protein, and were the average values of at least three determinations.

2.10. Microbiological assays

The microbiological analyses were as follows: a total amount of 10 g of muscle, from at least 3 different packages, was collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 ml of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min in a Stomacher blender (model Colworth 400, Seward, London, UK), appropriate dilutions were prepared for the following microorganism determinations: (i) total bacterial counts (TBC) on spread plates of Iron Agar (Scharlab, Barcelona, Spain), 1% NaCl incubated at 15 °C for 3 days; (ii) H_2S-producers organisms, as black colonies, on pour plates of Iron Agar incubated at 15 °C for 3 days; (iii) luminescent bacteria on spread plates of Iron Agar 1% NaCl incubated at 15 °C for 5 d; (iv) total aerobic mesophiles on pour plates of Plate Count Agar, PCA (Oxoid) incubated at 30 °C for 72 h; (v) Pseudomonas on spread plates of Pseudomonas Agar Base (Oxoid) with added CFC (Cetrimide, Fucidine, Cephalosporine) supplement for Pseudomonas spp. (Oxoid) incubated at 25 °C for 48 h; (vi) Enterobacteriaceae on double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h [after first adding 5 ml of Tryptone Soy Agar (Merck, Darmstadt, Germany) and incubating at room temperature for 1 h]; (vii) lactic acid bacteria on double-layered plates of MRS Agar (Oxoid) incubated at 30 °C for 72 h. All microbiological counts

are expressed as the log of the colony-forming units per gram (log cfu/g) of sample. All analyses were performed in triplicate.

2.11. Total volatile basic nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) determinations were carried out in triplicate over the storage period using the method of Antonacopoulos & Vyncke (1989). Analyses were performed at least in triplicate, and results were expressed as mg TVB-N/100 g muscle.

2.12. Thiobarbituric acid reactive substances (TBARS)

Samples were analysed by the method of Vyncke (1970). A standard curve was prepared using 1,1,3,3-tetraethoxypropane. Analyses were performed at least in triplicate, and results were expressed as μmol malondialdehyde (MDA) per kg of muscle.

2.13. pH

Approximately 5–10 g of muscle were homogenised with distilled water (1:2, w/v). After 5 min at ambient temperature, pH was determined with a pHm93 pH-meter and a combined pH electrode (Radiometer, Copenhagen, Denmark). The experiments were repeated at least in triplicate.

2.14. Statistical analysis

Statistical tests were performed using the SPSS® computer programme (SPSS Statistical Software v.18, Inc., Chicago, IL.) One-way analysis of variance was carried out. The difference of means between pairs was resolved by means of confidence intervals using a Tukey-*b* test at a level of significance of $p \leqslant 0.05$.

3. Results and discussion

3.1. Colour analysis

Colour measurement results expressed in terms of L^* (lightness), a^* (redness) and b^* (yellowness) parameters of both raw salmon muscle and differently processed muscles are shown in Table 1. As expected, both pressurised and cooked samples showed significant ($p \leqslant 0.05$) increases in L^* values and decreases in a^* values compared with the raw muscle, as well as presenting a cooked-like appearance in all cases. The lightness of pressurised muscle was not affected by the pressurisation temperature, which was, significantly lower ($p \leqslant 0.05$) than in the cooked muscle. When covered with film a noticeable decrease in lightness was observed, which was more pronounced when high pressure treatment was carried out at 40 °C. High pressure at both temperatures induced a smaller decrease in redness than by cooking. Significant differences in a^* values ($p \leqslant 0.05$) were found as a function of the pressurising temperature and film covering. When high pressure was applied at 5 °C, the presence of the film led to higher redness whereas the opposite was observed at 40 °C. All processed samples exhibited significant increases ($p \leqslant 0.05$) in b^* values compared to the raw muscle, which also contributed to their cooked-like appearance. In this case, pressurising temperatures did not produce any differences, however, yellowness increased slightly in muscle covered with film.

Consistent with our results, thermally treated salmon muscle had previously been reported as presenting considerably higher lightness and lower redness when compared with muscle pressurised at 300 MPa (Yagiz et al., 2009). On the other hand, increased L^* values and decreased a^* values as a result of applying high hydrostatic pressure on fish muscle is well documented (Chevalier, Le Bail, & Ghoul, 2001; Matser, Stegeman, Kals, & Bartels, 2000), and more specifically so in the case of salmon muscle (Amanatidou et al., 2000; Yagiz et al., 2009). Covering samples with film significantly affected L^* and a^* values showing that the film had a certain preservative effect on the colour of the pressurised muscle. However, the slightly higher b^* values for the covered samples could be the result of yellow being transferred from the film.

Table 1
Colour parameters (L^*, a^*, b^*) of muscle subjected to different processing treatments.

Treatment	L^*	a^*	b^*
R	41.55 ± 0.12^a	16.82 ± 0.03^a	9.29 ± 0.04^a
C	74.71 ± 0.58^b	9.50 ± 0.35^b	14.91 ± 0.45^b
HP5	65.64 ± 0.44^c	11.04 ± 0.02^c	12.20 ± 0.49^c
HPF5	60.70 ± 0.86^d	13.71 ± 0.62^d	17.65 ± 0.76^d
HP40	66.39 ± 0.25^c	12.12 ± 0.73^e	12.35 ± 0.42^c
HPF40	56.31 ± 0.31^e	10.44 ± 0.32^{bc}	14.45 ± 0.07^b

R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.
Different letters (a,b,c,...) in the same column indicate significant differences ($p \leqslant 0.05$).

3.2. Protein solubility

The percentage of salt-soluble protein in raw salmon muscle was high (73.75%) as would be expected for fresh fish (Table 2). However, all processed samples showed a pronounced reduction in salt-soluble protein compared with the raw muscle, especially the cooked muscle which had values of around 7%. High pressure treatment at both 5 and 40 °C produced ~40% decrease in salt-soluble protein, with no significant variations ($p > 0.05$) as a result of pressure temperature or film covering. Changes in muscle protein solubility are a measure of protein denaturation, as solubility is decreased due to the formation of insoluble protein aggregates that can no longer be extracted. Thus, the observed lower protein solubility suggested substantial denaturation of proteins, induced by either high pressure processing or cooking. Our results are in agreement with Carlez, Borderías, Dumay and Cheftel (1995) who found, by measuring the total enthalpy of denaturation, that high pressure processing of bream surimi (300 MPa, 5 °C, 15 min) caused much less protein denaturation (23%) than thermal treatment at 90 °C for 30 min (67%).

3.3. Apparent viscosity

Measurement of the apparent viscosity of a fish muscle homogenate has been proposed as an indicator of protein quality, and has been reported to be more sensitive than protein solubility (Barroso, Careche, & Borderias, 1998). Apparent viscosity values of muscle homogenates, with and without a previous filtration/centrifugation step, are presented in Table 2, and were obtained from both raw muscle and differently processed muscles. As expected, all processed samples showed a dramatic reduction in viscosity compared to the raw fish, and this can be attributed to extensive protein aggregation. No significant differences ($p > 0.05$) were observed between cooked muscle and muscle pressurised at 5 °C, with or without a film covering. However, when high pressure treatment was carried out at 40 °C, a significant ($p \leqslant 0.05$) decrease in viscosity was observed, revealing additional heat-induced protein denaturation. By comparing the viscosity values registered with and without filtration, no differences were found in the cooked muscle. However, upon filtration, the muscles pressurised at 5 °C and 40 °C exhibited a ~55% and ~23% decrease respectively in viscosity. The results for apparent viscosity were not related

Table 2
Protein functionality parameters of muscle subjected to different processing treatments.

Treatment	Protein solubility (%)	Viscosity (cp) with filtering	Viscosity (cp) without filtering	Carbonyl group (nm/mg protein)
R	73.54 ± 2.00^{a}	11140 ± 135.27*	11140 ± 135.27*	0.087 ± 0.009^{a}
C	6.73 ± 0.73^{b}	47.92 ± 1.07^{a}	50 ± 2.46^{a}	0.654 ± 0.044^{bc}
HP5	30.29 ± 0.94^{c}	27.84 ± 3.20^{b}	48.61 ± 2.50^{a}	0.567 ± 0.026^{bd}
HPF5	31.36 ± 0.74^{c}	25.92 ± 1.90^{b}	47.5 ± 3.27^{a}	0.490 ± 0.037^{d}
HP40	30.20 ± 0.90^{c}	5.83 ± 0.83^{c}	26.66 ± 2.84^{b}	0.728 ± 0.061^{c}
HPF40	31.14 ± 0.84^{c}	6.16 ± 1.36^{c}	26.12 ± 2.71^{b}	0.610 ± 0.057^{b}

R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.
Different letters (a,b,c, ...) in the same column indicate significant differences ($p \leq 0.05$).
* Value not included in the analysis of variance.

with those for salt-soluble protein, especially in the case of the cooked sample, which registered extremely low protein solubility but viscosity comparable to HP5, and even slightly higher than HP40. Such differences could be due to the distinct nature and size of protein aggregates obtained under cooking and high pressure conditions. The nature of the heat-induced aggregates did not interfere with the filtration process, most likely because they are strong small aggregates. However, pressurising at 5 °C led to large protein aggregates being retained in the gauze, possibly as a result of higher protein unfolding favouring subsequent protein–protein interactions. The viscosity values of the muscle pressurised at 40 °C suggested an intermediate state between high pressure- and heat-denaturation as denoted by the strong aggregation and presence of large aggregates. Covering the muscle with film did not affect apparent viscosity when pressurising at either 5 °C or 40 °C.

3.4. SDS-PAGE

The electrophoretic profiles of the salt-soluble protein fractions extracted from the raw muscle and the muscle processed under different conditions are shown in Fig. 1. In accordance with the pronounced percentage decrease in soluble protein in the cooked muscle, the electrophoretic profile was characterised by the nearly total disappearance of the majority of bands. The band at ~200 kDa, corresponding to the myosin heavy chain (MHC), was hardly visible, whereas the bands at ~105 kDa and ~45 kDa, tentatively assigned to α-actinin and actin respectively, as well as a pool of lower molecular weight proteins, in which troponins and myosin light chains (MLC) are included, disappeared completely, denoting extensive heat-induced protein aggregation of the main myofibrillar proteins. Specifically, α-actinin denaturation could be largely involved in the strong muscle colour changes produced by heating, since it has recently been shown to be the target myofibrillar protein where astaxanthin, which is mainly responsible for the intense red colour of salmon, is bound (Tintchev et al., 2009).

The salt-soluble protein fractions from all pressurised muscles presented a great number of bands, resembling to some extent the profile of the raw muscle, and revealing a considerably lower degree of protein denaturation than that of the cooked muscle. Slight differences could be observed with respect to the pressurisation temperature, but none could be observed as far as the film covering was concerned. The profile corresponding to the muscle pressurised at 40 °C (with and without film) was characterised by the practical disappearance of protein bands in the range between 200 and 63 kDa, including the MHC and to some extent, the α-actinin. This effect was less pronounced when the muscle was pressurised at 5 °C, indicating slightly lower protein aggregation within this molecular weight range. The intensity of the actin band decreased markedly with high pressure treatment, compared to the raw sample, although more markedly when pressure was applied at 5 °C, suggesting a higher degree of actin denaturation when pressure was applied at low temperature. The protein at ~63 kDa, tentatively assigned to tropomyosin, and proteins below 45 kDa, were scarcely affected by high pressure as denoted by the preserved intensity of the corresponding bands, suggesting strong resistance of tropomyosin, troponins, MLC as well as sarcoplasmic proteins to high pressure treatment. The importance of the role of

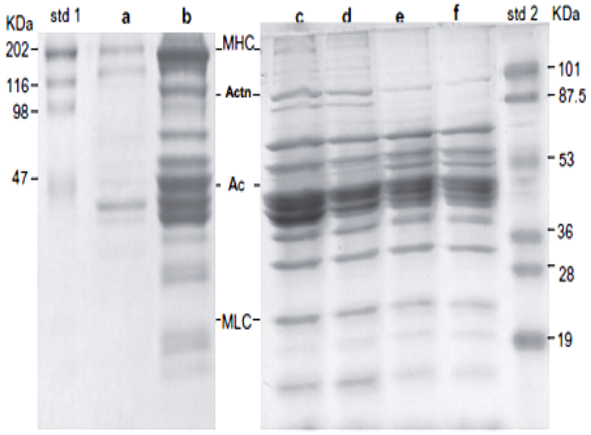


Fig. 1. SDS-PAGE of proteins solubilised in 0.8 M NaCl of raw salmon muscle and muscle subjected to different processing treatments. (a) C, muscle cooked at 90 °C for 10 min; (b) R, raw muscle; (c) HP5, muscle pressurised at 5 °C for 10 min; (d) HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; (e) HP40, muscle pressurised at 40 °C for 10 min; (f) HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min. std 1: standard of high molecular weight; std2: standard of low molecular weight; MHC: myosin heavy chain, Actn: alpha actinin, Ac: actin, MLC: myosin light chain.

MHC and actin polymerisation, as opposed to troponins, tropomyosin and MLC in pressure-induced protein aggregation has been previously reported (Angsupanich et al., 1999; Carlez et al., 1995).

3.5. FTIR spectroscopy

The FTIR spectra between 4000 and 1000 cm^{-1} of raw salmon muscle as well as muscle subjected to the different processing conditions are depicted in Fig. 2. Generally, all spectra exhibited similar features to spectra acquired from raw salmon myofibres as reported by Böcker et al. (2008). The amide A band (~3100–3600 cm^{-1}), attributed fundamentally to OH and NH stretching vibrations, exhibited a broadened absorbance in consonance with the high water content in the muscle. In fact, the greatest contribution of this band in raw muscle, as opposed to processed muscles, could be largely indicative of a higher water-holding capacity.

The amide I band, located in the region between 1600–1700 cm^{-1}, arises predominantly from C=O stretching vibrations, being weakly coupled with in-plane N-H bending and C-N stretching vibrations. Studying changes in the amide I band was put forward as a helpful tool for determining specific secondary structures within proteins (Jackson & Mantsch, 1995). Spectral changes in the amide I region have been associated with myofibrillar protein conformational changes as a result of either heating pork or salting salmon muscle (Bertram, Kohler, Böcker, Ofstad, & Andersen, 2006; Böcker et al., 2008). In the present study, the amide I band showed maximum IR absorbance at 1639 cm^{-1} in raw muscle, whereas it shifted to lower frequency values in the pressurised muscles (~1631 cm^{-1}), and more markedly in the cooked one (1623 cm^{-1}). To enhance the spectral resolution, a second derivative spectrum (Fig. 3) was used to investigate the amide I region (1700–1600 cm^{-1}). The bands located at 1695 cm^{-1}, 1683 cm^{-1}, 1668 cm^{-1}, 1658 cm^{-1}, 1652 cm^{-1}, 1635 cm^{-1}, 1625 cm^{-1}, 1618 cm^{-1} and 1612 cm^{-1} coincided for the most part with those described previously for raw Atlantic salmon muscle (Böcker et al., 2008). According to these authors, the major peak at 1652 cm^{-1} was indicative of the contribution of the α-helical structure to protein conformation. As shown in Fig. 3, this peak was diminished in the cooked sample compared with the raw muscle. In contrast, all pressurised muscles showed increased intensity for this peak, especially when high pressure took place at 5 °C without film covering (HP5). A similar trend was observed regarding IR intensity at 1683 cm^{-1}, although in this case no differences were found among high pressure treatments. The peak at 1683 cm^{-1} was related to changes in intramolecular antiparallel β-sheet structures in salted and smoked salmon muscle (Carton et al., 2009). The increase in α-helix and β-sheet structures in all pressurised samples was attributed to the denaturing effect of high pressure, leading to protein unfolding. Pressure levels above 200 MPa are known to cause protein unfolding and reassociation of subunits from dissociated oligomers, inducing changes in both secondary and tertiary structures (Lullien-Pellerin & Balny, 2002). An appreciable loss of loop structures could also be found in the cooked muscle, as denoted by the diminished intensity at 1658 cm^{-1}. Pressure-induced changes in loop structures were much less evident; nevertheless, these structures tended to decrease slightly in comparison with raw muscle when high pressure was applied at 5 °C, whereas the opposite was found to be the case at 40 °C. On the other hand, the cooked muscle exhibited a considerable increase in peak intensity at both 1695 cm^{-1} and ~1625 cm^{-1}, the latter together with a noticeable shift towards lower frequency values, denoting increased intramolecular aggregation of β-sheet structures. Moreover, the higher intensity at 1618 cm^{-1} in the cooked sample would also indicate β-sheet aggregation at the intermolecular level (Carton et al., 2009). The muscles subjected to high pressure showed both intramolecular and intermolecular aggregation of β-sheet structures, as shown by the increased intensity at 1695 cm^{-1}, 1625 cm^{-1} and 1618 cm^{-1}, when compared with the raw muscle, but less pronounced than in the cooked sample. Differences among all pressurised samples were in general scarce, although HP40 (muscle pressurised at 40 °C without film) showed a slight tendency to exhibit higher intermolecular β-sheet aggregation. The band at 1667 cm^{-1}, which was tentatively assigned to non-hydrogenated C=O groups, reportedly increased in salmon muscle after salting (Carton et al., 2009); however, this band was scarcely affected by either cooking or high pressure treatments.

