UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos

TESIS DOCTORAL

Development of edible and active film coatings from marine products
Desarrollo de recubrimientos activos comestibles a partir de productos del mar

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Nuria Blanco Pascual

Directoras

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DEVELOPMENT OF EDIBLE AND ACTIVE FILM COATINGS FROM MARINE PRODUCTS

DESARROLLO DE RECUBRIMIENTOS ACTIVOS COMESTIBLES A PARTIR DE PRODUCTOS DEL MAR

DOCTORAL THESIS

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Department of Nutrition Food-Science and Technology
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Institute of Food Science, Technology and Nutrition
Spanish National Research Council
Instituto de Ciencia y Tecnología de Alimentos y Nutrición
Consejo Superior de Investigaciones Científicas

DEVELOPMENT OF EDIBLE AND ACTIVE FILM COATINGS FROM MARINE PRODUCTS

DESARROLLO DE RECUBRIMIENTOS ACTIVOS COMESTIBLES A PARTIR DE PRODUCTOS DEL MAR

Thesis report submitted by Nuria Blanco Pascual to qualify for the PhD degree at the Complutense University of Madrid
Under the supervision of M. Carmen Gómez Guillén, PhD, and M. Pilar Montero García, PhD INSTITUTE OF FOOD SCIENCE, TECHNOLOGY AND NUTRITION (ICTAN-CSIC)
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Memoria que presenta Nuria Blanco Pascual para optar al grado de Doctor por la Universidad Complutense de Madrid
Bajo la dirección de la Dra. M. Carmen Gómez Guillén y la Dra. M Pilar Montero García, INSTITUTO DE CIENCIA Y TECNOLOGÍA DE ALIMENTOS Y NUTRICIÓN (ICTAN-CSIC)
Madrid, 2013

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CERTIFY/CERTIFICAN:

That the present Thesis Report entitled **"Development of edible and active film coatings from marine products",** submitted by **NURIA BLANCO PASCUAL** to qualify for the **PhD** degree, has been carried out at the Institute of Food Science, Technology and Nutrition, ICTAN (CSIC) under their supervision, and that, once accomplished, they grant their permission to defend the dissertation in a public examination by the corresponding Thesis committee.

Que la presente Memoria titulada **"Desarrollo de recubrimientos activos comestibles a partir de productos del mar"**, presentada por **NURIA BLANCO PASCUAL** 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.

In witness thereof, the parties hereby sign the present document in Madrid on the 2nd of September of 2013.

Y para que así conste a los efectos oportunos, firman la presente certificación en Madrid, a 2 de septiembre de dos mil trece.

Dr. Mª Carmen Gómez Guillén
PhD supervisor/
Directora de la Tesis Doctoral

Dr. Mª Pilar Montero García
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“If you don't know where you are going any road can take you there”

Lewis Carroll, *Alice in wonderland*

A aquellos que forman parte de lo que soy

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TABLE OF CONTENTS

Abbreviations

Abbreviation	Concept
E	Extract
F	Film
FS	Film-forming solution
C	Concentrate/d
S	Solubilized
M	Mastocarpus
L	Laminaria
A	Ascophyllum
H	Hydrolysate
p	phenolic content
c	sodium carbonate extraction
h	sodium hydroxide extraction

Extracts/Films ***Dosidicus gigas***	**Description**
water-S	Muscle protein solubilized in water
salt-S	Muscle protein solubilized in salt
alkaline-S	Muscle protein solubilized at pH10
acidic-S	Muscle protein solubilized at pH3
alkaline-C	Muscle protein solubilized at pH10 and concentrated by isoelectric precipitation
acidic-C	Muscle protein solubilized at pH3 and concentrated by isoelectric precipitation
alkaline-C4	Films stored for 4 months
acidic-C4	Films stored for 4 months

Extracts/Films Seaweeds	Description
M1	Mastocarpus First aqueous antioxidant extraction (4 ºC)
M2	Mastocarpus Second aqueous antioxidant extraction (45 ºC)
M3	Mastocarpus First carrageenan rich extraction (91 ºC)
M4	Mastocarpus Second carrageenan rich extraction (91 ºC)
H	Mastocarpus Phenolic-partially removed hydrolysate (50 ºC)
Hp	Mastocarpus Phenolic-containing hydrolysate (50 ºC)
Lc	Laminaria Sodium carbonate alginate rich extraction (75 ºC)
Lh	Laminaria Sodium hydroxide alginate rich extraction (75 ºC)
Ac	Ascophyllum Sodium carbonate alginate rich extraction (21 ºC)
Ah	Ascophyllum Sodium hydroxide alginate rich extraction (75 ºC)
C	Microcapsules filled by demineralized water
Cp	Microcapsules filled by 1% peptide solution

Concept Unification	
Abbreviation 1 experimental work	**Abbreviation 2 general discussion**
water-E	water-S extract
salt-E	salt-S extract
alkaline-E	alkaline-S extract
acidic-E	acidic-S extract
water-F	water-S film
salt-F	salt-S film
alkaline-F chapter 6	alkaline-S film
acidic-F chapter 6	acidic-S film
alkaline concentrate/extract chapter 7	alkaline-C extract
acidic concentrate/extract chapter 8	acidic-C extract
alkaline-F chapter 7	alkaline-C film
acidic-F chapter 7	acidic-C film
alkaline-F 4	alkaline-C4 film
acidic-F 4	acidic-C4 film
FM3	M3 film
FM4	M4 film
FM3+M4	M3+M4 film
FM3-M4+M1	M3+M4+M1 film
F-Hp0 chapter 9	M3+M4-10 film
F-Hp15 chapter 9	M3+M4-10-Hp15 film
F-Hp30 chapter 9	M3+M4-10-Hp30 film
F chapter 11	Lc-3 film
F-C chapter 11	Lc-3-C film
F-Cp chapter 11	Lc-3-Cp film

Resumen / Abstract [1]

[1] Este breve resumen es parte del cuerpo de la Tesis. El resumen ampliado se presenta al final del manuscrito, en cumplimiento de las directrices de la normativa de desarrollo del Real Decreto 99/2011, de 28 de enero, que regula los estudios de doctorado en la Universidad Complutense de Madrid (UCM) (BOUC nº 14, de 21 de diciembre de 2012) y de acuerdo con las especificaciones establecidas por la Comisión de Doctorado de la UCM.

This short abstract is part of the main Thesis report. The extended abstract is included, at the end of the manuscript, in fulfilment of the directives of the regulation of development of the Real Decreto 99/2011, de 28 de enero, which regulates the studies of doctorate at the Universidad Complutense de Madrid (UCM) (BOUC nº14, de 21 de diciembre de 2012) and in agreement with the specifications established by the Commission of Doctorate of the UCM.

Resumen

En el presente trabajo se desarrollaron películas activas y comestibles de proteínas y polisacáridos, a partir de recursos de origen marino, como son los desechos del procesado industrial de cefalópodos (*Dosidicus gigas*) y algas infrautilizadas (*Mastocarpus stellatus, Laminaria digitata* y *Ascophyllum nodosum)*.

Con este fin, se extrajeron las proteínas musculares de desechos de la retirada de pieles de *Dosidicus gigas,* y extractos no refinados de carragenato y alginato a partir de algas rojas (*Mastocarpus stellatus*) y pardas (*Laminaria digitata* y *Ascophyllum nodosum*). A partir de los biopolímeros extraídos de productos marinos, se desarrollaron películas y se caracterizaron físico-químicamente para explorar sus distintas aplicaciones alimentarias potenciales.

Las proteínas de *D. gigas* se recuperaron mediante dos métodos: i) solubilizando en medio acuoso, salino, alcalino y ácido y ii) concentrando mediante la precipitación isoeléctrica de las proteínas solubilizadas en medio alcalino y ácido. Mientras que solubilizando se consigue un uso integral de la materia prima, concentrando se restringe a las proteínas susceptibles de precipitar, lo cual garantizaría la eliminación de la mayoría de compuestos nitrogenados no deseados. En los medios alcalino y ácido se obtuvieron los más altos rendimientos; y al concentrarlos se recuperaron proteínas estructuralmente mejor preservadas, y por lo tanto más funcionales.

Los extractos solubilizados y concentrados de músculo de *D. gigas* fueron buenas materias primas para el desarrollo de películas comestibles, con total manejabilidad, transparentes y con total absorción de luz ultravioleta. Las películas de extracto solubilizado en agua presentaron alta barrera al agua; si bien estas películas junto con las obtenidas solubilizando con sal, no fueron microbiológicamente estables ni presentaron tan buenas propiedades mecánicas como las elaboradas con extractos obtenidos a pH alcalino o ácido. Los extractos solubilizados en pH alcalino dieron lugar a proteínas parcialmente desnaturalizadas que originaron películas más resistentes al agua, a la tracción y perforación mecánica. Estas propiedades mejoraron mucho con el concentrado alcalino, probablemente debido a un aumento de interacciones proteína-proteína. Los extractos solubilizados en pH ácido sufrieron hidrólisis proteica y formaron películas más plastificadas y deformables. El concentrado ácido ayudó a la estabilidad proteica, mejorando discretamente sus propiedades de barrera al agua. Tras 4 meses de conservación, las películas de concentrado alcalino y ácido sufrieron una agregación proteica que se tradujo en una pérdida de interacciones proteína-proteína y de propiedades en las películas alcalinas y una ligera mejora de las ácidas.

Con el objetivo de aprovechar al máximo todos los componentes de *M. stellatus* y facilitar la obtención y caracterización extractos acuosos e hidrolizados con propiedades activas (antioxidante y antihipertensora); este alga se sometió a dos tipos de extracciones: i) Extracción acuosa escalonada con carragenato solubilizado y ii) Hidrólisis con precipitación de carragenato.

En la extracción acuosa escalonada de *M. stellatus* se obtuvieron dos extractos poliméricos ricos en el híbrido κ/ι-carragenato y en proteínas, con buena capacidad filmogénica: M3 y M4; y dos extractos antioxidantes: M1 y M2. M3 presentó características químicas más parecidas al carragenato comercial,

mientras que M4 presentó un alto contenido en otros compuestos presentes en el alga tales como proteínas. M1 presentó mayores rendimientos de extracción y actividad antioxidante que M2.

En la hidrólisis de *M. stellatus* se obtuvieron dos hidrolizados con actividad antioxidante y antihipertensora: H (con el contenido fenólico parcialmente desechado) y Hp (con contenido fenólico completo). Mientras que H presentó la mayor capacidad inhibidora de la enzima convertidora de angiotensina (ECA), Hp fue el hidrolizado con mayor capacidad antioxidante y mayor rendimiento de extracción.

A partir de los dos extractos ricos en polisacáridos (M3 y M4), se desarrollaron distintas películas. Mientras que M3 fue adecuado para elaborar películas transparentes y flexibles, M4 originó una matriz más resistente al agua y a la fuerza mecánica debido a la mayor proporción de uniones proteína-carragenato. La mezcla de M3 y M4 dio lugar a películas con propiedades intermedias que aumentaron considerablemente su resistencia al agua y mecánica al disminuir la proporción de plastificante en su formulación.

Dado su mayor rendimiento de extracción y su mayor actividad antioxidante, M1 y Hp se seleccionaron como compuestos activos para su incorporación en el desarrollo de películas antioxidantes. M1 y Hp presentaron un alto contenido en compuestos azufrados, lo que podría ser debido a una pequeña fracción de carragenato extraído de manera colateral y/o la presencia de ficobiliproteínas con actividad antioxidante. A pesar de que la actividad antioxidante de M1 fue menor que la de Hp, su incorporación en las películas dio lugar a la mayor actividad antioxidante, probablemente debido a una interacción más eficiente de Hp con las hélices de carragenato y el plastificante que disminuyó su capacidad de interaccionar con agentes oxidantes. La incorporación de M1 mejoró la resistencia al agua y mecánica de las películas y la de Hp tuvo un efecto plastificante que mejoró la elongación por tracción de estas, aunque dependiendo de la proporción de Hp añadida las propiedades filmogénicas se vieron afectadas de forma diferente.

En las algas pardas *L. digitata* y *A. nodosum, s*e diseñó una nueva extracción con hidróxido sódico y se comparó con la tradicionalmente llevada a cabo con carbonato sódico en cada especie. Se analizaron diversos métodos de extracción, con el objetivo de suavizar al máximo las condiciones ácidas del pre-tratamiento y optimizar tanto la temperatura como el tipo de tratamiento alcalino. Estos extractos, además de contener alginato en mayor o menor medida degradado, contendrían la mayor parte del resto de compuestos de cada alga. De cada tipo de extracción, se eligieron aquellos extractos que presentaron mejores rendimientos y capacidad filmogénica.

El tratamiento con NaOH recuperó mayor proporción de alginato no degradado con un alto contenido en unidades de gulurónico, lo cual facilitó la formación de interacciones poliméricas en la película. Sin embargo, la extracción con Na_2CO_3 recuperó un alto porcentaje de alginato degradado y unidades de ácidos urónicos dispersos, lo cual facilitó sus interacciones con el plastificante. El tipo de tratamiento alcalino condicionó las diferencias entre películas como barrera al agua, mientras que la especie de alga influyó en sus propiedades mecánicas. El extracto obtenido con Na_2CO_3 de Ascophyllum dio lugar a películas transparentes y flexibles, mientras que el extracto obtenido con NaOH de Laminaria formó la película más fuerte y menos permeable al vapor de agua.

La presencia de pigmentos como la fucoxantina, y de polisacáridos sulfatados como el fucoidan, confirió actividad antioxidante a las películas. La película obtenida con el extracto de NaOH en Ascophyllum tuvo mayor capacidad antioxidante que el resto de películas desarrolladas a partir de algas pardas, probablemente debido a la presencia de una mayor proporción de compuestos sulfatados. Esta actividad destacó por ser parecida a la obtenida en Mastocarpus al añadir hidrolizado antioxidante en su fórmula.

Dado que la adición directa de hidrolizados en películas comestibles puede ir en detrimento de sus propiedades filmogénicas, se planteó un nuevo método de incorporación de péptidos activos extraídos de las túnicas de *D. gigas* usando la técnica de microencapsulación por inyección, mediante impresión en una solución de ácido esteárico y cera de carnauba, siguiendo el modelo *core-shell.* Se obtuvo una eficiencia de encapsulación del 84.7 ± 3.4% y con una ratio de material de encapsulación:péptido de 13,3:1. Las cápsulas fueron homogéneas, con un diámetro de 110-140 µm, y más estables a bajas temperaturas y pH 7. Se desarrolló un método de inclusión durante la elaboración de películas de extracto en Na_2CO_3 de Laminaria evitando la aglomeración y rotura de las microcápsulas, obteniendo películas más resistentes y deformables a la tracción mecánica y más impermeables al vapor del agua.

Todas las películas desarrolladas en el presente trabajo, podrían ser susceptibles de formar parte de la presentación final de productos alimenticios, ya sea como parte del envase o como ingrediente propio del diseño del alimento.

Abstract

The present work has studied the development of edible and active protein and polysaccharide films from resources of marine origin, such as waste from the cephalopod processing industry (*Dosidicus gigas*) and underutilized seaweeds (*Mastocarpus stellatus, Laminaria digitata* and *Ascophyllum nodosum)*.

With this aim, muscle proteins recovered from the waste resulting from the *D. gigas* skinning process, and carrageenan and alginate unrefined extracts from red (*Mastocarpus stellatus*) and brown (*L. digitata* and *A. nodosum*) seaweeds were obtained. Films were developed from the biopolymers extracted from marine products, and they were physicochemically characterized to explore for different potential applications in the food industry.

D. gigas proteins were recovered by two methods consisting in: i) solubilization under aqueous, salt, alkaline and acidic conditions, and ii) concentration by isoelectric precipitation of the proteins solubilized under alkaline and acidic conditions. Whereas an integral use of the raw material is achieved with the solubilization method, the concentration method is restricted only to those proteins susceptible to precipitation, which would guarantee the removal of most of the undesirable nitrogenated compounds. Alkaline and acidic conditions maximized the extraction yields; and when extracts were concentrated, more structurally preserved proteins, and thereby more functional, were recovered.

Both solubilized and concentrated extracts obtained from *D. gigas* muscle were good raw materials for edible film development, absolutely easy to handle, transparent, and with total ultraviolet light absorption. The water solubilized extract films showed a high water barrier, although these films, together with those obtained by salt solubilization, failed to be microbiologically stable and did not result in as good mechanical properties as those made of extracts obtained under alkaline or acidic pH.

Alkaline solubilized extracts elicited partially denatured proteins which resulted in films with more water resistance, tensile and puncture strength. These properties were considerably improved in alkaline concentrated films, probably due to an increased number of protein-protein interactions. Acidic solubilized extracts underwent protein hydrolysis and resulted in more plasticized and malleable films. The acidic concentrated extraction contributed to protein stability and slightly improved the film water barrier properties. After 4 months of storage, alkaline and acidic concentrated films suffered protein aggregation, which entailed a loss of protein-protein interactions to the detriment of the properties in alkaline films, and a slight improvement of the acidic films properties.

With the aim of exploiting to the maximum all the *M. stellatus* components and helping to obtain and characterize aqueous extracts and hydrolysates with active properties (antioxidant and antihypertensive), two different types of extractions were performed: i) Stepped aqueous extraction with solubilized carrageenan, and ii) Hydrolysis with carrageenan precipitation.

M. stellatus stepped aqueous extraction resulted in two polymer extracts, rich in κ/ι-carrageenan hybrid and proteins, with a good filmogenic capacity: M3 and M4; and two antioxidant extracts: M1 and M2. M3 showed chemical characteristics resembling commercial carrageenan, while M4 presented a high

content of other seaweed compounds such as proteins. M1 showed higher extraction yields and antioxidant activity than M2.

Two antioxidant and antihypertensive hydrolysates were obtained in the *M. stellatus* hydrolysis: H (with partially removed phenolic contents) and Hp (with full phenolic contents). Whereas H showed the highest angiotensin-converting enzyme (ACE) inhibitory capacity, Hp presented the highest antioxidant capacity and extraction yield.

Based on the two extracts rich in polysaccharides (M3 and M4), different films were developed. While M3 was suitable to develop transparent and flexible films, M4 produced a matrix with more water resistance and mechanical strength due to a higher proportion of protein-carrageenan bonds. The M3 and M4 mixture resulted in intermediate film properties that considerably increased its mechanical strength and water resistance when lowering the plasticizer ratio in the formula.

Given their higher extraction yield and antioxidant activity, M1 and Hp were selected as active ingredients for antioxidant film development. M1 and Hp presented a high sulfated compounds content, which might be due to a small carrageenan fraction extracted collaterally, and/or the presence of phycobiliproteins with antioxidant activity. Even though M1 antioxidant activity was lower than Hp's, its inclusion in the films resulted in the highest antioxidant activity, which was probably due to a more efficient interaction between Hp peptides and the film's carrageenan helices and plasticizer, lowering its capacity to react with oxidant agents. The inclusion of M1 in the films improved their mechanical strength and water resistance; and Hp addition had a plasticizing effect that improved their tensile elongation, but depending on the Hp ratio included the filmogenic properties were affected in a different way.

A new alkaline extraction, using sodium hydroxide, was designed in *L. digitata* and *A. nodosum* brown seaweeds, and it was compared in each species with the traditional sodium carbonate extraction procedure. With the purpose of softening as much as possible the pretreatment acidic conditions and optimizing both the temperature and the type of alkaline treatment, various extraction procedures were analysed. Apart from an alginate content degraded to a greater or lesser extent, these extracts would also contain most of the rest of each seaweed compounds. From each extraction method, those extracts presenting the best yields and filmogenic capacity, were selected.

A higher proportion of non-degraded alginate with a high content in guluronic units was recovered with the NaOH treatment, which contributed to form polymer interactions in the film. However, a high percentage of degraded alginate and dispersed uronic acid units was recovered with Na_2CO_3 extraction, which contributed to their interaction with the plasticizer. The type of alkaline treatment determined the water barrier differences among films, while the seaweed species affected the mechanical properties of the films. The Ascophyllum Na_2CO_3 extract resulted in transparent and flexible films, while the Laminaria NaOH extract produced the strongest and least water vapour permeable film.

The film antioxidant activity was conferred by the presence of pigments like fucoxanthin and sulfated polysaccharides such as fucoidans. The film obtained with the Ascophyllum NaOH extract had the highest antioxidant capacity among all the brown seaweed films developed, probably due to the presence of a higher proportion of sulfated compounds. This activity was emphasized for its resemblance to that previously reported in Mastocarpus films when adding antioxidant hydrolysate to the formula.

Since the direct addition of hydrolysates in edible films could be detrimental to their filmogenic properties, a new method of incorporation of bioactive peptides extracted from *D. gigas* tunics was applied by using the microencapsulation inkjet technology, through printing in a stearic acid and carnauba wax solution, following the core-shell model. An encapsulation efficiency of 84.7 ± 3.4% and a ratio of 13.3:1 microencapsulation material:peptide were obtained. The microcapsules were homogeneous, with a 110-140 μm diameter, and more stable at low temperatures and pH 7. In order to avoid microcapsule agglomeration and breakage during the development of films from Laminaria Na_2CO_3 extract, a new and specific film inclusion procedure was developed, obtaining higher water vapour impermeability, a better tensile strength, and more malleable films.

All films developed in the present work might be susceptible of use in the final food serving presentation, whether as part of the packaging material or as an ingredient by itself in the food design.

I. Introduction

1.1. Exploitation of marine resources

1.1.1. Introduction

The development of different strategies for a sustainable use of live marine resources has been one of the world's latest concerns, not only focussing on the importance of the natural resources exploitation to satisfy human necessities, but also in the maintenance of their potentiality for future generations (Caddy & Griffiths, 1995).

Spain is a fishery country, with the highest European fishery production (14% of the total), mainly dedicated to human consumption (40 kg/person/year in Spain, while in Europe the average is 20 kg/person/year), but typical resources have been decreasing for years. 80% of the capture is sold in the market as fresh fish, but the processing industry has gained weight in the latest years (FAO 2010), which has entailed different consequences.

Moreover, in the Spanish Atlantic coast, many different seaweed species are currently harvested for industrial purposes; however they are underutilized due to the fact that the direct consumption is limited. On the other hand, seaweed phycocolloid extraction does not consider the extraction of other nutrients in parallel, since it is not focussed on making direct use of both the phycocolloids and the nutrients of the algae (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010; Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Pereira, Critchley, Amado, & Ribeiro-Claro, 2009; Rupérez & Toledano, 2003).

1.1.2. Fishing Industry Impact

1.1.2.1. Processing Industry

The fishery sector has different problems such as an over-exploited aquifer, restrictions and dependence on foreign fishing grounds, and contamination. Nowadays, a lot of waste is produced in the processing industry of sea products, mostly protein that is normally recovered to produce animal feed and fertilizers, or directly dismissed as waste. This material is a source of environmental pollution and due to its ecological, economic and social-cultural impact, there is a need to increase its exploitation (Cuclas, 1997).

Environmental regulations are progressively getting stricter, and so it is going to result impossible to maintain the current waste level generated in the future. The fish processing industry needs to be capable of producing high quality byproducts under controlled environmental-friendly methods, taking advantage of the waste recovery no matter what destination, either for human consumption or not.

Solid residues can reach 50-75% of the catch (Shahidi, 1994) and the use of the same for food applications would result in a greater efficiency and less dependency on capture fishery products and byproducts. In order to obtain value-added products and to eliminate the pollution generated from discarded material, the waste from the fishing industry has been studied to obtain different edible raw materials, such as collagen, gelatine, surimi, hydrolysates and lipids (Rustad, Storrø, & Slizyte, 2011), to ensure an added value and to minimize discards during processing.

1.1.2.2. *Dosidicus gigas*

Cephalopod catches have considerably increased from 2002 to 2008, being Spain, Italy and Japan the largest consumers and importers of these species. The jumbo squid (*Dosidicus gigas*) (Fig. 1) is the largest and most abundant squid species found in the pelagic zone of the eastern Pacific, from the coast of Chile to Oregon (Nigmatullin, Nesis, & Arkhipkin, 2001), and it is usually imported to Spain due to its high percentage of edible portion (60-80%) and its suitability for industrial processing either fresh, sliced in fillets or separated by parts (tentacles, mantle…), canned, frozen (the cephalopod as a whole or in parts), yielding byproducts such as strips and rings from *surimi* or mince.

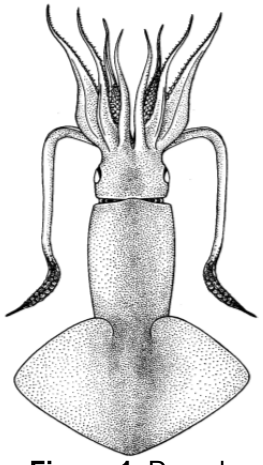

Figure 1. Dorsal view of *Dosidicus gigas.* Species Catalogue FAO *1984. # 125,* VOL. 3.

However, the byproducts obtained from squid processing, including viscera, mantles and tentacles among others, represent up to 60% of the total weight. Fortunately, a number of studies have reported this waste as an excellent raw material to obtain important co-products with high commercial value such as gel-based products (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; De la Fuente-Betancourt, García-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sánchez, 2009), gel-emulsions (Felix-Armenta, Ramírez-Suarez, Pacheco-Aguilar, Diaz-Cinco, Cumplido-Barbeitia, & Carvallo-Ruiz, 2009), *surimi* (Campo-Deaño, Tovar, Jesús Pombo, Teresa Solas, & Javier Borderías, 2009), and also other collagen-based products from skins and tunics (Denavi, Pérez-Mateos, Anon, Montero, Mauri, & Gómez-Guillén, 2009; Giménez, Alemán, Montero, & Gómez-Guillén, 2009; Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011).

D. gigas muscle differs in its structure from fish and mammals muscles in that it is surrounded by several sheets of connective tissue (tunics) and composed of 75-84% of water, 13-22% of crude protein, 0.1-2.7% of lipids, and 0.9-1.9% of minerals (Sikorski & Kolodziejska, 1986). During the mantle skinning, a great amount of muscle (~30-40%) is discarded, being necessary to develop methods for protein recovery and to promote its use.

Muscle proteins from *D. gigas* mantle contain three main fractions: sarcoplasmic (about 15% of the total), collagen (about 3%) and myofibrillar (about 80%) (Sikorski & Kolodziejska, 1986). These muscle proteins can be solubilized by different procedures, such as a simple water homogenization due to its high protein solubility (~85%) (De la Fuente-Betancourt, García-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sánchez, 2009), under low ionic strength (Sánchez-Alonso, Careche, & Borderías, 2007), and under acidic (1-3) or alkaline (9-11) pH conditions (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009).

D. gigas contains about 37% of non-proteinaceous compounds included in its estimated nitrogenous components, which are mainly physiological trimethylamine oxide, metabolism products

(NH_4Cl), and other amines (Marquez-Rios, Moran-Palacio, Lugo-Sánchez, Ocano-Higuera, & Pacheco-Aguilar, 2007; Sikorski & Kolodziejska, 1986).