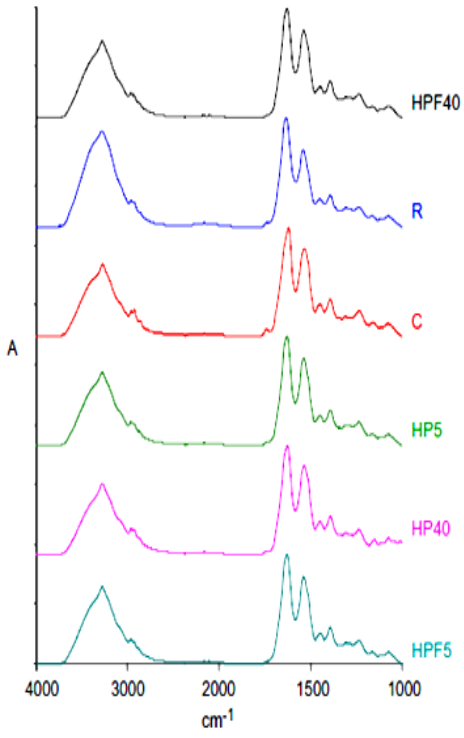


Fig. 2. FTIR spectra of raw salmon muscle and muscle subjected to different processing treatments. R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for

3.6. Dynamic oscillatory study

The degree of protein denaturation and/or aggregation was also assessed by dynamic oscillatory studies. The raw muscle, as well as the muscles which had been processed under different conditions, were subsequently solubilised with NaCl (salted ground muscle) and the viscoelastic properties of the resulting doughs were determined. The extent of protein solubilisation after homogenisation with salt would depend on the degree and type of muscle protein denaturation/aggregation resulting from the processing conditions, and would influence the viscoelastic behaviour of the corresponding dough.

Fig. 4 represents the mechanical spectra, in terms of elastic (G'), viscous (G'') and complex (G^*) moduli as a function of the angular frequency at 10 °C, of the different salted ground muscles, which had previously been subjected to a short setting period of 90 min at the same temperature. In all cases, elasticity prevailed over vis-

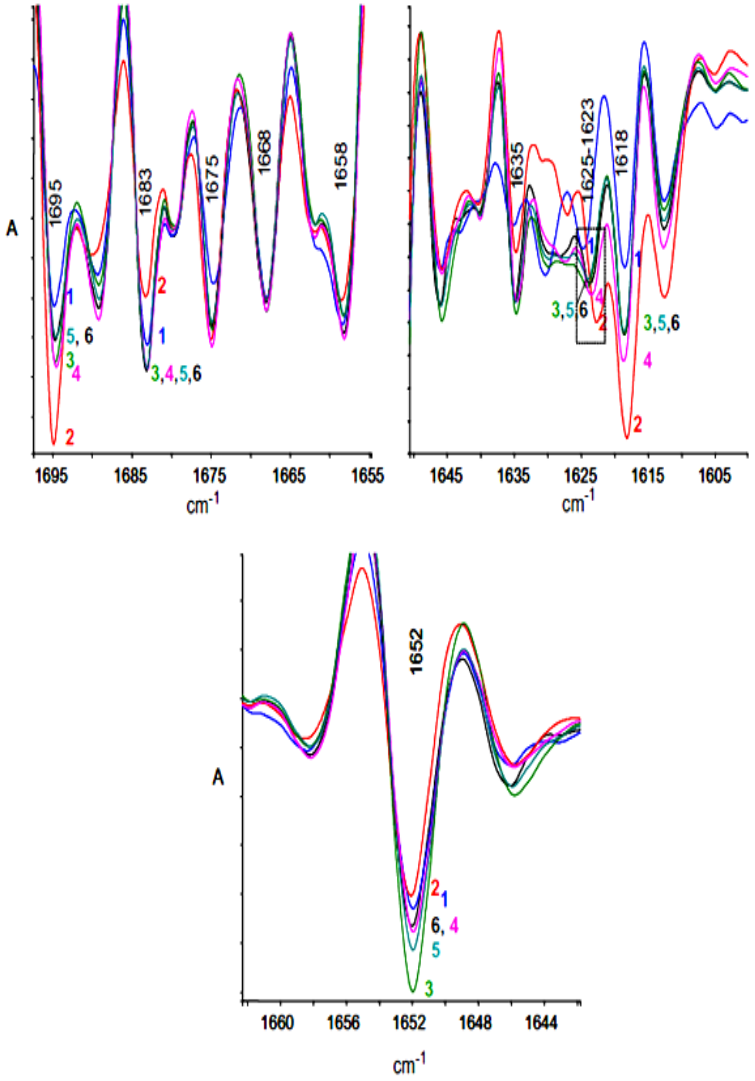


Fig. 3. Second derivative of Amide I band (1700–1600 cm^{-1}) from FTIR spectra of raw salmon muscle and muscle subjected to different processing treatments. (1) R, raw muscle; (2) C, muscle cooked at 90 °C for 10 min; (3) HP5, muscle pressurised at 5 °C for 10 min; (4) HP40, muscle pressurised at 40 °C for 10 min; (5) HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; (6) HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.

resulting doughs could be considered as self-aggregated disordered colloidal networks with the consistency of a viscoelastic gel (Badii & Howell, 2002). The G' values in the samples pressurised at 5 °C were very close to the raw muscle G' value, especially when covered with film (HPF5) (Fig. 4a). The elastic modulus of the salted ground muscle increased noticeably when the samples had been pressurised at 40 °C, increasing more markedly in the samples with film (HPF40), being highest in the cooked sample. A similar trend was found for G^* as well as for G'', the latter being clearly lowest in the raw sample (Fig. 4b and c). This would confirm a higher degree of protein aggregation in the cooked muscle, followed by the muscle which had been pressurised at 40 °C, as compared to the raw muscle and muscle pressurised at 5 °C. Phase angle (δ) values were significantly ($p \leqslant 0.05$) lower in the raw sample (Table 3), indicating better gel consistency, which could be attributed to higher self-aggregation due to a more preserved protein conformation. On the other hand, values of δ were higher ($p \leqslant 0.05$) in salted ground muscle from all pressurised samples, compared with the cooked one, suggesting a more disordered protein network in the former.

The frequency dependence of G', G'' and G^* at 10 °C varied considerably among the different samples. Table 3 shows other viscoelastic parameters, derived from G', G'' and G^* mechanical spectra, which had been calculated after fitting the power law, as previously described (Campo & Tovar, 2008). According to Zhou and Mulvaney (1998) and Campo and Tovar (2008), G'_0 and G''_0 would indicate the resistance of the material to elastic and viscous deformation, respectively, at an angular frequency of 1 rad/s. The difference $G'_0 - G''_0$ and An are magnitudes related with the viscoelastic gel strength, as well as with the strength of rheological unit interaction (Campo & Tovar, 2008; Gabriele, De Cindio, & D'Antona, 2001). Both $G'_0 - G''_0$ and An were highest ($p \leqslant 0.05$) in the cooked sample, followed by the HPF40 sample, while all the other pressurised samples registered values closer to the raw muscle. The higher viscoelastic gel strength in the cooked sample is attributed to the presence of a more aggregated protein resulting from the heat

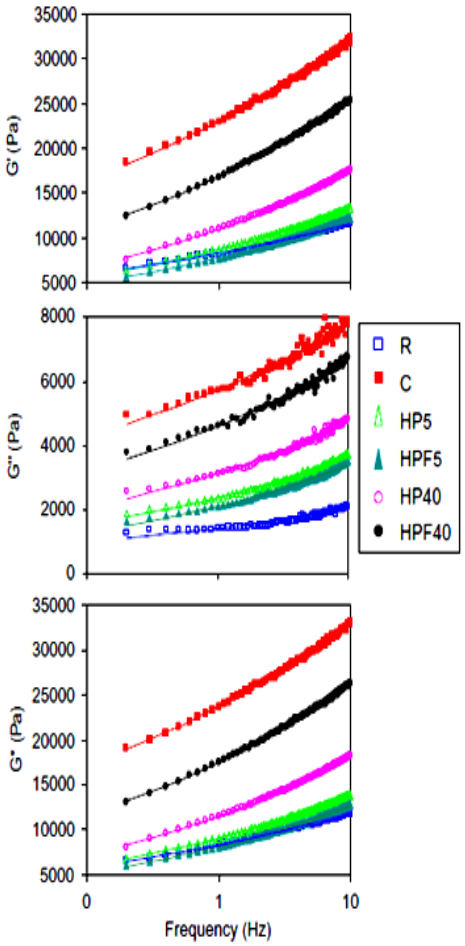


Fig. 4. Elastic modulus (G', Pa), viscous modulus (G'', Pa) and complex modulus (G^*, Pa) as a function of the angular frequency of salt-ground muscles at 10 °C with a prior setting time of 90 min. R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.

treatment, which in turn led to stronger protein interactions (mainly disulphide bonds), which were not destroyed after solubilisation with NaCl. In general, this effect was not observed in pressurised samples, especially if the high pressure treatment was carried out at 5 °C, suggesting the predominance of non-covalent interactions in pressure-induced protein aggregation. In particular, hydrogen bonds have reportedly been largely involved in cod myofibrillar protein denaturation at high pressure levels above 200 MPa, with additional stabilization by disulphide bonds (Angsupanich et al., 1999).

The power law exponent n' is related to structural stability and protein network conformation in the studied samples: the higher the n' values, the higher the instability of the matrix against frequency changes (Campo & Tovar, 2008). As shown in Table 3, n' was significantly higher ($p \leqslant 0.05$) in the pressurised samples when compared with both raw and cooked samples, indicating that the pressurised protein network was more unstable, irrespective of pressurisation temperature. This suggests a considerably lower level of conformational order in the salted ground muscle leading to higher matrix discontinuity as a result of high pressure treatment. This confirmed once again, that the protein interactions in the pressurised muscle were weaker than in the cooked one. Moreover, the structure is considerably more unstable than in the raw muscle, again confirming noticeable pressure-induced changes in protein conformation. Among pressurised samples, no significant differences were found in n' values ($p > 0.05$) due to pressurisation temperature or film covering.

Fig. 5 shows the thermal aggregation ability of the different salted ground muscles in terms of the elasticity modulus (G'), the viscosity modulus (G'') and phase angle (δ) as a function of heating temperature (from 10 °C to 90 °C). As expected, the raw muscle presented the highest thermal aggregation capacity, as deduced from the sharp increase in both G' and G'' values at temperatures from 45 °C upwards. This would indicate extensive protein network stabilization produced by the formation of heat stable bonds. Furthermore, the pronounced phase angle peak values between 30 and 45 °C revealed noticeable myofibrillar protein conformational changes during this temperature interval, which could be attributed to moderate heat-induced protein unfolding as well as to some muscle autolytic activity, which might be more active at these temperatures. Similar results were previously reported for thermal aggregation properties of squid mantle proteins (Gómez-Guillén, Martínez-Alvarez, & Montero, 2003). The viscoelastic behaviour of the salted ground muscle from the cooked sample revealed that no protein unfolding took place at moderate temperatures, which was consistent with most of the protein aggregation having taken place during the previous cooking treatment. Moreover, the possible contribution of muscle autolytic activity might also have been hindered by previous cooking. The sharp increase in both G'' and phase angle at temperatures above 70 °C was attributed mainly to extensive protein dehydration at such high temperatures, revealing the water-holding inefficacy of the protein network. Regarding high pressure treatments, the thermal aggregation capacity was considerably diminished in all pressurised muscles compared to the raw muscle, as revealed by the considerably lower G' and G'' values reached at the end of the heating ramp. Furthermore, conformational protein changes at ~30 °C were only observed when samples had been pressurised at 5 °C, and to a much lesser extent than with the raw muscle. This effect can once again be largely attributed to the protein denaturing effect of high

Table 3
Rheological parameters derived from the frequency sweep test of the different salt-ground muscles at 10 °C with a prior setting time of 90 min.

	$G'_0 \times 10^3$	$G''_0 \times 10^3$	$(G'_0 - G''_0) \times 10^3$	$A_n \times 10^3$	n'	δ (°)
R	6.03 ± 0.05^a	1.04 ± 0.04^a	4.99 ± 0.56^a	6.13 ± 0.51^a	0.132 ± 0.027^a	9.44 ± 0.25^a
C	16.47 ± 0.27^b	4.38 ± 0.00^b	12.09 ± 0.27^b	17.05 ± 0.27^b	0.144 ± 0.005^a	13.75 ± 0.37^b
HP5	5.67 ± 0.14^a	1.58 ± 0.00^c	4.09 ± 0.14^c	5.89 ± 0.13^a	0.185 ± 0.007^b	15.13 ± 0.24^c
HPF5	5.06 ± 0.15^c	1.29 ± 0.03^d	3.77 ± 0.18^c	5.23 ± 0.14^c	0.190 ± 0.005^b	14.93 ± 0.04^c
HP40	6.90 ± 0.25^d	2.07 ± 0.02^e	4.83 ± 0.39^a	7.21 ± 0.35^d	0.202 ± 0.018^b	15.41 ± 0.49^c
HPF40	11.04 ± 0.22^e	3.28 ± 0.03^f	7.72 ± 0.26^d	11.52 ± 0.19^e	0.181 ± 0.009^b	14.91 ± 0.01^c

R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.
Different letters (a,b,c, ...) in the same column indicate significant differences ($p \leqslant 0.05$).

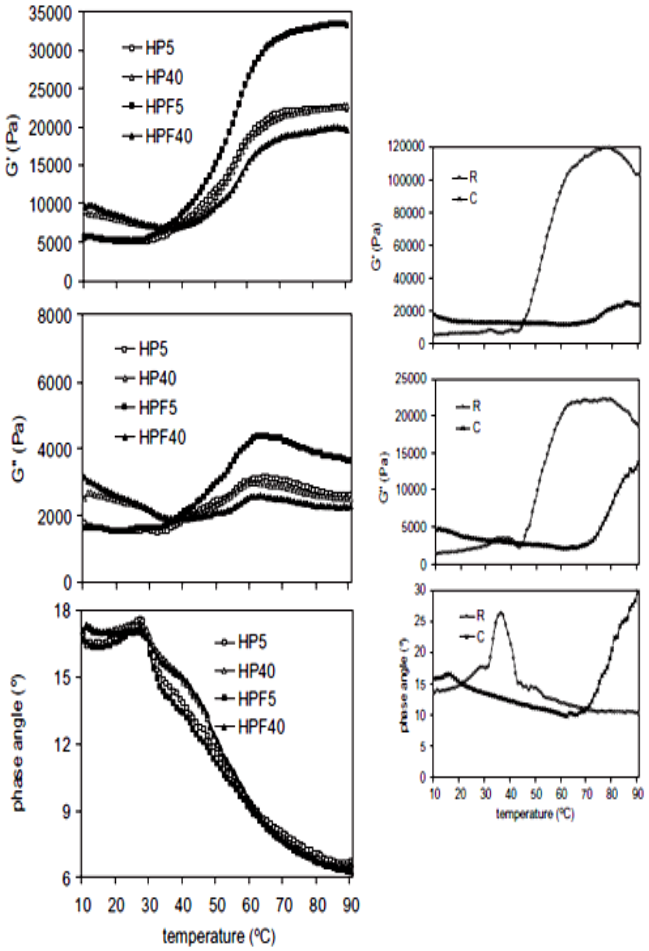


Fig. 5. Elastic modulus (G', Pa), viscous modulus (G'', Pa) and phase angle (°) as a function of temperature of salt-ground muscles from 10 °C to 90 °C. R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.

pressure, being higher at 40 °C than at 5 °C. With regard to the effect of covering muscle with film, the highest G' values were registered by the HPF5 sample. This finding confirmed that film could prevent protein denaturation to some extent when high pressure treatment was applied at 5 °C (not a denaturing temperature); however, this effect was not observed at 40 °C.