Myosin is the most important protein in the giant squid myofibrillar fraction, followed by actin and paramyosin. *Dosidicus gigas* myosin has a low stability due to its high enzymatic activity, especially under acidic pH, and paramyosin can represent 25% of the myofibrillar portion (Sikorski & Kolodziejska, 1986).

Obviously, the yield of the myofibrillar recovery could be affected by other factors, for example the raw material conditions (proteolysis degree, ammonia compounds), the protein extraction methods, and so on. The high metalloprotease content (Campo-Deaño, Tovar, Jesús Pombo, Teresa Solas, & Javier Borderías, 2009; Gómez-Guillén, Hurtado, & Montero, 2002; Moral, Morales, Ruíz-Capillas, & Montero, 2002) might be useful for promoting protein autohydrolysis, and the resulting water soluble compounds susceptible to be recovered could be used as food ingredients (Lian, Lee, & Park, 2005). It is well known that the presence of hydrolyzed proteins in *D. gigas* muscle results in a quality devaluation, thereby constituting an abundant resource susceptible to be used in the development of co-products.

The ability to solubilise fish myofibrillar proteins under extreme acidic (pH 2-3) (Hultin & Keelleher 1998a) or alkaline (pH 10.5-11.5) (Hultin & Keelleher 1998b) conditions, in combination with a subsequent isoelectric protein precipitation (pH 4.8-5.5) leads to the highest recovery of highly functional myofibrillar proteins, discarding most of the sarcoplasmic and non-proteinaceous content. There are not many studies regarding cephalopod protein recovery (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009), and it is important to take into account the different protein behaviours depending on the species, given that muscle protein solubilities are also different. For example, *D. gigas* muscle is totally soluble under low ionic strength (Borderías, Careche, & Sánchez Alonso, 2005) while Todadores needs a really high ionic strength (Moral, Morales, Ruíz-Capillas, & Montero, 2002).

It would be interesting to study the myofibrillar protein recovery from *D. gigas* muscle adhered to the mantles as a waste in the processing industry; and to carry out the analysis of its filmogenic capacity, due to its potential as raw material for coating applications. So far, muscle and/or myofibrillar protein films from *D. gigas* have never been developed before despite they would represent an interesting alternative use; and no information regarding its film forming capability, or the adequate extraction methods to obtain the film properties of interest, is currently available.

1.1.3. Seaweeds

1.1.3.1. Seaweeds: An underused resource

Seaweeds have been traditionally incorporated into Pacific and Asian foods for hundreds of years, but they were not included in western diets until a few years ago (Rinaudo, 2008). In recent decades, and due to the algae richness in polysaccharides (high fibre content), proteins (Fleurence, 1999), minerals and vitamins (Mabeau & Fleurence, 1993), their low lipid content (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010), and their ability to generate a wide range of secondary metabolites with biological activity(Plaza, Cifuentes, & Ibáñez, 2008), there has been an increase in the direct

consumption of seaweeds as food products and in their use to obtain functional compounds (Plaza, Cifuentes, & Ibáñez, 2008; Shahidi, 1994). Moreover, seaweeds are an excellent source of bioactive substances like sulfated-polysaccharides, peptides, polyphenols and pigments with biological activities, such as antioxidant (Cardozo, Guaratini, Barros, Falcão, Tonon, Lopes, et al., 2007; Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012).

Regarding their colour, macroalgae are classified in three types: red (Rhodophyceae), brown (Phaeophyceae) and green (Chlorophyceae) seaweeds. Both red and brown seaweeds are the most commonly used for human consumption, being considered novel foods in Spain, and, in general, lacking specific European regulations (Gómez Ordóñez, 2013).

The Spanish coasts are full of different red and brown underutilized algae species, which are easy to cultivate and present rapid growth and a production susceptible to be controlled by manipulating the cultivation conditions. *Mastocarpus stellatus* (Rhodophyceae), *Laminaria digitata* and *Ascophyllum nodosum* (Phaeophyceae) are some of the most abundant species existing in the north-western Spanish coast, and unlike other countries where they are a source of wealth for many companies, their exploitation is still far away from its real potential. In Europe, algae are traditionally used for the production of additives (phycocolloids) or in animal nutrition, but rarely promoted for integral consumption due to their strong sea-flavour. Apart from their high mineral and protein contents or the functional properties of their polysaccharides, these organisms have shown the potential to be natural sources of ingredients with different biological activities, making it necessary to study new ways of production at an industrial scale and, if possible, of developing an integral extraction procedure (Plaza, Cifuentes, & Ibáñez, 2008).

The development of a more appropriate, fast, cost-effective and environmental-friendly extraction process to isolate different compounds, together with a more attractive food presentation format, would be desirable in order to promote seaweed consumption and novel applications of the different compounds.

On the other hand, human life styles and eating habits have led to increasing cases of chronic diseases, and to a growing interest in developing health products. Seaweeds could constitute an excellent raw material for this purpose due to their previously described useful properties (Ferraces-Casais, Lage-Yusty, de Quirós, & López-Hernández, 2012; Gupta & Abu-Ghannam, 2011; Holdt & Kraan, 2011). Moreover, the therapeutic properties of the seaweeds would make them a suitable source for the development of new functional food products (Mohamed, Hashim, & Rahman, 2012).

Figure 2. *Mastocarpus stellatus* (A) *Laminaria digitata* (B) and *Ascophyllum nodosum* (C) seaweeds.

Among the most common and consumed Spanish algae species (Fig. 2), red seaweeds as *Mastocarpus stellatus* and brown seaweeds as *Laminaria digitata* and *Ascophyllum nodosum* (though the latest is consumed in a lesser extent) are the most representative for their abundance in the Atlantic coast and their low heavy metal content, well within the human consumption limits (Holdt & Kraan, 2011).

1.1.3.2. Seaweeds: Chemical composition

1.1.3.2.1. Phycocolloids

The most important characteristic of algae is their high fibre content, in which the soluble fraction is mainly composed of phycocolloids. Phycocolloids are special high molecular weight polysaccharides produced by several seaweed species, stored in the cell walls, which are normally extracted to be used as thickeners or gelling polymers. Each seaweed type differs from the others in their specific cell wall structural and storage polysaccharides composition. Rhodophytas contain neutral sugars such as agar, carrageenan and a few other sulfated polysaccharides, while Phaeophytas produce laminaran, fucoidan and alginate (Rinaudo, 2008). Soluble fibres are known for having a beneficial effect on the health of the stomach, the faecal bulking capacity and the digestive transit, which indirectly would help to prevent gastrointestinal chronic ailments (Mohamed, Hashim, & Rahman, 2012). A high dietary fibre intake can help to reduce the incidence of diabetes, heart disease, and the risk of cancer, while phycocolloids have shown hypocholesterolaemic activity (Jiménez-Escrig & Sánchez-Muñiz, 2000).

1.1.3.2.1.1. Carrageenan

Carrageenans are high molecular weight sulfated D-galactans composed of repeating disaccharide units alternating 3-linked β-D-galactopyranose and 4-linked α-D-galactopyranose or 4-linked 3,6-anhydrogalactose, depending on the source and extraction conditions. Carrageenans are classified according to their sulfation patterns and the presence of α-D-galactopyranose or 4-linked 3,6-anhydrogalactose units (Jiao, Yu, Zhang, & Ewart, 2011).

There are 15 different carrageenan structures, being kappa (κ), iota (ι) and lambda (λ) forms the most relevant. The κ-carrageenan is produced by alkaline elimination from its precursor μ-carrageenan (-(1⟶3)-β-D-galactopyranose-4-sulfate-(1⟶4)-3,6-anhydro-α-D-galactopyranose-(1⟶3)-). The ι-carrageenan has an additional sulfate group on C2 (O), resulting in two sulfates per disaccharide repeating unit, and it is produced by alkaline elimination from its precursor ν-carrageenan (-(1⟶3)-β-D-galactopyranose-4-sulfate-(1⟶4)-3,6-anhydro-α-D-galactopyranose-2-sulfate-(1⟶3)-). The λ-carrageenan is converted into the alternative form θ-carrageenan (theta-carrageenan) by alkaline elimination, but at a much slower rate than that causing the production of ι-carrageenan and κ-carrageenan, and has three sulfate groups per disaccharide unit with the third one at the C6 position of the 4-linked residue (-(1⟶3)-β-D-galactopyranose-2-sulfate-(1⟶4)-α-D-galactopyranose-2,6-disulfate-(1⟶3)).

Natural carrageenan is typically present as mixtures of different hybrid types, such as κ/ι-hybrids, k/μ-hybrids or ν/ι-hybrids (Fig. 3) (Jiao, Yu, Zhang, & Ewart, 2011).

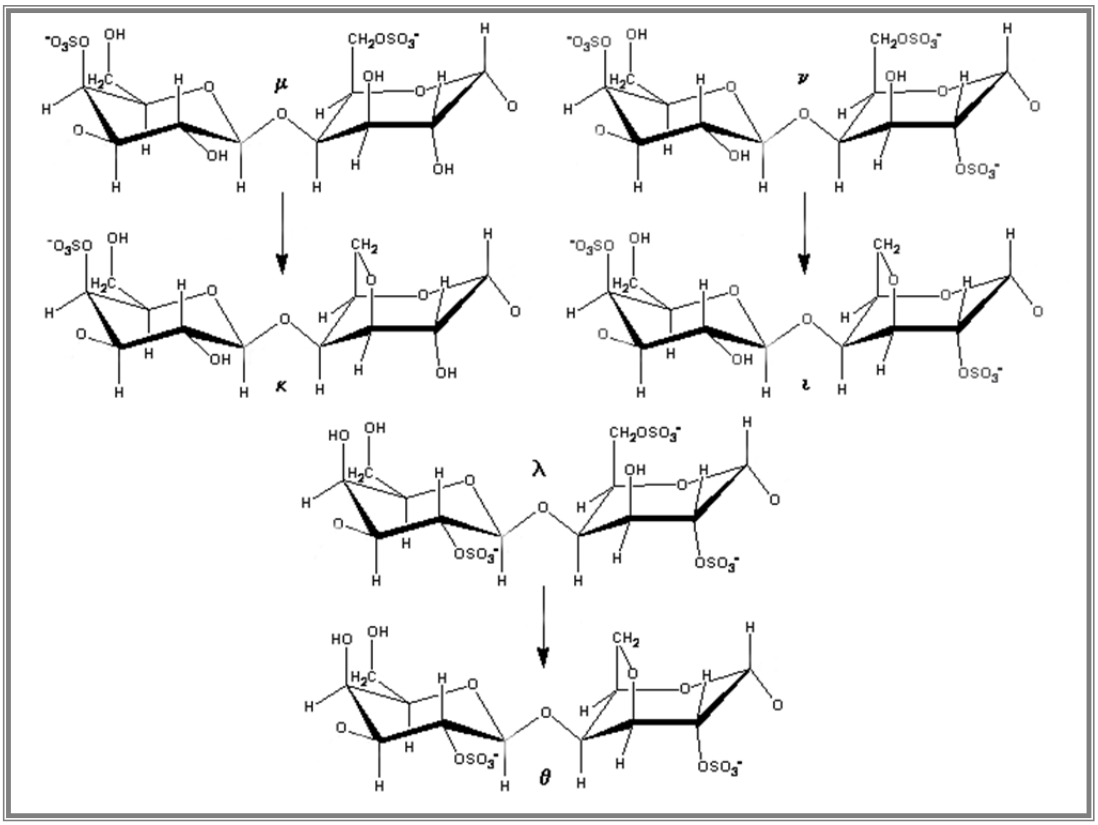

Figure 3. Structural formula of μ, κ, ν, ι, λ and θ-carrageenan.

Carrageenans are highly flexible molecules which wind around each other to form double-helical structures. These double-helical structures confer good functional properties, being the gelling capacity the most highlighted among others (Hilliou, Larotonda, Sereno, & Gonçalves, 2006; Ridout, Garza, Brownsey, & Morris, 1996). Gel formation, in κ- and ι-carrageenans, involves a first stage of helix formation over the cooling of a hot solution (Fig. 4), followed by a second stage of gel-induction and gel-strengthening aided by K^+ or Ca^{2+} cations respectively, which do not only contribute to helix formation, but also support the subsequent formation of aggregating linkages between the helices, originating the junction zones (Fig. 5).

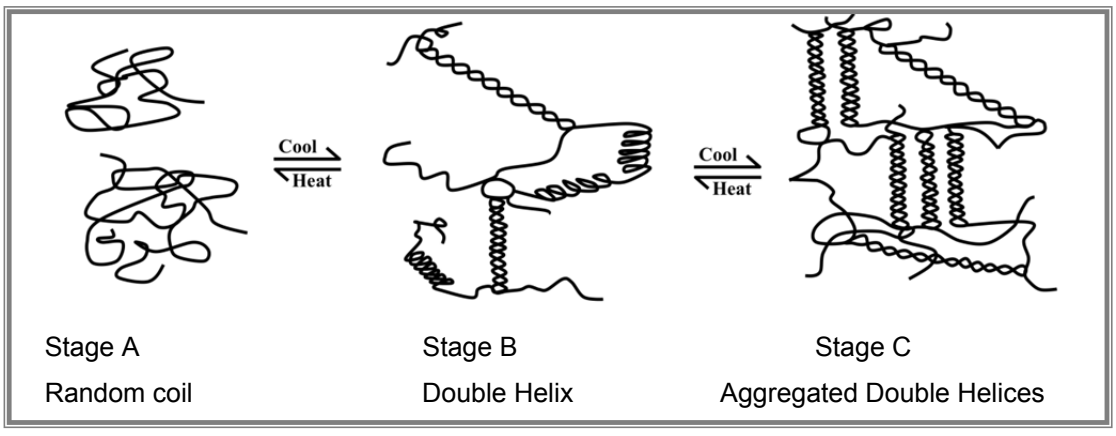

Figure 4. Models of conformational transition of κ-carrageenan and ι-carrageenan (Wu & Imai, 2012).

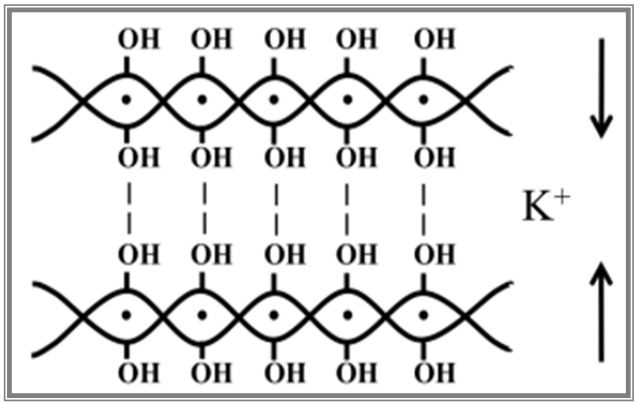

Figure 5. The gelation mechanism of κ-Carrageenan cross-linked by K^+ ions (Wu & Imai, 2012).

Depending on their composition and configuration, carrageenans present different rheological properties as thickeners, gelling agents and stabilizers widely applied in the food, pharmaceutical and cosmetic industries. κ and ι-carrageenan are gelling agents, while λ-carrageenan is a thickener. The κ-type forms gels that are hard, strong and brittle, whereas ι-carrageenan forms soft and weak gels (Van De Velde, Peppelman, Rollema, & Tromp, 2001). The lack of linkage in the anhydrogalactose residue of μ and ν-carrageenan gives rise to different conformations, which difficult gel formation (Pereira & Van De Velde, 2011).

Carrageenans have also shown good filmogenic properties (Han & Kim, 2008; Karbowiak, Ferret, Debeaufort, Voilley, & Cayot, 2011), which will be more extensively mentioned in the following sections.

1.1.3.2.1.2. Alginate

Commercial alginate is normally found in two forms, alginate salt from the sodium salt extraction (more stable and soluble at pH 5.5-10), or transformed into alginic acid (less stable) by treatment with diluted acidic solutions (Rinaudo, 2008).

Alginates are linear block copolymers formed by 1,4-linked β-D-mannuronic acid and α-L-guluronic acid (G). These two uronic acid units form three types of blocks. G blocks contain L-guluronic units, M blocks contain D-mannuronic units and MG blocks are formed by alternation of both units (Leal, Matsuhiro, Rossi, & Caruso, 2008).

Due to their linear structure and their high molecular weight, alginates can form strong films and good fibres when solidifying, being their gel formation capacity their main characteristic (Draget, Skjåk-Bræk, & Stokke, 2006; Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013). M blocks form linear chains, while G blocks form loop structures. Thereby, two G block chains aligned side-by-side would result in a diamond structure with a gap suitable to accommodate Ca^{2+} and form a dimeric gel structure (Fig. 6) ("egg box model" proposed by Grant, 1973).

According to the G and M blocks proportion, alginate gel properties will be different, being M/G ratio a common parameter used for characterization. The calcium complex formation not only depends on the composition (M/G ratio) and distribution of M and G units in the chains, but also on the stiffness of the alginate chains (Rinaudo, 2008). Generally, low M/G ratios lead to stronger and more rigid gels, while a high M/G ratio results in more elastic and soft structures (Draget, Skjåk-Bræk, & Stokke, 2006). This is

the reason why alginates have the ability to retain water and are used as stabilizers, thickeners and gelling agents in the food industry.

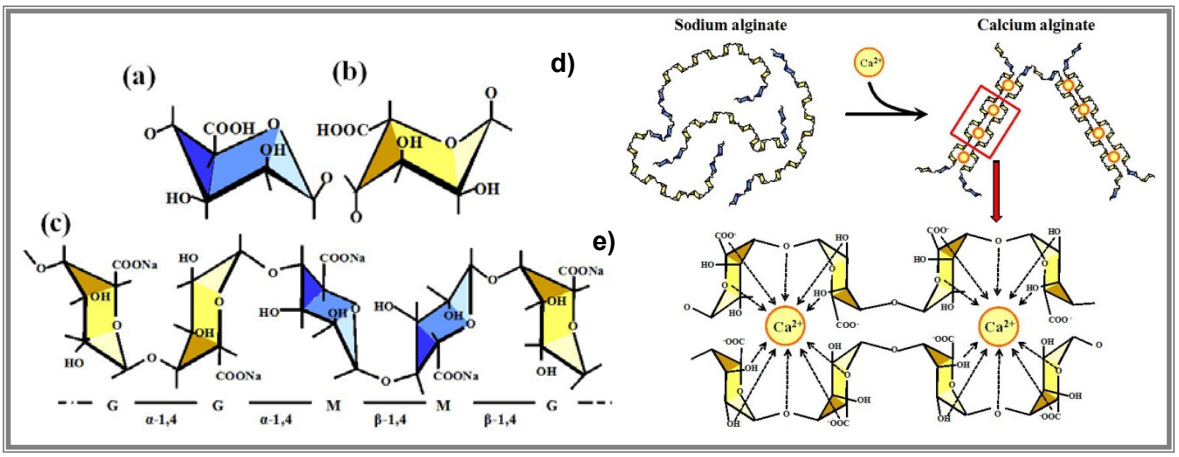

Figure 6. Alginate composition: (a) β-D-mannuronic acid. (b) α-L-guluronic acid. (c) Structural formula of sodium alginate molecule, (d) gelation of α-L-guluronic acid junction with calcium ions and (e) binding of divalent cations to alginate: the "Egg-box" model (Kashima & Imai, 2012).

Regarding alginate film forming properties, there are many studies about alginate film development with and without cation complexation (Ca^{+2}) (Nakamura, Nishimura, Hatakeyama, & Hatakeyama, 1995; Russo, Malinconico, & Santagata, 2007), and also as an ingredient in composite films (Paşcalau, Popescu, Popescu, Dudescu, Borodi, Dinescu, et al., 2012; Salmieri & Lacroix, 2006).

1.1.3.2.2. Fucoidans and laminarans

Fucoidans are sulfated polysaccharides (fucans) from brown algae that can reach 40% of their weight (Holdt & Kraan, 2011). Despite there are several studies about fucoidans composition (Li, Lu, Wei, & Zhao, 2008), some particularities remain unknown. Fucoidans consist in a fucose backbone according to which they are classified into two groups. One group includes the fucoidans from *Laminaria digitata* and have (1-3)-linked α-L-fucopyranose residues as their central chain, while the second group, where *Ascophyllum nodosum* is included, have repeating (1-3)- and (1-4)-linked α-L-fucopyranose residues (Jiao, Yu, Zhang, & Ewart, 2011).

Laminarans are the main storage polysaccharide in brown seaweeds, being abundant in *L. digitata* and *A. nodosum* species (36%) (Devillé, Gharbi, Dandrifosse, & Peulen, 2007; Holdt & Kraan, 2011). Formed by (1-3)-β-D-glucose units with different (1-6)-β-ramifications (Rioux, Turgeon, & Beaulieu, 2007a), they are mainly used as food fibre (Devillé, Gharbi, Dandrifosse, & Peulen, 2007).

Nevertheless, no bibliographic references have been found regarding the potential technological applications of fucoidans and laminarans.

1.1.3.2.3. Other compounds

Algae also contain a multitude of bioactive compounds that might have antioxidant and antihypertensive properties (Plaza, Cifuentes, & Ibáñez, 2008). These active compounds are mainly

pigments (e.g. phycobilins and fucoxanthin), minerals and peptides that have shown benefits against degenerative diseases (Mohamed, Hashim, & Rahman, 2012).

Regarding the protein content, the higher amount has been found in green and red seaweeds (10-47%), compared to brown seaweeds (3-15%), representing aspartic and glutamic acids the largest part of the amino acid fraction. Rhodophytas specifically contain a high amount (1-10%) of phycobiliproteins (bonded to phycoerythrin and phycocyanin pigments) (Heo, Park, Lee, & Jeon, 2005; Lordan, Ross, & Stanton, 2011) as part of the phycobilins, which have shown bioactivity as antioxidants (Fleurence, 1999; Mohamed, Hashim, & Rahman, 2012).

Seaweeds are also an important source of different minerals (8–40% content) such as sodium, potassium, calcium, magnesium and phosphorous and other micronutrients, which might contribute to their food applications; and carotenoids, especially fucoxanthin and β-carotene in brown seaweeds and α-tocopherol in red seaweeds, which would improve their antioxidant character (Holdt & Kraan, 2011).

Seaweeds are considered low fat raw materials (<4%) and polyphenols have shown very low concentrations in green and red seaweeds (<1%) (Mabeau & Fleurence, 1993) compared to brown seaweeds (<14%) (Holdt & Kraan, 2011).

1.1.3.3. Seaweeds: Extraction procedures

The traditional commercial carrageenan extraction is normally carried out under strong alkaline conditions, at near boiling point temperature, for several hours (Rinaudo, 2008); but it has been observed that using only high temperatures (85 ºC) during 3 h the lower molecular weight “native” phycocolloid was extracted at pH 7 (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009).

Commercial alginate extraction is traditionally carried out by using sodium carbonate to obtain sodium alginate, or by acidic dilution to obtain alginic acid (Rinaudo, 2008), and at temperatures that can vary from 21 ºC (Vauchel, Kaas, Arhaliass, Baron, & Legrand, 2008) to 70 ºC (Rioux, Turgeon, & Beaulieu, 2007b).

Seaweeds have proven to be a good peptide and polyphenol source (Patel, Pawar, Mishra, Sonawane, & Ghosh, 2004; Plaza, Cifuentes, & Ibáñez, 2008; Rupérez, 2002), and it would be very interesting to develop an extraction procedure in which not only phycocolloids were extracted, but also other compounds that in previous studies have shown antioxidant activity, such as lipophilic compounds like chlorophylls and carotenoids or hydrophilic compounds like polyphenols (Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001). So far, most of the studies have been performed at a laboratory level, and there is no known industrial scale extraction procedure.

Sulfated-polysaccharides, peptides and polyphenols have shown biological activities in previous studies, such as antioxidant and antihypertensive (Cian, López-Posadas, Drago, Sánchez De Medina, & Martínez-Augustin, 2012; Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011; Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012; Ngo, Wijesekara, Vo, Van Ta, & Kim, 2011). In general, sulfated polysaccharide (carrageenan and fucoidan) bioactivity is related to the still not well established interaction between different factors such as sulfation degree, sulfate groups distribution and molecular weight (Jiao,

Yu, Zhang, & Ewart, 2011; Li, Lu, Wei, & Zhao, 2008). However, carrageenan antioxidant properties appeared to be positively correlated to the sulfate content (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

The cell wall rigidity and structural complexity of the seaweeds represent a major obstacle for the efficient extraction of the intracellular bioactive constituents. Food processing could transform seaweeds into food products having a specified nutritional and sensorial quality, making them more available to consumers. pH and temperature variations usually lead to structure modifications that may sometimes not be reversible, but that can favour other characteristics.

Rhodophytas have a high protein content, mainly composed of bioactive phycobiliproteins (Heo, Park, Lee, & Jeon, 2005) and other wall proteins that might be more efficiently extracted by an enzymatic treatment (Joubert & Fleurence, 2008; Martínez, Sánchez, Ruíz-Henestrosa, Rodríguez Patino, & Pilosof, 2007). Hydrolysis has also previously resulted in both antihypertensive and antioxidant extracts (Cian, Martínez-Augustin, & Drago, 2012; Wang, Jónsdóttir, Kristinsson, Hreggvidsson, Jónsson, Thorkelsson, et al., 2010).

It has also been reported that the extraction of bioactive compounds from several brown algae may be improved by enzymatic extraction (Heo, Park, Lee, & Jeon, 2005), and that byproducts obtained from alginate extraction, such as fucoidan and laminaran, have also shown antioxidant activity (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

Depending on the extraction procedure, the percentage of compounds extracted, their integrity and composition can be modified. In some cases, specific extraction conditions can favour the extraction of one element, but damage other compounds integrity, affecting their activity or functionality; however, in other cases, more extreme conditions are needed to obtain all the compounds of interest. For example, the customary carrageenan extraction is usually performed at 85 ºC during 3 h (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009), which would damage the possible bioactivity of other compounds; hence carrageenan extraction should be modified in order to allow the subsequent extraction of bioactive compounds.

It would be interesting and necessary to combine a series of extraction methods to progressively extract one by one all the compounds of interest, and thereby achieve a total seaweed extraction for different food technological applications, increasing the commercial phycocolloid extraction yields (20-30%) (Istini, Ohno, & Kusunose, 1994), and offering alternative seaweed food presentations and applications that might boost their consumption.

Conventional water and solvent extractions usually have low selectivity, low extraction efficiency, and pose the problem of the solvent residue (Herrero, Cifuentes, & Ibáñez, 2006). A good technological alternative would be the hydrolytic enzyme-assisted seaweed extraction. The application of enzymatic digestions to proteins and/or polysaccharides can improve the extraction yield and the release of secondary plant metabolites and, depending on the component to be extracted, different enzymes would be utilized (Wang, et al., 2010). Hydrolytic extraction would disrupt the cell wall structure, increasing the extraction of complex storage materials inside the same, and also the cell wall protein release, which is

commonly difficult to accomplish due to the presence of the cell wall anionic polysaccharides (Joubert & Fleurence, 2008; Martínez, Sánchez, Ruíz-Henestrosa, Rodríguez Patino, & Pilosof, 2007). Therefore, the benefits of hydrolysis would be the protein digestibility improvement, the enhancement of the bioactive activity with the breakdown of high-molecular-weight polysaccharides and proteins (Siriwardhana, Kim, Lee, Kim, Ha, Song, et al., 2008), the total yield extraction increase, and the contribution to the extraction of substances with antioxidant properties or functional biopeptides (Fleurence, 1999).