3.7. Protein oxidation

Carbonyl group content, as a measure of protein oxidation in the different muscles, is presented in Table 2. In the fresh raw sample, the carbonyl group content was lowest (0.087 nmol/mg protein), but protein oxidation increased significantly ($p \leqslant 0.05$) in all treated samples, reaching maximum values in the muscle pressurised at 40 °C (HP40) and in the cooked muscle (0.728 and 0.654 nmol/mg protein, respectively). The muscle pressurised at 5 °C HP5 showed a carbonyl content significantly lower ($p > 0.05$) than that recorded with the HP40 treatment. In the latter, however, covering the muscle with film led to a significant reduction ($p \leqslant 0.05$) in carbonyl content. The high pressure and cooking treatments used in the current study have been shown to produce significant myofibrillar protein denaturation. Moreover, haem proteins would most likely have been denatured as well at the pressure levels used and become more pro-oxidative. Therefore it is possible that the increase in protein carbonyl groups during pressurisation and cooking could have been due to catalysis by haem proteins, which are known to induce protein oxidation in fish muscle (Eymard, Baron, & Jacobsen, 2009). For film-covered samples, slightly lower carbonyl content was detected compared to non-covered samples, showing that the film offers some protection against protein oxidative changes. This protection was found to be significant ($p \leqslant 0.05$) in the case of HPF40 treatment. Both the antioxidant and oxygen barrier properties of gelatin–lignin composite film may have contributed to the reduction of protein oxidation in the film-covered samples.

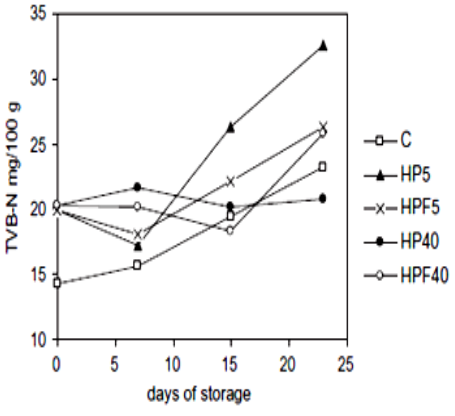
3.8. Lipid oxidation

The TBARS method was used to monitor changes in the level of secondary lipid oxidation products in salmon muscle as a result of the different processing treatments, and during the subsequent 23 d of chilled storage (Table 4). The TBARS value of the raw salmon was 3.22 ± 0.19 μmol MDA/kg muscle, which was in agreement with the low values reported by Andersen et al. (1990) (2.8 μmol MDA/kg muscle) and Yagiz et al. (2009) (3.78 μmol MDA/kg muscle). The cooking treatment induced a pronounced increase in TBARS, whereas the high pressure treatment at 5 °C (with or without film) did not modify significantly the level of lipid

Table 4
TBARS (μmol MDA/ kg muscle) of muscle subjected to different processing treatments and stored at 7 °C for 23 d.

Treatment	Days of chilled storage			
	1	7	15	23
C	$11.20^{a/x}$	$13.91^{a/y}$	$7.06^{a/z}$	$7.07^{a/z}$
HP5	$3.34^{b/x}$	$2.78^{b/x}$	$1.85^{b/x}$	$8.40^{b/y}$
HPF5	$3.32^{b/x}$	$2.14^{b/yz}$	$1.49^{b/y}$	$2.38^{c/z}$
HP40	$1.32^{c/x}$	$2.64^{b/y}$	$1.35^{b/x}$	$4.27^{b/z}$
HPF40	$6.30^{d/x}$	$8.58^{c/y}$	$1.05^{b/z}$	$2.19^{c/z}$

C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.
Different letters (a,b,c,...) in the same column indicate significant differences ($p \leqslant 0.05$) as a function of treatment; different letters (x,y,z,...) in the same row indicate significant ($p \leqslant 0.05$) differences as a function of storage time.



Lots	Days of storage			
	0	7	15	23
C	a/x	a/x	c/y	b/y
HP5	a/x	ab/x	a/xy	a/y
HPF5	a/x	abc/x	b/xy	b/y
HP40	a/x	c/x	bc/x	b/x
HPF40	a/x	bc/x	c/x	b/y

Fig. 6. Total basic volatile nitrogen microbial (mg TBV-N/100 g) in muscle subjected to different processing treatments and stored at 7 °C for 23 d. C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min. Different letters (a,b,c,...) in the same column indicate significant differences ($p \leqslant 0.05$) as a function of treatment; different letters (x,y,z,...) in the same row indicate significant differences ($p \leqslant 0.05$) as a function of storage time.

oxidation or even reduce it slightly when pressurising at 40 °C. Unexpectedly, the gelatin–lignin film increased TBARS levels in HPF40, however, this effect tended to disappear during chilled storage. The level of TBARS remained practically unchanged in HP5 and HP40 samples for the first 15 d of chilled storage, consistent with a presumably high level of carotenoids, which has been said to act as a strong endogenous antioxidant system in salmon muscle (Andersen et al., 1990). In contrast to the present work, Gómez-Estaca et al. (2009) found a significant increase in TBARS after pressurising salmon carpaccio at 300 MPa/7 °C/15 min. The different results could be put down to the sample size (very thin slices *vs* thick fillet portions) as well as to the duration of treatment (15 min *vs* 10 min). TBARS value increased drastically at day 23 of chilled storage in uncovered pressurised muscle, especially in HP5. However this increase was significantly ($p \leqslant 0.05$) minimised by film covering, suggesting a possible inhibitory effect against lipid oxidation of lignin, as reported by Ugartondo et al. (2009).

3.9. pH

The salmon registered a pH of 6.5 on day 0 and then remained practically constant even after cooking, applying the film or high pressure treatment. During storage all the lots evolved in the same way, reaching around 6.6 on day 23.

3.10. Total volatile basic nitrogen

TVB-N in the raw salmon was 19.1 mg TVB-N/100 g muscle, whereas Aubourg et al. (2007) on the other hand found 11.2 mg TVB-N/100 g muscle in Coho salmon on day 0. Some changes were observed after applying the various treatments. Thus, total volatile compounds decreased after cooking while the lots treated with high pressure, in combination or not with the film, barely changed the TVB values (Fig. 6). This fact can be attributed to the release of exudates as a consequence of protein aggregation during the cooking process. Throughout the storage period, TVB production increased in all lots, but the limit of 30 mg TVB-N/100 g of muscle (the established limit for fresh fish) was only exceeded by the HP5 lot ($p \leqslant 0.05$). The lignin film helped to reduce basic compound production in the lot pressurised at 5 °C (Fig. 6). However, this behaviour was not observed in lots pressurised at lower temperatures ($p \leqslant 0.05$).

3.11. Microbiological analysis

The microbial counts are shown in Table 5. In spite of the high microbial load of raw salmon (≈7 cru/g on Iron Agar spread plates), the number of micro-organisms was reduced after high pressure treatment. Thus, the total viable bacteria in Iron Agar was reduced to about 4 or 5 log cru/g when the high pressure treatment was at 40 °C or 5 °C, respectively. In the case of total aerobic mesophiles there was also a reduction in the number of micro-organisms by approximately 3 logarithmic cycles. Pseudomonas spp. and Enterobacteriaceae were below the detection limit after high pressure treatment while lactic acid bacteria registered a lower reduction (1.5 log cycle). This was to be expected if we take into account the higher resistance of gram-positive flora to high pressure treatment. Black colonies (presumptive *Shewanella putrefaciens*, López-Caballero, Martínez-Álvarez, Gómez-Guillén, & Montero, 2007) were not detected in the raw material before treatment nor after treatment. *S. putrefaciens* sensitivity to high pressure was previously described by López-Caballero, Pérez-Mateos, Montero, and Borderías (2000). Yagiz et al. (2009) obtained total counts with reductions of 3 log cycles and counts even below the detection limits for Atlantic salmon after pressurisation at 150 and 300 Mpa/15 min at room temperature. In this same species, reduction in the total count for *S. putrefaciens* and lactic acid bacteria were reported by Amanatidou et al. (2000) after high pressure treatment (150 MPa, 10 min, 5 °C). Luminescent colonies, considered as presumptive *Photobacterium phosphoreum* (López-Caballero, Álvarez, Sánchez-Fernández, & Moral 2002), were also reduced after high pressure treatment (Table 5). Paarup, Sánchez-Fernández, Peláez, and Moral (2002) reported that the number of luminescent colonies decreased after pressurisation of squid at 300 MPa for 15 min at room temperature, though not so sharply as in our work. Pressure resistance, psychrofilic characteristics and luminous properties of these marine vibrios have been described previously (Paarup et al., 2002). The cooking process reduced the numbers of all the micro-organisms studied, which remained below the detection limit throughout the period studied.

Table 5
Microbial counts (log UFC/g) in muscle subjected to different processing treatments and stored at 7 °C for 23 d.

Treatment	Days of chilled storage			
	1	7	15	23
Total viable count (iron agar 1% NaCl)				
C	$<2^{c}$	$<2^{e}$	$<2^{d}$	$<2^{d}$
HP5	$2.00^{a/x}$	$5.70^{a/y}$	$7.88^{a/z}$	$7.74^{a/z}$
HPF5	$2.00^{a/x}$	$5.10^{b/y}$	$7.20^{b/z}$	$7.37^{b/z}$
HP40	$3.33^{b/w}$	$3.87^{c/x}$	$5.67^{c/y}$	$8.22^{c/z}$
HPF40	$3.33^{b/x}$	$3.04^{d/x}$	$5.43^{c/y}$	$8.26^{c/z}$
H_2S-producers micro-organisms				
C	<2	$<2^{c}$	$<2^{d}$	$<2^{d}$
HP5	<2	4.08^{a}	5.08^{a}	5.08^{a}
HPF5	$<2^{x}$	$4.71^{b/y}$	$5.28^{a/z}$	$5.49^{a/z}$
HP40	<2	$<2^{c}$	2.30^{b}	2.30^{b}
HPF40	$<2^{x}$	$<2^{c/x}$	$3.39^{c/y}$	$3.39^{c/y}$
Luminous colonies				
C	<2	$<2^{e}$	$<2^{e}$	<2
HP5	$<2^{x}$	$5.70^{a/y}$	$7.60^{a/z}$	$<2^{x}$
HPF5	$<2^{x}$	$4.77^{b/y}$	$6.47^{b/z}$	$<2^{x}$
HP40	$<2^{x}$	$2.15^{c/y}$	$2.15^{c/y}$	$<2^{x}$
HPF40	$<2^{x}$	$2.93^{d/y}$	$4.04^{d/z}$	$<2^{x}$
Pseudomonas spp.				
C	<2	$<2^{d}$	$<2^{d}$	<2
HP5	$<2^{x}$	$<2^{d/x}$	$2.30^{a/y}$	2.00^{y}
HPF5	$<2^{w}$	$3.82^{b/x}$	$4.98^{b/y}$	2.00^{z}
HP40	$<2^{x}$	$2.42^{c/y}$	$2.00^{a/y}$	2.00^{y}
HPF40	$<2^{x}$	$2.39^{c/y}$	$3.59^{c/y}$	3.00^{y}
Lactic acid bacteria				
C	$<1^{c}$	$<1^{e}$	$<1^{c}$	$<1^{c}$
HP5	$2.52^{a/w}$	$3.98^{a/x}$	$7.06^{a/y}$	$7.48^{a/z}$
HPF5	$2.52^{a/x}$	$4.84^{b/y}$	$6.44^{b/x}$	$7.15^{a/z}$
HP40	$2.02^{b/x}$	$1.80^{c/x}$	$5.25^{b/y}$	$8.57^{b/z}$
HPF40	$2.02^{b/w}$	$3.58^{a/x}$	$5.51^{b/y}$	$8.25^{b/z}$
Enterobacteriaceae				
C	<1	$<1^{a}$	$<1^{a}$	$<1^{a}$
HP5	<1	$<1^{a}$	$<1^{a}$	$<1^{a}$
HPF5	$<1^{x}$	$2.04^{b/y}$	$3.03^{b/z}$	$2.74^{b/z}$
HP40	<1	$<1^{a}$	$<1^{a}$	3.00^{b}
HPF40	$<1^{x}$	$1.00^{c/x}$	$4.18^{c/y}$	$3.48^{b/y}$
Total mesophilic counts				
C	$<1^{b}$	$<1^{e}$	$<1^{c}$	$<1^{c}$
HP5	$2.74^{a/x}$	$4.13^{a/y}$	$7.15^{a/z}$	$7.64^{a/z}$
HPF5	$2.74^{a/x}$	$4.83^{b/y}$	$7.19^{a/z}$	$7.32^{a/z}$
HP40	$2.63^{a/x}$	$2.50^{c/x}$	$5.84^{b/y}$	$8.48^{b/z}$
HPF40	$2.63^{a/w}$	$3.46^{d/x}$	$5.73^{b/y}$	$8.35^{b/z}$

R, raw muscle; C, muscle cooked at 90 °C for 10 min; HP5, muscle pressurised at 5 °C for 10 min; HPF5, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min; HP40, muscle pressurised at 40 °C for 10 min; HPF40, muscle covered with gelatin–lignin film and pressurised at 5 °C for 10 min.
Different letters (a,b,c,...) in the same column indicate significant differences ($p \leqslant 0.05$) as a function of treatment; different letters (x,y,z,...) in the same row indicate significant ($p \leqslant 0.05$) differences as a function of storage time..

The number of micro-organisms increased during storage ($p \leqslant 0.05$). The HP treatment temperature (5 or 40 °C) affected these micro-organisms differently depending on the type of organism involved. Thus, the total counts of viable, aerobic and lactic acid bacteria were lower at 5 °C on day 23, even though the counts for these micro-organisms were less at 40 °C up to the 15th day of storage. However, *S. putrefaciens* and *P. phosphoreum* concentrations decreased more at 40 °C, which seems logical if we take into account the psychrofilic character of these micro-organisms and *P. phosphoreum* sensitivity to non-chilling temperatures. This fact explains the difference of almost 2 logarithmic cycles between the surface counts obtained for PCA and those of Iron Agar (Table 5). Differences in total microorganism counts depending on the medium and incubation temperature have already been described (López-Caballero et al., 2000).

Although in recent years, the effect of high pressure on micro-organisms has been published extensively, literature on high pressure treatment combined with films has been less abundantly reported. Previous work in our laboratories has demonstrated the antimicrobial effect on fish storage of combining high pressure treatment with films (Gómez-Estaca et al., 2007). However, to our knowledge there is no information about the effect of gelatin films incorporating lignin on the spoilage flora of fish, when used in conjunction or not with high pressure. In this study the effect of film on the growth of the micro-organisms studied has not been very noticeable since the counts are similar for batches with or without film (Table 5).

4. Conclusions

HPP was found to produce a noticeably lower overall degree of protein aggregation compared with conventional heating treatment, especially when performed at 5 °C instead of 40 °C. FTIR analysis and dynamic oscillatory studies provided complementary information about the protein unfolding effect of HPP. High pressure processing at 5 °C in combination with the gelatin–lignin film was found to preserve redness colouration and minimally prevent protein denaturation and oxidation, without jeopardising the appearance of a ready-to-eat product. The film reduced the level of lipid oxidation arising during advanced stages of chilled storage; however it was ineffective at inhibiting microbial growth.

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6 Discusión integradora

En la actualidad, multitud de alimentos se envasan para su manejo, transporte, conservación, etc. Existen materiales diversos que se utilizan para el envasado, pero destaca el uso de los plásticos, que genera gran cantidad de desechos no biodegradables (Kirwan et al., 2003). La gelatina es un material biopolimérico y biodegradable que se presenta como alternativa para el envasado de alimentos. La gelatina procede de distintas fuentes. En la literatura se recogen multitud de trabajos que utilizan gelatina bovina debido a sus excelentes propiedades, sin embargo su utilización se rechaza en algunas ocasiones por razones socioculturales, religiosas o por transmisión de enfermedades (EET) (Gómez-Guillén et al., 2009). Es por ello que el estudio de gelatinas de distintos orígenes, como la gelatina de piel de pescado, adquiere gran interés.

La lignina y el lignosulfonato son residuos industriales susceptibles de ser utilizados para la formulación de materiales filmogénicos por su naturaleza polimérica, así como por su biodegradabilidad, baja citotoxicidad, alta capacidad antioxidante, etc. (Ugartondo et al., 2009; Ugartondo et al., 2008). La lignina se encuentra como subproducto en la industria del papel, pero al mismo tiempo está presente en todos los vegetales y forma parte de la fibra dietética (Elleuch et al., 2011). La alta miscibilidad que presenta con la gelatina ha propiciado un estudio en profundidad de optimización y caracterización de las mezclas resultantes. Las películas con las mejores propiedades se han aplicado a filetes de sardina y porciones de salmón para mejorar su conservación en estado refrigerado. Los resultados más sobresalientes se discuten a continuación.

6.1 *Desarrollo y aplicación de películas de gelatina y lignosulfonato*

En esta primera etapa del estudio se ha utilizado una gelatina de peces de aguas frías, en comparación con gelatina bovina, así como varios tipos de lignosulfonato (en distintos ratios), con la finalidad de obtener la composición óptima para la formación de la película. En todos los casos, la suma de gelatina y lignosulfonato fue de 4 g de matriz en 100 ml de disolución y se añadió glicerol y sorbitol como plastificantes, cada uno en proporción 15 % sobre el peso de la matriz.

La película con las mejores propiedades físicas se sometió a un estudio de estabilidad durante 28 días a temperatura ambiente bajo dos sistemas modelo: a) en ambiente de aire húmedo y b) en contacto con aceite de girasol. A continuación, se llevó a cabo un estudio de conservación de filetes de sardina refrigerados recubiertos con la película de elección, sola o combinada con un tratamiento de alta presión.

6.1.1 Estudio de composición y ratio óptimo de gelatina-lignosulfonato

6.1.1.1 Formulación de películas de gelatina de baja capacidad gelificante y lignosulfonato

Las gelatinas de peces de aguas frías se caracterizan por presentar escasa capacidad gelificante y baja temperatura de transición térmica (gelificación/fusión). Dichas gelatinas poseen buena capacidad filmogénica; sin embargo, las películas resultantes ofrecen muy baja resistencia al agua, pues son extremadamente solubles (Pérez-Mateos et al., 2009). Una práctica habitual para que sus propiedades (mecánicas, de barrera, etc.) mejoren es la mezcla con otros compuestos. El lignosulfonato presenta alta solubilidad en agua y aparente miscibilidad con la gelatina. Posee grupos muy polares sobre un esqueleto hidrofóbico que le confieren una elevada actividad como surfactante (Telysheva et al., 2001), por lo cual se ha utilizado conjuntamente con otros biopolímeros con la finalidad de mejorar la resistencia al agua y las propiedades físicas de películas de naturaleza tanto proteica como polisacárida (Baumberger et al., 1997; Huang et al., 2003). La gelatina, por su parte, presenta un contenido balanceado de aminoácidos hidrofílicos e hidrofóbicos, que igualmente le confieren interesantes propiedades de superficie, y además presenta numerosos grupos reactivos con capacidad para interaccionar con el medio y con los compuestos adyacentes. Dichas interacciones condicionan en gran medida las propiedades de las películas compuestas, así como de las soluciones filmogénicas previo al secado.

6.1.1.1.1 Interacción gelatina-lignosulfonato en la solución filmogénica

El comportamiento viscoelástico de las soluciones filmogénicas es un factor importante a tener en cuenta, ya que condiciona la homogeneización y el manejo de las mezclas en todas las etapas previas al moldeo y secado de las películas. Las interacciones que tienen lugar en presencia del solvente (agua en este caso), condicionan las propiedades de fluencia de la solución y su comportamiento en función del incremento de temperatura, factores que determinan que, tras el secado, la película tenga una apariencia lisa y homogénea.

Se han formulado soluciones filmogénicas a partir de gelatina de peces de aguas frías (F) y proporciones crecientes de un lignosulfonato cálcico-magnésico de alto contenido en azúcar (LS). La matriz constituida por un 100% de gelatina constituye la mezcla 100-0. Mediante sustitución parcial de la gelatina por cantidades crecientes de lignosulfonato se obtuvieron las mezclas 85-15, 80-20, 75-25, 70-30 y 65-35.

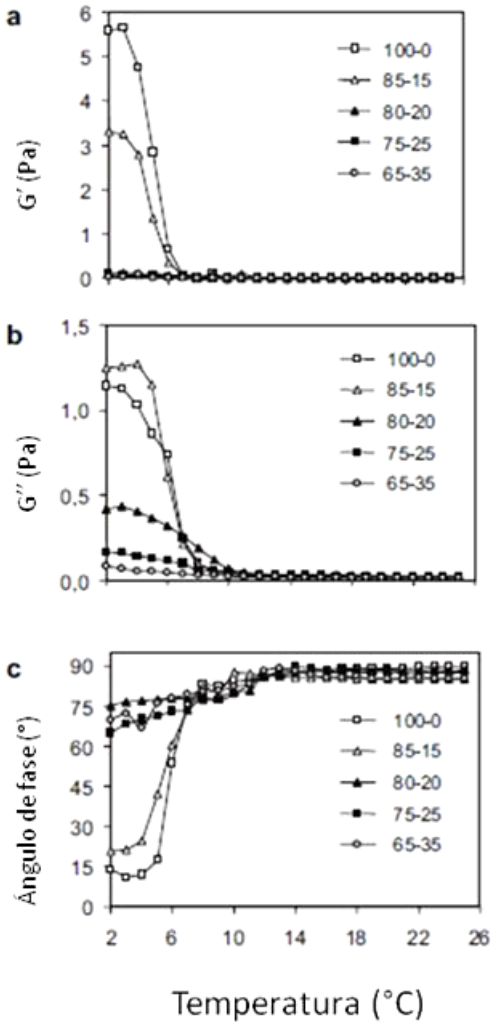


Figura 7. Estudio reológico de las soluciones filmogénicas F-LS. a) Módulo elástico, G'. b) Módulo viscoso, G''. c) Ángulo de fase.

La baja capacidad gelificante de la gelatina de pescado se pone de manifiesto a través de los valores extremadamente bajos del módulo elástico (G'), tal y como revela la figura 7. Ello se debe principalmente al bajo contenido en prolina y sobre todo en hidroxiprolina, comparado con la composición que tienen las gelatinas de bovino o las procedentes de peces de aguas cálidas. Estos aminoácidos originan puentes de hidrógeno, necesarios para el establecimiento y crecimiento de los puntos de nucleación responsables de la formación en frío de triples hélices a partir de las cadenas polipeptídicas de la gelatina (Gilsenan & Ross-Murphy, 2000). La solución filmogénica sin LS forma un gel débil que funde a una temperatura superior a 6 °C. La sustitución del 15 % del contenido de gelatina por LS produce una reducción en los parámetros viscoelásticos de G' y de G'' y aumento en el ángulo de fase, sugieriendo un grado de interacción notable entre ambos compuestos que produce una interferencia significativa en la capacidad de gelificación de la gelatina. Dicho efecto es tanto

más acusado cuanto mayor es la proporción de lignosulfonato en la mezcla. El estudio de la viscosidad pone de manifiesto el alto grado de interacción entre la gelatina y el lignosulfonato. Sin embargo, no desvela información sobre la naturaleza de dichas interacciones.

6.1.1.1.2 Interacción gelatina-lignosulfonato en la película

Las películas se obtuvieron mediante un procedimiento de moldeo o "casting" a partir de las soluciones filmogénicas previamente descritas. Tras el secado y previo al análisis, es necesario un periodo de almacenamiento en condiciones estándar de temperatura y humedad relativa (acondicionamiento), hasta alcanzar un estado lo más próximo posible a un equilibrio termodinámico, que permita una adecuada comparativa entre las distintas muestras. Las condiciones estándar empleadas para el acondicionamiento de las películas fueron 22 ^{o}C y 58 % de humedad relativa.

6.1.1.1.2.1 Estudio por espectroscopía Infrarroja con transformada de Fourier (ATR-FTIR)

Para el caso concreto del estudio por FTIR, las películas deben contener el mínimo porcentaje de humedad posible, ya que la técnica presenta gran interferencia con las moléculas de agua. Por este motivo, las películas se acondicionan en atmósfera de aire seco con gel de sílice. La espectroscopía infrarroja permite evidenciar cambios en la proteína, fundamentalmente a nivel de su estructura secundaria, a la vez que puede identificar posibles interacciones químicas a través de puentes de hidrógeno o de enlaces covalentes.

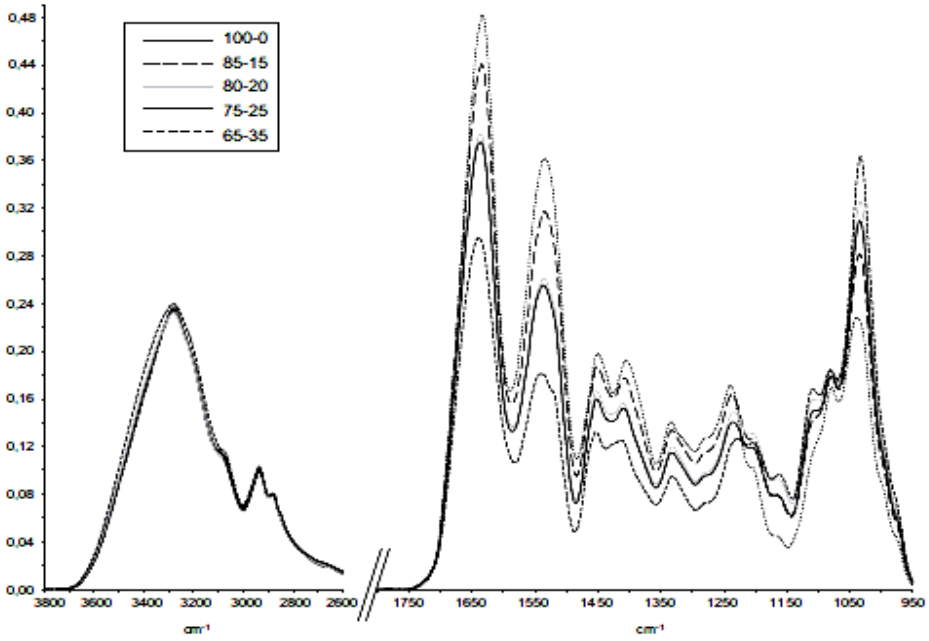


Figura 8. Espectro realizado con ATR-FTIR de las películas basadas en las mezclas de películas de F-LS a distintos ratios.

El grado de interacción entre los dos biopolímeros (gelatina de pescado y lignosulfonato) se evaluó observando cambios en la intensidad o la frecuencia de los principales picos de absorción. La amida A (banda asociada a la vibración por estiramiento del enlace N-H en la amida) de la gelatina presenta pocos cambios en presencia del LS y sólo se observó una pequeña modificación en la mezcla 65-35. **La ausencia de algún cambio en la amplitud y frecuencia de la banda sugiere que la posible interacción a través de enlaces de hidrógeno a este nivel sea insignificante**. Por otro lado, la amida I (banda asociada a la vibración por estiramiento de los grupos C=O de la proteína) mostró que sin adición de LS o adicionado a una concentración del 25 % o menor, no se produce un aparente desplazamiento hacia números de onda menores, lo cual confirma que **es poco probable una interacción significativa gelatina-LS mediante puentes de hidrógeno.** A continuación, se calculó la segunda derivada de la amida I y en el espectro resultante (figura 7) se observó que la adición de LS causó cambios en la conformación de las cadenas polipeptídicas de la gelatina, dando lugar a una **disminución de la presencia de hélices α libres, "random coils" y estructuras desordenadas** por la disminución de la intensidad de los picos a ~1651 cm^{-1} y ~1645-1635 cm^{-1} (Payne & Veis, 1988).

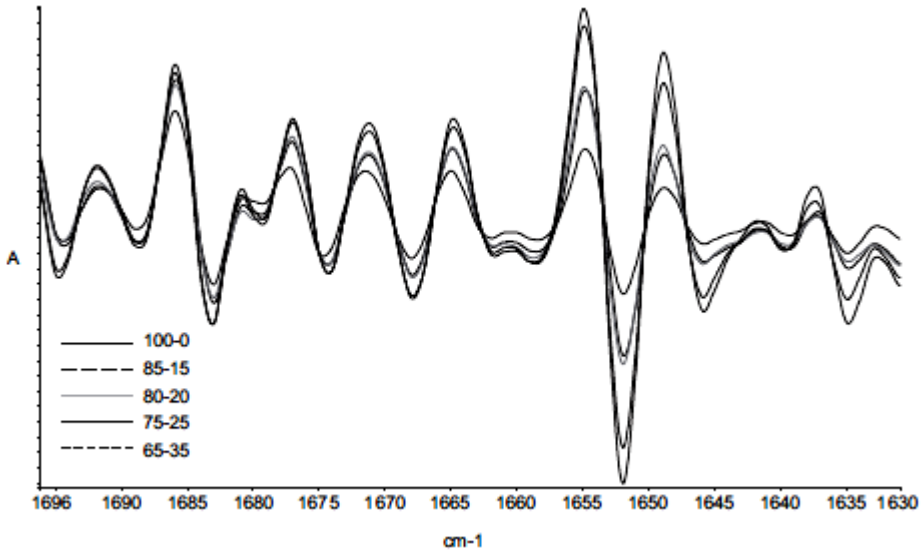


Figura 9. Segunda derivada de la amida A del espectro FTIR de las películas basadas en las mezclas de F-LS a distintos ratios.

También se observó que en la mezcla 80-20, y con concentraciones mayores de LS, se produce una interferencia con la agregación intermolecular de las hélices α de colágeno y de las láminas β por la reducción de la intensidad de las bandas a 1695 cm^{-1} y 1675 cm^{-1} (Muyonga, Cole & Duodu, 2004). Las láminas β sirven como puntos de nucleación para la formación y crecimiento de las triple hélices (Prystupa & Donald, 1996) y por lo tanto, **el LS dificulta la formación de dichos puntos de nucleación**, en clara correspondencia con los resultados del estudio de viscoelasticidad**.** Los efectos del LS sobre la conformación de las cadenas polipeptídicas de la gelatina se atribuyen principalmente

a interacciones inespecíficas de tipo físico, debidas a la elevada actividad de superficie del LS. Estudiando la Amida II (banda asociada con la vibración de flexión del grupo N-H y vibración de estiramiento del grupo C-N), se observó una disminución en su intensidad y un desplazamiento hacia números de onda mayores con la incorporación de cantidades crecientes de LS, lo cual indica un incremento en el nivel de hidratación proteica, acompañado de una disminución en la interacción proteína-proteína (Yakimets, Wellner, Smith, Wilson, Farhat & Mitchell, 2005).

6.1.1.1.2.2 Estudio por calorimetría diferencial de barrido (DSC)

Se realizó un análisis térmico de las películas mediante calorimetría diferencial de barrido (en inglés, DSC). Como se observa en la figura 10, se produce una tendencia a disminuir los valores de Tg (temperatura de transición vítrea) cuando se añade LS, pero a partir del ratio 70:30 esta tendencia se invierte, por tanto las muestras con cantidades elevadas de LS pasan a tener carácter antiplastificante. Este comportamiento se ha descrito previamente en la literatura (Huang et al., 2003) cuando se mezcló LS con proteínas de soja, pues a bajas concentraciones de LS se produce un efecto de entrecruzamiento físico que induce un efecto de plastificación, pero a mayores concentraciones se origina el efecto contrario, lo que sugiere que el LS queda disperso en la matriz a modo de relleno. Por ello, se concluye que concentraciones bajas o moderadas de LS dan lugar a cierta separación de microfases, permitiendo así incrementar el volumen libre y produciendo un descenso en la Tg; cuando la concentración de LS aumenta, se da un efecto de impedimento estérico que disminuye la capacidad de movimiento del biopolímero, lo que da lugar a un aumento en la Tg.