1.1.3.4. Seaweeds as functional food ingredients and technological applications

Apart from their antioxidant activity, seaweed hydrolysates have aroused great interest due to the fact that they have shown ACE (angiotensin-1-converting enzyme) inhibitory activity (Mohamed, Hashim, & Rahman, 2012). The ACE is involved in the blood pressure regulation and its inhibition helps to reduce hypertension, cardiovascular disease risk and other related conditions.

There are currently many applications of these viable compounds isolated from algae, and focus is being given to controlled cultivation to produce valuable new products at a large scale. The main polysaccharide applications are as a source of dietary fibre (Jiménez-Escrig & Sánchez-Muñiz, 2000), and hydrocolloid cosmetic, pharmaceutical and food industrial applications (Lordan, Ross, & Stanton, 2011). Bioactive carotenoids, polyphenols, minerals, vitamins and fatty acids play an invaluable role in the drug discovery process, demonstrating the promising pharmaceutical application of their ability to produce metabolites (Cardozo, et al., 2007) due to their reported bioactivity (Lordan, Ross, & Stanton, 2011).

The food addition of edible seaweed-derived ingredients might be a good strategy to introduce new food products in the functional food market. The functional food concept lies in the improvement of the general body condition and the reduction of illness and disease risk, providing health benefits beyond the basic nutritional value (Shahidi, 2004).

The expression *"Seaweed is more than the wrap that keeps rice together in sushi"* (Holdt & Kraan, 2011) perfectly describes the actual algae situation. Either as an *haute cuisine* ingredient, in a nutraceutical format or as coating material, seaweeds extracts might constitute a suitable raw material for the design of new food products such as edible films, and for getting consumers to approach seaweeds consumption. At the moment, both alginate and carrageenan films have been widely studied (Campos, Gerschenson, & Flores, 2011), but there are no previous studies describing a total seaweed use for film development, or presenting edible films as a possible way of algae consumption.

1.2. Edible and Biodegradable films

1.2.1. Films and coating materials

Packaging plays an important role in the food industry, since it is responsible for, not only containing food products, but also their preservation and quality maintenance, their appearance and convenience, and for protecting the food and providing the product information to the consumer (European Parliament and Council Directive 94/62/EC).

The most common food packaging materials are glass, wood, metal, plastics, paper and other flexible packages such as coatings and adhesives. Synthetic plastic packaging materials are not biodegradable and, consequently, they represent an environmental issue, not only because of their manufacturing work and incineration, but also due to their rapid production and waste accumulation (Raheem, 2013). Altogether, and in combination with the possible migration of non-desirable or even toxic compounds, such as additives or monomers (Nerín de la Puerta, 2009), from the plastic to the food product, this has led to the emergence of a great social-political interest in the development of novel materials alternative to the traditional plastics, wherein biopolymers extracted from natural sources constitute a good option to obtain less contaminant and biodegradable food packaging.

The bibliography widely discloses two edible and/or biodegradable packaging types: films and coatings (Petersen, Væggemose Nielsen, Bertelsen, Lawther, Olsen, Nilsson, et al., 1999). The main difference between films and coatings is that films are stand-alone, formed separately from anything and not dependent on their intended use. Films can be used as solid covers, wraps or separation layers. On the contrary, coatings refer to the formation of a film directly over the surface of the final product, so that it becomes part of it, and it may improve or protect said final product (Gennadios, 2002). This work memory will focus on film development; therefore, further information will be specifically about films.

Lately, there has been an increasing research interest in the development of edible and/or biodegradable film components from different materials such as proteins, polysaccharides, lipids, and resins, and for different applications in the food processing, pharmaceutical and agricultural industries (Kester & Fennema, 1986). Among these materials, some of them have been extensively studied because of their relative abundance, film forming abilities, and nutritional qualities.

It is currently difficult to think about edible films as substitutes of non-edible packaging materials, but they might have complementary applications.

On the other hand, the combination of science and gastronomy in the last few years has opened the possibility of making high quality and healthy food for consumers, while optimizing the creativity to produce an innovative food design. Scientific packaging solutions could be used to solve practical problems, from an industrial point of view, but also to create food opportunities for edible films in the *nouvelle cuisine* (Arboleya, Olabarrieta, Luis-Aduriz, Lasa, Vergara, Sanmartín, et al., 2008) or as a substrate to entrap nutraceutical compounds (Cuq, Aymard, Cuq, & Guilbert, 1995; Gupta & Abu-Ghannam, 2011; Ngo, Wijesekara, Vo, Van Ta, & Kim, 2011; Rustad, Storrø, & Slizyte, 2011).

1.2.2. Edible and/or biodegradable film components

1.2.2.1. Edible and/or biodegradable film structural matrix

For film development, it is at least necessary a structural matrix component, which can be obtained from any animal or vegetable origin, such as the cephalopod industry waste or the underused seaweeds previously referred to.

The varied nature of the compounds that may be used as structural matrix, which are mainly of protein, polysaccharide and lipid origin, is responsible for the final film characteristics.

Therefore, films can be classified based on their formulation as:

- Hydrocolloids
  - Proteins
  - Polysaccharides
- Lipids

1.2.2.1.1. Hydrocolloids

Hydrocolloids have good aptitude to form a cohesive and continuous matrix with adequate mechanical properties due to their polymer chains association by hydrogen bonds, favoured by their chemical structure (Campos, Gerschenson, & Flores, 2011). The most common hydrocolloids used as biopolymers are proteins and polysaccharides. Mixtures of both are frequently used for film formulation. The formation of a hydrocolloid structural matrix requires a total or partial solubilization that enables the breakage of low energy intermolecular native bonds to provide a new polymer chain orientation and interactions with the rest of the film-solution components (Cuq, Gontard, Cuq, & Guilbert, 1998).

1.2.2.1.1.1. Protein films

Proteins can be of animal (albumin, whey proteins, gelatine, collagen, sarcoplasmic and myofibrillar proteins, etc.) or vegetable (wheat gluten, zein, soy protein, etc.) origin. Due to their high intermolecular binding potential, protein-based films can have good gas barrier, optical and mechanical properties compared to those made with polysaccharides and fat-based, but poor water vapour resistance (Campos, Gerschenson, & Flores, 2011; Krochta & DeMulderJohnston, 1997).

Proteins are formed by polar and non-polar amino acids that are able to form intermolecular linkages resulting in three-dimensional macromolecules matrixes stabilized by different interactions (electrostatic bonds, hydrogen bonds, covalent bonds, disulfide bonds, and Van der Walls forces) that are usually highly moisture sensitive (Krochta & DeMulderJohnston, 1997; Vroman & Tighzert, 2009).

Protein type defines matrix development. High molecular weight and fibrillar proteins (myofibrillar, collagen) normally result in wide nets with good mechanical properties, while low molecular weight and globular proteins (sarcoplasmic, soy) lead to more compact and less elastic and resistant nets (Gennadios, 2002).

In order to enhance matrix interactions, different strategies can be followed. For example, proteins with different characteristics can be mixed, like gelatine fibrillar protein and soy extracted globular protein, adding up their best features (Denavi, Pérez-Mateos, Anon, Montero, Mauri, & Gómez-Guillén, 2009). Another example would be the higher or lower sarcoplasmic protein presence in muscle protein films, which would modify the resulted film rheological and barrier properties; however results would be more dependent on the species than on the muscle composition itself (Artharn, Benjakul, & Prodpran, 2008; Sobral, dos Santos, & García, 2005).

Protein secondary, tertiary and quaternary structures can also be modified by physical and chemical agents (temperature, pH), changing their properties and sometimes optimizing protein configuration and interactions. These changes will condition protein film development (Gennadios, 2002).

Despite muscle proteins are likely to be used to develop novel restructured food products, edible films with muscle proteins have also been studied as a new possible use over the last few years.

1.2.2.1.1.1.1. Muscle protein films

Muscle proteins have been recovered lately from the fishing industry waste, and studied due to their film-forming ability and nutritional value (Hamaguchi, Weng, & Tanaka, 2007). At the moment muscle protein films have been developed from different marine species such as the Indo-Pacific blue marlin (*Makaira mazara*) (Hamaguchi, Weng & Tanaka, 2007), the round scad (*Decapterus punctatus*) (Artharn, Benjakul & Prodpran, 2008) or the squid (*Todadores pacificus*) (Leerahawong, Arii, Tanaka, & Osako, 2011). Remarkable differences would depend on the species and the protein myofibrillar/sarcoplasmic proportion (Artharn, Benjakul, & Prodpran, 2008).

1.2.2.1.1.2. Polysaccharide films

Hydrosoluble polysaccharides are normally used as stabilizers and gelling agents in the food industry, and have several origins such as, cellulose, starch, pectins, arabinoxylan and alginate and carrageenan gums (Guilbert & Gontard, 2005).

The properties of polysaccharide films depend on the polysaccharide structure, such as the hydrogen bonding level, molecular weight and polarity. They range in structure from linear to highly branched, repeating different monosaccharide building blocks, neutral (agar), negatively (carrageenan, alginate) or positively (chitosan) charged. The structural characteristics will define their solubility, gel capacity, other compounds compatibility (polysaccharides, proteins, minerals, lipids…) and filmogenic capacity.

As proteins, polysaccharide films normally have a good gas barrier capacity and moderate mechanical properties, but due to their hydrophilicity (high solubility and poor water vapour permeability) they do not present good water barrier properties (Kester & Fennema, 1986), being even less water resistant than protein films.

Biomacromolecules extracted from seaweeds, such as alginates, form strong and quite brittle edible films with poor water resistance; while carrageenan edible films have been less studied, forming fragile structures that are more commonly used as food coating materials (Campos, Gerschenson, & Flores, 2011).

1.2.2.1.1.2.1. Seaweed extracts films

Alginates and carrageenans, as biological polymers, and due to their gel formation capacity, have been widely exploited and studied regarding their good film forming ability (Hambleton, Perpiñan-Saiz, Fabra, Voilley, & Debeaufort, 2012; Han & Kim, 2008; Karbowiak, Hervet, Léger, Champion, Debeaufort, & Voilley, 2006; Nakamura, Nishimura, Hatakeyama, & Hatakeyama, 1995; Siddaramaiah, Swamy, Ramaraj, & Lee, 2008), but they are not the only compounds susceptible to be extracted and used in both red and brown algae.

It would be interesting to find new seaweed food applications through the development of improved seaweed extraction procedures resulting in a higher diversity of extracted compounds, and the production of novel effective filmogenic materials.

1.2.2.1.2. Lipid films

Waxes and fats were the first biopolymers used to cover food and extend preservation times (Gontard, Thibault, Cuq, & Guilbert, 1996). Currently, lipids are widely used either alone or in combination with other polymers as edible packaging, forming hydrophobic films used as barriers.

Lipid films display a different barrier efficiency depending on their chemical composition, polar groups presence, carbohydrated chain length and insaturation or acetylation degree, being ordered by decreasing effectiveness as waxes, shellacs, fatty acids and alcohols, acetyl glycerides, cocoa compounds and derivatives (Debeaufort & Voilley, 2009).

Despite lipid films are good water barriers because of their low polarity, they are also brittle and thick, not easy to stick onto hydrophilic surfaces, and sometimes showing a low gas permeability (Bourtoom, 2008).

Although lipids, such as waxes and fatty acids, have been mainly used as edible coating constituents, they do not have a good stand-alone filmmaking capacity; hence they are normally supported on a polysaccharide matrix. Lipids are added to hydrocolloid-based films to improve their characteristics such as the visual appearance or, most importantly, their water barrier characteristics (Campos, Gerschenson, & Flores, 2011).

1.2.2.1.2.1. Wax films

Natural food grade wax coatings are commonly found on cheese, vegetables and fruits surfaces to prevent moisture loss during the dry season, being insect waxes like bee wax, and plant waxes like candelilla and carnauba wax (which are permitted food additives in the European Union (E901-903)) examples of the most used for industrial applications (Milanovic, Manojlovic, Levic, Rajic, Nedovic, & Bugarski, 2010).

Waxes are interesting due to their good water barrier, their easiness to handle and biocompatibility properties; but they are also brittle and provide a candle-like flavour, thereby presenting difficulties in their food applications (Bhoyar, Morani, Biyani, Umekar, Mahure, & Amgaonkar, 2011). One of their possible uses might be as micro-encapsulation agents for spicy flavourings or active substances (Tharanathan, 2003). Concretely, carnauba wax is a very versatile material in the preparation of capsules (Fini, Cavallari, Rabasco Álvarez, & Rodríguez, 2011; Stojaković, Bugarski, & Rajić, 2012) and it would be interesting to carry out further studies about its food applications.

Biodegradable biopolymer mixtures can improve their intrinsic properties, such as their mechanical or water barrier characteristics. At the moment, many different copolymer blends of protein-polysaccharide, protein-lipid and polysaccharide-lipid nature have been described (Krochta & DeMulderJohnston, 1997). These combinations can be made by direct incorporation using an emulsion,

suspension or dispersion depending on the material, applying different layers or using different solvents to make them miscible (Bourtoom, 2008).

For example, lipid addition to hydrocolloid films can improve their oxygen and water barrier properties (Greener Donhowe & Fennema, 1992; Phan The, Debeaufort, Voilley, & Luu, 2009), or different hydrocolloid combinations could improve the mechanical properties of the films (Wang, Liu, Holmes, Kerry, & Kerry, 2007), providing many other different potential applications and film development possibilities.

1.2.2.2. Edible and/or biodegradable film additives

Edible films allow the addition of ingredients that sometimes improve their functionality, nutritional quality, or even their flavour and texture among others. Specifically, in film development, plasticizers are considered to play an important role, but there are many other additives that can determine the final application of edible films.

1.2.2.2.1. Plasticizers

Hydrocolloid films are normally brittle and barely flexible, therefore bringing up the need of addition of a plasticizer to improve their properties (Gennadios, 2002). Plasticizers reduce intermolecular forces and favour polymer chains mobility, therefore improving mechanical properties such as flexibility and extension capacity (Banker, Gore, & Swarbric.J, 1966). The plasticizer must be compatible with the main matrix component, being the most used ones low molecular weight compounds like polyols (sorbitol, glycerol, polyethylene glycol, and other glycerol derivatives), mono-, di- or polysaccharides and, sometimes, lipids.

As many authors have stated, the plasticizing effect is more evident when the plasticizer molecule is smaller and more hydrophilic (Gontard, Guilbert, & Cuq, 1993; Sobral, dos Santos, & García, 2005), being necessary to restrict its use, since an excessive addition can negatively affect the film barrier and mechanical properties.

Glycerol is the most common plasticizer utilized in film-forming solutions to prevent film brittleness (Karbowiak, Hervet, Léger, Champion, Debeaufort, & Voilley, 2006) and, due to its humidity absorption, it is normally used in combination with sorbitol to improve the resulting film mechanical and barrier properties at the expense of a loss of flexibility (Gennadios, 2002).

1.2.2.2.2. Other additives

Apart from plasticizers, other additives may be also added to the film forming solution in order to improve its technological properties, such as anti-sticking agents, wetting agents and surfactants (Huber, 2009). Surfactants are normally added to stabilize emulsions or different nature biopolymer mixes, and also as anti-foaming agents during the film making process (Huber, 2009; Kulkarni, Tomšič, & Glatter, 2011).

Likewise, there are also additives that are used to improve the flavour and nutritional properties of the films, like flavourings, colourants, nutrients and even nutraceuticals. Some additives are bioactive and

can confer said bioactivity to the film. The addition of these bioactive compounds can result in functional films, with antioxidative and antihypertensive properties that might improve their own preservation time, and even be transmitted to the food products.

1.2.3. Film applications and functionality

Films can play different roles useful within food products, that is act like a barrier, improve their quality and/or properties, protect specific parts, or work as food adhesives or additive carriers, among others (Debeaufort, Quezada-Gallo, & Voilley, 1998; Kester & Fennema, 1986).

As barriers, films have been studied for the prevention of the inside-outside transfer of different compounds such as moister regulators, gas scavengers (O_2, CO_2), the release or absorption of flavours and odours, solids (fats, carbohydrates and minerals) and radiations (UV, visible light) (Debeaufort, Quezada-Gallo, & Voilley, 1998; Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999).

Films can be part of the final products and used to improve their aspect, protect them from mechanical damages and also work as carriers of different active additives (Debeaufort, Quezada-Gallo, & Voilley, 1998).

Consumers are currently looking for minimally processed foods with natural substances that will provide health benefits and maintain good nutritional and sensory characteristics, being the addition of bioactive substances in edible film coatings a possible industrial application (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011).

The transport and release of different active compounds (antioxidants, antihypertensive, etc.) are important aspects to be studied in the development of edible films. Nowadays, microencapsulation is considered a possible solution for the transport and controlled release of functional and bioactive compounds in different conditions (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011).

1.2.3.1. Incorporation of bioactive compounds in films

Natural biodegradable polymers are much less stable than most synthetic materials, due to their sensitivity to microbial attacks under specific conditions. The possibility of including bioactive compounds could improve their preservation time and quality by addition of their activities.

Lately, there has been an increasing interest in finding naturally occurring compounds with activities as antioxidants, for example, as alternatives to synthetic products. Some of the natural antioxidant sources that have been studied are the aquatic plants (Wang, et al., 2010).

In the last decade, the new marine bioprocess technologies have allowed the isolation of substances with antioxidative properties or functional biopeptides by enzymatic hydrolysis (Fleurence, 1999). Concretely, seaweeds have proven to be a good peptide and polyphenol source (Patel, Pawar, Mishra, Sonawane, & Ghosh, 2004; Plaza, Cifuentes, & Ibáñez, 2008; Rupérez, 2002).

Antioxidants have been widely used as food additives both to improve lipid oxidation stability and to extend the product shelf life. But antioxidants can also be incorporated into edible films for polymer stabilization and protection from degradation.

The production of peptides through protein hydrolytic reactions seems to be one of the most promising techniques to obtain proteinaceous antioxidants, since peptides normally show higher antioxidant activity than proteins (Elias, Kellerby, & Decker, 2008). Recently, these hydrolysates have been incorporated in the development of biodegradable films to improve their functionality and physical properties (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009; Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010; Ikawa, Schaper, Dollard, & Sasner, 2003; Mokrejs, Janacova, Svoboda, & Vasek, 2010), and the potential use of films as antioxidant releasing packages, capable of improving food preservation, would be a rather interesting application.

Marine organisms represent a valuable source of new active substances in the field of the development of bioactive products. The molecular diversity of different marine peptides has been described, and information about their biological properties and mechanisms of action has also been provided (Aneiros & Garateix, 2004). Bioactive hydrolysates have been isolated from a variety of species of macroalgae, among other marine species, and they have been used to develop nutraceutical products with antihypertensive activity (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011; Lordan, Ross, & Stanton, 2011).

Due to the increase observed in the prevalence of chronic diseases during the last decade, mainly caused by oxidative stress and hypertension, a number of investigations have aimed at the identification of bioactive compounds from natural sources that have shown to be effective in preventing such ailments. These compounds would have a potential use as bioactive ingredients that could be incorporated into functional foods (Tierney, Croft, & Hayes, 2010). Functional foods, nutraceuticals and other natural health products are connected with health promotion and normally entail some difficulties to be developed. For example, the direct inclusion of bioactive compounds in the films carries different disadvantages such as the non-controlled release, the lack of control of the dosage and local effects, and the bioactive agent interactions with other film matrix components (Martín-Belloso, Rojas-Graü, & Soliva-Fortuny, 2009). Moreover, the stability of the functional compounds can be affected both by the processing conditions and the storage time (Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999), being the bioactive compound encapsulation a good alternative to preserve their activity during the film processing and storage.

On the other hand, from the technological point of view, the addition of some bioactive compounds to the filmogenic solution has shown detrimental to the filmogenic properties, therefore having some limitations (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009). Capsules might be a good carrier for the incorporation of a variety of compounds (bioactive compounds, minerals, flavours, etc.) in the films, without affecting so negatively the resulting filmogenic properties.

1.2.3.2. Microencapsulation of bioactive compounds

For many years, encapsulation has been used in the pharmaceutical industry, but its application in the food industry would be useful too. Encapsulation would consist in the incorporation of food ingredients in small capsules. Bioactive food components are subject to rapid inactivation or degradation. Encapsulation can be employed to improve the stability of the bioactive compounds under adverse environmental conditions (moisture, heat and other extreme conditions), and also the viability of the bioactive compounds by favouring their retention in the food product during the processing or storage,

and/or providing a controlled release of the encapsulated material under pre-established conditions (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011; Gibbs, Kermasha, Alli, & Mulligan, 1999; Milanovic, Manojlovic, Levic, Rajic, Nedovic, & Bugarski, 2010).

The bioactive components can be lipids, vitamins, peptides, fatty acids, antioxidants, minerals and also living cells like probiotics (de Vos, Faas, Spasojevic, & Sikkema, 2010). Peptide issues such as allergenicity or bitter off-flavours, as well as the capacity to alter the final food product texture and colour still need to be addressed (Elias, Kellerby, & Decker, 2008), but anyway, microencapsulation might be a good alternative. Microencapsulation would solve many limitations to the use of bioactive additives like peptides in different food products as it might mask undesirable flavours and reduce undesirable properties such as high volatility, hygroscopicity or reactivity with other food compounds (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011). The encapsulation procedure will depend on the type of peptide, its envisioned effect and the product that will serve as microcapsule vehicle (de Vos, Faas, Spasojevic, & Sikkema, 2010).

The most common encapsulation technologies are coacervation, spray drying, spray cooling, fluid bed coating and extrusion, and other more expensive technologies like liposome and cyclodextrin encapsulation. None of them can be considered suitable for application to any bioactive compound, because each bioactive compound has its own molecular structure which will define its polarity, solubility, etc. (de Vos, Faas, Spasojevic, & Sikkema, 2010).

The encapsulation procedure will be chosen depending on its compatibility with the bioactive component, the protection from degradation it offers to keep the bioactive compound functionality, and the packaging load efficiency. Unlike the pharmaceutical industry, food manufacturing costs will also affect the methodology elections since they need to be reduced as much as possible (de Vos, Faas, Spasojevic, & Sikkema, 2010; Gibbs, Kermasha, Alli, & Mulligan, 1999).

For example, **coacervation** requires lower levels of food ingredients, which reduces the cost. Coacervation is an efficient method that consists on emulsifying a material such as flavour oil into a dissolved gelling protein of an opposite charge, so a complex is formed (Bakan, 1969). **Spray drying** is a core dissolved in a dispersion of matrix material that is subsequently atomized in heated air, being an economical and effective method, where specialized equipment is not required. Spray drying is widely employed for flavours and dehydration such as powdered milk production (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007; Shahidi & Han, 1993). **Spray cooling/chilling** is a technology in which the spray drying opposite principle is applied, and usually has as outer material vegetable oils having different applications like dry soup mixes, high fat content foods and bakery products. Spray cooling/chilling has the disadvantage of special handling and storage conditions requirements (Gibbs, Kermasha, Alli, & Mulligan, 1999). The **extrusion** has the advantage of the total isolation by the wall material and is normally used for dry food applications such as drinks, cakes, cocktail and gelatine dessert mixes; and also to encapsulate flavours (Risch, 1988). **Fluidized bed** encapsulation is applicable to hot-melt coatings (oils, stearines, fatty acids, emulsifiers and waxes) or solvent-based coatings (starches, gums, maltodrextrins) to produce fortified foods, nutritional mixes and dry mixes (Gibbs, Kermasha, Alli, & Mulligan, 1999). **Liposomes** are dispersions of polar lipids in aqueous solutions that have more versatile properties and a lower fragility than capsules made of fat with other techniques.

Liposome encapsulation has also a high efficiency, it is easy to scale up and uses mild conditions (Mayer, Bally, Hope, & Cullis, 1986). And the encapsulation **in cyclodextrin** is used to envelop molecular structures by forming molecular inclusion complexes with a hydrophobic centre and an outer hydrophilic part (de Vos, Faas, Spasojevic, & Sikkema, 2010).

Microcapsules can be made of different encapsulating materials such as fats, starches, dextrins, alginates, proteins and lipids. There are also different encapsulation forms like for example a simple membrane coating (a wall), a multiwall structure (with the same or varying compositions) or numerous cores within the same walled structure; and microcapsules can be spherical or irregular shaped (Fig. 7) (Gibbs, Kermasha, Alli, & Mulligan, 1999).

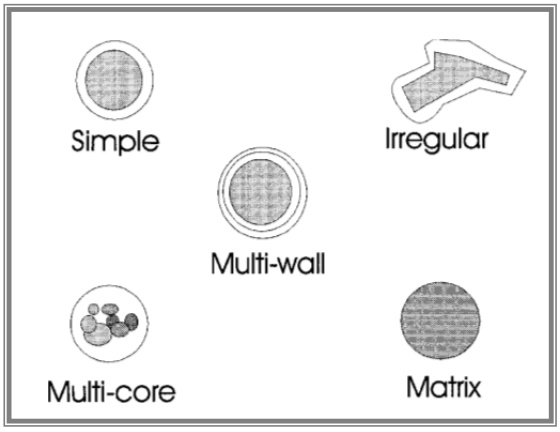

Figure 7. Different forms of capsules (Gibbs, Kermasha, Alli, & Mulligan, 1999).

Microencapsulation applications have shown a slow increase since the method could be too expensive and highly specific. Nevertheless, due to the fact that production volumes have increased in the last years, more cost-effective techniques have been developed, significantly increasing the number of encapsulated food products. New markets are being developed as new advances in encapsulation are reached. The high cost of production and the lack of food grade available materials are the main limitations of the current encapsulation methods, making necessary the development of new procedures (Gibbs, Kermasha, Alli, & Mulligan, 1999) to minimize these restrictions.

New inkjet and **printing** technologies have been developed in the last few years with the objective to lead to new and improved products (Houben, 2012). Inkjet heads have been used to print all kinds of materials, like monomers or metals. But inkjet technology can also be applied to the food industry as an alternative to conventional spray-drying and for the encapsulation of flavours and nutrients. This encapsulation printing technology (Houben, Rijfers, Brouwers, Eversdijk, & Van Bommel, 2009) would allow manufacturers to reduce their energy consumption and might be quite suitable for optimising the processing of sensitive ingredients. Encapsulation printing gives the possibility to convert liquids into powders and separate core and shell flows (Fig. 8). Compared to the rest of microencapsulation methods, this technique presents advantages such as the continuity and the mildness of the process, the wide range of materials that can be processed (aqueous, oils/waxes, polymers, solutions, dispersions) and the quality of the final products that are usually mono-dispersed core-shell microcapsules.