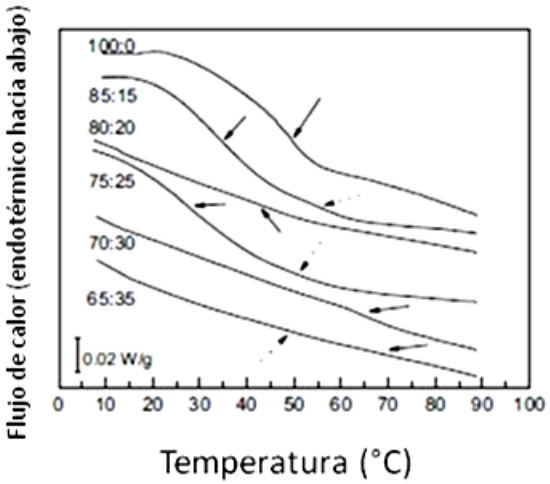


Figura 10. Gráfica obtenida al utilizar la técnica DSC para varias muestras basadas en gelatina de pescado y LS en varios ratios.

No se ha encontrado evidencia de interacción química significativa a través de puentes de hidrógeno o de enlaces covalentes entre la gelatina y el LS, por lo que se considera que éste actúa a modo de "filler" o relleno inespecífico de la matriz de gelatina, interfiriendo en la interacción

entre las cadenas polipeptídicas y favoreciendo una mayor interacción con las moléculas de agua, todo ello fuertemente condicionado por la proporción de lignosulfonato en la fórmula.

6.1.1.1.2.3 Propiedades físicas de las películas

Las películas de gelatina de pescado son transparentes e incoloras. Cuando se adiciona LS, las películas continúan siendo transparentes pero adquieren una tonalidad marrón. Los ratios 70-30 y 65-35 dan lugar a películas muy pegajosas, lo que dificulta su manipulación. Este inconveniente se consideró como un primer criterio de selección para escoger el mejor ratio.

Desde el punto de vista de las propiedades mecánicas, la película de gelatina sin LS presenta muy alta elongación a la ruptura y una fuerza de tensión menor a la obtenida en películas de gelatina bovina (Pranoto, Lee & Park, 2007) o de peces de aguas cálidas (Pérez-Mateos et al., 2009). La adición de LS no da lugar a cambios significativos en la fuerza de tensión pero sí disminuye el valor de elongación a la ruptura. Estos resultados concuerdan con el posible papel del LS a modo de relleno de la matriz, interfiriendo en la agregación de las cadenas de gelatina, de forma más intensa a altas concentraciones.

La adición de LS en un ratio de 80-20 o 75-25 da lugar a una disminución marcada en la solubilidad y un aumento en la permeabilidad al vapor de agua. El aumento de permeabilidad al vapor podría deberse a la existencia de cierta separación de microfases, como se ha indicado previamente. Estos resultados concuerdan con lo observado en los estudios de viscoelasticidad, FTIR y DSC, y sugieren que un porcentaje del 20% de LS en la matriz es el límite a partir del cual el LS interfiere negativamente en la reorganización de las cadenas polipeptídicas de la gelatina. Dicha interferencia provoca un impedimento físico de la gelatina para interactuar con el agua, lo cual puede ser responsable de la disminución de la solubilidad. Ello se debe a que el lignosulfonato y la gelatina son aparentemente miscibles en agua, dada su elevada solubilidad, pero a determinada concentración de lignosulfonato no son químicamente compatibles, pues predomina el impedimento estérico.

Estos datos concuerdan con un estudio realizado con LS y proteína de soja, donde también se observó una disminución en la solubilidad (Huang et al., 2003). Por otro lado, el alto contenido en azúcar del LS utilizado podría originar reacciones de glicación con la gelatina a nivel de los telopéptidos, contribuyendo a la reducción de la solubilidad (Delgado-Andrade, Seiquer, Nieto & Navarro, 2004). Dichas reacciones, no obstante, no pudieron evidenciarse por FTIR.

La adición de LS dio lugar a un aumento en la opacidad de la película resultante. Este hecho se debe a la naturaleza cromófora de la lignina, la cual puede proteger de la radiación ultravioleta (Ban et al., 2007). Comparando los distintos ratios utilizados, se observó un gran salto cualitativo en el nivel de opacidad para la mezcla 80-20, debido no sólo a la presencia del LS, sino a la interferencia inespecífica del LS, que causa cambios conformacionales en la matriz proteica.

A la vista de los resultados obtenidos, se seleccionó el ratio 80-20 como óptimo para producir una disminución sustancial de la solubilidad de la película, y consecuentemente para originar una **mayor resistencia al agua**, con el menor perjuicio en el resto de propiedades físicas.

En la tabla 1 se muestran las propiedades comparadas de la película de gelatina (100-0) y la de F-LS (80-20).

Tabla 1. Comparativa de propiedades físicas de una película F-LS 100-0 y 80-20.

Propiedad	100-0	80-20	Acción del LS
Tg	~ 50°C	~ 42°C	
FT x 10^6 (N/m^2)	5,39 ± 0,42[a]	4,76 ± 0,58[a]	
EAR(%)	546 ± 7[a]	426 ± 32[b]	
Solubilidad (%)	92,5 ± 1,55[a]	54,54 ± 2,26[b]	
PVA (g mm/h Pa cm^2)	2,60 ± 0,14[a]	5,54 ± 0,89[b]	
Opacidad (Abs 600nm/mm)	0,39 ± 0,02[a]	1,93 ± 0,30[b]	

FT: Fuerza de tensión. EAR: elongación en la ruptura. PVA: permeación al vapor de agua.

Distintas letras (a,b) en la misma fila indican diferencias significativas (p<0,05) entre muestras.

La incorporación de LS en un ratio 80-20 produce un descenso moderado en la Tg y también en la elongación de la película, que con gelatina de especies de aguas frías es extraordinariamente alta. La fuerza de tensión no se altera significativamente. El principal efecto adverso es un aumento de la permeabilidad al vapor de agua, que cobra una importancia secundaria teniendo en cuenta la reducción tan significativa de la solubilidad. El incremento en la opacidad va en detrimento de la apariencia física de la película, sin embargo, produce un efecto de barrera positivo para proteger el alimento de la posible oxidación inducida por la exposición a la luz.

6.1.1.2 Evaluación del efecto del tipo de gelatina y de lignosulfonato en las propiedades físicas de la película

Una vez establecido el mejor ratio de F-LS (80-20), se continuó profundizando en la búsqueda de la formulación óptima de la película mediante un estudio comparativo de *películas de gelatina de*

peces de aguas frías (F) (baja capacidad gelificante) y *gelatina bovina (B)* (alta capacidad gelificante), utilizando distintos lignosulfonatos:

- *LS cálcico-magnésico de alto contenido en azúcar (LSa)*
- *LS cálcico-magnésico de bajo contenido en azúcar (LSb)*
- *LS sódico de bajo contenido en azúcar (LSc*)

Las películas con gelatina bovina (B) presentan **mayor fuerza de tensión** y **menor elongación a la ruptura** que las producidas a partir de gelatina de pescado (F). Esto se debe a que el contenido en prolina e hidroxiprolina es mucho mayor en B que en F y los anillos pirrolidínicos de los iminoácidos imponen restricciones conformacionales, impartiendo un cierto grado de rigidez molecular. Las diferencias encontradas en función del tipo de lignosulfonato utilizado son poco acusadas. El LSc destacó por su capacidad de aumentar la tensión y disminuir la elongación, en especial al mezclarse con la gelatina bovina (película B-LSc). Este efecto se puede atribuir al bajo contenido en azúcares y la naturaleza monovalente del catión Na^+ del LSc, que obstaculiza en menor medida las fuertes interacciones proteína-proteína de la matriz de gelatina bovina. Sin embargo, como contrapartida produce un incremento en la **permeabilidad al vapor de agua** de dicha película.

Considerando los valores de **solubilidad en agua**, el tipo de LS no da lugar a cambios significativos, aunque sí los da el tipo de gelatina utilizada: las películas de gelatina de pescado tienen una solubilidad mucho mayor que las de gelatina bovina, y ello se debe una vez más al mayor contenido en iminoácidos de la gelatina bovina, que produce un mayor grado de entrecruzamiento de las cadenas polipeptídicas. La **opacidad** de las películas de gelatina bovina es significativamente superior a las de la gelatina de pescado, independientemente del tipo de LS utilizado.

El **estudio reológico** de propiedades viscoelásticas de las soluciones filmogénicas reveló que el **LSa (alto contenido en azúcar) es el que interfiere más** en la capacidad de renaturalización de las cadenas polipeptídicas, pues se observó, en ambos tipos de gelatina, una disminución en el módulo elástico y un aumento en el ángulo de fase. En cambio, LSc produjo el efecto contrario en la mezcla con gelatina, confirmando una mayor capacidad de la misma para establecer interacciones inter e intramoleculares.

Por último, se evaluó el comportamiento de las distintas soluciones filmogénicas a 30 °C y se dedujo que ninguna gelatina pierde la condición de termorreversibilidad (por sus altos valores de ángulo de fase y bajos valores de módulo viscoso) y que, independientemente del LS o gelatina utilizados, no se establecen interacciones covalentes significativas entre ambos biopolímeros, ya que se produce una fusión completa del gel en todos los casos.

6.1.1.3 Estudio de propiedades antioxidantes, antimicrobianas y de citotoxicidad del lignosulfonato

Al margen del papel del lignosulfonato como agente de refuerzo de la matriz de gelatina, el interés principal de este compuesto, dada su naturaleza polifenólica, es su utilización como agente antioxidante, para el diseño de un material de envasado activo.

Se evaluó la **capacidad antioxidante** de los tres lignosulfonatos estudiados y no se encontraron diferencias significativas en los valores de IC_{50} (83-97 µg/mL) obtenidos con el método del DPPH. La actividad antioxidante de las ligninas en general está influída por características estructurales, principalmente por la presencia de grupos funcionales como los grupos hidroxílicos fenólicos y por el peso molecular (Pan, Kadla, Ehara, Gilkes & Saddler, 2006; Ugartondo et al., 2008). Los tres LS evaluados tienen un peso molecular similar, lo que da lugar a una actividad antioxidante parecida. Otro factor que influye es el contenido en carbohidratos, los cuales pueden interactuar al formar puentes de hidrógeno con los grupos fenólicos, aunque este hecho no da lugar a cambios significativos en la capacidad de secuestro del radical DPPH de LSa, LSb o LSc.

En conclusión, **todos los LS presentan una actividad antioxidante notoria y muy similar entre sí.**

Por otro lado, y enfocado al posible uso del LS para el envasado activo de alimentos, se realizaron estudios de **citotoxicidad** de los tres tipos de LS. Se observó un efecto citotóxico a concentraciones muy elevadas de LS (IC_{50} = 1480-1745 µg/mL), sin diferencias significativas entre ellos, de manera que se pudo concluir que **el LS funciona bien como antioxidante a concentraciones no citotóxicas**.

Por último, se evaluó la **capacidad antimicrobiana** de los LS ante diversos microorganismos. Los resultados no fueron favorables salvo en algunos casos concretos, como por ejemplo ante *D. hansenii* CECT 11364, presentando LSc el mayor porcentaje de inhibición, o hacia *S. aureus* CECT 240 y *B. thermosphacta* CECT. En la literatura se describe la actividad antimicrobiana o antifúngica de lignina kraft o extractos ácidos de lignosulfonatos (Dizhbite et al., 2004) (Cruz et al., 2001). Los LS son surfactantes y ello puede inducir alguna actividad antimicrobiana, aunque también se sabe que ciertos LS pueden acelerar la reproducción microbiana por la presencia de azúcares (Lou, Qiu, Yang & Pang, 2004). Los resultados obtenidos con los tres LS estudiados indican que **su actividad capacidad antibacteriana es bastante limitada, si bien presentan ligera capacidad antifúngica.**

Teniendo en cuenta los resultados y que el lignosulfonato cálcico-magnésico de alto contenido en azúcar (LSa) está expresamente autorizado para el consumo animal, la mezcla formada por gelatina bovina (B) y LSa, en un ratio de 80:20, fue la más idónea para obtener una película de gelatina – lignosulfonato de uso alimentario con las mejores propiedades mecánicas, de resistencia al agua y de barrera a la luz. Si bien el lignosulfonato contribuye a un acusado descenso en la solubilidad de las películas de gelatina de pescado (de aguas frías), dichas películas son notable-

mente menos resistentes al agua que con gelatina bovina, por lo que no suponen una alternativa eficiente para el envasado de alimentos de humedad alta o intermedia, como es el caso del pescado.

6.1.2 Estabilidad físico-química y funcional de películas de gelatina y lignosulfonato

A diferencia de los plásticos convencionales derivados del petróleo, las películas biopoliméricas son inestables y pueden sufrir con el tiempo cambios en sus propiedades físico-químicas debido a fenómenos de agregación por envejecimiento o "ageing". En la medida que dichas películas sean más o menos proclives a sufrir estos procesos, el tiempo de almacenamiento previo a su utilización puede verse limitado. Por otro lado, la resistencia y funcionalidad de la película también se pueden ver notablemente afectadas por las condiciones del medio circundante, que varían a su vez dependiendo del alimento y tipo de aplicación a la que vayan destinadas.

A modo de ejemplo se evaluó la estabilidad de las películas de gelatina bovina (B, 100-0) y de gelatina bovina – lignosulfonato (B-LSa, 80:20) durante 28 días de almacenamiento a temperatura ambiente (22 ^{o}C) en dos sistemas modelo: 1) en atmósfera de aire con humedad controlada (58 %) y 2) en contacto con aceite expuesto al aire y la luz (condiciones oxidativas).

6.1.2.1 Estabilidad físico-química de las películas de gelatina y lignosulfonato

A diferencia de las películas conservadas en aire húmedo, las películas de gelatina conservadas en aceite de girasol experimentan en los primeros dos días de exposición un aumento significativo de la fuerza de tensión. **El contacto con el aceite produce un efecto antiplastificante**, disminuyendo la elongación a la rotura de la película. La naturaleza hidrofóbica del aceite en contacto con los residuos hidrofílicos expuestos de las cadenas polipeptídicas afectan las propiedades de superficie de la película a modo de refuerzo, por agregación de la proteína. Además, las posibles interacciones químicas entre la gelatina y determinados productos de oxidación del aceite también podrían contribuir a reforzar la película (Aewsiri, Benjakul et al. 2011). En el caso de las películas compuestas**, la naturaleza surfactante del LS interfiere la agregación intermolecular de la gelatina**, y por tanto, contrarresta el efecto reforzante del aceite en superficie. Con el transcurso del tiempo, ambos tipos de películas conservadas en aceite no mostraron cambios importantes en la fuerza de tensión. Por el contrario, las películas de gelatina conservadas en aire húmedo presentaron un ligero aumento de la fuerza y disminución de la elongación con el tiempo, por agregación proteica (envejecimiento), que no se puso de manifiesto en la película con LS.

La adición de LS disminuyó los valores de permeabilidad al vapor de agua de modo significativo en el caso de las películas conservadas en aceite al inicio de la exposición. Independientemente del medio de conservación, la permeabilidad experimentó una ligera fluctuación a las dos semanas

de almacenamiento en todos los casos, si bien aumentó hasta alcanzar valores similares a los iniciales.

Ya se comentó con anterioridad que las películas con LS tienen un color marrón y su presencia produce un aumento en la opacidad debido a su naturaleza cromófora, que es protectora frente a la radiación ultravioleta (Ban et al., 2007). Durante el almacenamiento, la opacidad aumentó en las películas de 100 % gelatina conservadas en aire húmedo debido a fenómenos de agregación de la gelatina. Sin embargo, el nivel de opacidad en las películas con LS permanece inalterado debido a que interfiere la agregación proteica. Por otro lado, la exposición al aceite ocasionó un aumento de la opacidad en las películas de 100 % gelatina - posiblemente también debida a fenómenos de entrecruzamiento - que, sin embargo, permaneció estable frente al tiempo, al igual que en las películas con LS.

Las películas de 100 % gelatina pierden su estructura, se hinchan, se reblandecen y tienden a formar un gel amorfo en contacto con el agua; sin embargo, en presencia del LS mantienen su forma original transcurridas más de 24 h. Esta característica es muy importante, ya que permite su utilización sobre alimentos húmedos. El periodo de 28 días de almacenamiento en condiciones de aire húmedo no provoca cambios significativos en la solubilidad en agua de las películas, con o sin lignosulfonato, si bien se produce alguna pequeña fluctuación en los primeros 14 días.

La película de gelatina conservada en aceite presenta menor solubilidad que la conservada en aire, mientras que al hacer la misma comparación en las películas con LS las solubilidades son similares. En general, las películas conservadas en aceite muestran una menor solubilidad durante todo el periodo de conservación respecto a las mantenidas en aire, una vez más debido a la modificación de propiedades de superficie inducido por la presencia del aceite.

La tendencia general que se observa en las propiedades físico-químicas de la película durante el periodo de almacenamiento podría resumirse de la siguiente manera:

- El LS dificulta la tendencia natural de la gelatina a sufrir procesos de agregación o entrecruzamiento por envejecimiento, los cuales, en cualquier caso, no se muestran con intensidad.

- El contacto con el aceite produce modificaciones a nivel de tensión superficial y de posibles fenómenos de agregación de la gelatina en la película, lo que induce cambios significativos en las propiedades mecánicas y en la solubilidad.

-Las propiedades de las películas con lignosulfonato no cambian sustancialmente al cabo de 28 días de almacenamiento en las condiciones estudiadas, si bien se producen ligeras fluctuaciones en los primeros 14 días.

6.1.2.2 Propiedades antioxidantes y estabilidad funcional de películas de gelatina y lignosulfonato

La capacidad antioxidante de las películas se determinó mediante dos metodologías diferentes: capacidad reductora del hierro (método FRAP) y secuestro de radicales libres (método ABTS). En ambas técnicas es importante resaltar la importancia de la solubilidad de las películas pues la actividad antioxidante se determina sobre la fracción de película solubilizada, que es la responsable de la liberación de los compuestos activos. **Las películas de B-LS mostraron un incremento muy pronunciado en los valores de capacidad antioxidante** con respecto a las películas de 100 % gelatina, independientemente del medio de conservación. Estos resultados eran esperables dada la elevada actividad antioxidante del lignosulfonato (Dizhbite et al., 2004), el cual presenta además alta capacidad para difundir de la película.

Durante el tiempo de almacenamiento, la película con lignosulfonato no perdió su capacidad antioxidante. Los posibles efectos de agregación de la gelatina por efecto del tiempo o por el contacto con el aceite son mínimos en presencia del lignosulfonato, y en consecuencia, no afectan a la capacidad de la película para liberar los compuestos activos.

Es importante destacar que el aceite es un medio en el que la película mantiene buenas propiedades, lo que indica que las películas van a podrán aplicar a alimentos con alto contenido graso. Por ello y teniendo en cuenta la gran estabilidad y excelentes propiedades de las películas de gelatina y lignosulfonato, se realizó un estudio de aplicación de estas películas sobre filetes de sardina para su conservación.

6.1.3 Aplicación de películas de gelatina y lignosulfonato para la conservación de sardinas refrigeradas

El pescado es un alimento muy perecedero debido a la alta tasa de crecimiento bacteriano y a los efectos químicos del oxígeno atmosférico (Özogul et al., 2004). La refrigeración es el método de elección para la conservación de pescado fresco, que se puede complementar con otros métodos como alta presión, envasado a vacío, atmósferas modificadas, etc. La sardina es una especie altamente susceptible de sufrir fenómenos de oxidación por su alto contenido en ácidos grasos poliinsaturados, razón por la cual el envasado activo se presenta como una alternativa interesante (Mohan et al., 2012). La película de gelatina y lignosulfonato mostró alta resistencia al agua y buena funcionalidad en contacto con aceite, por lo que su aplicación a un alimento de humedad moderadamente elevada y alto contenido graso, como son los filetes de sardina, puede resultar adecuada. Se elaboraron películas de acuerdo a la composición óptima seleccionada (gelatina bovina y lignosulfonato cálcico-magnésico de alto contenido en azúcar, en proporción 80:20), y se utilizaron para recubrir los filetes de sardina. Con fines comparativos, se ensayaron los siguientes sistemas de conservación a temperatura de refrigeración:

- filetes de sardina cubiertos con película (SP)
- filetes de sardina tratados con alta presión (300 MPa, 5 ºC, 10 min) (SAP)
- filetes de sardina cubiertos con película y tratados con alta presión (300 MPa, 5 ºC, 10 min) (SPAP).
- filetes de sardina envasados a vacío (SV).

Los distintos lotes de pescado se almacenaron en una cámara de refrigeración a 7 ± 2 ºC durante 17 días, y se realizaron análisis periódicos del músculo para el estudio de vida útil. La película mantuvo su integridad en contacto con el pescado durante todo el periodo de almacenamiento, permitiendo su retirada con facilidad y sin romperse.

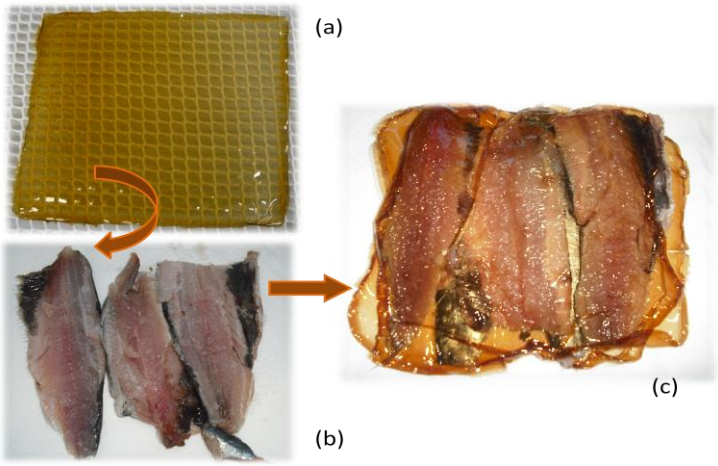


Figura 11. Imágenes de película B:LSa 80:20 (a), filetes de sardina (b) y filetes de sardina cubiertos con película B:LSa 80:20 y alta presión (c).

6.1.3.1 Evolución de parámetros físico-químicos

La evolución del pH en el pescado depende de diversos factores como la liberación de fosfato inorgánico o amoniaco por la degradación enzimática del ATP (Sikorski, Kolakowska & Kurt, 1990), la producción de ácido láctico por bacterias ácido lácticas principalmente o de nitrógeno básico volátil total (NBVT) por microorganismos responsables del deterioro. El pH permanece muy estable durante la conservación, siendo muy similar entre los lotes.

El nitrógeno básico volátil total (NBVT) es un índice objetivo de la calidad del pescado y su contenido se incrementa por la generación de compuestos básicos procedentes del metabolismo de ciertas bacterias. El lote SP alcanza valores por encima del límite de aceptabilidad establecido en la Unión Europea (35 mg NBVT/100g músculo) a partir del día 11 de conservación. Los lotes SP y SV evolucionan de forma semejante hasta el día 8 de conservación, lo que sugiere que el papel de la película durante la conservación de la sardina puede ser similar a la del envasado a vacío. Si bien la

película de gelatina-lignisulfonato no previene totalmente la formación de compuestos básicos, su producción se reduce de forma significativa en combinación con tratamientos de alta presión, lo que está en consonancia con los resultados descritos por Ojagh et al., (2011).

La peroxidación lipídica puede producir daño o muerte celular en los tejidos (Miki, Tamai, Mino, Yamamoto & Niki, 1987), y en el caso del pescado produce rancidez y cambios texturales por agregación de las proteínas miofibrilares. El malonaldialdehído es un producto secundario de la oxidación de los lípidos, que se puede valorar a través de la reacción con el ácido tiobarbitúrico (TBA). **La película de gelatina-lignosulfonato aplicada sobre el músculo de sardina no previene la oxidación de la grasa de forma más efectiva que un envasado a vacío convencional**. Por el contrario, los niveles de TBA en el músculo presurizado permanecen bajos durante todo el periodo de almacenamiento y especialmente en el caso de los filetes con tratamiento combinado de película y alta presión (lote SPAP). Se estima que **la alta presión favorece la liberación de los compuestos antioxidantes de la película** que actúan sobre el músculo de sardina.

Con relación al color del músculo tras 8 días de almacenamiento se produjo un **oscurecimiento de la sardina recubierta con película** y también en la envasada a vacío, asociado a fenómenos de oxidación de la mioglobina que normalmente se correlacionan positivamente con la oxidación de lípidos (Chaijan, Benjakul, Visessanguan & Faustman, 2006). Dicho oscurecimiento, sin embargo, no tuvo lugar en las muestras tratadas con alta presión (sin o con película). **Los filetes recubiertos con película y presurizados mostraron un incremento en la tendencia al color amarillo** (parámetro b*), probablemente debido a transferencia de compuestos de la película al músculo favorecido por la alta presión.

6.1.3.2 Evolución del crecimiento microbiano

Durante la conservación, la sardina cubierta con la película (SP) obtuvo los mayores recuentos. El envasado a vacío previno ligeramente el crecimiento de los microorganismos estudiados, especialmente del género *Pseudomonas* spp, debido a su naturaleza aerobia estricta. La aplicación de alta presión reduce el crecimiento de microorganismos, si bien es el tratamiento combinado de alta presión-película el que los inhibe de forma significativa, aumentando la vida útil en términos microbiológicos hasta los 17 días. Si bien es conocido el poder antibacteriano de la lignina (Muzzarelli, Boudrant, Meyer, Manno, Demarchis & Paoletti, 2012), el efecto de la película de gelatina-lignosulfonato no se manifiesta durante la conservación en refrigeración. La vida útil de los lotes SP y SV se estima en 8 días. Estos resultados están en concordancia con lo obtenido en el apartado anterior, donde los menores valores de NBVT y TBA corresponden al lote SPAP.

Un tratamiento combinado de recubrimiento con película y alta presión resultó más efectivo que el envasado a vacío para prevenir la oxidación lipídica y el deterioro microbiano del músculo de sardina durante la conservación en refrigeración.

6.2 *Desarrollo y aplicación de películas de gelatina y lignina*

La lignina, al igual que el lignosulfonato, se considera un polímero renovable, no tóxico y de carácter biodegradable (Ban et al., 2007). Por diferencias en los procesos de extracción, la lignina carece de la abundancia de grupos sulfónicos hidrofílicos altamente polares presentes en el lignosulfonato, y en consecuencia es insoluble en agua, pero soluble en soluciones alcalinas (pH ≥ 10). La lignina presenta características adecuadas para su utilización en la formulación de recubrimientos y mezclas por su pequeño tamaño de partícula (menor que el lignosulfonato), hidrofobicidad y capacidad de formar mezclas estables (Park, Doherty & Halley, 2008). Presenta alta capacidad antioxidante (Ugartondo et al., 2009), antimicrobiana (Dong et al., 2011) y una baja citotoxicidad (Ugartondo et al., 2008). En el estudio anterior, la gelatina de peces de aguas frías mostró una buena capacidad filmogénica; sin embargo, la baja resistencia al agua de las películas resultantes representa un factor muy limitante para su aplicación en alimentos húmedos, en comparación con la gelatina de bovino. Para el presente estudio se ha recurrido al empleo de una gelatina de peces de aguas cálidas, de alta capacidad gelificante por su alto contenido en prolina e hidroxiprolina. Estas gelatinas suponen una alternativa interesante a las gelatinas de bovino por no presentan rechazo religioso, sociocultural o sanitario (Avena-Bustillos et al., 2006; Gómez-Guillén, Turnay, Fernández-Díaz, Ulmo, Lizarbe & Montero, 2002). Para llevar a cabo este estudio, se ha utilizado una gelatina comercial de piel de pescado de aguas cálidas (principalmente pez gato "catfish") y dos tipos de lignina: Lignina 1000 (L1000) y Lignina 2400 (L2400), que se diferencian básicamente en que L1000 tiene menor densidad aparente y mayor temperatura de ablandamiento. Las interacciones producidas en la matriz entre ambos biopolímeros, así como las características de las películas G (100 % gelatina), G-L1000 (85 % gelatina – 15 % lignina L1000) y G-L2400 (85 % gelatina – 15% lignina L2400) se discuten a continuación. La elección del ratio gelatina: lignina se ha basado en los resultados del estudio anterior, adaptando la formulación a las propiedades intrínsecas de composición y solubilidad de la lignina en polvo.

6.2.1 Formulación idónea de películas complejas de gelatina y lignina

6.2.1.1 Interacción gelatina-lignina en la solución filmogénica (SF)

Las soluciones filmogénicas se prepararon utilizando 4 gramos de matriz, donde la proporción fue de 85 % de gelatina, 15 % de lignina (85-15). Se añadieron glicerol y sorbitol en una proporción del 15 % de peso de matriz, para cada uno. Se aumentó el pH hasta alcanzar un pH ~11 con el objetivo de disolver la lignina. Las soluciones filmogénicas resultantes se estudiaron mediante viscoelasticidad dinámica oscilatoria para determinar el grado de interferencia de las ligninas en la reestructuración de las cadenas polipeptídicas en estructura de triple hélice de la gelatina.

Tabla 2. Propiedades viscoelásticas de las SF basadas en gelatina y L1000 o L2400 en un ratio 85:15.

Gelatina-Lignina	G' $_{5°C}$ (Pa)	G'' $_{5°C}$ (Pa)	a. f. $_{5°C}$ (°)	Tgel (°C)	Tf (°C)
G	852 ± 25	11,30 ± 0,23	0,91 ± 0,01	15	26
G-L1000	1,65 ± 0,05	1,81 ± 0,05	40,55 ± 2,03	6	18
G-L2400	10,10 ± 0,30	2,25 ± 0,09	12,70 ± 0,25	7	18

G': módulo elástico. G'': módulo viscoso. a.f.: ángulo de fase. Tgel: temperatura de gelificación. Tf: temperatura de fusión.

Observando la tabla 2, la SF pierde drásticamente la capacidad gelificante al incorporarse la lignina, tal y como demuestra el aumento del ángulo de fase y la disminución pronunciada de los módulos elástico y viscoso. La lignina L1000 provoca una mayor interferencia con la gelatina, posiblemente debido a su menor densidad en comparación con L2400, que permite un contacto más intenso con las cadenas polipeptídicas.

Al comparar las mezclas de gelatina de peces de aguas cálidas con lignina y de gelatina bovina con lignosulfonatos, se observó un menor ángulo de fase y mayores módulos viscoso y elástico en el segundo caso. La gelatina bovina y la gelatina de peces de aguas cálidas tienen propiedades muy similares (Gilsenan et al., 2000), por lo que estos resultados indican que el **lignosulfonato provoca menor interferencia en la matriz de gelatina que la lignina**.

6.2.1.2 Interacción gelatina-lignina en la película

Las películas con ambos tipos de lignina se obtuvieron a partir de las soluciones filmogénicas mediante moldeo o *casting*, en las mismas condiciones que las empleadas en el experimento anterior. Una vez formadas, se acondicionaron en humedad relativa de 58% durante 4 días, salvo para el estudio de espectroscopía infrarroja (FTIR), que se utilizó una atmósfera seca con gel de sílice.

6.2.1.2.1 Estudio por espectroscopía FTIR

El espectro de infrarrojo de las películas se detalla en la figura 12. La incorporación de lignina produce un descenso en la intensidad de las bandas de amida A, I, II y III (~3283 cm^{-1}, ~1631 cm^{-1}, ~ 1543 cm^{-1}, ~ 1238 cm^{-1} respectivamente), que se atribuyen en primer lugar a un efecto de dilución de la proteína; además, la lignina también produjo cambios conformacionales, pues se dan pequeñas variaciones en el número de onda al que absorbe la amida I, lo cual indica una ruptura de los puentes de hidrógeno en los grupos C=O de los polipéptidos de la gelatina.

Se ha descrito que la lignina interacciona con proteínas de soja de modo similar (Huang et al., 2003). L2400 origina un mayor grado de hidratación proteica, que se deduce al observar un ligero aumento en la amplitud de la amida A, así como un ligero aumento en su número de onda. Ambos

tipos de ligninas provocan una fuerte interferencia en la formación de puentes de hidrógeno entre el agua y los residuos imido de la gelatina (Payne et al., 1988), que concuerda con lo observado en el estudio reológico, donde la lignina impedía la correcta generación de la triple hélice. En el estudio previo realizado con gelatina y lignosulfonato, se observó que la adición de LS no ocasiona modificaciones en la formación de puentes de hidrógeno por lo que se establece **que ambos tipos de lignina dan lugar a un mayor grado de interferencia con la gelatina de la película que el lignosulfonato**.