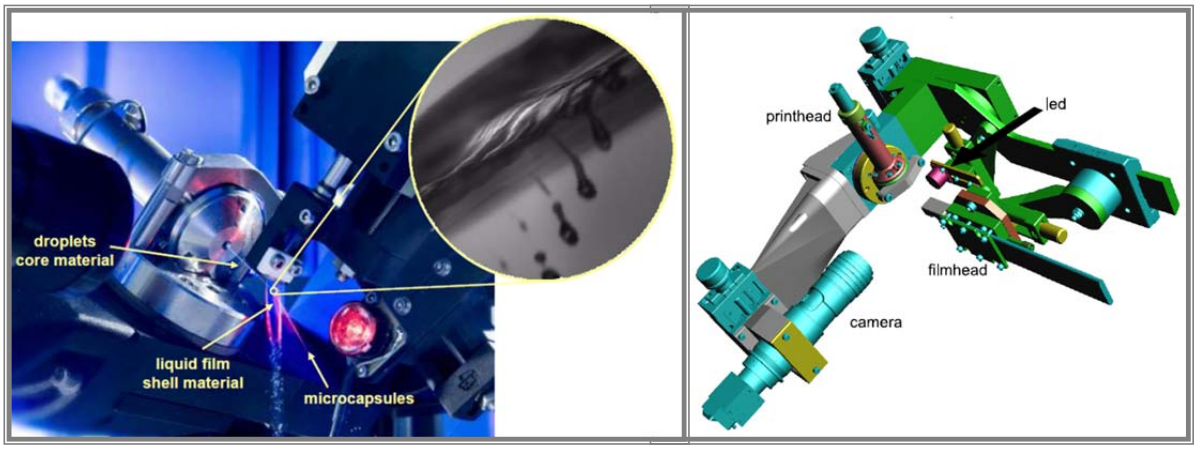

Figure 8. Encapsulation printer (picture provided by TNO, photography by Verse Beeldwaren, Eindhoven). Encapsulation of micron sized droplets 3D scheme setup (RBJ Koldeweij, University at Twente 2010).

Other types of microcapsules might also be processed, such as multilayer shells and different complexes obtained by gelation, demixing of emulsions or precipitation (Fig. 9).

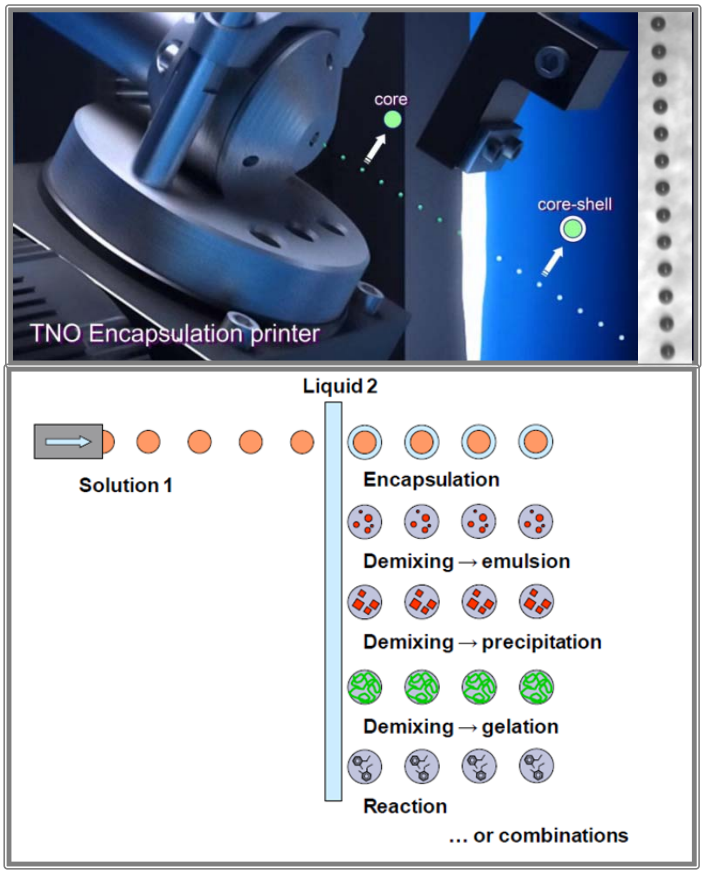

Figure 9. Encapsulation printing possibilities (Graphics by Twisted, Eindhoven, and TNO and drawing extracted from (Houben, Rijfers, Brouwers, Eversdijk, & Van Bommel, 2009)).

1.3. Legislation

Edible films could be considered food co-products, food ingredients, food packaging and food additives (Debeaufort, Quezada-Gallo, & Voilley, 1998), being regulated for each functionality:

In the European Union, edible films are considered a special active part of the food, and legally, they have to be regarded as foodstuff, along with the food packed in the film, thus having to fulfil the general requirements for food. As food or food ingredient, edible films have to be secure, food grade and non-toxic. The Regulation (EC) No. CE 853/2004 of the European Union (European Commission, 2004) lays down the specific rules for the hygiene of food stuffs of animal origin, taking into account the manufacture details and authorized raw materials, including live marine gastropods intended for human consumption and processed fishery products. These rules should be applied to the animal protein material used in film development.

Regarding seaweeds, France was the first European country to regulate the use of seaweeds for human consumption as vegetables and condiments (Mabeau & Fleurence, 1993; Rupérez & Saura-Calixto, 2001). Apart from the list of seaweed species authorised for human consumption, maximum allowed levels of toxic minerals have been also defined for all edible algae (Holdt & Kraan, 2011). Seaweeds do not have specific regulations in other European countries, but are considered food and food ingredients. According to the Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 about novel foods and novel food ingredients, seaweeds were considered novel foods in Europe (European Commission, 1997). The specific country legislations might limit their introduction in the market. There is a lack of European specific regulations about seaweed human consumption, they are just ruled by the general food regulations or, in some cases, they are not even considered food (Rupérez & Saura-Calixto, 2001). In Spain there is still no legislation about seaweed consumption, their derivatives or contaminant level limitations, being currently in progress a first Real Decreto draft about seaweed commercialisation (Circular A/079/13).

As food packaging, edible films need to fit in the Regulation 1935/2004/EC on materials and articles intended to come into contact with food (EU Commission), ensuring that no material in contact with food can elicit any chemical reaction that might change their composition or organoleptic properties.

With respect to the compounds that can be incorporated into edible films, additives are considered under the Commission Regulation (EU) No. 231/2012 of 9 March 2012 laying down specifications for the food additives listed (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011), and including sorbitol (E-420), glycerol (glycine, E-422) and polysorbate 80 among others.

These ingredients can be active, being necessary to follow the Regulation CE 450/2009 Commission Regulation (EC) No. 450/2009 of 29 May 2009 on active and intelligent materials and articles intended to come into contact with food (Campos, Gerschenson, & Flores, 2011). This regulation describes the active compounds that can be released in both the film and the food product; however this regulation does not include edible packaging materials.

Besides, there is no specific European legislation for efficacy and health claims of new functional foods in the EU Novel Foods legislation (258/97/EC).

Regarding food safety, it is expected that legislation will become even more complicated, and will keep constantly changing and lacking in clarity. In addition, other licences might be required to market new bioactive edible films derived from cephalopod proteins and seaweeds.

II. Hypothesis

In recent years, consumers have become more demanding with respect to food products, requesting new organoleptic characteristics and serving presentations aside from the mere eating need. In this regard, the present work has focused on studying new edible resources in order to obtain new raw materials and novel food presentations. In this research area, two possible new edible material sources have been chosen in order to increase its exploitation: i) food industry waste ii) underused edible sources. Both of them present certain limitations such as cultural restrictions and the lack of specific uses, being necessary to undertake good exploitation practices.

Among all food industrial waste, the remains originated in the fishing industry have been a matter of concern worldwide, due to the environmental contaminant effect, which at the same time represents extraordinary losses of potential nutrients. The waste from *Dosidicus gigas* industrial process is rich in muscle and connective tissues, and both are good sources for the extraction of food ingredients and/or additives.

From all the underutilized edible sources, seaweeds are quite underexploited in Western countries basically due to cultural diet habits. Among all seaweeds, some red and brown species have been chosen to be studied, due to their nutritional value and low contaminant content. *Mastocarpus stellatus*, *Laminaria digitata* and *Ascophyllum nodosum* are abundant in the Atlantic coast, and are already commercialized by the industry, which ensures their safety.

Marine proteins and polysaccharides with characteristics similar to that of the extracts obtained in this study have been widely studied, especially for their functional properties, although the sources and extraction methods are not always the same. Furthermore, on occasion, they have also been applied to the development of filmogenic matrixes.

Edible films have been studied lately as an alternative to the synthetic packaging materials under certain circumstances, representing an emerging research area due to the need to reduce plastic residues. However, there are only a few studies available regarding the application of edible films as part of the final food products instead of part of the packaging materials designed to extend the shelf-life of said food products. Moreover, those edible films could improve the final product quality.

Therefore, *Dosidicus gigas* waste and the selected seaweeds could be used to develop novel food products. Not only was the extraction of macromolecules interesting, but also the derived active compounds obtained. These compounds could be used as additives in the development of edible films. Nevertheless, the extraction of active compounds is normally performed following long and tedious procedures that are rarely susceptible to being scaled up to an industrial level. The main limitation of bioactive compounds is their reactivity with the environment, thereby decreasing their activity. Due to this limitation, some studies have explored different systems of encapsulation for bioactive compound protection.

Based on the possibility of obtaining edible biopolymers and active compounds from the previously described undervalued sources, the present work raised the possibility of optimizing the extraction of food grade edible biopolymers and active compounds, having both of them the potential to be incorporated in the development of edible films as ingredients and/or additives. Extracts would be susceptible of constituting edible films as a food product itself, being part of the coating materials or developing active films or coatings, depending on the composition. Due to the strong flavour of some marine products, as seaweeds, edible films may offer a new way of consumption through a novel serving presentation. In this search for new food sources, the latest studies have paid special attention to functional food development. In this regard, the extracted active compounds could be used in film formulation in order to improve the functionality, either by direct addition to the film forming solutions or carried into microcapsules.

III. Objectives

The aim of this thesis was to develop edible and active protein and polysaccharide films, from resources of marine origin, such as waste from the cephalopod processing industry and underutilized seaweeds.

To this end, the following partial objectives were considered:

1. Valorization of polymer materials recovered from different marine resources to produce muscle protein extracts from the waste from *Dosidicus gigas* industrial processing, and carrageenan and alginate unrefined extracts from red (*Mastocarpus stellatus*) and brown (*Laminaria digitata* and *Ascophyllum nodosum*) seaweeds.

2. Development of films based on biopolymers extracted from marine products, and their further physicochemical characterization to explore for different applications in the food industry.

3. Production and characterization of aqueous extracts and hydrolysates with active properties (antioxidant and antihypertensive) from the waste from *D. gigas* industrial processing and *M. stellatus*, to be utilized as functional ingredients.

4. Development of antioxidant films by the incorporation of active compounds into the biopolymer matrixes.

5. Microencapsulation of bioactive peptides extracted from *D. gigas* tunics by the *core-shell* method using the inkjet printing technology, characterization of the microcapsules and its addition to edible films.

IV. Experimental design

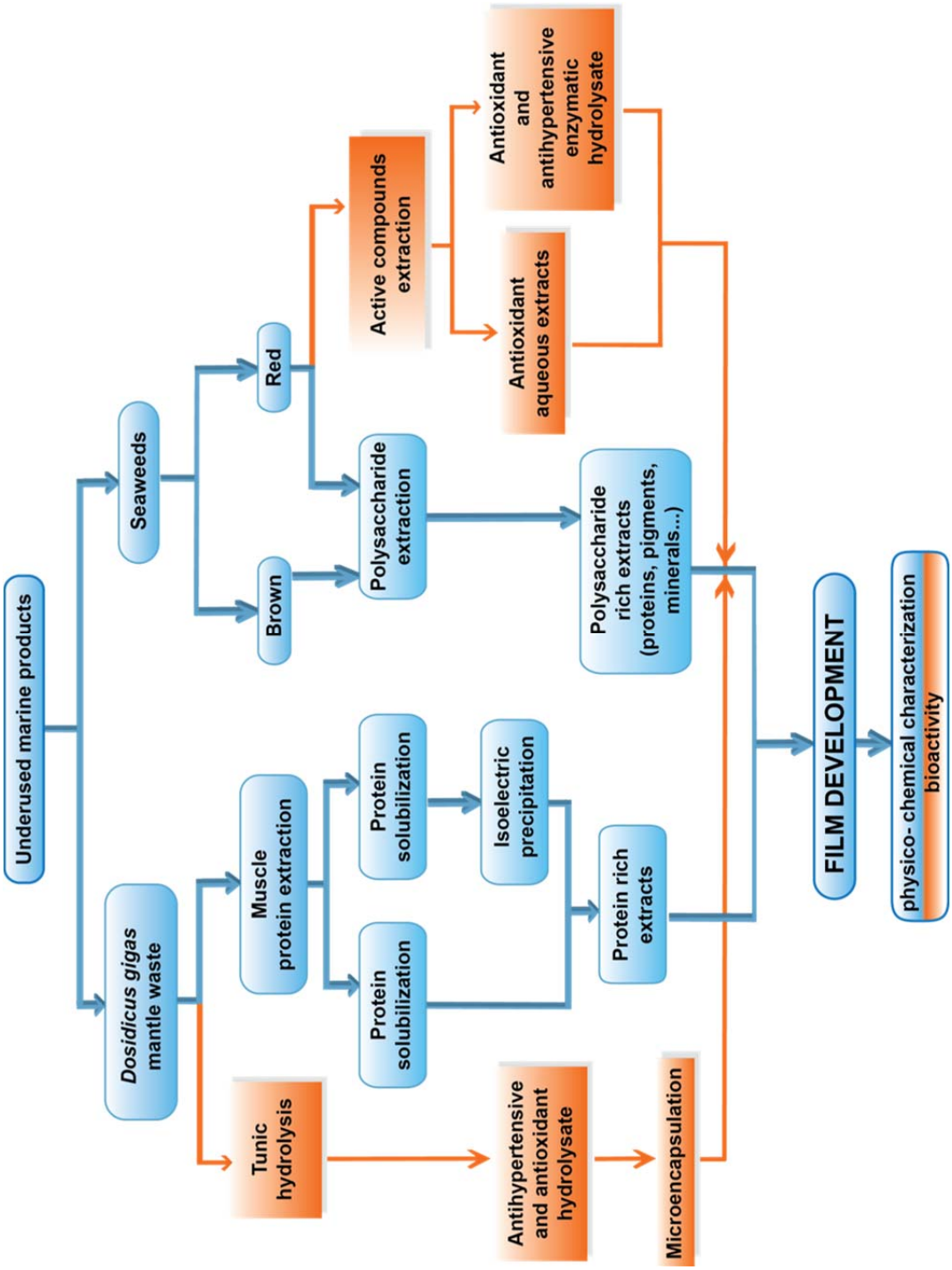

Figure 1. Experimental design.

V. Work plan

1. EFFECT OF DIFFERENT PROTEIN EXTRACTS FROM *DOSIDICUS GIGAS* MUSCLE ON EDIBLE FILM DEVELOPMENT

This chapter partially deals with objectives 1 and 2.

Objective 1. Valorization of polymer materials recovered from *Dosidicus gigas* industrial waste to produce muscle protein extracts.

Objective 2. Development of films based on muscle proteins extracted from *Dosidicus gigas* mantles, and their further physicochemical characterization to explore for different applications in the food industry.

2. JUMBO SQUID (*DOSIDICUS GIGAS*) MYOFIBRILLAR PROTEIN CONCENTRATE FOR EDIBLE PACKAGING FILMS AND STORAGE STABILITY

This chapter partially deals with objectives 1 and 2.

Objective 1. Valorization of polymer materials recovered from *Dosidicus gigas* industrial waste to produce muscle protein concentrates.

Objective 2. Development of films based on myofibrillar protein concentrates extracted from *Dosidicus gigas* mantles, and their further physicochemical characterization to explore for different applications in the food industry.

3. INTEGRAL *MASTOCARPUS STELLATUS* USE FOR ANTIOXIDANT EDIBLE FILM DEVELOPMENT

This chapter shows a novel use of red seaweeds and their application as edible active film products.

Objective 1. Valorization of polymer materials recovered from *Mastocarpus stellatus* to produce carrageenan unrefined extracts from red seaweeds.

Objective 2. Development of films based on biopolymers extracted from *Mastocarpus stellatus*, and their further physicochemical characterization to explore for different applications in the food industry.

Objective 3. Production and characterization of aqueous extracts with antioxidant properties from *M. stellatus*, to be utilized as functional ingredients.

Objective 4. Development of antioxidant films by the incorporation of active compounds into the biopolymer matrixes.

4. ENZYME-ASSISTED EXTRACTION OF K/I-HYBRID CARRAGEENAN FROM *MASTOCARPUS STELLATUS* FOR OBTAINING BIOACTIVE INGREDIENTS AND THEIR APPLICATION FOR EDIBLE ACTIVE FILMS DEVELOPMENT

The integral use of red seaweeds is achieved in this chapter, exploiting the bioactive potential of their components and obtaining ingredients for food applications, such as antihypertensive hydrolysates or edible films.

Objective 1. Valorization of polymer materials recovered from *Mastocarpus stellatus* to produce carrageenan unrefined extracts from red seaweeds.

Objective 2. Development of films based on biopolymers extracted from *Mastocarpus stellatus*, and their further physicochemical characterization to explore for different applications in the food industry.

Objective 3. Production and characterization of hydrolysates with active properties (antioxidant and antihypertensive) from *M. stellatus*, to be utilized as functional ingredients.

Objective 4. Development of antioxidant films by the incorporation of active compounds into the biopolymer matrixes.

5. ANTIOXIDANT FILM DEVELOPMENT FROM UNREFINED EXTRACTS OF BROWN SEAWEEDS *LAMINARIA DIGITATA* AND *ASCOPHYLLUM NODOSUM*

This chapter studies the use of brown seaweeds for the development of active edible films. Conventional and novel extractions are compared in order to obtain the most functional filmogenic material.

Objective 1. Valorization of polymer materials recovered from *Laminaria digitata* and *Ascophyllum nodosum* to produce alginate unrefined extracts from brown seaweeds.

Objective 2. Development of films based on biopolymers extracted from *Laminaria digitata* and *Ascophyllum nodosum*, and their further physicochemical characterization to explore for different applications in the food industry.

6. PEPTIDE MICROENCAPSULATION BY CORE-SHELL PRINTING TECHNOLOGY FOR EDIBLE FILM APPLICATION

This chapter deals with the microencapsulation of bioactive compounds (peptides) to be included into edible films, in order to provide a functional carrier system and to protect them from different environmental pH conditions.

Objective 2. Development of films based on biopolymers extracted from *Laminaria digitata*, and their further physicochemical characterization to explore for different applications in the food industry.

Objective 5. Microencapsulation of bioactive peptides extracted from *D. gigas* tunics by the *core-shell* method using the inkjet printing technology, characterization of the microcapsules and its addition to edible films.

VI. Effect of different protein extracts from *Dosidicus gigas* muscle on edible film development

Effect of different protein extracts from *Dosidicus gigas* muscle on edible film development

Blanco-Pascual, N., Fernández-Martín, F. and Montero, P. (2013). Effect of different protein extracts from *Dosidicus gigas* muscle co-products on edible films development. *Food Hydrocolloids***.** 33, 118-131.

6.1. Abstract

The waste produced in the processing industry of *Dosidicus gigas* muscle, is a good source of polymer material for film developing. The objective of this work was to compare different ways of protein recovery to find the best conditions of material for edible film developing. The proteins were recovered by water, salt, alkaline and acidic solubilization. The highest protein solubilization was obtained at alkaline conditions (≥70%) and DSC confirmed a partial denaturalization in saline and alkaline solution and total at acidic conditions, while SDS-PAGE confirmed a hydrolysis effect at pH3. According to FTIR, the loss of secondary structure at pH10 led to a stronger bonding film network and the hydrolysis at pH3 resulted in more protein-plasticizer and protein-water interactions. Both alkaline and acidic conditions led to transparent and microbiologically stable films, the alkaline-film being more water resistant and with less protein release in water contact. Both alkaline- and acidic-films resulted in the more flexible and more resistant, especially at alkaline conditions. While salt-extract did not improve any mechanical property of the corresponding film compared with water-film, both films presented the lower solubility and the more water resistance but were not microbiologically stable and had poorer mechanical properties.

6.2. Introduction

Nowadays, in the processing industry of fishery products a lot of waste are produced, mostly protein, that is a source of environmental pollution. In order to get value-added products and eliminate pollution from discarded material, numerous strategies to create new alternatives are studied (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; De la Fuente-Betancourt, García-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sánchez, 2009).

This is the case of Jumbo squid (*Dosidicus gigas*) which is the largest and most abundant squid species found in the pelagic zone of eastern Pacific, from Chile up to Oregon coasts (Nigmatullin, Nesis, & Arkhipkin, 2001). The percentage of the edible portion of cephalopods is exceptionally high, between 60 and 80% of their total weight.

In the last decades many products have been developed from *Dosidicus gigas* muscle, such as gel-based products (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; De la Fuente-Betancourt, García-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sánchez, 2009) and gel-emulsion products (Felix-Armenta, Ramírez-Suarez, Pacheco-Aguilar, Diaz-Cinco, Cumplido-Barbeitia, & Carvallo-Ruiz, 2009; M. C. GómezGuillén, Borderías, & Montero, 1997), *surimi* and mince (Campo-Deaño, Tovar, Jesús Pombo, Teresa Solas, & Javier Borderías, 2009; C. GómezGuillén, Solas, Borderías, & Montero, 1996; M. C. GómezGuillén, Borderías, & Montero, 1997) and also other products have been made from skins and tunics (collagen, gelatine) (Denavi, Pérez-Mateos, Anon, Montero, Mauri, & Gómez-Guillén, 2009; Giménez, Alemán, Montero, & Gómez-Guillén, 2009; Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). The use of the Dosidicus mantle muscle as a polymer material to film developing is a good asset, ensuring an added value and minimizing the discards during their process.

Edible and biodegradable films can be developed from different materials such as proteins, polysaccharides, lipids, and resins (Krochta, 2002). Among these materials, some proteins have been

extensively studied because of their relative abundance, film-forming abilities, and nutritional qualities (Hamaguchi, Weng, & Tanaka, 2007).

At the moment muscle protein films have been developed from different marine species such as the bigeye (*Priacanthus tayenus*) (Chinabhark, Benjakul & Prodpran, 2007), the Indo-Pacific blue marlin (*Makaira mazara*) (Hamaguchi, Weng & Tanaka, 2007), the round scad (*Decapterus punctatus*) (Artharn, Benjakul & Prodpran, 2008) or the squid (*Todadores pacificus*) (Artharn, Benjakul, & Prodpran, 2008; Chinabhark, Benjakul, & Prodpran, 2007; Hamaguchi, Weng, & Tanaka, 2007; Leerahawong, Arii, Tanaka, & Osako, 2011; Paschoalick, García, Sobral, & Habitante, 2003); finding differences depending on the species and the protein myofibrillar/sarcoplasmic proportion (strong protein-protein interaction), such as a decreasing of films solubility when the sarcoplasmic content increased or a higher elongation at break when sarcoplasmic proportions under 20% (Artharn, Benjakul, & Prodpran, 2008). Obviously other factors could be affected, for example the raw material conditions (proteolysis degree, ammonia compounds), protein extraction methods and so on. Other authors observed that film-forming by myofibrillar proteins produce a continuous matrix while the sarcoplasmic fraction tend to develop an added superimposed network (P. J. D. Sobral, dos Santos, & García, 2005) or could be placed in the space left in the myofibrillar protein matrix due to their globular structure and small size. Denavi, Pérez-Mateos, Anon, Montero, Mauri, and Gómez-Guillén (2009) found the same behaviour with soy protein in a gelatine matrix.

So far, muscle protein films from giant squid have never been developed before despite it would be an interesting alternative use. Several methods have been used to solubilized muscle proteins of *Dosidicus gigas*, it can be performed by a simple homogenization changing the water proportion used because its myofibrillar protein is highly water-soluble, or by solubilizing myofibrillar proteins at low ionic strength (Sánchez-Alonso, Careche, & Borderías, 2007), and at low (1-3) or high (9-11) pH values (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; De la Fuente-Betancourt, García-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sánchez, 2009; Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009). However, no information regarding the film ability of *Dosidicus gigas* muscle neither the adequate extraction methods to obtain film properties of interest have been described before.

The ability to solubilize myofibrillar proteins at extreme acidic (pH 2-3) (Hultin & Keelleher 1998a) or alkaline (pH 10.5-11.5) (Hultin & Keelleher 1998b) conditions and a subsequent isoelectric protein precipitation (pH 4.8-5.5) leads to the highest recovery of highly functional myofibrillar proteins.

The objective of this study was to evaluate the film forming ability of the *Dosidicus gigas* muscle proteins. For this purpose both different methods of protein extraction and properties of the resulting protein edible films were tested.

6.3. Materials and Methods

6.3.1. Materials

Muscle proteins were recovered from the frozen mantle of giant squid (*Dosidicus gigas*), which was caught in the coast of Peru in January 2009. Following capture, the specimens were gutted and

mantles separated from tentacles and frozen on board to -20 ºC. The frozen mantles were shipped to the industrial plant PSK Océanos S.A. (Pozuelo de Alarcón, Madrid, Spain) and, after processing fishing byproducts, the pieces of discarded mantles were frozen to -20 ºC and then sent to our Institute, where were kept frozen as raw material until analysis.

Analytical grade HCl, NaOH, NaBr, glycerol and sorbitol, and food-standard NaCl were from Panreac Química S.A. (Montplet and Estaben S.A., Montcada i Reixac, Barcelona, Spain). DOW 1510 silicon antifoaming agent was from DOW Corning Europe (Brussels, Belgium).

6.3.2. Methods

6.3.2.1. Proximate analysis

Moisture, fat and ash content of the raw material were determined according to official methods (A.O.A.C., 1995). Total nitrogen content was determined by Dumas´ method (A.O.A.C., 2000) using a combustion oven apparatus (Model FP-2000, Leco Corporation, St Joseph, MI, USA). Nitrogen-to-protein conversion factor of 6.25 was employed quantify total protein content. Analysis was performed at least in triplicate, and results expressed as percentages.

6.3.2.2. Microbiological assays

Raw material (10 g) or each film (1 g) were aseptically weighed and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 9 ml of buffered 0.1% peptone water (Oxoid, Basingtoke, UK), and four decimal dilutions were made. The total number of mesophilic microorganisms was determined with plate count agar (PCA, Merck) following the pour plate method, incubating at 30 ºC for 72 h. The number of *enterobacteriaceae* microorganisms was also determined in the films, using double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 ºC for 48 h. All microbiological counts were determined at least in triplicate and expressed as the log of the colony-forming units per gram of sample (log CFU/g).

6.3.3. Muscle protein extraction

Squid mantles were kneaded in a vacuum homogenizer (Stephan UM5, Stephan u Söhne GmbH & Co., Hameln, Germany) at temperatures lower than 10 ºC. Distilled water was added in the 1:1 (v:w) proportion, and also DOW 1510 at 1 drop/100 mL to reduce the foam appearance. Extractions were carried out by using four different media: H_2O, 0.1M NaCl, pH 10, and pH 3. Water extraction (water-E) was performed at pH 6.50 ± 0.05. For saline extraction (salt-E), 0.1M NaCl was added in 1% proportion with a final pH of 6.58 ± 0.05. For alkaline and acidic extractions, NaOH and HCl dilutions were respectively added until reaching pH 10.0 ± 0.2 (alkaline-E) and pH 3.0 ± 0.2 (acidic-E). Two homogenization cycles (30 s at 1500 rpm and 90 s at 3000 rpm each one) were necessary to completely homogenize the muscle. The tunic rests were removed manually and the muscle extract was kept under 5 ºC during less than 2 hours until film preparation. A portable pH-meter series 3 Star Orion with an electrode pH ROSS (Thermo Fisher Scientific Inc., Landsmeer, Netherlands) was used for pH measurements.

6.3.4. Film preparation

Protein film-forming solutions (2% w/v) were prepared by the same method as in muscle extraction. A plasticizer mixture (glycerol and sorbitol at the same proportion) was added at 50% (w/w) of the total protein.

The pH of the film-forming solutions (FS) were 6.59 ± 0.05 for water-FS, 6.54 ± 0.05 for salt-FS, 9.63 ± 0.05 for alkaline-FS, and 3.39 ± 0.05 for acidic-FS. Finally, film-forming solutions were filtered to remove air bubbles, and 50 mL aliquots were then cast into methacrylate plates (120x120 mm) (Plexiglas® GS Röhm GmbH & Co. KG, Darmstadt, Germany) through a gauze for exhaustive bubbles removing. Plates were left for 21-23 h at 4.0 ± 0.5 ºC and 85 ± 5% RH prior to further drying in a ventilated oven (FD 240 Binder, Tuttlingen, Germany) at 45.0 ± 0.8 ºC and 12 ± 3% of relative humidity (RH) for 21-23 h. All films were conditioned at 58.0 ± 0.2% RH and 22 ± 1 ºC for 4 days prior to analysis.

6.3.5. Protein solubility

Protein concentration in extracts and film-forming solutions was determined with the BCA kit (Meridian RD., Rockford, IL, 61101 USA) (Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano et al., 1985). Corresponding water-soluble protein fractions were expressed at least in triplicate as the percentage of solubilized protein with respect to total muscle protein, which was determined according to A.O.A.C. (Association of Official Analytical Chemists, 2000) and expressed as percentages in wet basis.

6.3.6. Electrophoretic analysis (SDS-PAGE under reducing conditions)

Extracts and film-forming solutions were mixed with a 2-fold concentrated loading buffer (2% SDS, 7% mercaptoethanol and 0.002% bromophenol blue) adjusting with distilled water to reach a final concentration of 2 mg/mL protein. Samples were heat-denatured 5 min at 95 ºC and analysed by PAGE-SDS under reducing conditions using 10% Mini-PROTEAN TGX^{TM} gels in a Mini Protean II unit (Bio-Rad Laboratories SA, Alcobendas, Madrid, Spain) at 25 mA/gel. Protein bands were stained with Coomassie brilliant Blue R250. Precision Plus Protein Dual Xtra standards from 2 kD to 250 kD were used as markers (Bio-Rad Laboratories SA, Alcobendas, Madrid, Spain).

For electrophoretic profile of water-soluble film proteins, approximately 0.4 g of the films were placed in falcon tubes with 10 mL distilled water and shaken at 100 rpm in an orbital shaker at 22 ºC for 24 h. The solution was then filtered through Whatman # 1 filter paper to discard the remaining un-dissolved material and the recovered water solution analysed like in the extracts and film-forming solutions.

Protein solubility was determined at least in triplicate by BCA and expressed in mg/mL.

6.3.7. Thermal properties

Calorimetric analysis of extracts and films were performed using a differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated by running high purity indium (melting point, 156.4 ºC; melting enthalpy, 28.44 J/g). Samples of around 10-15 mg of extracts and protein films were weighed within ± 0.002 mg by an electronic balance (Model

ME235S Sartorius, Goettingen, Germany) and then tightly encapsulated in aluminium hermetic pans. An empty pan was used as reference. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 90 ºC at a heating rate of 10 ºC/min. Peak temperatures (T_{peak}, ºC) and denaturation enthalpies (ΔH) were measured at least in triplicate, the last data being normalized to dry matter content (J/g_{dm}) after desiccation of each particular capsule.

6.3.8. Film determinations

6.3.8.1. Total volatile basic nitrogen (TVB-N)

TVB-N determinations were carried out at least in triplicate by using the method of (Ojagh, Nunez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011). Results were expressed in dry basis as mg TVB-N/100 g film.

6.3.8.2. Moisture content

It was determined at least in triplicate by drying samples of around 0.5 g at 105°C for 24 h, according to A.O.A.C. (Association of Official Analytical Chemists, 1995). Water content was expressed as a percentage of the total weight.

6.3.8.3. Water activity

It was measured at least in triplicate placing circles cut at exactly the same shape of the equipment containers on each film stuck to the bottom, with a Lab MASTER-aw equipment (Novasina AG, Lachen, Switzerland) at constant temperature of 25 ºC.

6.3.8.4. Thickness

It was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 4-6 random locations in 15 films for each treatment as described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

6.3.8.5. ATR-FTIR spectroscopy

Film infrared spectra between 4000 and 650 cm^{-1} using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA) as was described by Ojagh, Nunez-Flores, López-Caballero, Montero, and Gómez-Guillén (2011). Data were recorded at least in triplicate and were processed using the Spectrum software calculating the second derivative.

6.3.8.6. Light absorption and transparency

The light barrier properties and transparency of the films were calculated at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 200 to 700 nm following the method described by Pérez-Mateos, Montero, and Gómez-Guillén (2009). Transparency was calculated by the equation.

$$\text{Transparency} = -\log(T_{600}/x)$$

where T_{600} is the light transmission (T) at 600 nm, and x is the film thickness (mm).

6.3.8.7. Colour

The colour parameters *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were measured using a Konica Minolta CM-3500d colourimeter (Konica Minolta, Madrid, Spain). D65 illuminant (Daylight) and D10º standard observer were used. Measurements were taken at a number of 5 locations in different film portions and each reported value was the mean of at least 11 measurements.

6.3.8.8. Water vapour permeability

It was determined at least in triplicate following the method described by Sobral et al. (2001) at room temperature and in a dissecator with distiller water (100% RH). RH increment was calculated every hour during 7 hours using the following equation: $w \cdot x \cdot t^{-1} \cdot A^{-1} \cdot \Delta P^{-1}$ where *w* is the gained mass (g), *x* is the film thickness (mm), *t* is the time (h), *A* is the film area exposed (cm^2) and ΔP is water vapour partial pressure difference between the atmosphere and silica gel (2642 Pa at 22 ºC). Results were expressed in $g \cdot mm \cdot h^{-1} \cdot cm^{-2} \cdot Pa^{-1}$.

6.3.8.9. Water solubility

Film circumferences of 40 mm in diameter were placed in plastic containers with 50 mL distilled water and placed at 22 ºC for 24 h. The solution was then filtered through Whatman # 1 filter paper to recover the remaining undissolved film, which was desiccated at 105 ºC for 24 h. Film solubility *FS* (%) was calculated using the expression $[(W_o - W_f)/W_o] \times 100$, where W_o was the initial weight of the film expressed as dry matter and W_f was the weight of the undissolved desiccated film residue. All tests were carried out at least in triplicate.

6.3.8.10. Water resistance

Films were fixed onto the opening of calibrated cells (area 15.90 cm^2) and the cells placed in desiccators and exposed over distilled water. Distilled water (5 mL) was poured over the film surface. The film deformation due to the water effect, the time when the water started to leak and the time when the film broke were annotated. All tests were carried out at least in triplicate.

6.3.8.11. Mechanical properties

Tensile and puncture tests were run using a texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) (58% RH and room temperature) controlled by the Texture Exponent Software (Texture Technologies and by Stable Micro Systems, Ltd., Scarsdale, NY, USA), using a 5 kg load cell. Tensile test: At least three probes were cut rectangular (100 mm x 20 mm), leaving initial grips separation (l_0) of 60mm and using cross-head speed of 100 mm/min. The tensile strength (*TS,* MPa) (break force/initial cross-sectional area) and elongation at break $[(l_{break} - l_0)/l_0] \times 100$, (*EAB,* %), were determined from the stress *vs* strain curves at the breaking point, and the elastic modulus or Young's modulus (*Y,* MPa) calculated as the slope of the linear initial portion (elastic response zone) of the curve $(l_{break} - l_0)/l_0$. Puncture test: Films of 100X100 mm were fixed in a 35 mm diameter cell and punctured to breaking point with a round-ended stainless steel plunger (5 mm) at a cross-head speed of 100 mm/min, for breaking force (*F*, N), and breaking deformation (*D*, %) data according to Sobral, Menegalli, Hubinger, and Roques (2001), which were carried out at least in triplicate at room temperature and keeping the samples at 58% RH until the text performance.

6.3.8.12. Microstructure

Low temperature scanning electron microscopy (LowT-SEM) (Oxford CT1500 Cryosample Preparation Unit, Oxford Instruments, Oxford, England) was used to examine representative film cross sections. Samples were mounted with an optical coherence tomography (OCT compound Gurr ®) and mechanically fixed onto the specimen holder and cryo-fractured after mounted as described by Gómez-Guillén, Ihl, Bifani, Silva, and Montero (2007).

6.3.9. Statistical analysis

Statistical tests were performed using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, Illinois, USA) for one-way analysis of variance. The variance homogeneity was made using the Levene test or, the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

6.4. Results and discussion

The muscle used as raw material showed 83.46 ± 1.21% moisture, 0.80 ± 0.01% fat, 0.87 ± 0.05% ash, and 14.87 ± 0.31% protein, of which 37.10 ± 1.35% was water-soluble protein. Total viable bacteria count was 4.4·log CFU/g. This load was lower than the allowed limits for fresh and frozen fishery products (m = 5.7·log CFU/g) (International Commission on Microbiological Specifications for Foods, 1986), which indicated a relatively good quality for waste from the processing of squid.

6.4.1. Protein extracts (E) and film-forming solutions (FS)

6.4.1.1. Protein solubility

Table 1 shows protein solubility data for each extraction. The highest solubility was obtained at pH10 (alkaline-E) while water extraction (water-E) caused the lowest solubility. Extractions at pH3 (acidic-E) and 0.1M NaCl (salt-E) produced similar ($P>0.05$) protein solubility, being slightly lower than at alkaline conditions. Palafox, Cordova-Murueta, del Toro, and García-Carreno (2009) reported similar protein solubility in giant squid muscle at basic (11) and acidic (3) pHs.

However, Sánchez-Alonso, Careche, and Borderías (2007) reported considerably lower protein solubility (~55%) in giant squid muscle extracted with 0.1M NaCl. De la Fuente-Betancourt, García-Carreno, Del Toro, and Cordova-Murueta (2009) have also observed a high functionally, evaluated as foaming and emulsifying properties, of giant squid muscle protein at alkaline pH (10-11), reaching the highest stability; however water extraction was not as good.

Regarding film-forming solutions, protein solubility at 0.1M NaCl (salt-FS) was lower than before but similar to water extraction (water-FS), which may be due to the dilution effect from 0.1M to 0.04M whereas pH remained similar in the extracts ($P\leq0.05$). This dilution also caused a total solubilization (100%) at pH10 (alkaline-FS), while pH3 (acidic-FS) obtained a limited but rather high protein solubility (~83%). Hamaguchi, Weng, and Tanaka (2007) also observed higher solubility (percentages not given) at pH values of 1-4 and 10-12 in films forming solutions of 2% of blue marlin (*Makaira mazara*) muscle protein and 1% glycerol than pH values between 4 and 10 .

	SP (%)	
Sample	Extract (E)	Film-forming solution (FS)
water-	60.39 ± 2.00 a/x	56.70 ± 2.12 a/x
salt-	65.41 ± 0.79 b/x	54.60 ± 0.62 a/y
alkaline-	70.11 ± 0.50 c/x	100.82 ± 1.20 b/y
acidic-	66.07 ± 2.57 b/x	83.03 ± 4.07 c/y

Table 1. Water-soluble protein (SP, %) in water (water-E), salt (salt-E), alkali (alkaline-E) and acid (acidic-E) extracts, and corresponding film-forming solutions water-FS, salt-FS, alkaline-FS and acidic-FS. Results are the mean ± standard deviation. Two-ways ANOVA: Different letters (a, b, c) in the same column indicate significant differences among the different treatments (P≤0.05). Different letters (x, y) in the same row indicate significant differences among extracts and film-forming solutions (P≤0.05).

Solubility differences between protein extracts and film-forming solutions were detected (P≤0.05) in all cases except in water extraction.

6.4.1.2. Electrophoretic patterns

Molecular weight protein distribution of extracts and film-forming solutions are shown in Figure 1. Similar behaviour was observed in all extracts, mainly in those in water and salt. A different degradation pattern was appreciated depending on the pH; in alkaline pH a higher band density at high molecular weight (including myosin and paramyosin) was observed. In acid-aided processes a high proportion of myosin heavy chains (MHC) (205 kDa) disappeared, and paramyosin (P) (108 kDa) and actin (A) (45 kDa) band intensities decreased; meanwhile higher band intensities between 50-75 kDa of light meromyosin (LMM) and below 45 kDa were observed possibly due to protein degradation by endogenous enzymes and the low pH. The fact that the solubility capacity behaved in different ways along the pH scale (De la Fuente-Betancourt, García-Carreno, Del Toro, & Cordova-Murueta, 2009) suggests that the degradation level might affect the functional properties. Others authors observed a reduction of MHC and a consequently increase of LMM and HMM (heavy meromyosin) which may indicate a stronger metalloprotease activity at pH3 (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008). This would tend to support the idea that pH induces conformational changes in protein structures making them more liable to enzyme hydrolysis (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008). Additionally, more intense bands were detected below the 50 kDa region, which is the expected location for derivatives from such hydrolysis. Degraded myofibrillar fragments due to acid treatment were also found in acid-aided protein recovery from enzyme-rich pacific whiting (Choi & Park, 2002), where numerous new and low-molecular-weight bands appeared and even actin band was degraded into two bands. On the other hand, Ramírez-Suarez, Ibarra-León, Pacheco-Aguilar, Lugo-Sánchez, García-Sánchez, and Carvallo-Ruiz (2008) found a disappearance of the 50-58 and 85 kDa bands, which they attributed to a cross-linking caused by endogenous transglutaminase (TGase) enzyme action (although the pH was not optimum), forming dimmers and trimers with an approximate molecular weight of 153 kDa.

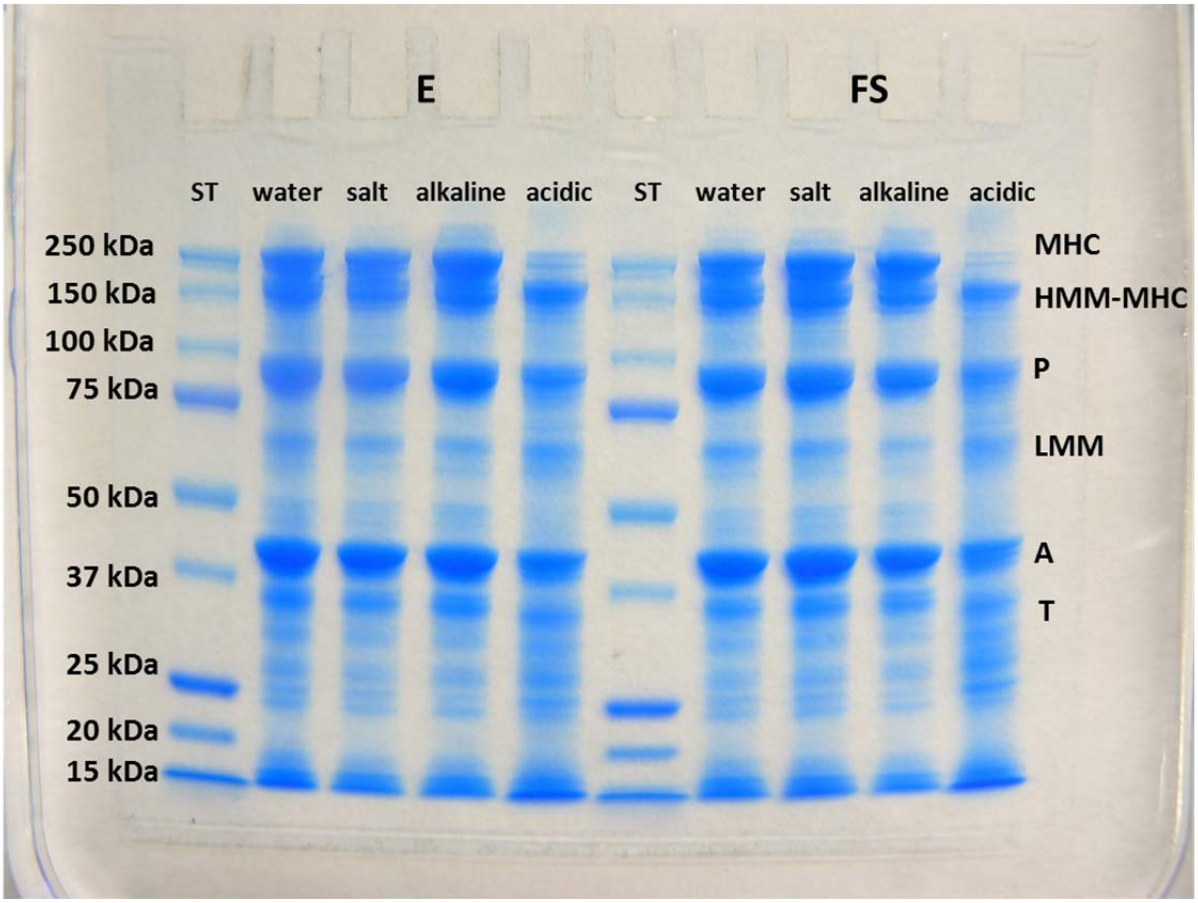

Figure 1. SDS-PAGE patterns under reducing conditions of extracts (E) and film-forming solutions (FS). Left: water-E, salt-E, alkaline-E and acidic-E extracts. Right: water-FS, salt-FS, alkaline-FS and acidic-FS film-forming solutions. ST: Standard; MHC: Myosin heavy chain; HMM-MHC: Heavy meromyosin-myosin heavy chain; LMM: Light meromyosin; P: Paramyosin; A: Actin; T: Tropomyosin.

However, it was not clear whether the reduction of myosin heavy chain resulted from the degradation of myosin by acidic proteases or acid hydrolysis. Extracts (E) and corresponding film forming solutions (FS) presented highly similar electrophoretic behaviours (Fig. 1).

6.4.1.3. DSC

DSC thermograms of extracts (E) had the typical profiles shown in Figure 2: water-E, salt-E and alkaline-E depicted two main (rounded shaped) endothermic transitions at the ranges of 45 − 50 ºC (mainly myosin) and 70-80 ºC (mostly actin), with an overlapping zone in between (paramyosin, collagen (nearly inexistent) and sarcoplasmic proteins), that resembled the general pattern of actomyosin systems. Acidic-E had however a flat trace with no sign of transitions.

Respective T_{peak} (ºC) values were: 51.08 ± 0.77 and 77.52 ± 0.33 in water-E; 46.63 ± 0.46 and 74.64 ± 0.39 in salt-E; 45.54 ± 1.92 and 69.86 ± 1.79 ºC in alkaline-E. Transition temperatures were significantly different except for the low transition data in salt-E and alkaline-E which were not significantly different. Corresponding ΔH (J/g_{dm}) values were 5.05 ± 0.48, 4.70 ± 0.68, and 4.21 ± 0.71 respectively for water-E, salt-E, and alkaline-E extracts. Denaturation enthalpies were not significantly different among the extracts.

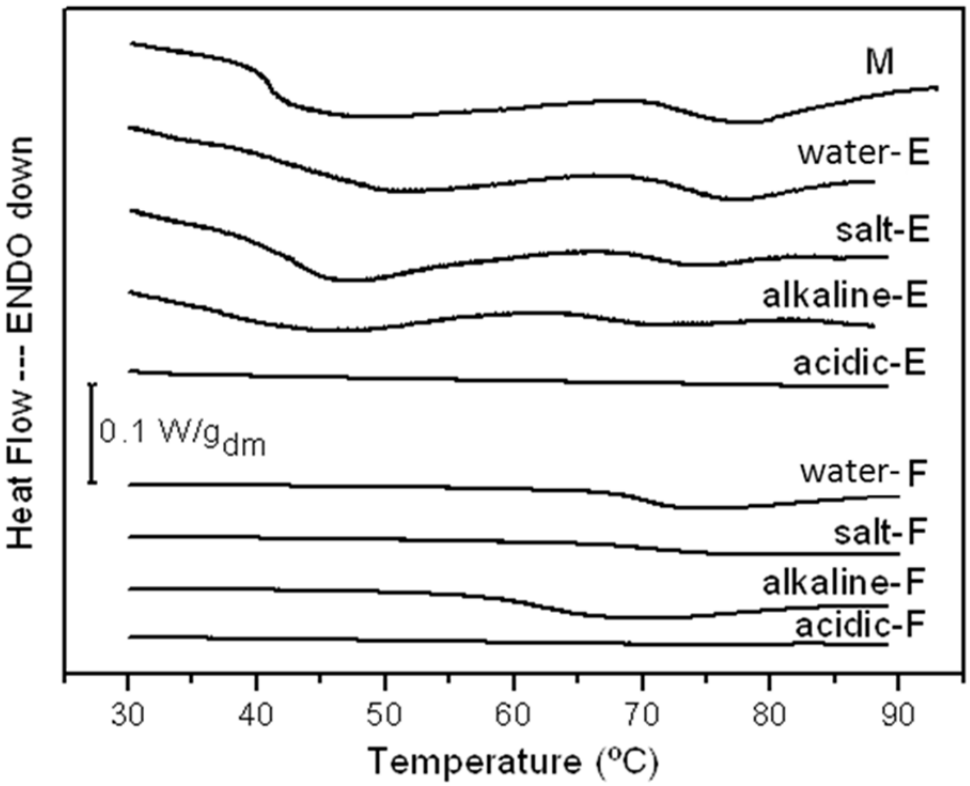

Figure 2. DSC of extracts (E) and Films (F). Top: *Dosidicus gigas* muscle (M), water-E, salt-E, alkaline-E and acidic-E extracts. Bottom: water-F, salt-F, alkaline-F and acidic-F films.

DSC traces of water-E, salt-E and alkaline-E closely resembled typical profiles of frozen muscle proteins from dissected mantles of giant squid (*D. gigas*) of likely the same source and processing than current material (Fernández-Martín, 1998) (Fig. 2, line M). Corresponding thermal denaturation data were: 47.28 ± 0.52 ºC and 77.60 ± 0.43 ºC for the main transitions T_{peak} endotherms, with around 60/40 enthalpy ratio and total ΔH of 8.16 ± 0.12 J/g_{dm}. These data recorded usual grinding/freezing and frozen-storage effects on myofibrillar proteins and differed to previous reports by others on different cephalopod species and experimental conditions (Hastings, Rodger, Park, Matthews, & Anderson, 1985; Paredi, Tomas, Crupkin, & Anon, 1996; Ramírez Olivas, Rouzaud Sández, Haard, Pacheco Aguilar, & Ezquerra Brauer, 2004). From the calorimetric point of view, water-E extracts seemed to consist of considerably structure-preserved proteins (~62%) relative to those of frozen mantle muscle of giant squid (Fernández-Martín, 1998). It is well known that squid proteins differ from fish and mammals proteins of being more water-soluble, and more prone to thermal denaturation. Despite the general fact that proteins are more soluble in salt (depending on ionic strength) at normal pH, salt-E yield (~58%) was not significantly different than that of water-E concerning enthalpy data, but transition temperatures underwent a significant down-shifting, in agreement to generally described salt effects on myofibrillar proteins. Regarding extracts at high pH (alkaline-E), the yield (~52%) was not significantly lower (~10%) than the above case and consisted of mainly preserved myosin and paramyosin, and considerably degraded high-thermostable proteins (sarcoplasmic proteins and actin) which underwent, as expected, a great reduction in the transition temperatures. These findings seemed to be accordant to the high functionality ascribed to this protein fraction by De la Fuente-Betancourt, García-Carreno, Del Toro, and Cordova-Murueta (2009). It is worth nothing that the intermediate endothermic DSC zone appeared reduced with respect to water-E

in salt-E and, even more, in alkaline-E (Fig. 2), which could likely be due to a significant suppression of sarcoplasmic proteins solubility in the presence of high salt concentration (Kim, Yongsawatdigul, Park, & Thawornchinsombut, 2005), and their partial unfolding during alkaline extraction since some sarcoplasmic proteins are alkali stable according to Tadpitchayangkoon, Park and Yongsawatdigul (2010). It is also well known that low pH treatments may produce a high protein recovery but with great structural degradations, as in acidic-E (Totosaus, Montejano, Salazar, & Guerrero, 2002) depending on the pH level. Additionally, sarcoplasmic proteins are also significantly suppressed in solubility at an acidic pH (Kim, Yongsawatdigul, Park, & Thawornchinsombut, 2005). Effects of processing by pH-shifting (3 and 11) in giant squid have been recently reported by Palafox, Cordova-Murueta, del Toro, and García-Carreno (2009). References on thermal data of giant squid subjected to acid treatment are very scarce however since the only precedent was found in Campo-Deaño, Tovar, Borderías, and Fernández-Martín (2011): two cases were reported on frozen giant squid mantle processed at ~pH 5 under different conditions, with the result of different protein yields and denaturation effects; considerably higher acidic conditions may likely cause entire protein denaturation, as in current acidic-E.

These thermal results on extracts confirmed the respective solubility data but could not necessarily match with the corresponding electrophoretic patterns due to the different protein nature (native and denatured respectively).

6.4.2. Film (F) properties

6.4.2.1. Light barrier properties

Light transmission in UV and visible ranges at selected wavelength of 200-700 nm as well as transparency are shown in Table 2. Generally, films exhibited the lower transmission in the UV range (200-280 nm), irrespective of pH or NaCl presence, which could decrease lipid oxidation in food system.

These results are consistent with earlier works (Artharn, Benjakul, & Prodpran, 2008; Benjakul, Artharn, & Prodpran, 2008; Hamaguchi, Weng, & Tanaka, 2007; Shiku, Hamaguchi, Benjakul, Visessanguan, & Tanaka, 2004) reporting that fish muscle protein films had very good UV barrier properties, owing to their high content of aromatic amino acids that absorb UV light. This is interesting because most of synthetic polymer films do not prevent the passage of UV light above 280 nm (Shiku, Hamaguchi, Benjakul, Visessanguan, & Tanaka, 2004; Shiku, Hamaguchi, & Tanaka, 2003).