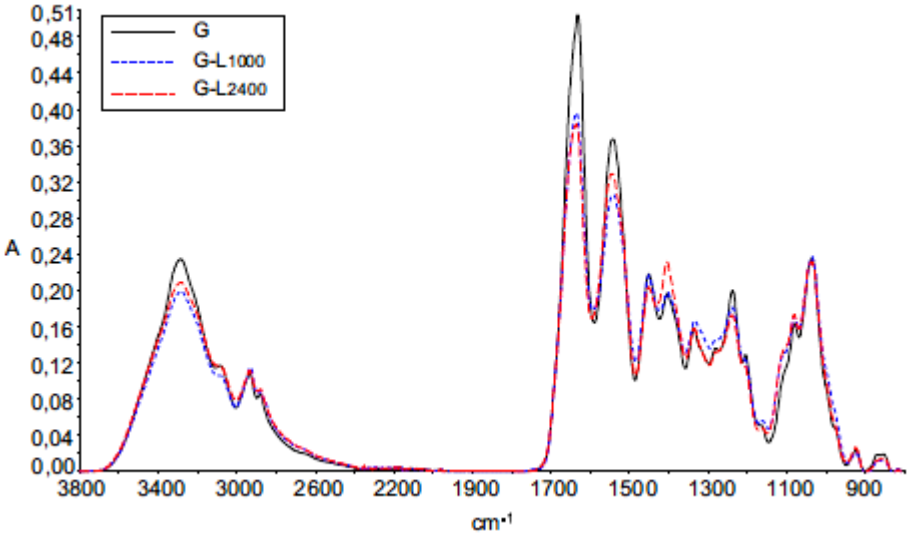


Figura 12. Espectros de las películas de gelatina y de gelatina con L1000 o L2400 (85:15), obtenidos mediante espectroscopía FTIR.

6.2.1.2.2 Estudio por calorimetría diferencial de barrido (DSC)

Las ligninas y sus derivados se han utilizado en otras ocasiones para mejorar las propiedades de algunos materiales (Cui et al., 2007; Feldman et al., 2001; Huang et al., 2003) y han originado cambios en las propiedades físicas, provocando un efecto plastificante al mezclarse con almidón (Wu et al., 2009), o bien de refuerzo al utilizarse junto con la celulosa (Rohella, Sahoo, Paul, Choudhury & Chakravortty, 1996).

Se realizó un estudio térmico de calorimetría diferencial de barrido de las películas con ambos tipos de lignina. Se observó un gran descenso en la temperatura de transición vítrea (Tg) al incorporar la lignina, siendo L1000 la que da lugar a una menor temperatura (-63,4 ± 0,8 °C frente a -54,9 ± 0,6 °C), por lo que se deduce un cierto **efecto plastificante de la lignina**. Por otro lado, se obtiene una segunda Tg a valores muy inferiores que los que presenta la muestra G, que de nuevo le corresponde a la muestra G-L1000. El hecho de aparecer una segunda Tg indica cierta inmiscibilidad entre la lignina (hidrofóbica) y la gelatina (hidrofílica) y por tanto puede haber una **microseparación de fases** permitiendo que la gelatina tenga más espacio para su movilidad. En el experimento también

se observó en el experimento anterior con gelatina y lignosulfonato. La informaciónque se obtiene a partir del estudio de calorimetría diferencial sugiere que **la lignina induce cambios conformacionales en las cadenas polipeptídicas de la gelatina y modifica el efecto plastificante del agua en la película.**

6.2.1.2.3 Propiedades físicas de las películas

Al estudiar las propiedades mecánicas y físico-químicas de las películas, la lignina provocó una disminución en la fuerza de tensión y un aumento en la elongación a la ruptura, de forma más intensa con L2400, al comparar con una película sin lignina (G). Se confirma que la lignina ejerce una acción plastificante y que las moléculas libres de agua contribuyen a este efecto, lo que concuerda con un aumento en la actividad de agua, especialmente con L2400. Esta lignina no dio lugar a cambios significativos en la solubilidad, mientras que con L1000 se produjo una disminución significativa. La permeabilidad al vapor de agua aumentó con el uso de L2400. Todo indica que **el mayor efecto plastificante inducido por L2400 hace que la red polimérica sea menos densa y más permeable, y por lo tanto, menos resistente al agua que con la lignina L1000.** En la tabla 3 quedan resumidas algunas de las propiedades estudiadas.

Tabla 3. Resumen de las propiedades físicas en una película de gelatina y gelatina-lignina (G-L1000 y G-2400).

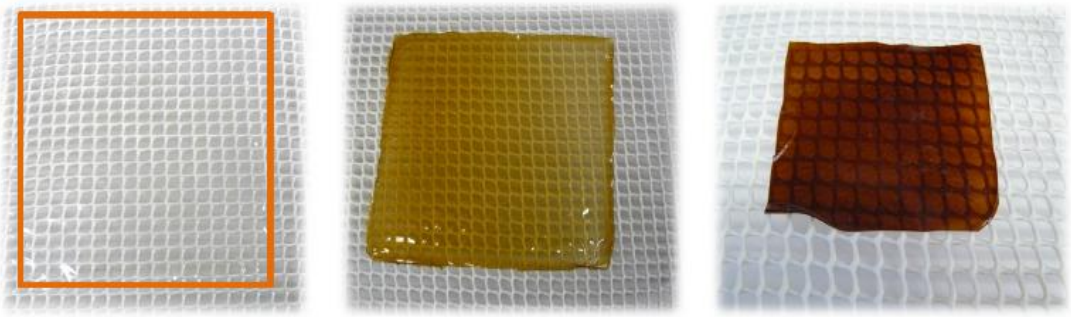
Muestra	FT x 10^{6} (N/m^2)	EAR(%)	Solubilidad en agua (%)	PVA x 10^{-8} (g mm/h Pa cm^2)	Actividad de agua
G	$16,44\pm0,18^{a}$	$136,6\pm39,8^{a}$	$44,29\pm0,97^{a}$	$2,06\pm0,10^{a}$	$0,375^{a}$
G-L1000	$12,13\pm0,48^{b}$	$316,5\pm19,7^{b}$	$39,38\pm1,73^{b}$	$2,17\pm1,73^{a}$	$0,390^{b}$
G-L2400	$7,51\pm0,05^{c}$	$362,8\pm39,6^{b}$	$46,52\pm1,66^{a}$	$4,58\pm0,53^{b}$	$0,467^{c}$

FT: Fuerza de tensión. EAR: elongación en la ruptura. PVA: permeación al vapor de agua. TTV: Temperatura de transición vítrea. Diferentes letras (a,b,c) en la misma columna indican diferencias significativas ($p<0,05$) entre las muestras.

6.2.1.2.4 Propiedades ópticas

Para completar el estudio físico se realizaron pruebas de opacidad y de colorimetría a las películas formadas por gelatina y lignina, teniendo como referencia una película de 100 % gelatina. Al igual que el lignosulfonato, la lignina tiene alta capacidad de protección frente a la radiación ultravioleta, sola y en combinación con otros compuestos (Ban et al., 2007) (Mishra et al., 2007). Los valores de opacidad de las películas con lignina fueron muy superiores a los de la película de gelatina. **La**

opacidad de la película G-L1000 es aproximadamente el doble que la que se obtiene al utilizar L2400, y muy superior en ambos casos a la de las películas con lignosulfonato.



(a) (b) (c)

Figura 13. Imágenes de película de gelatina de bovino (a), gelatina de bovino y LSa (b) y gelatina de peces de aguas cálidas y L1000 (c).

En la Figura 13 se compara el color y el grado de transparencia de las películas de gelatina, gelatina – lignosulfonato y gelatina – lignina.

La lignina en estado natural presenta una fuerte coloración marrón, que hace que las películas formadas conserven cierta transparencia pero con un intenso color marrón. Así, los índices de referencia para la colorimetría (L*, a*, b* y el ángulo de matiz "hue" y el valor cromático) revelan que la película GL2400 tiene más componente marrón que GL1000, en ambos casos mucho mayor al de la película de 100 % G. Por tanto, las películas con lignina pueden contribuir a la prevención de oxidación lipídica inducida por radiación ultravioleta debido a su alto efecto barrera frente a la luz.

La lignina L1000 confiere a las películas de gelatina mayor resistencia mecánica, menor solubilidad, menor permeabilidad al vapor y mayor grado de opacidad que la L2400.

6.2.2 Propiedades antioxidantes de películas de gelatina-lignina.

Una vez revisadas las propiedades físicas de las películas de gelatina y lignina se evaluó su capacidad antioxidante. Es importante destacar que, al igual que con las películas de G:LS, la actividad antioxidante se refiere a la fracción soluble en agua, que es la que tiene la capacidad para liberar los compuestos activos. **A pesar de la menor solubilidad de la película G-L1000 en comparación con G-L2400, la primera presentó mayor capacidad antioxidante** en las dos pruebas realizadas (capacidad reductora de hierro, *FRAP* y capacidad de secuestro de radicales, *ABTS*). Al igual que lo descrito para las películas de gelatina-lignosulfonato, las películas de gelatina-lignina pueden estar muy indicadas para conservar alimentos con alta tendencia a oxidarse. Estas películas presentan una capacidad antioxidante comparable a otras películas adicionadas de otras sustancias, como por ejemplo las

películas de gelatina y borraja (Gómez-Estaca et al., 2009) o gelatina y orégano o romero (Gómez-Estaca, Montero, Fernández-Martín, Alemán & Gómez-Guillén, 2009).

En la tabla 4 se compara la actividad antioxidante de películas de gelatina con lignina o con lignosulfonato.

Tabla 4. Comparativa de capacidades antioxidantes de la mejor película testada de G:LS y las películas de gelatina y lignina.

Película	ABTS (mg vit C/g película)	FRAP (mol Fe^{2+}/g película)
G: LSa (80:20) (día 7)	7,22 ± 0,41	570,20 ± 7,70
G: L_{1000} (85:15) (día 4)	27,82 ± 2,19	258,97 ± 8,83
G: L_{2400} (85:15) (día 4)	15,31 ± 0,88	180,20 ± 5,71

Teniendo en cuenta los resultados comparados en la tabla 4 se observa claramente que **las películas con lignina tienen mayor capacidad de secuestro de radicales libres (ABTS), en especial con lignina L1000, mientras que la película con lignosulfonato presenta mayor capacidad reductora (FRAP)**, en buena parte debido al alto contenido en azúcares presentes en el preparado comercial de LSa. Las gelatinas utilizadas son distintas, pero ninguna posee capacidad antioxidante significativa, por lo que este efecto es provocado únicamente por la presencia de L o LS.

6.2.3 Estudio de propiedades antioxidantes, antimicrobianas y citotoxicidad de la lignina

De ambos tipos de lignina estudiados, la lignina L1000 es la que confiere las propiedades físicas y funcionales más convenientes a la película. La lignina L1000 tiene una elevada capacidad secuestrante del radical DPPH (IC_{50} = 36,5 µg/mL), pero inferior al trolox (derivado soluble de la vitamina E). Al igual que el lignosulfonato, L1000 presenta citotoxicidad a concentraciones muy elevadas (IC_{50} = 631 µg/mL), por lo que **se puede utilizar L1000 como antioxidante a concentraciones no citotóxicas.**

Se realizó un estudio con L1000 para determinar su capacidad antimicrobiana frente a 26 cepas de colección, entre ellas bacterias Gram-positivas y Gram-negativas, mohos y levaduras y no se observó ningún efecto inhibidor del crecimiento de estos microorganismos. Las cepas testadas fueron las mismas utilizadas para evaluar la actividad antimicrobiana de los lignosulfonatos previamente evaluados, los cuales sí que mostraron una cierta actividad. Esta diferencia de actividad puede guardar relación con los distintos pH de los lignoderivados: el LS tiene pH ácido mientras que L1000 se ha evaluado a pH neutro o básico (no es soluble a pH ácidos) y este factor puede hace que la actividad antimicrobiana de los ácidos fenólicos pierdan efectividad (Wen et al., 2003).

La capacidad funcional del lignosulfonato y la lignina, que se detalla en la tabla 5.

Tabla 5. Capacidad antioxidante, antimicrobiana y citotoxicidad de LSa, LSb, LSc y L1000.

Sustancia	Capacidad secuestrante ddel radical DPPH (IC_{50} µg/ml)	Citotoxicidad en fibroblastos de ratón (IC_{50} µg/ml)	Inhibición en crecimiento bacteriano
LSa	92,6±8,1	1480±114	*D. hansenii* ++, *S. aureus* +, *B. thermosphacta* +
LSb	83,4±2,5	1696±55	*D. hansenii* ++, *S. aureus* +, *B. thermosphacta* +
LSc	97,5±6,4	1745±16	*D. hansenii* ++, *S. aureus* +, *B. thermosphacta* +
L1000	36,5±5,5	631±92	No inhibición

++: porcentaje de inhibición del 5-10 %. +: porcentaje de inhibición de < 5 %.

Al realizar esta comparación, se deduce que L1000 es la más antioxidante pues es necesaria una menor concentración para atrapar la mitad de los radicales de DPPH que en el caso de los LS. La citotoxicidad, sin embargo, es mayor que para cualquiera de los LS estudiados, ya que se requiere menor concentración para producir un 50% de muerte celular. Sin embargo, los LS presentan cierta capacidad antimicrobiana, aunque leve, y como se comentó anteriormente, también confieren un mayor poder reductor a la película.

6.2.4 Aplicación de películas de gelatina y lignina para la conservación de salmón refrigerado

Las películas de gelatina (peces de aguas cálidas) y lignina (L1000) (proporción 85-15, *G-L*) se utilizaron para un estudio de conservación en refrigeración de salmón procesado por alta presión. La alta presión permite alargar la vida útil de los alimentos conservando al mismo tiempo los micronutrientes y compuestos responsables del sabor (Knorr, 2000). Como efecto colateral, la presión induce cambios de coloración y opacidad en el músculo de determinadas especies, como el salmón, confiriéndole un aspecto de producto cocido, por degradación oxidativa de pigmentos y desnaturalización de proteínas (Gómez-Estaca, López-Caballero, Gómez-Guillén, López De Lacey & Montero, 2009; Yagiz, Kristinsson, Balaban, Welt, Ralat & Marshall, 2009). Sin embargo, para el diseño de platos

semi-preparados o listos para el consumo, la alta presión puede representar una alternativa interesante al tratamiento térmico para evitar la degradación de nutrientes (aminoácidos, vitaminas) y otros compuestos activos.

Se planteó un estudio de conservación en refrigeración durante 23 días de salmón recubierto con película y tratado por alta presión a dos temperaturas (5 y 40ºC), y se comparó con un tratamiento térmico a 90ºC. Para ello se realizaron los siguientes lotes:

- Salmón sin tratar **(S)**
- Salmón cocinado a 90 ºC **(C)**
- Salmón tratado por alta presión (300 MPa – 5 ºC - 10 min) **(AP5)**
- Salmón recubierto con película de G-L y tratado por alta presión (300 MPa – 5 ºC - 10 min) **(PAP5)**
- Salmón tratado por alta presión (300 MPa – 40 ºC - 10 min) **(AP40)**
- Salmón recubierto con película de G-L y tratado por alta presión (300 MPa – 40 ºC - 10 min) **(PAP40)**

6.2.4.1 Análisis de parámetros físico-químicos

6.2.4.1.1 Análisis del color

El músculo presurizado presentó cambios menos acusados de color con respecto al producto crudo que el músculo tratado por calor. Estos resultados están en concordancia con otros trabajos donde se observó que el pescado sometido a tratamiento térmico adquiere mayor luminosidad y menor tonalidad rojiza que el pescado crudo (Chevalier, Le Bail & Ghoul, 2001). La temperatura de presurización produce diferencias notables en los parámetros de color, dependiendo de si el pescado va recubierto o no por la película. La aplicación de película con alta presión, en especial con el tratamiento a 40ºC, disminuye notablemente el aumento de luminosidad del músculo en comparación con el horneado. Sin embargo, **el tratamiento de película con alta presión a 5ºC preserva mejor el color rojizo característico del salmón**, aunque incrementa ligeramente la tonalidad amarilla.