Although every film showed high transparency, they became more transparent at pH3 (acidic-F) and pH10 (alkaline-F) (0.73 and 1.14 respectively) than in water (water-F) and salt (salt-F), as shown in Table 2. The lack of pigments in the muscle might favour the transparency of the films. Artharn, Benjakul, and Prodpran (2008) observed that higher proportion of solubilized myofibrillar protein gave place to higher transparency, which is in agreement with the present study. Shiku, Hamaguchi, and Tanaka (2003) claimed that blue marlin muscle protein films prepared at acid (2-3) or alkaline (11-12) pH led to more stable protein networks, with superior transparency close to synthetic films.

Film	T at selected wavelengths						Transparency
	200	280	350	400	500	600	700	
water-F	0.01 ± 0.001 a	0.01 ± 0.003 a	13.51 ± 0.86 a	23.49 ± 1.87 a	35.83 ± 3.77 a	39.63 ± 4.15 a	41.59 ± 3.94 a	3.48 ± 0.42 a
salt-F	0.01 ± 0.001 a	0.02 ± 0.006 a	19.98 ± 1.28 b	30.52 ± 1.91 b	40.51 ± 2.51 ac	43.78 ± 2.62 a	45.16 ± 2.62 a	3.40 ± 0.44 a
alkaline-F	0.01 ± 0.001 a	0.01 ± 0.001 a	15.26 ± 1.67 a	41.64 ± 2.44 c	77.63 ± 3.32 b	87.42 ± 3.34 b	89.60 ± 3.47 b	1.14 ± 0.26 b
acidic-F	0.01 ± 0.001 a	0.01 ± 0.001 a	20.04 ± 3.76 b	33.81 ± 3.25 b	45.95 ± 2.93 c	50.91 ± 3.42 c	53.82 ± 3.94 c	0.73 ± 0.05 b

Table 2. Light transmission (T, %) at several wavelengths (nm) and Transparency ($-\log(T_{600}/x)$) of water-F, salt-F, alkaline-F and acidic-F films. Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films ($P \leq 0.05$).

*L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish) values are shown in Table 3, where it is revealed that all films had low lightnesses (~35). In general, films had a lack of reddish tendency (+*a**). Acidic-F specimens had the higher *a** value while alkaline-F had the lowest *a** value (P≤0.05). Alkaline-F specimens had the most yellowish tendency (+*b**) and acidic-F type the least (P≤0.05). No significant differences (P>0.05) were found between water-F and salt-F films. A possible reason which could explain the yellowish tendency at pH10 might be a higher solubilized myofibrillar protein proportion in the corresponding film-forming solution (Artharn, Benjakul, & Prodpran, 2008).

Data obtained in this study seemed to indicate that giant squid protein films are highly transparent, UV barrier and have the adequate colour for their use as see-through packaging or coating materials.

Film	*L**	*a**	*b**
water-F	34.44 ± 0.24 a	-1.00 ± 0.05 a	0.06 ± 0.01 a
salt-F	35.98 ± 0.64 b	-1.15 ± 0.13 ab	0.10 ± 0.1 a
alkaline-F	34.23 ± 0.12 a	-1.24 ± 0.03 b	1.26 ± 0.07 b
acidic-F	35.93 ± 0.87 b	-0.69 ± 0.03 c	-0.56 ± 0.09 c

Table 3. L^*, a^* and b^* of water-F, salt-F, alkaline-F and acidic-F films.
Results are the mean ± standard deviation. One-way ANOVA: Different letters in the same column indicate significant differences among the different films (P≤0.05).

6.4.2.2. ATR-FTIR

Figure 3A shows ATR-FTIR spectroscopic patterns (4000-800 cm^{-1}) of water-F, salt-F, alkaline-F and acidic-F films. Factors as pH and NaCl led to important changes in the spectra. The Amide A band (~3300 cm^{-1}) and amide B (~3079 cm^{-1}), attributed fundamentally to N-H stretching of protein vibrations, with contribution from O-H stretching of intermolecular hydrogen bonding, are related to free water. Salt-F, acidic-F and alkaline-F showed a slight shift to the lower wavenumbers of amide A (~3280-3273 cm^{-1}), specially the last one. This was possibly caused by the higher formation of hydrogen bonding interaction between polymer molecules in the film, causing higher hydration at alkaline conditions. In this study, films contained glycerol as one of the plasticizers; as a consequence, some water might be bound to the film protein network as Hoque, Benjakul, and Prodpran (2010) stated.

The amide I band, located in the region ~1650 cm^{-1}, arises predominantly from C=O stretching vibrations, being weakly coupled with in-plane N-H bending and C-N stretching vibrations. Spectral changes in the amide I region have been associated with myofibrillar protein conformational changes and widely used for the spectroscopic analysis of the secondary structure of proteins (Bertram, Kohler, Bocker, Ofstad, & Andersen, 2006; Bocker, Ofstad, Bertram, Egelandsdal, & Kohler, 2006; Ojagh, Nunez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011; Palaniappan & Vijayasundaram, 2008).

To enhance the spectral resolution, a second derivative spectrum (Fig. 3B.) was used to investigate the amide I region (1700-1600 cm^{-1}). Alkaline-F showed the highest wavenumber at ~1655 cm^{-1}, which indicates a higher denaturation due to the loss of α-helical structure (secondary structure). In this case no difference was found among water-F, salt-F, and acidic-F (~1651 cm^{-1}). Alkaline-F also

showed intra-molecular aggregation of β-sheet structures (~1683 cm^{-1} at pH10 and 1682 cm^{-1} the rest), but less pronounced than in the α-helix band, which may indicate a more organized structure.

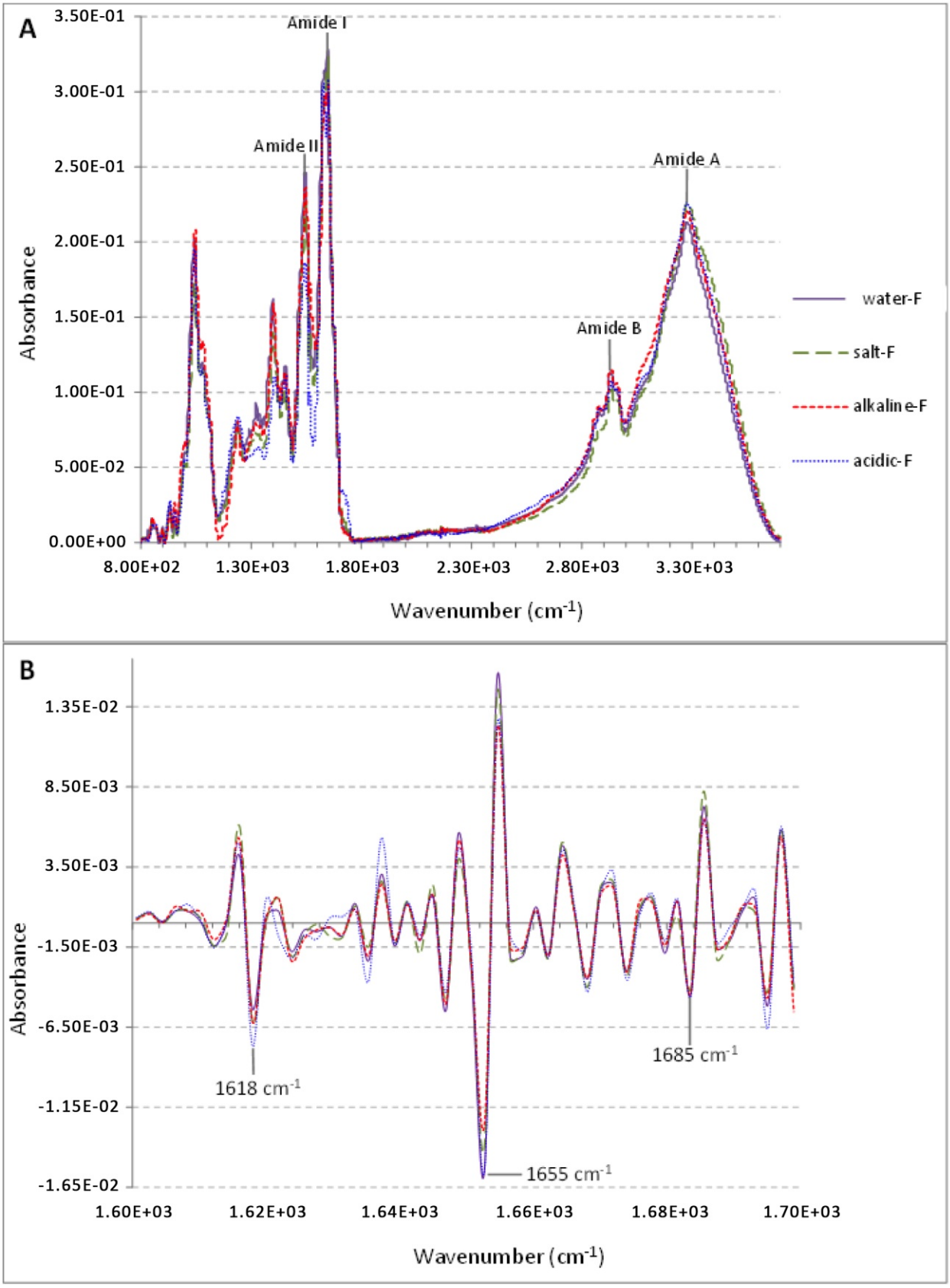

Figure 3. A. ATR-FTIR spectra of water-F, salt-F, alkaline-F and acidic-F films. B. Second derivative of Amide I band (1700-1600 cm^{-1}) from FTIR spectra of water-F, salt-F, alkaline-F and acidic-F films.

However, salt-F had the tendency to exhibit higher intermolecular β-sheet aggregation (~1618 cm^{-1}), which might have relation to nonhydrogenated C=O groups (Bocker, Kohler, Aursand, & Ofstad, 2008).

The amide II bands (~1545 cm^{-1}) (Fig. 3A) represent N-H bending vibrations coupled to C-N stretching vibrations. Generally, the lower wavenumber showed the existence of hydrogen bonds, with stronger hydrogen bonded peptide groups and collagen absorbing. Amide II band is less susceptible to secondary structure changes, but more affected by hydration. Regarding the extraction procedure, changes in these bands might be affected by the collagen rests. It is known that acidic pH condition helps collagen solution and protein hydrolysis, as there has been shown in the SDS-PAGE, producing short protein fragments more suitable for hydrogen bonding. While amide II band remained at ~1548 cm^{-1} in alkaline-F, it decreased to ~1539 cm^{-1} in acidic-F which may be due to the reduction of the number of non-bonded peptide groups caused by more extensive hydrogen bonding between the protein and glycerol (Chunli, Stading, Wellner, Parker, Noel, Mills et al., 2006), causing a higher hydration.

The peak situated around ~1000-1100 cm^{-1} might be related to possible interactions arising between plasticizer (OH groups of glycerol and sorbitol) and film structure (Bergo & Sobral, 2007). Acidic-F presented the lowest wavenumber (~1040 cm^{-1}), which were related to the possible extra interactions between short protein fragments and the plasticizers. On the other hand, alkaline-F presented the highest wavenumber (~1043 cm^{-1}) could be associate to less interaction with plasticizers.

Fourier-transform infrared study indicated that pH had some differences in functional groups and inter- and intra-molecular interaction, resulting in more protein-plasticizer and protein-water interactions at pH3 and more protein-protein interactions at pH10, which is due to the higher secondary structure lost reported at alkaline conditions, and the protein hydrolysis found at pH3.

6.4.2.3. Microbiological index

Total viable bacteria count was 6.52 log CFU/g in conditioned water-F and 6.57 log CFU/g in conditioned salt-F, which were higher than the limits in similarly conditioned edible gelatine films (3.7 log CFU/g). This is due to environmental conditions: time, temperature during processing and, particularly, the nature of extracts that are very suitable for the microbial growth. On the contrary, counts in conditioned alkaline-F and acidic-F (2.5 log CFU/g and 2.86 log CFU/g respectively) were lower than the recommended limits in the fishery products (5 log CFU/g). Regarding *enterobacteriaceae* counts, it was 4.13 log CFU/g in water-F, while only 0.4 log CFU/g grew in salt-F, and the growth was totally inhibited at pH10 (alkaline-F) and pH3 (acidic-F).

The nitrogen of total volatile bases (TVB-N) quantified in marine products had also been utilized as indicator of bacterial spoilage for some fish species, where amounts over 30 mg/100 g of muscle are considered the maximum allowed. In this study, the values observed were from 1135 to 274.5 mg N/100 g both raw material and film. Marquez-Rios, Moran-Palacio, Lugo-Sánchez, Ocano-Higuera, and Pacheco-Aguilar (2007) related the high level found (243.7 to 278.8 mg N/100 g *Dosidicus gigas* muscle) to the intrinsic high level of NH_4Cl, which acts as a physiological tool to regulate squid buoyancy. Therefore, the high TVB-N value detected in this species cannot be utilized as a quality/spoilage index for squid muscle material since it is not an exclusive consequence of bacterial activity.

Concerning their use as food packaging, only alkaline-F and acidic-F preparations would be considered harmless adequate to avoid microbial growth; however addition of an antimicrobial edible agent should be needed in the case of water-F and salt-F preparations.

6.4.2.4. Physical properties

The water activity evolution of the different films along the conditioning process at 58% RH and 20 °C for 3 days followed the typical profiles asymptotically. Acidic-F was the only film practically getting the RH equilibration (0.56 ± 0.01) and it seemed clear that the rest needed considerably longer conditioning times (salt-F 0.53 ± 0.03 and alkaline-F 0.54 ± 0.01), water-F in particular (0.50 ± 0.03). Moisture contents were however not significantly different among the films (Table 4, first column) except for alkaline-F which showed higher value, which confirms the elevated hydrogen bonding stated in the Amide A FTIR results. Since the plasticizer amount and the plasticizer/protein ratio were the same in all the films, it seemed that any different behaviour exhibited by the films may be likely due to different conformational states of corresponding proteins derived from the different extraction method used and the amount of hydrogen and protein bonds developed during the drying step. The highest film moisture (Table 4) was found at pH10 ($P \leq 0.05$) which might have been influenced by the higher formation of hydrogen bonding interaction between polymer molecules as it has been seen by FTIR (Amide A).

Film thicknesses examined in this study ranged between 108.8-126.8 μm (Table. 4). Salt-F and acidic-F were thicker ($P \leq 0.05$) and no significant difference was found between water-F and alkaline-F. This difference might be caused by the protein-protein, protein-water and protein-plasticizer interactions and the different protein size resulting in different compaction. Alkaline pH led to a better film aspect and compact thickness (Bourtoom, 2009), which might justify the higher density reached at pH10; and probably films made at pH3 were thicker due to the presence of more interactions with plasticizers.

Protein films have been associated with high water vapour permeability (*WVP*), which is caused by the high number of hydrophilic groups (Hamaguchi, Weng, & Tanaka, 2007). Despite the thickness was higher in salt- and acidic-F, the salt crystallization (Leerahawong, Arii, Tanaka, & Osako, 2011) and the hydrolysis caused by pH 3 increased their water vapour permeability ($P \leq 0.05$) (Table 4). A higher solubilized sarcoplasmic (globular and lighter) and the loss of secondary structure protein fraction at pH10 might improve the network matrix by filling the little holes left in the myofibrillar aggregation structure.

Depending on the raw material, protein film water vapour permeability was different (Paschoalick, García, Sobral, & Habitante, 2003), other author did not find significant difference between alkaline and acidic treatments; such as in 2% Indo-Pacific blue marlin (*Makaira mazara*) muscle protein and 1% glycerol film (Hamaguchi, Weng, & Tanaka, 2007; Iwata, Ishizaki, Handa, & Tanaka, 2000; Shiku, Hamaguchi, & Tanaka, 2003). Alkaline and acidic treatment did not lead to low water vapour permeability, contrary to Bourtoom (2009) findings. Adding different concentration of organic salts (0-10%) in films with 4% squid (*Todadores pacificus*) muscle protein and 2% glycerol, Leerahawong, Arii, Tanaka, and Osako (2011) did not observe a permeability reduction.

Film solubility increased with pH treatment and more drastically with NaCl ($P \leq 0.05$). Despite the significant differences, film solubility resulted in the same range and the differences were not higher than 7%. Current films were dried at 45 ºC for 21-23 h which might boost hydrophobic and covalent bonds.

Covalent bonds are mainly formed at higher temperatures but longer exposure times may also provide suitable conditions at lower temperatures (Gómez-Guillén, Montero, Solas, & Borderías, 1998). Moreover, sarcoplasmic proteins presence entails remained enzymes, one of those muscle endogenous transglutaminase (TGase) which catalyses the formation of a covalent bond between a free amine group and the gamma-carboxyamide group of protein- or peptide-bound glutamine at low and moderate temperatures (25-40 ºC), depending on the breed (Montero, López-Caballero, Pérez-Mateos, Solas, & Gómez-Guillén, 2005) which might also cause a reduction in water vapour permeability. From the total release material, only 2-4% was protein in water-, salt-, and alkaline-F, and 12% in acidic-F (Table 4), which indicated that the rest should mainly be plasticizer. The acidic treatment led to a higher protein release due to the increase of the network free volume (Cuq, Gontard, Cuq, & Guilbert, 1997). Nevertheless the results showed more interactions with plasticizers only in acidic-F. Shiku, Hamaguchi, and Tanaka (2003) observed in alkaline and acidic films with 1% Indo-Pacific blue marlin (*Makaira mazara*) myofibrillar proteins and 0,5% glycerol dried at 25 ºC for 24 h, that pH affected secondary, hydrophobic and hydrogen bonds, which might also lead to a weaker network compared to water-F.

Film	Moisture content (%)	Thickness (μm)	Film solubility (%)	Protein release (%)	*WVP* ($\times 10^{-7}$ g m^{-1} s^{-1} Pa^{-1})
water-F	21.35 ± 1.19 a	109.0 ± 9.0 a	40.75 ± 0.51 a	4.41 ± 0.91 a	1.35 ± 0.05 a
salt-F	22.63 ± 0.97 a	126.8 ± 7.4 b	47.67 ± 0.38 b	2.37 ± 0.26 b	1.77 ± 0.03 b
alkaline-F	27.42 ± 0.56 b	108.8 ± 8.7 a	45.48 ± 0.78 c	3.11 ± 0.01 c	1.55 ± 0.02 c
acidic-F	20.99 ± 1.36 a	125.6 ± 6.5 b	42.95 ± 0.56 d	12.98 ± 0.28 d	1.71 ± 0.04 b

Table 4. Thickness, moisture content, film solubility, protein release and water vapour permeability (*WVP*) of water-F, salt-F, alkaline-F and acidic-F films.
Results are the mean ± standard deviation. One-way ANOVA: Different letters in the same column indicate significant differences among the different films (P≤0.05).

Figure 4 illustrates the electrophoretic patterns of the proteins released in water, where water-F presented the lowest band intensities. This might mean a higher loss of sarcoplasmic proteins and plasticizers proportion instead of myofibrillar proteins, which seems to be strongly aggregated and was not released from the matrix.

Both water- and salt-F showed bands below 50 kDa, which could be due to a sarcoplasmic protein release. While salt-F showed Tropomyosin (30-35 kDa) and actin (45 kDa) soft bands, they were not visible in water-F, which indicated changes and/or interactions in proteins as a consequence of salting (Llorca, Hernando, Pérez-Munuera, Quiles, Larrea, & Lluch, 2007) making water-F more insoluble. 17 kDa band corresponds to light chains of myosin (Llorca, Hernando, Pérez-Munuera, Quiles, Larrea, & Lluch, 2007), and was hardly visible in water-F but quite intense in salt- and alkaline-F, which indicated myosin degradation to little fragments. Alkaline-F also showed the light chain of myosin, which might have come from LMM which, in turn, showed lower band intensity in respective alkaline-FS, leading to a different cross-linking pattern and forming weaker bonds facilitating their release in water contact. The acidic-FS protein hydrolysis previously observed both in the extract and DSC results, led to the weakest network in acidic-F, releasing more protein material, including a large amount of tropomyosin and even actin and paramyosin. Acidic-F retained more plasticizers and water, which may explain the unique

swelling effect caused when they came in contact with water, partially losing its network integrity but not getting fully dissolved. Kristinsson and Hultin (2003) also found that, as a result of the HMM dissociation, the relative viscosity and hydrodynamic volume were higher at acidic than at alkaline pH. According to the protein solubility determined by BCA, the maximum release of water-soluble proteins was observed in acidic-F, indicating a higher release of proteins than plasticizers; while alkaline-F and salt-F showed the lower values, possibly due to a higher proportion of plasticizers lost, being slightly higher in alkaline-F due to the denaturalization effect of alkaline conditions.

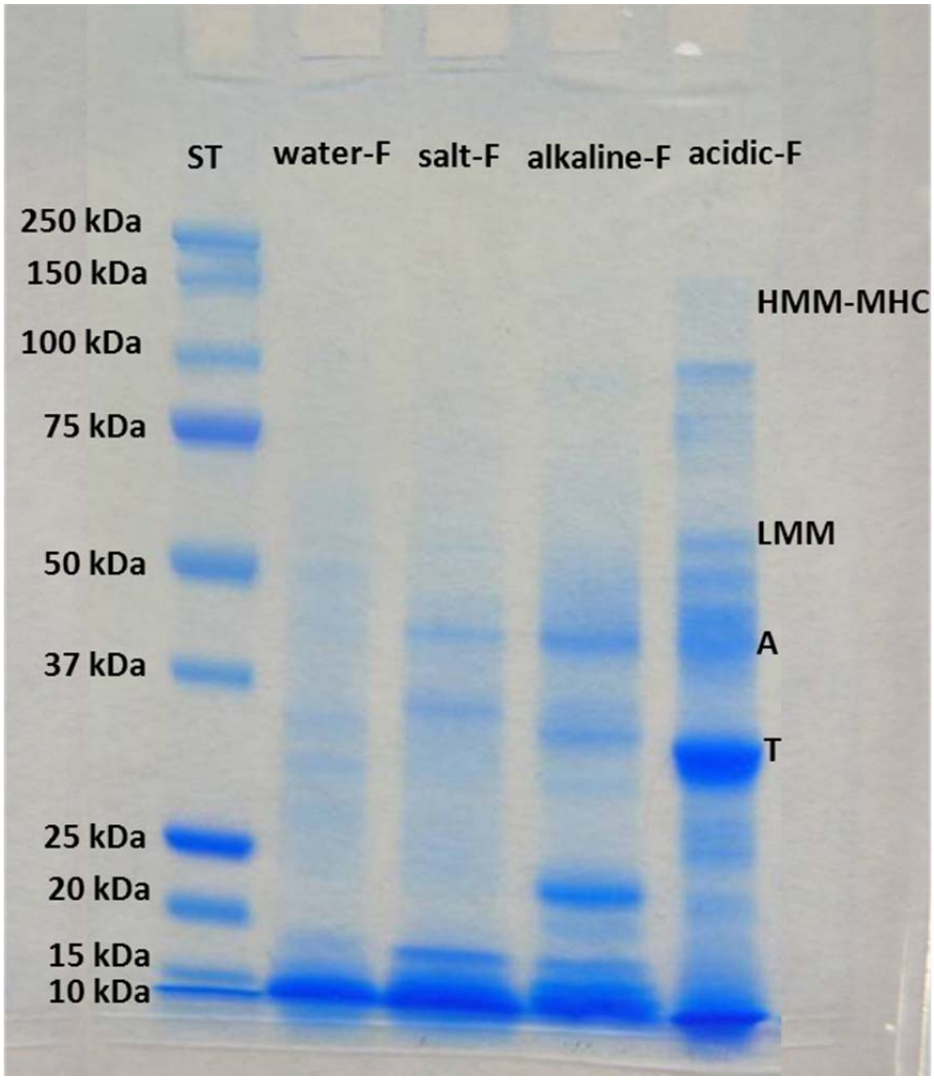

Figure 4. SDS-PAGE patterns under reducing conditions of proteins released in water from water-F, salt-F, alkaline-F and acidic-F films. ST: Standard; MHC: Myosin heavy chain; HMM-MHC: Heavy meromyosin-myosin heavy chain; LMM: Light meromyosin; P: Paramyosin; A: Actin; T: Tropomyosin.

Table 5 describes film water resistance. Water-F samples did not break and resisted more time before water soaked through. Water-F, salt-F and alkaline-F presented short and similar elongation while acidic-F rapidly stretched until breakage. Experimental results could not be compared to others because the inexistence of previous data. This can be explained by the fact that protein interactions were modified

	water-F								salt-F				alkaline-F			acidic-F						
Elongation time (h)	0.13	0.50	4	24	51	120	192	288	0.05	0.10	1	24	0.08	0.13	25	0.02	0.03	0.05	0.06	0.07	0.08	0.1
Elongation (cm)	0.3± 0.01	0.7± 0.1	1± 0.1	11.3± 0.5	1± 0.01	0.5± 0.01	0.3± 0.01	0.1± 0.01	0.3± 0.01	0.5± 0.02	1± 0.1	0.7± 0.02	0.5± 0.02	1± 0.2	1.3± 0.1	0.5± 0.01	1± 0.1	1.33± 0.1	1.4± 0.2	1.7± 0.1	1.8± 0.1	2± 0.2
Water filtration time (h)	5 ± 0.5								2 ± 0.3				0.17 ± 0.03			No filtration						
Breakage time (h)	Unbreakable								50 ± 2				48 ± 1			0.09 ± 0.01						

Table 5. Water resistance parameters: Elongation time (h), Elongation (cm), Water filtration time (h) and Breakage time (h), for water-F, salt-F, alkaline-F and acidic-F films
Results are the mean ± standard deviation.

Alkaline-F showed significantly highest (P≤0.05) tensile strength (*TS*) (Table 6), followed by water-F and salt with an intermediate *TS* value, although salt- values were not always significantly different from acidic-F. Nevertheless, tensile values were lower than the results obtained in other studies on different muscle protein and plasticizer percentages from various raw materials, such as 1% glycerol and 2% Purple-spotted bigeye (*Priacanthus tayenus*) myofibrillar protein films at acidic and alkaline pH (*TS* ~3.5 MPa) (Chinabhark, Benjakul, & Prodpran, 2007), 0.5% glycerol and 1% Indo-Pacific blue marlin (*Makaira mazara*) myofibrillar protein films (8-16.7 MPa) (Shiku, Hamaguchi, & Tanaka, 2003) or ~1% glycerol and 2% Nile Tilapia (*Oreochromis niloticus*) myofibrillar protein films at acidic pH (2-10 MPa) (Sobral, dos Santos, & García, 2005). Both species had a very superior muscle texture compared to *Dosidicus gigas*', which is extremely tender, meaning that intrinsic characteristics might influence their mechanical resistance.

Film	*TS* (MPa)	*EAB* (%)	*Y* (MPa)	*F* (N)	*D* (%)
water-F	1.36 ± 0.32 a	1.82 ± 0.28 a	83.8 ± 16.9 a	6.59 ± 0.44 a	8.88 ± 0.59 a
salt-F	0.97 ± 0.33 ac	1.90 ± 0.89 a	51.0 ± 16.7 b	4.80 ± 0.66 a	8.52 ± 2.50 a
alkaline-F	3.10 ± 0.53 b	12.09 ± 3.25 b	55.0 ± 11.9 ab	15.78 ± 0.46 b	24.06 ± 2.36 b
acidic-F	0.85 ± 0.60 c	13.28 ± 1.68 b	34.0 ± 4.9 b	6.78 ± 1.02 a	12.69 ± 2.96 a

Table 6. Tensile strength (*TS*), elongation at break (*EAB*), Young's modulus (*Y*), puncture force (*F*) and puncture deformation (*D*) of water-F, salt-F, alkaline-F and acidic-F films.
Results are the mean ± standard deviation. One-way ANOVA: Different letters in the same column indicate significant differences among the different films (P≤0.05).

The elongation at break values (Table 6) showed notable differences between those with pH shifting and the others without it, being much higher in alkaline-F and acidic-F. These results seem to refute the common belief that tensile strength and elongation at break are inversely related in edible protein films (Kester & Fennema, 1986; Krochta & DeMulderJohnston, 1997). Experimental results could not be compared to others because the inexistence of previous data, but showed lower elongation than previous studies in fish muscle protein films, such as 1% glycerol and 2% Indo-Pacific blue marlin (*Makaira mazara*) muscle protein films (74.6 ± 7.4%) (Hamaguchi, Weng, & Tanaka, 2007), and 0.3-1.3% glycerol and 2% Nile Tilapia (*Oreochromis niloticus*) muscle protein films (30-90%) (Sobral, dos Santos, & García, 2005); but neither of those who compared acidic and alkaline pH found differences between their elongation at break.

As far as Young's modulus is concerned (Table 6), the highest elasticity corresponded to water-F (P≤0.05) while acidic-F obtained the lowest one (P≤0.05). Despite the alkaline-F and acidic-F flexibility, their stretchiness was not high, and water-F had less plasticity than alkaline- and acidic-F but more elasticity. These results might suggest that water-F might have stronger protein interactions and weaker interactions with the plasticizer and water than acidic-F, as it has been seen in FTIR results. These results were much higher than those in 1% glycerol and 2% Nile Tilapia (*Oreochromis niloticus*) muscle proteins at acidic pH (5-10 MPa) (Sobral, dos Santos, & García, 2005).

Regarding puncture deformation (*D*) no significant differences were observed between water-F, salt-F, acidic-F films (Table 6), even higher than the tensile elongation at break: while in acidic-F the

percentage was similar to the *EAB*. Respect to the puncture force (*F*) (Table 6), a similar behaviour to tensile test was detected, with the only significant difference in alkaline-F which showed the highest resistance to the puncture (P≤0.05). In general, deformation capacity was greater (2-9%) than the observed by other authors in 1% glycerol and 2% Nile Tilapia (*Oreochromis niloticus*) muscle protein films elaborated at acidic pH (Sobral, dos Santos, & García, 2005; Sobral, García, Habitante, & Monterrey-Quintero, 2004), or 0.3-1.3% glycerol and 1% Nile Tilapia (*Oreochromis niloticus*) myofibrillar protein films at pH 2.7 (Paschoalick, García, Sobral, & Habitante, 2003; Sobral, 2000). Puncture force values at acidic conditions were however similar.

Artharn, Benjakul, and Prodpran (2008) found that higher sarcoplasmic protein solubilized proportion in film-forming solution reduced tensile strength while higher myofibrillar protein solubilized proportion increased strength due to its fibrillar structure and cross-linking capacity. Sarcoplasmic protein with low molecular weight would make possible their dispersion and insertion between myofibrillar proteins during drying, weakening myofibrillar protein-protein interactions and favouring network migrations (Orliac, Rouilly, Silvestre, & Rigal, 2002; Shiku, Hamaguchi, & Tanaka, 2003; Sobral, dos Santos, & García, 2005); which might be the reason for the highest elasticity in water-F. The low tensile strength obtained at pH3 (acidic-F) is related to less protein-protein interactions and more protein-plasticizer and protein-water interactions due to the hydrolysis caused by acidic conditions. Leerahawong, Arii, Tanaka, and Osako (2011) found that films with 2% glycerol and 4% squid (*Todadores pacificus*) myofibrillar protein showed salt crystallization from 0.5% NaCl addition, which affected film physical properties. This may justify the mechanical behaviour obtained with 0.4% NaCl films, not being remarkable in any test.

These findings were consistent with the low-temperature scanning electron microscopy (LowT-SEM) images (Fig. 5) of the current series of edible films. Water-F and salt-F films presented a similar cross section and occasionally showed some bacterial growth as mentioned before.

However, salt-F showed a more laminar structure while water-F seemed denser compact and disorganized. This water-F structure might favour the water resistance, film low solubility and low water vapour permeability. Salt-F surface had phosphate crystals, by its tendency to catch cations in detriment to NaCl.

Dosidicus mantle is usually treated with phosphates during the process, and it is present in all muscles, although the crystals only are formed in presence of NaCl. This irregular salt-F surface aspect was congruent with the findings of Leerahawong et al. (2011) who hypothesized that it should be due to the salt crystallization produced from 0.5% NaCl concentration in 4% muscle protein squid (*Todadores pacificus*) and 2% glycerol films.

Despite the similar cross section appearance at 500X, alkaline-F tended to be more compact, evidencing tubular structures and little globules where the cut was irregular, indicating a strong and resistant structure. Higher protein network density might cause the reduction of thickness showed in water-F and alkaline-F films. Interestingly, acidic-F showed more resistance to the cut due to its high plastic behaviour causing a rough cross section probably affected by that difficulty. Despite their irregular cross section, acidic-F was homogeneous and less compact than water-F and alkaline-F films. This

plastic behaviour and appearance might be due to the high proportion of protein-water and protein-plasticizer interactions.

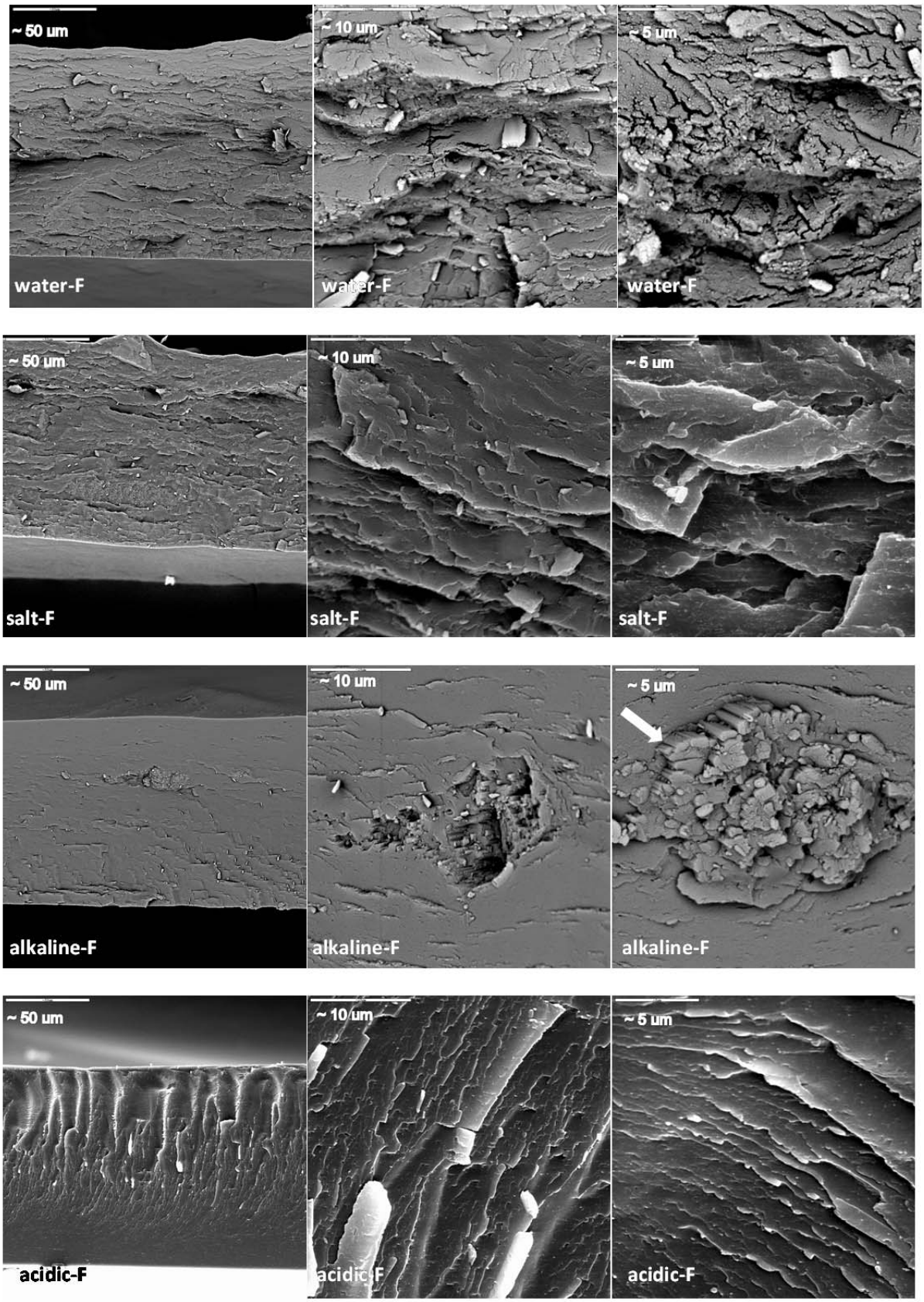

Figure 5. Low Temperature-Scanning Electron Microscopy cross section images of water-F, salt-F, alkaline-F and acidic-F films.

6.4.2.5. DSC

Typical DSC traces of the films (F) are also shown in Figure 2: a clear endothermic transition was evident with maximum temperatures at around 70 − 80 ºC in the cases of water-F, salt-F and alkaline-F, while acidic-F obviously presented no transitions. It was consistent with the thermal behaviour of corresponding mother extracts (E) but with some additional effects: First, the presence of plasticizers may induce some crystalline inhibiting effects; secondly, higher denaturation was expected in low-thermostable proteins such as myosin, since the long drying time period at 45 ºC.

Thermal denaturation data were: 74.57 ± 0.18 ºC and 1.19 ± 0.06 J/g_{dm} in water-F; 76.47 ± 0.42 ºC and 0.40 ± 0.03 J/g_{dm} in salt-F; 69.32 ± 0.16 ºC and 2.88 ± 0.06 J/g_{dm} in alkaline-F. Transition temperatures T_{peak} and corresponding transition enthalpies ΔH were, on each side, significantly different among the films. Interesting to note is that salt-F was the most affected by processing in that film drying caused NaCl precipitation (LowT-SEM), suggesting that a reduced salt effect in decreasing transition temperatures may likely be derived as well as an increased plasticization (free-water) and subsequent vitrification effects in the system. These results match with the mechanical film behaviour previously discussed: higher interaction with plasticizer in acidic conditions led to plastic behaviour (a better elongation but less resistant); whereas in alkaline-F less denaturized proteins resulted in better mechanical behaviour.

Similarly to extracts E, thermal results on films F generally conformed to respective solubility data but did not necessarily match corresponding electrophoretic patterns.

6.5. Conclusion

The present study emphasizes on alkaline- and acidic-films. Water-F presented high water-resistance, lower solubility and water vapour permeability, which might be interesting for some particular applications, but both water-F and salt-films were not microbiologically stables and did not show as good mechanical properties as alkaline and acidic-F. Alkaline pH led to a higher unfolded myosin inducing changes in the structure and also showed intramolecular aggregation, hence there were more functional groups available. This effect led to the highest water-soluble protein fraction in the film-forming solution, therefore an easier molecular orientation and hence a more mechanically resistant film.

On the other hand, pH3 induced protein hydrolysis also enhancing its solubility in film-forming solution but leading to a weaker network due to more plasticizer-protein interactions, additionally increasing its hygroscopicity. Besides acidic-film had not as good behaviour in contact with water as alkaline film, both of them were transparent, a good UV barrier and had good mechanical properties.

6.6. References

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VII. Jumbo squid (*Dosidicus gigas*) myofibrillar protein concentrate for edible packaging films and storage stability

Jumbo squid (*Dosidicus gigas*) myofibrillar protein concentrate for edible packaging films and storage stability

Blanco-Pascual N., Fernández-Martín F. and Montero P. *Dosidicus gigas* myofibrillar protein concentrates for edible packaging films and storage stability. *LWT - Food Science and Technology*. Submitted 6th March, 2013.

7.1. Abstract

Properties and storage stability of two different jumbo squid myofibrillar protein-based films were investigated. Myofibrillar proteins were extracted by isoelectric precipitation after acidic and alkaline solubilization, obtaining the same extraction yield. During these extractions, not only was the edible fraction, discarded during the mantle skinning, recovered but also was the fishy flavour produced by the nitrogen and other undesirable compounds removed; although some sarcoplasmic proteins were simultaneously lost. In alkaline-Concentrate (C), myosin unfolding led to water resistant films, less water vapour permeable and more mechanical resistant than acidic–Films (F); whereas acidic-C protein hydrolysis resulted in more transparent and soluble films, with higher protein release (~80 g/L). During 4 months of storage, some structure reorganization occurred, and both films incremented their yellowish tendency, especially the acidic-F, which was attributed to a Maillard reaction with the plasticizers. After storage time, water solubility increased in C-films. While acidic-F aggregation led to a protein release reduction and tensile strength improvement; alkali-F became weak and brittle, loosing transparency. C-films offered different filmogenic properties, being promising biodegradable packaging materials.

7.2. Introduction

During the last decade, there has been an increasing research interest in edible and biodegradable packaging films, and some proteins have been extensively studied due to their filmogenic capacity (Gennadios, 2002). Myofibrillar proteins play a functional role in foods, as they produce viscoelastic gel matrixes ,entrap water and form strong flexible films (Krochta & DeMulderJohnston, 1997).

Jumbo squid (*Dosidicus gigas*) is the largest and most abundant squid species found in the pelagic zone of the eastern Pacific, from Chile up to Oregon coasts (Nigmatullin, Nesis, & Arkhipkin, 2001), and it is normally imported to numerous countries, mainly Europe and Asia, due to its high percentage of edible portion (600-800 g/kg) and its suitability for industrial processing. Jumbo squid processing waste represents up to 200-400 g/kg of the total weight, which includes viscera, rests of muscle, tunics and tentacles among others. A number of studies have reported jumbo squid muscle as an excellent raw material to obtain co-products with high commercial value such as gel-based products (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008), gel-emulsion products (Felix-Armenta, Ramírez-Suarez, Pacheco-Aguilar, Diaz-Cinco, Cumplido-Barbeitia, & Carvallo-Ruiz, 2009), *surimi* (Campo-Deaño, Tovar, Jesús Pombo, Teresa Solas, & Javier Borderías, 2009) and also other collagen-based products from tunics (Denavi, Pérez-Mateos, Añón, Montero, Mauri, & Gómez-Guillén, 2009).

During the skinning of the mantle, a great amount of muscle (~300-400 g/kg) is discarded, which could be reutilized by different protein recovery methods. The ability to solubilize myofibrillar proteins at extreme acidic (pH 2-3) (Hultin & Keelleher, 1998a) or alkaline (pH 10.5-11.5) (Hultin & Keelleher, 1998b) conditions and a subsequent isoelectric protein precipitation (pH 4.8-5.5) leads to the highest recovery of highly functional myofibrillar proteins.

Muscle proteins have been lately recovered from the waste of the fishing industry to study their film-forming ability and nutritional value (A. Artharn, Benjakul, & Prodpran, 2008; Hamaguchi, Weng, & Tanaka, 2007). Due to their high intermolecular binding potential, protein-based films can have good optical and mechanical properties but poor water vapour resistance (Krochta & DeMulderJohnston, 1997).

Myofibrillar isoelectric precipitated protein films from jumbo squid and from other cephalopods have never been developed before, although Leerahawong, Arii, Tanaka, and Osako (2011), Leerahawong, Tanaka, Okazaki, and Osako (2011) and Blanco-Pascual, Fernández-Martín, and Montero (2013) studied the properties of *Todadores pacificus* and *Dosidicus gigas* muscle films respectively. However, some compounds should be removed in order to improve the product versatility and mantain good properties for its comsuption; for instance, the reduction of its NH_4Cl content (Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009).

Due to the film components nature, molecular changes and reorganization can be expected to take place over time. Stability is scarcely known in edible films and it can be significantly different depending on matrixes composition. There are only a few studies regarding myofibrillar/muscle films stability (Artharn, Prodpran, & Benjakul, 2009) and none regarding jumbo squid.

The aim of the study was to study the jumbo squid myofibrillar protein concentrate as a potential material for film development and to evaluate film stability at short term (four months). For those purposes, two different types of high quality protein concentrates (acidic and alkaline) were obtained.

7.3. Materials and methods

7.3.1. Materials

Frozen muscle proteins were recovered from mechanical mantle skinned waste of jumbo squid *surimi* process industry (PSK Océanos S.A. Pozuelo de Alarcón, Madrid, Spain).

Analytical grade HCl, NaOH, NaBr, glycerol and sorbitol were from Panreac Química S.A. (Montplet and Estaben S.A., Montcada i Reixac, Barcelona, Spain). DOW 1510 silicon antifoaming agent was from DOW Corning Europe (Brussels, Belgium).

7.3.2. Methods

7.3.2.1. Myofibrillar protein concentrate

The mantles were kneaded in a vacuum homogenizer (Stephan UM5, Stephan u Söhne GmbH & Co., Hameln, Germany) with the blades mixer tool, at temperatures lower than 10 ºC, with distilled water at 1:1 (v:w) proportion and DOW 1510 at 1 drop/100 mL(≤ 10 ºC). For alkaline and acidic extractions, pH was adjusted to 10.0 ± 0.2 and 3.0 ± 0.2 with NaOH and HCl respectively (pH-meter series 3 Star Orion and electrode pH ROSS, Thermo Fisher Scientific Inc., Landsmeer, Netherlands). Two homogenization cycles (30 s at 1500 rpm and 90 s at 3000 rpm) were carried out. Tunics were manually discarded and myofibrillar proteins were precipitated at isoelectric pH (4.8) and centrifuged at 5000 rpm for 5 min (4 ºC)

using a Sorvall Evolution RC Centrifuge (Thermo Fisher Scientific Inc., Landsmeer, Netherlands). Alkaline and acidic concentrate (C) yields were calculated by percentage of wet material weight.

7.3.2.2. Film preparation

Film-forming solutions (FS) of protein concentrates (25 g/L) were mixed with glycerol and sorbitol at the same proportion at 400 g/kg of total protein at pH10 ± 0.05 (alkaline-FS) or pH3 ± 0.05 (acidic-FS) adjusted with NaOH and HCl respectively. Film-forming solutions were filtered and cast into methacrylate plates (Plexiglas® GS Röhm GmbH & Co. KG, Darmstadt, Germany) to obtain films around 100-110 µm of thickness. Plates were left for 21-23 h at 4 ± 0.5 ºC and 85 ± 5% relative humidity (RH) prior to further drying in an oven at 45 ± 0.8 ºC and 12 ± 3% RH for 21-23 h (FD 240 Binder, Tuttlingen, Germany). All films were conditioned at 58 ± 0.2% RH and 22 ± 1 ºC for 4 days prior to analysis, reaching almost the same moisture in alkaline (190.3 ± 9.9 g/kg) and acidic-F (206.1 ± 5.4 g/kg).

7.3.2.3. Protein concentrates solubility and film forming solutions

Soluble protein, in concentrated extracts and film-forming solutions, was determined by BCA (Meridian RD., Rockford, IL, 61101 USA) (Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, et al., 1985). The corresponding water-soluble protein fractions were expressed at least in triplicate as grams of protein solubilized with respect to kg of total muscle protein (A.O.A.C. 2000) in wet basis.

7.3.2.4. Electrophoretic analysis (SDS-PAGE)

Alkaline and acidic extracts before isoelectric precipitation and FSs SDS-PAGE electrophoretic analysis and the electrophoretic profile of water-soluble film proteins were performed at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

7.3.2.5. Thermal properties

Calorimetric analysis of concentrates and films were performed using a differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA) as described in Blanco-Pascual, Fernández-Martín, and Montero (2013). Additionally, glass transition temperatures, Tg (ºC), were determined in triplicate by the midpoint method.

7.3.2.6. Film determinations

7.3.2.6.1. Thickness

It was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan) averaging the values of 6-8 random locations in 15 films per treatment as described by Pérez-Mateos, Montero, and Gómez-Guillén (2009) confirming values of 107.63 ± 11.48 and 106.09 ± 6.62 µm for alkaline-Film (F) and acidic-F respectively.

7.3.2.6.2. ATR-FTIR spectroscopy

Infrared spectra between 4000 and 650 cm^{-1} were recorded at least in triplicate using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA) as was described by Ojagh, Nunez-Flores, López-Caballero, Montero, and Gómez-Guillén (2011).

7.3.2.6.3. Light absorption and film transparency

Light barrier properties and transparency were calculated at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths (200-700 nm) following the method described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

7.3.2.6.4. Colour measurements

Lightness (*L**), redness (*a**), and yellowness (*b**) were measured following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

7.3.2.6.5. Water resistance

Film water resistance elongation (cm) in time (h), water filtration time (h) and Breakage time (h) were measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013)

7.3.2.6.6. Water vapour permeability (*WVP*)

WVP was determined at least in triplicate following the method described by Sobral (2000).

7.3.2.6.7. Water solubility

Film solubility was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

7.3.2.6.8. Mechanical properties

Tensile and puncture tests were run at least in triplicate using a texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) as was described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

7.3.2.6.9. Microstructure

Low temperature scanning electron microscopy (LowT-SEM) (Oxford CT1500 Cryosample Preparation Unit, Oxford Instruments, Oxford, England) was used to examine representative film surfaces and cross sections as described by Gómez-Guillén, Ihl, Bifani, Silva and Montero (2007).

7.3.2.7. Film stability during for four months of storage

The remaining films were stored in the desiccators at 58% relative humidity and 22 ± 2 ºC for 4 months. ATR-FTIR, DSC, thickness, light transmission, transparency, colour, solubility, tensile strength, elongation at break and Young's elastic modulus test were performed at least in triplicate.

7.3.3. Statistical analysis

Statistical tests were performed using IBM SPSS statistics (SPSS Statistical Software Inc., Chicago, Illinois, USA) for one-way analysis of variance. The variance homogeneity was made using the Levene test or the Brown-Forsythe test when variance conditions were not fulfilled. Paired comparisons

were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

7.4. Results and discussion

7.4.1. Protein concentrates (C) and film-forming solutions (FS) characterization

Soluble protein for each C and the corresponding FS are shown in table 1. High protein solubility and no significant differences were found between concentrates. This high jumbo squid muscle protein solubility has being attributed to the partial unfolding of myosin caused by extreme pH conditions (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009).

	SP (g/kg)	
Sample	Concentrate (C)	Film-forming solution (FS)
Alkaline-	920.1 ± 31.0 a/x	804.4 ± 18.3 a/y
Acidic-	890.7 ± 35.1a/x	796.3 ± 32.0 a/y

Table 1. Water-soluble protein (SP, g/kg) in alkaline-C and acidic-C concentrates (C), and corresponding film-forming solutions (FS) alkaline-FS and acidic-FS.
Results are the mean ± standard deviation. One-way ANOVA: Different letters, a and b in the same column indicate significant differences among the different pH. Different letters, x and y in the same row indicate significant differences among concentrates and film-forming solutions ($P \leq 0.05$).

Alkaline and acidic FS protein solubility was not significantly different either (~800 g/kg) ($P \leq 0.05$), being significantly lower than the respective concentrates. This loss of protein solubility might have been caused by a higher protein degradation after precipitate solubilization (De la Fuente-Betancourt, García-Carreno, Del Toro, & Cordova-Murueta, 2009), and/or could favour more protein-plasticizer interactions.

Molecular weight protein distribution of solubilized extracts, before concentration, and FS are shown in Figure 1. Alkaline myosin heavy chain (MHC), paramyosin (P) or actin (A) intensity bands did not show signs of degradation. Acidic MHC (205 kDa) almost disappeared, whereas the band intensities of heavy meromyosin-myosin heavy chain (HMM-MHC) (150 KDa), P (108 kDa), light meromyosin (LMM) (75 kDa) and A (45 kDa) were reduced. Besides, acidic samples depicted more intense bands below the 50 kDa region, which is the expected location of new bands after hydrolysis. Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, and García-Sánchez (2008) observed that acidic pH induces conformational changes in protein structure, making them more liable to acidic proteases, therefore causing a reduction of MHC band. Blanco-Pascual, Fernández-Martín, and Montero (2013) found similar electrophoretic patterns for alkaline and acidic muscle protein solubilization.

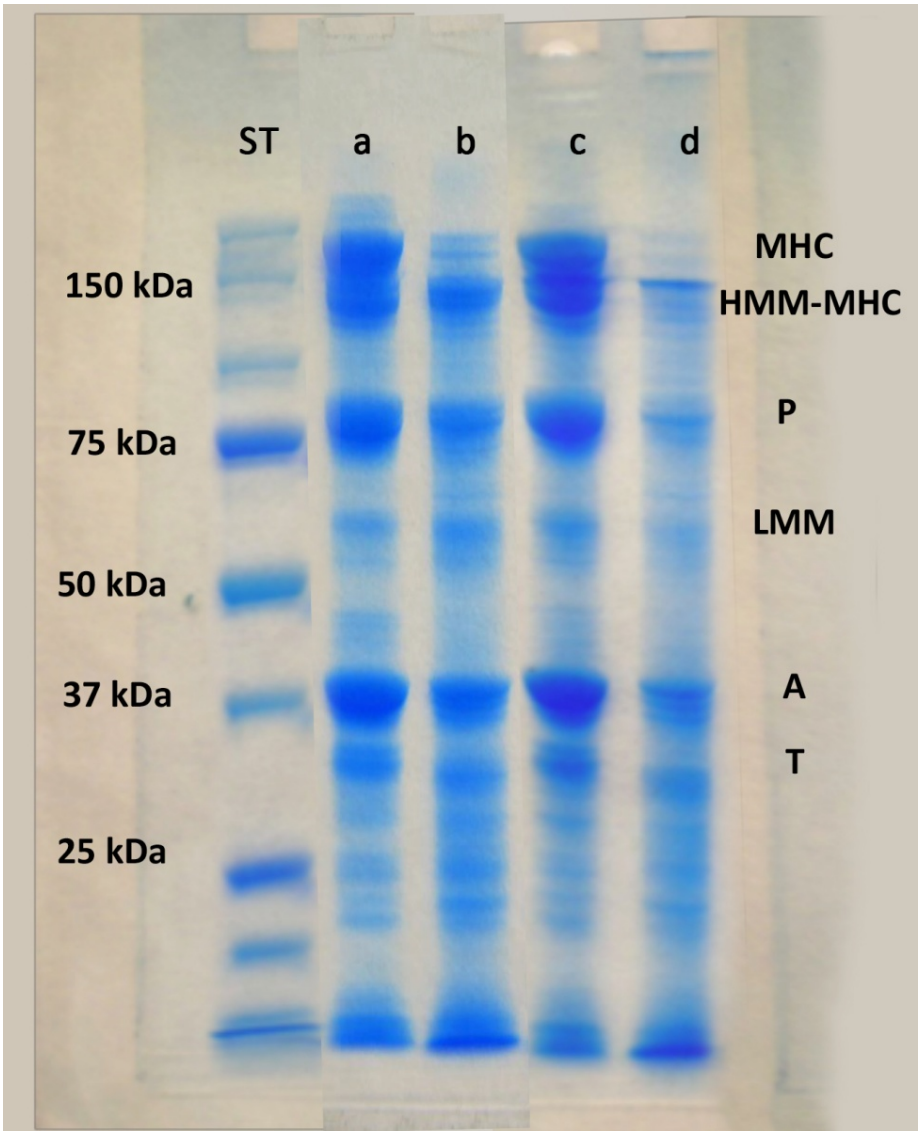

Figure 1. SDS-PAGE patterns of alkaline (a) and acidic (b) extracts before isoelectric precipitation and alkaline (c) and acidic (d) film-forming solutions. ST: Standard; MHC: Myosin heavy chain; HMM-MHC: Heavy meromyosin-myosin heavy chain; LMM: Light meromyosin; P: Paramyosin; A: Actin; T: Tropomyosin.