6.2.4.1.2 Funcionalidad proteica

6.2.4.1.2.1 Solubilidad

La solubilidad proteica es un índice que se utiliza para evaluar el estado funcional de la proteína muscular, asociando la agregación de proteínas con pérdida de solubilidad en solución salina. **La alta presión provoca una disminución de la solubilidad proteica notablemente menor que el tratamiento térmico**, independientemente de la temperatura de presurización o de la presencia o no de la película.

6.2.4.1.2.2 Viscosidad aparente

La viscosidad aparente es un indicador de la calidad funcional del músculo más sensible que la solubilidad de las proteínas (Barroso, Careche, Barrios & Borderias, 1998). La viscosidad disminuyó de forma muy acusada en el músculo procesado (alta presión o calor), debido a fenómenos de agregación proteica, en concordancia con lo observado en la solubilidad. El estudio de viscosidad, con y sin filtración, revela diferencias notables en el tamaño de los agregados proteicos, no perceptibles en el estudio de solubilidad. Así, **el tratamiento térmico produce agregados fuertes y de menor tamaño que el tratamiento de alta presión**. La mayor temperatura de presurización reduce el tamaño de los agregados, sin embargo, la presencia o no de película no induce cambios significativos.

6.2.4.1.2.3 Estudio electroforético

El estudio electroforético de la fracción soluble en sal de las muestras presurizadas revela un mayor número de bandas que en el lote horneado, lo cual concuerda con un menor grado de desnaturalización o agregación proteica. La película no dio lugar a cambios significativos, al contrario que la temperatura de presurización. Las proteínas de peso molecular inferior a 45 kDa (tropomiosina, troponinas, cadenas ligeras de miosina y proteínas sarcoplásmicas) presentan alta resistencia a la presión. Sin embargo, las bandas correspondientes a las cadenas pesadas de miosina, α-actinina y actina pierden intensidad en el músculo presurizado a 40 ^{o}C, indicando que **el tratamiento con alta presión a 5 ^{o}C produce menor agregación proteica que a 40 ^{o}C**.

6.2.4.1.2.4 Espectroscopía FTIR

El músculo crudo presenta mayor capacidad de retención de agua que cualquiera de los procesados, coincidiendo con un pico de absorción de la amida A de mayor amplitud. Se llevó a cabo un estudio en profundidad de la Amida I, dado que se trata de una herramienta muy útil para observar cambios conformacionales en la estructura secundaria de las proteínas miofibrilares (Bertram, Kohler, Böcker, Ofstad & Andersen, 2006; Böcker, Kohler, Aursand & Ofstad, 2008; Jackson & Mantsch, 1995). El pico de absorción de la Amida I sufre un desplazamiento evidente hacia valores más bajos de número de ondas en todas las muestras procesadas, más acusado en el caso de la muestra tratada por calor, lo que confirma una **mayor agregación proteica inducida por calor**. Se realizó una segunda derivada para mejorar la resolución espectral y se observó que la contribución de α-hélices libres (1652 cm^{-1}) mostró un pico de absorción **menor** en el caso de la muestra horneada comparado con la muestra sin tratar, mientras que en los lotes presurizados este pico tuvo **mayor** absorción, en especial en el lote presurizado a 5 ^{o}C sin película. A 1683 cm^{-1}, pico atribuido a estructuras de lámina-β (Carton, Bocker, Ofstad, Sørheim & Kohler, 2009), la tendencia fue similar aunque no se observaron diferencias tan acusadas entre las muestras presurizadas. El incremento de estructuras α-hélice o lámina-β en las muestras presurizadas se atribuye al **despliegue de las cadenas proteicas por el efecto desnaturalizante de la alta presión** (Lullien-Pellerin & Balny, 2002). Por el con-

trario, el espectro IR de la muestra tratada con calor registró mayor intensidad a 1695 cm^{-1},1625 cm^{-1} y 1618 cm^{-1} lo que indica un incremento mayor en la agregación intra e intermolecular de las láminas-β (Carton et al., 2009), en comparación con las muestras presurizadas. En general, **ni la presencia de la película ni la temperatura de presurización indujeron cambios acusados en los espectros de absorción infrarroja**.

6.2.4.1.2.5 Estudio reológico

Por último, el grado de agregación/desnaturalización proteica también se evaluó mediante reología del músculo homogeneizado con NaCl (aspecto de masa en estado de sol). El grado de solubilización del músculo con sal depende del estado general de agregación proteica y va a influir el comportamiento reológico de las masas. Se realizó un primer ensayo de barrido de frecuencias, para evaluar la consistencia y grado de cohesión interna de las distintas masas a temperatura constante de 10 ^{o}C. El comportamiento del módulo elástico *G'* de las muestras presurizadas a 5 ^{o}C fue muy similar al de la muestra cruda, en especial cuando va recubierto por la película. Esto indica que **la muestra recubierta con película y presurizada a 5 ^{o}C es la que presenta menor alteración de la proteína muscular**. En todos los lotes presurizados el módulo elástico fue menor que en el lote horneado, lo cual confirma el **mayor estado de agregación del músculo tratado por calor**. El estudio de la capacidad de agregación térmica de la proteína, realizado a través de un barrido de temperaturas, confirmó el mejor estado funcional de la proteína en el caso de las muestras recubiertas y presurizadas en frío.

6.2.4.1.2.6 Oxidación proteica

El contenido de grupos carbonilo, como medida de la oxidación de las proteínas, también se estudió en todos los lotes. Las muestras procesadas (por alta presión o calor) sufrieron un aumento muy acusado de grupos carbonilo, producto de la oxidación de proteínas. A parte de la proteína miofibrilar, las proteínas hemo también sufren desnaturalización por presión y/o calor, y tornarse más pro-oxidativas, favoreciendo el incremento de grupos carbonilo (Eymard, Baron & Jacobsen, 2009). La muestra recubierta por película y tratada por alta presión a 5 ^{o}C destacó por presentar el contenido más bajo de grupos carbonilo, por lo que **el tratamiento combinado con película y presurización en frío previene en cierta medida de la oxidación proteica asociada al procesado**. Este efecto se debe en parte a la capacidad antioxidante de la lignina, así como a cierta capacidad de barrera frente al oxígeno de la película.

Los resultados de la evaluación de parámetros físicos-químicos del salmón procesado quedan resumidos en la tabla 6. A la vista de los resultados obtenidos, **el tratamiento combinado con película de gelatina – lignina y alta presión en frío induce una menor alteración proteica en las muestras procesadas.**

Tabla 6. Resumen de los parámetros físicos y químicos de los lotes de salmón sometidos a distintos tratamientos (comparación con el lote de salmón crudo).

Técnica	Información relevante	Tratamiento de elección
Colorimetría	La alta presión provoca menos cambios que el tratamiento con calor, en especial a 5 ºC. La película minimiza los cambios en el color.	Alta presión a 5 ºC con película
Solubilidad	La alta presión produce menor agregación proteica que el horneado. La temperatura de presurización y la presencia de película no provocan cambios significativos	Alta presión
Viscosidad aparente	El tratamiento térmico produce agregados fuertes y de menor tamaño que con alta presión. La presurización a 40 ºC induce mayor agregación que a 5 ºC. La película no da lugar a diferencias significativas.	Alta presión a 5 ºC
Estudio electroforético	Menor grado de agregación en los lotes presurizados que en el horneado. La película no origina cambios significativos. A 5 ºC hay menores cambios que a 40 ºC	Alta presión a 5 ºC
FTIR	Las muestras procesadas pierden capacidad de retención de agua. Hay mayores cambios en agregación de estructuras de α-hélices y láminas-β a nivel intra e intermolecular en lote tratado por calor que en lotes presurizados.	Alta presión
Reología	La película reduce notablemente la desnaturalización proteica en los lotes presurizados a 5 ºC, pero menos a 40 ºC.	Alta presión a 5 ºC con película
Oxidación proteica	La alta presión a 40 ºC produce mayor oxidación de proteínas que el horneado, sin embargo, el tratamiento combinado con película y alta presión a 5 ºC produce la menor oxidación.	Alta presión a 5 ºC con película

6.2.4.2 Evaluación de parámetros químicos y microbiológicos durante la conservación

Una vez evaluados los efectos de los distintos tratamientos sobre el músculo de salmón se realizó un estudio de conservación durante 23 días en refrigeración.

6.2.4.2.1 Oxidación lipídica

El tratamiento por calor produce un aumento de la oxidación lipídica mayor que el tratamiento de alta presión, sin embargo, no tiende a aumentar durante el periodo de almacenamiento. Los valores de TBA permanecen bajos en todos los lotes presurizados durante los primeros 15 días debido a que el salmón posee alto nivel de carotenoides, los cuales son un fuerte sistema endógeno antioxidante (Andersen et al., 1990). Al final del almacenamiento, sin embargo, **la oxidación lipídica tiende a aumentar en los lotes presurizados sin película**. Este hecho confirma la hipótesis de que la

alta presión puede favorecer la liberación de los compuestos antioxidantes presentes en la película hacia el alimento.

6.2.4.2.2 pH y NBVT

El pH permaneció prácticamente constante en todos los lotes durante la conservación en refrigeración.

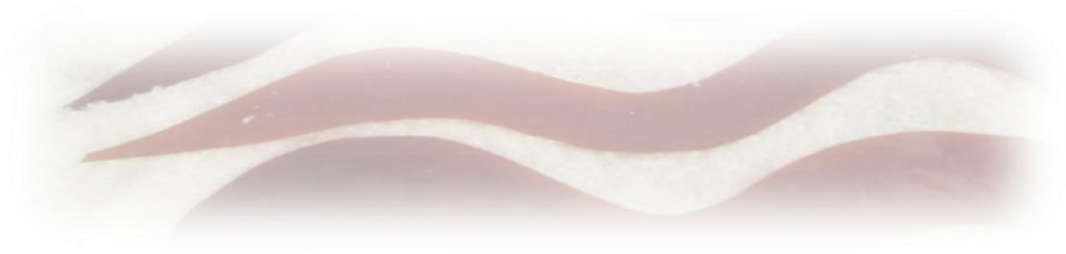
Inicialmente, el tratamiento térmico redujo el Nitrógeno Básico Volátil Total (NBVT), a diferencia de lo ocurrido tras el tratamiento con alta presión, con o sin película, que apenas mostraron diferencias. Este hecho se atribuye a la pérdida en la capacidad de retención de agua y consecuente aumento de exudado debido a la agregación proteica que tiene lugar durante el horneado. Los valores de NBVT se incrementaron en todos los lotes durante la conservación, y el lote AP5 superó los valores máximos autorizados por la Unión Europea para el pescado fresco. **El tratamiento combinado con película de gelatina-lignina y alta presión en salmón redujo eficazmente el incremento en compuestos básicos.** Este hecho también se observó durante la conservación en refrigeración de sardina.

6.2.4.2.3 Evaluación de crecimiento bacteriano

El tratamiento de alta presión redujo la carga microbiana en todos los lotes, en algunos casos incluso por debajo del límite de detección (ej. *Pseudomonas* spp. y *Enterobacteriaceae)*. La flora láctica se redujo en menor medida probablemente por la mayor resistencia de las bacterias Gram-positivas a la presión. Durante la conservación se observó un incremento en el número de microorganismos, y el comportamiento de los lotes fue distinto dependiendo de la temperatura de tratamiento y del tipo de microorganismo. Así, las colonias luminiscentes (presuntos *P. phosphoreum*) y organismos productores de H_2S disminuyeron durante el tratamiento de alta presión a 40 °C posiblemente por su carácter psicrotrofo. A diferencia de lo observado durante la conservación de la sardina, la presencia de película no mejora el efecto del tratamiento de alta presión en el salmón a las temperaturas ensayadas.

El tratamiento térmico y la alta presión con película a 40 °C y 5 °C resultan tratamientos adecuados para aumentar la vida útil de salmón.

7 Conclusiones / Conclusions



7.1 *Conclusiones*

PRIMERA. El lignosulfonato puede incorporarse en una proporción óptima de 80 % gelatina – 20 % lignosulfonato para aumentar la resistencia al agua de películas de gelatina de peces de aguas frías, que son extremadamente solubles, y para mejorar sus propiedades de barrera a la luz con el mínimo perjuicio de sus propiedades mecánicas. Dichas películas, sin embargo, son menos resistentes al agua que las de gelatina bovina independientemente del tipo de lignosulfonato.

SEGUNDA. La utilización de gelatina de peces de aguas cálidas adicionada de lignina L1000, en una proporción de 85 % gelatina – 15 % lignina, origina una película de resistencia al agua comparable a la de gelatina bovina y lignosulfonato, pero de mayor resistencia mecánica, mayor barrera a la luz y menor permeabilidad al vapor.

TERCERA. A nivel estructural, tanto el lignosulfonato como la lignina actúan preferentemente a modo de relleno físico de la matriz de gelatina e interfieren, con mayor intensidad en el caso de la lignina, en la interacción entre las cadenas polipeptídicas, favoreciendo una mayor interacción de las mismas con las moléculas de agua.

CUARTA. La capacidad antioxidante de la lignina L1000 es superior al doble de la de cualquier lignosulfonatos estudiados, pero también lo es su citotoxicidad. No obstante, ambos tipos de compuestos producen películas con alta capacidad antioxidante a concentraciones no citotóxicas, por lo que se estiman potencialmente aptas para uso alimentario, a pesar de no presentar actividad antimicrobiana efectiva.

QUINTA. El lignosulfonato dificulta la tendencia natural de la película de gelatina a sufrir procesos de agregación, razón por la cual las propiedades físicas y antioxidantes de las películas complejas no se modifican sustancialmente al cabo de 28 días de conservación.

SEXTA. El tratamiento combinado de alta presión (300 MPa – 5 ^{o}C – 10 min) y recubrimiento con película de gelatina – lignosulfonato resulta más efectivo que el envasado a vacío para prevenir la oxidación lipídica y el deterioro microbiano del músculo de sardina durante la conservación en refrigeración.

SÉPTIMA. El tratamiento combinado de alta presión (300 MPa – 5 ^{o}C – 10 min) y recubrimiento con película de gelatina – lignina previene parcialmente la oxidación y agregación proteica del músculo de salmón y mantiene de forma más eficaz su color característico, en comparación con un tratamiento térmico convencional.

7.2 *Conclusions*

FIRST. The lignosulphonate may be incorporated in an optimal ratio of 80% gelatin - 20% lignosulfonate to increase the water resistance of cold water fish gelatin films, which are extremely soluble, and to improve their light barrier properties with the minimum damage in their mechanical properties. Such films, however, were less water resistant than those developed from bovine gelatin, regardless of the lignosulphonate type.

SECOND. The use of warm water fish gelatin and lignin L1000, added in a proportion of 85% gelatin - 15% lignin, led to a film with water resistance comparable to that made of bovine gelatin and lignosulphonate, but with higher strength and lower water vapor permeability.

THIRD. Structurally, both lignin and lignosulphonate acted preferably as a physical filler in the gelatin matrix and interfered, with higher intensity in the case of lignin, in the self-aggregation of the polypeptide chains, favoring greater gelatin-water interactions.

FOURTH. The antioxidant capacity of lignin L1000 was approximately 2.5 times higher than any lignosulphonate studied, but also was its cytotoxicity. However, both types of compounds produced films with high antioxidant capacity at non-cytotoxic concentrations, and therefore they were potentially suitable for food use, despite presenting no effective antimicrobial properties.

FIFTH. The lignosulphonate hampers the natural tendency of the gelatin film to suffer aggregation processes, which explained why the physical and antioxidants properties of the complex films did not change substantially after 28 days of storage.

SIXTH. A combined treatment of high pressure (300 MPa - 5 ^{o}C - 10 min) and covering with gelatin- lignosulphonate film was more effective than vacuum packaging to prevent lipid oxidation and microbial spoilage of sardine muscle during chilled storage.

SEVENTH. A combined treatment of high pressure (300 MPa - 5 ^{o}C - 10 min) and covering with gelatin-lignin film partially prevented protein aggregation and oxidation of salmon muscle, and maintained more effectively its characteristic color in comparison with a conventional heat treatment.

8 Bibliografía

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