Figure 2 shows typical DSC traces of concentrates: alkaline-C and acidic-C depicted a single (rounded shaped) endothermic transition at practically the same range of 40 − 75 ºC, with acceptable preservation of some myosin and less preserved actin proteins (Wright, Leach & Wilding, 1977). Respective T_{peak} (ºC) were 56.7 ± 1.4 and 53.9 ± 1.6, not significantly different. Corresponding denaturation enthalpies ΔH (J/g_{dm}) were 1.59 ± 0.23 and 3.41 ± 0.24, being significantly smaller for alkaline-C. These traces were essentially different to alkaline-E and acidic-E extracts (prior to isoelectric precipitation) (Blanco-Pascual, Fernández-Martín, & Montero, 2013) resembling typical actomyosinic profiles of frozen giant squid (*D. gigas*) muscle proteins (Fernández-Martín, 1998). Isoelectric precipitation induced some equalization in the concentrates, showing similar profiles, but acidic-C exhibited significantly higher ΔH. This contrasted with respective acidic-E showing great protein degradation as usual in myofibrillar acidic-shift processing (Totosaus, Montejano, Salazar, Guerrero, 2002).

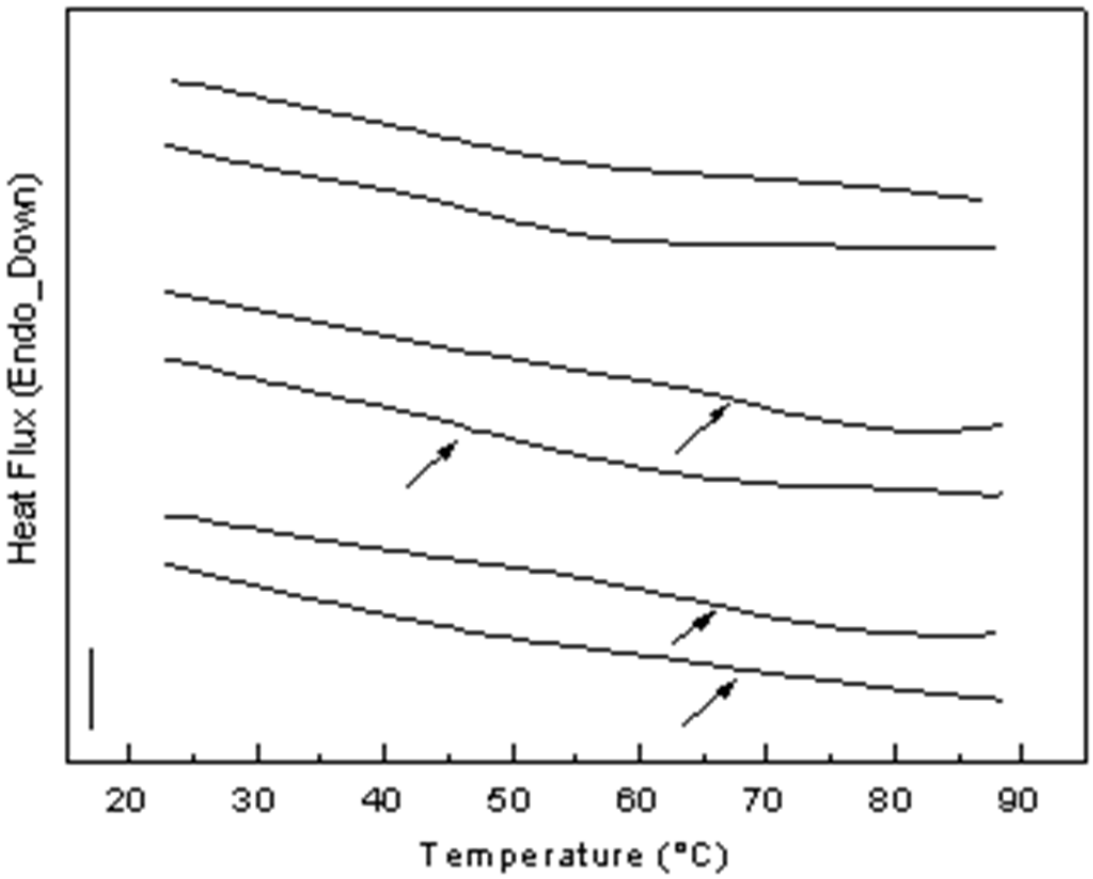

Figure 2. Typical normalized DSC traces of concentrates C, fresh films F and aged films F4. Lines from top to bottom: alkaline-C, acidic-C, alkaline-F, acidic-F, alkaline-F4, acidic-F4. Bar indicates ordinate scale (0.02 W/g_{dm}). Arrows roughly indicate Tg zones.

7.4.2. Film properties

7.4.2.1. Light barrier properties

Light transmission in UV and visible ranges as well as Transparency are shown in Table 2. Films scarcely exhibited transmission to light in the UV range; which could be taken into consideration in order to prevent food oxidation. These results are consistent with earlier works (Leerahawong, Arii, Tanaka, & Osako, 2011; Leerahawong, Tanaka, Okazaki, & Osako, 2011) where cephalopod protein film were very good UV barriers, becoming gradually poor above the wavelength of 300 nm.

Acidic-F was more transparent than alkaline (Table 2). Both concentrated-F transmitted more visible light than films previously formulated with jumbo squid alkaline and acidic solubilized muscle proteins (Blanco-Pascual, Fernández-Martín, & Montero, 2013), attributable to the lack of certain sarcoplasmic proteins. High film transparency might have been favoured by the high protein solubility and the protein denaturation caused by acidic and alkaline conditions; concretely at pH3, transparency could have been promoted by the presence of protein fragments. (Leerahawong, Tanaka, Okazaki, & Osako, 2011), *Todadores pacificus* muscle protein films became more transparent when protein solubility was improved by higher amount of organic salts.

Film	T at selected wavelengths							Transparency
	200	280	350	400	500	600	700	
Alkaline-F	0.01 ± 0.01 a/x	0.01 ± 0.01 a/x	32.54 ± 0.44 a/x	53.43 ± 0.30 a/x	70.36 ± 0.42 a/x	75.50 ± 0.50 a/x	77.45 ± 0.63 a/x	0.95 ± 0.03 a/x
Acidic-F	0.01 ± 0.01 a/x	0.01 ± 0.01 a/x	14.48 ± 1.57 b/x	40.44 ± 0.52 b/x	76.10 ± 1.87 b/x	85.94 ± 2.55 b/x	88.27 ± 2.67 b/x	0.6 ± 0.01 b/x
Alkaline-F 4	0.01 ± 0.01 a/x	0.01 ± 0.01 a/x	29.67 ± 0.48 a/y	51.92 ± 0.80 a/y	69.63 ± 0.37 a/x	74.76 ± 0.24 a/x	76.33 ± 0.60 a/x	1.16 ± 0.03 a/y
Acidic-F 4	0.01 ± 0.01 a/x	0.01 ± 0.01 a/x	12.03 ± 0.95 b/x	37.35 ± 1.33 b/y	72.16 ± 0.57 a/y	81.41 ± 0.72 b/y	83.43 ± 1.14 a/y	0.56 ± 0.08 b/x

Table 2. Light transmission (T %) at several wavelengths (nm) and Transparency ($-\log(T_{600}/x)$ of alkaline and acidic-F films at day four and after four months of storage (4).
Results are the mean ± standard deviation. One-way ANOVA: Different letters, a and b indicate significant differences among the different pH. Different letters, x and y indicate significant differences in each treatment among the fourth day and the fourth month ($P \leq 0.05$). The higher value indicated that the film was less transparent.

*L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish) values are shown in Table. 3. This table reveals that films had low lightness (~34) and lack of both reddish (+*a**) and yellowish (+*b**) tendencies, being suitable to be used as see-through film coating packaging materials.

Film	*L**	*a**	*b**
Alkaline-F	34.73 ± 0.27 a/x	-0.93 ± 0.05 a/x	-0.51 ± 0.01 a/x
Acidic-F	33.66 ± 0.15 b/x	-0.93 ± 0.04 a/x	0.07 ± 0.11 b/x
Alkaline-F 4	34.96 ± 0.41 a/x	-0.85 ± 0.15 a/x	0.84 ± 0.23 a/y
Acidic-F 4	33.35 ± 0.06 b/y	-1.33 ± 0.02 b/y	4.07 ± 0.18 b/y

Table 3. L^*, a^*, and b^* of alkaline-F and acidic-F films at day four and after four months of storage (4). Results are the mean ± standard deviation. One-way ANOVA: Different letters, a and b indicate significant differences among the different pH. Different letters, x and y indicate significant differences in each treatment among the fourth day and the fourth month (P≤0.05).

7.4.2.2. Infrared spectroscopy

Infrared spectroscopic patterns (4000-800 cm^{-1}) of alkaline and acidic-F are shown in figure 3a. The Amide A band (~3300 cm^{-1}) and amide B (~3079 cm^{-1}) are related to free water. Acidic-F Amide A wavenumber was slightly higher 3276.82 cm^{-1} than alkaline-F (3275.4 cm^{-1}), probably due to more hydrogen interactions between water, plasticizers and acidic proteins.

Spectral changes in the amide I (1700-1600 cm^{-1}) region have been associated with myofibrillar protein conformational changes and widely used for the spectroscopic analysis of the protein secondary structure (Bertram, Kohler, Bocker, Ofstad, & Andersen, 2006; Bocker, Ofstad, Bertram, Egelandsdal, & Kohler, 2006; Ojagh, Nunez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011; Palaniappan & Vijayasundaram, 2008). To enhance the spectral resolution, Amide I second derivative spectrum was performed (Fig. 3b). Little differences between protein secondary structures were found. Acidic-F showed a bigger loss of α-helical structure (1652.11 cm^{-1}) and more intramolecular aggregation (1683.33 cm^{-1}) than alkaline-F (1652.11 cm^{-1} and 1682.16 cm^{-1} respectively), probably tending to a more aggregated protein structure. Acidic-F aggregation might present higher difficulty in forming intermolecular aggregations (1617.16 cm^{-1}), while the unfolding of alkaline-F protein possibly favoured intermolecular protein interactions (1618.20 cm^{-1}) (Blanco-Pascual, Fernández-Martín, & Montero, 2013).

The peak located around 1000-1100 cm^{-1} might be related to the possible plasticizer hydrogen bonding (OH groups of glycerol and sorbitol) (Bergo & Sobral, 2007). This peak presented the lowest wavenumber (1039.65 cm^{-1}) at pH3 (pH10 1043.11 cm^{-1}), which was related to the possible extra linkage between short protein fragments caused by hydrolysis and the plasticizers.

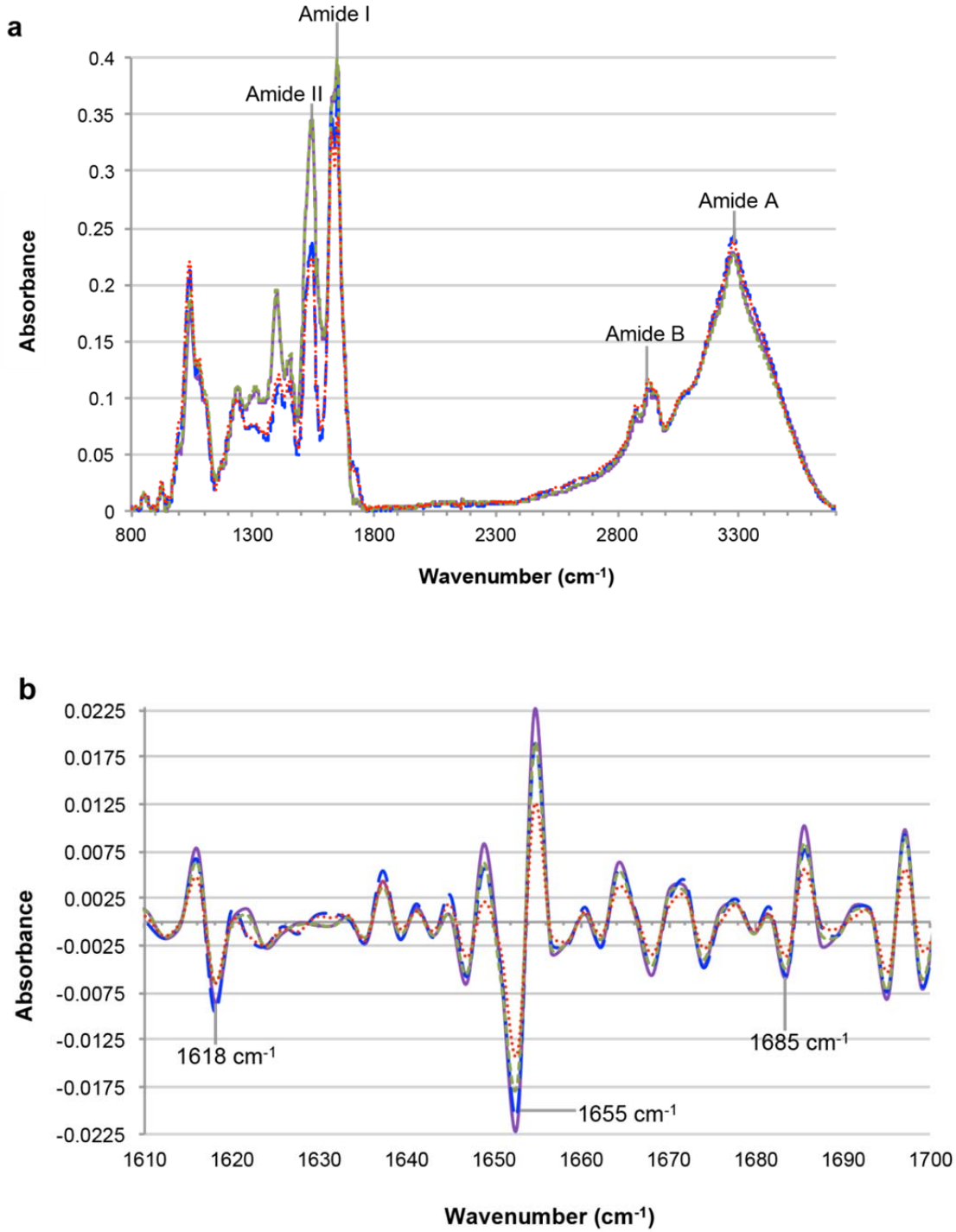

Figure 3. a. ATR-FTIR spectra of alkaline-F(——) and acidic-F(— —) after conditioning time, and of alkaline-F 4 (– – – –) and acidic-F 4 (.....) after four months of storage. b. Second derivative of Amide I band (1700-1600 cm-1) from FTIR spectra of alkaline-F and acidic-F after conditioning time and after four months (4).

7.4.2.3. Physical properties

Water vapour permeability (*WVP*) was significantly lower (P≤0.05) in alkaline-F (1.13 ± 0.07 x10^{-7} g m^{-1} s^{-1} Pa^{-1}) than in acidic-F (1.82± 0.05 x10^{-7} g m^{-1} s^{-1} Pa^{-1}). Protein films are often associated with high *WVP*, probably caused by the high number of hydrophilic groups (Hamaguchi, Weng, & Tanaka, 2007). Acidic-F might have more plasticizer interactions, increasing the matrix free volume and becoming less

dense, hence more permeable (Cuq, Gontard, Cuq, & Guilbert, 1997; Sobral, 2000). *WVP* results were in the same range as jumbo squid muscle films (Blanco-Pascual, Fernández-Martín, & Montero, 2013).

Table 4 shows that film solubility was not different between alkaline and acidic-F (P≤0.05). Low protein losses indicate that plasticizers were the first fractions to be released from the films. pH3 released around 70 g/kg more protein than pH10, suggesting alkaline-F stronger protein interactions. As films were dried at 45 ºC for 21-23 h, it might boost hydrophobic and covalent bonds, which are mainly formed at higher temperatures, but longer exposure times may also provide suitable conditions (Gómez-Guillén, Montero, Solas & Borderías 1998).

Film	Film solubility (g/kg)	Protein release (g/kg)	*TS* (x10^6 Pa)	*EAB* (%)	*Y* (x10^6 Pa)
Alkaline-F	266.5 ± 6.3 a/x	13.3 ± 00.3 a/x	1.54 ± 0.23 a/x	10.14 ± 4.82 a/x	184.53 ± 53.45 a/x
Acidic-F	305.2 ± 23.4 a/x	88.6 ± 04.2 b/x	0.96 ± 0.10 b/x	8.08 ± 2.47 a/x	155.88 ± 10.57 b/x
Alkaline-F 4	440.9 ± 21.7 a/y	23.1 ± 0.5 a/y	1.61 ± 0.24 a/x	1.53 ± 1.08 a/y	206.56 ± 66.41 a/x
Acidic-F 4	382.1 ± 18.2 b/y	68.4 ± 3.1 b/y	1.46 ± 0.27 a/y	11.75 ± 5.46 b/x	182.32 ± 2.92 a/y

Table 4. Film solubility, protein release, tensile strength (*TS*), elongation at break (*EAB*) and Young's modulus (*Y*), of alkaline-F and acidic-F films at day four and after four months of storage (4).
Results are the mean ± standard deviation. One-way ANOVA: Different letters, a and b indicate significant differences among the different pH. Different letters, x and y indicate significant differences in each treatment among the fourth day and the fourth month (P≤0.05).

Figure 4 illustrates the electrophoregram of the film protein release in water. Alkaline-F presented the lowest band intensity, confirming the plasticizer release. Acidic-F depicted more proteinic material, mainly T (30-35 kDa) and also A (45 kDa), P (108 KDa) and some bands of higher molecular weight. Acidic short protein fragments led to lower cross-linking pattern and allowed their release in water contact. Bands below tropomyosin were probably originated in MHC dissociation (Kristinsson & Hultin, 2003), finding another light chain of myosin intense band (20 kDa), which might have come LMM.

Alkaline-F was the most resistant, resulting in the highest tensile strength (*TS*) (P≤0.05) (Table 4), while elongation at break (*EAB*) was not significantly different. As far as Young's modulus is concerned (Table 4), the highest value corresponded to alkaline-F (P≤0.05), being in line with *TS* results and needing more strength to breakage due to its high stiffness. Regarding puncture deformation, films were more deformable by perforation and, as *EAB* results, no significant differences were observed between alkaline (17.42 ± 5.13%) and acidic-F (12.68 ± 2.12%). Films also showed more strength to the drilling, maintaining the same pattern as *TS,* with higher force in alkaline-F (20.88 ± 1.94 N) than acidic-F (13.76 ± 1.92 N) (P≤0.05). Isoelectric concentration of both alkaline and acidic solubilized extracts notably improved film elastic modulus values and puncture force (Blanco-Pascual, Fernández-Martín, & Montero, 2013).

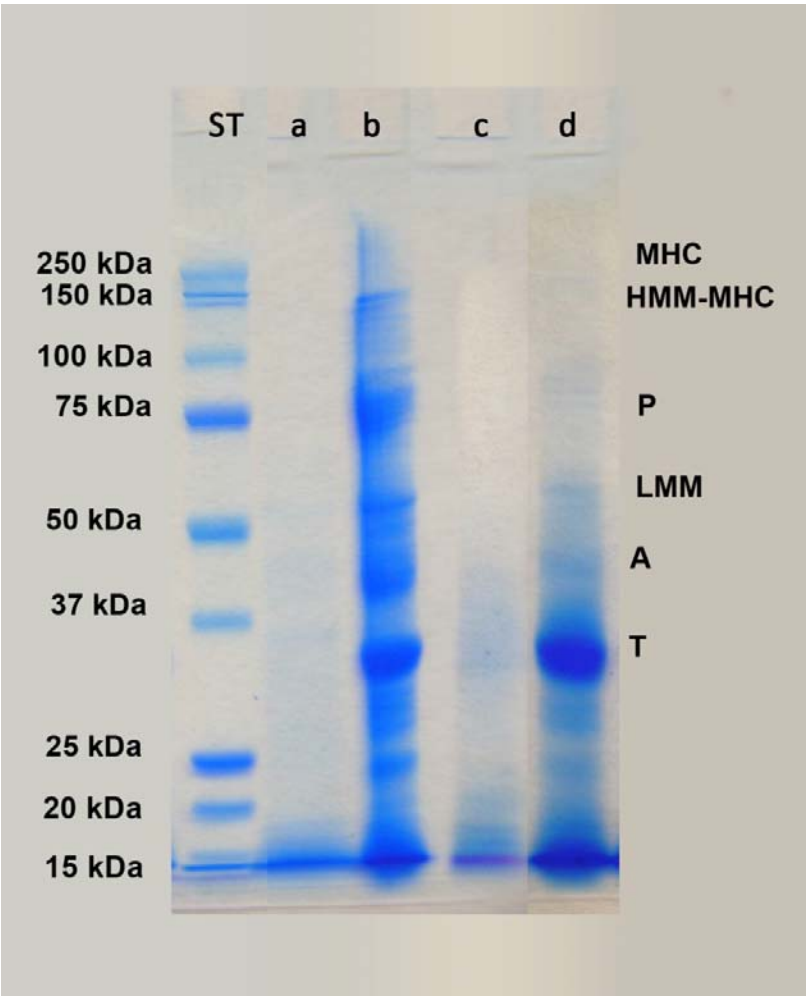

Figure 4. SDS-PAGE patterns of proteins released in water from alkaline-F (a) and acidic-F (b) at day four and after four months of storage (c and d respectively). ST: Standard; MHC: Myosin heavy chain; HMM-MHC: Heavy meromyosin-myosin heavy chain; LMM: Light meromyosin; P: Paramyosin; A: Actin; T: Tropomyosin.

Table 5 describes film water resistance. Both films avoided water filtration.

			Alkaline-F		
Elongation time (h)	0.03	0.16	1	5	24
Elongation (cm)	0.3 ± 0.01	0.45 ± 0.02	0.73 ± 0.02	0.9 ± 0.1	1 ± 0.1
Water filtration time (h)			No filtration		
Breakage time (h)			Unbreakable		
			Acidic-F		
Elongation time (h)	0.01	0.03	0.06	0.08	0.15
Elongation (cm)	0.5 ± 0.1	1 ± 0.1	1.5 ± 0.1	2 ± 0.1	2.5 ± 0.2
Water filtration time (h)			No filtration		
Breakage time (h)			40		

Table 5. Water resistance parameters: Elongation time (h), Elongation (cm), Water filtration time (h) and Breakage time (h), for alkaline-F and acidic-F films.
Results are the mean ± standard deviation.

Whereas alkaline-F hardly changed its shape and was unbreakable during 15 days, acidic-F rapidly elongated and stayed steady until breakage at 40 hours. Acidic protein hydrolysis affected protein distribution and resulted in a weaker film, with more interactions with plasticizers. Previous studies already showed that protein interactions were modified at alkaline and acidic treatment due to the proteinic chain extension and degradation (Bourtoom, 2009; Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; De la Fuente-Betancourt, García-Carreno, Del Toro, & Cordova-Murueta, 2009), possibly favouring more protein-protein disulphide (S-S) covalent bonds at alkaline conditions.

The cross-sectional images of both films (Figure 5) showed a homogeneous and organized myofibrillar protein structure (a and b), more structured at pH10 (c, d, e, f, g and h), which is in accordance with its higher mechanical and water resistance. Surface images (g and h) showed some vesicles or droplets homogeneously distributed in acidic-F, which might be attributed to its high water and plasticizer linkage.

7.4.2.4. DSC

Figure 2 shows typical DSC traces of Films (F): very small endothermic transitions in the temperature range of 60 − 85 ºC for alkaline-F and 40 − 75 ºC for acidic-F. Thermal denaturation data were 74.9 ± 1.4 ºC and 0.45 ± 0.06 J/g_{dm} in alkaline-F; 59.9 ± 1.4 ºC and 0.25 ± 0.06 J/g_{dm} in acidic-F, implying big denaturing processes, particularly in acidic-F as usual. Additionally to the plasticizers crystalline-inhibiting effects, current systems should have undergone major denaturation levels on myosin and low-thermostable components because of films drying at 45 ºC. Coexisting respective Tg (ºC) data were 66.9 ± 2.1 and 47.1 ± 3.4, overlapped with residual denaturation effects. Acidic-F may have undergone a bigger protein-matrix plasticization, consistently with FTIR results.

7.4.3. Stability study

Both films were a stable barrier to UV light (Table 2), becoming slightly more effective to superior wavelengths after four months, especially in acidic-F. Acidic-F showed stable values of transparency after four months, whereas alkaline-F suffered a loss of clarity (Table 2). The improvement of light transmission was in accordance with the colour results (Table 3), where films increased their yellowish tendency (+a^*), which was considerably more accused in acidic-F. This effect might be triggered by a Maillard reaction, comprising a condensation between free amino groups of amino acids, peptides and proteins, and carbonyl groups of glycerol and sorbitol, favouring glycation process, hence forming brown pigments. At acidic or alkaline pH, the endogenous proteases would be activated, hydrolyzing the proteins (especially acidic) and providing free amino group for the reaction. Nevertheless, these films presented better optical properties than previous results in 20 g/kg *Todadores pacificus* muscle films with 200 g/kg protein of glycerol or sorbitol after 1 month of storage (Leerahawong, Tanaka, Okazaki, & Osako, 2012).

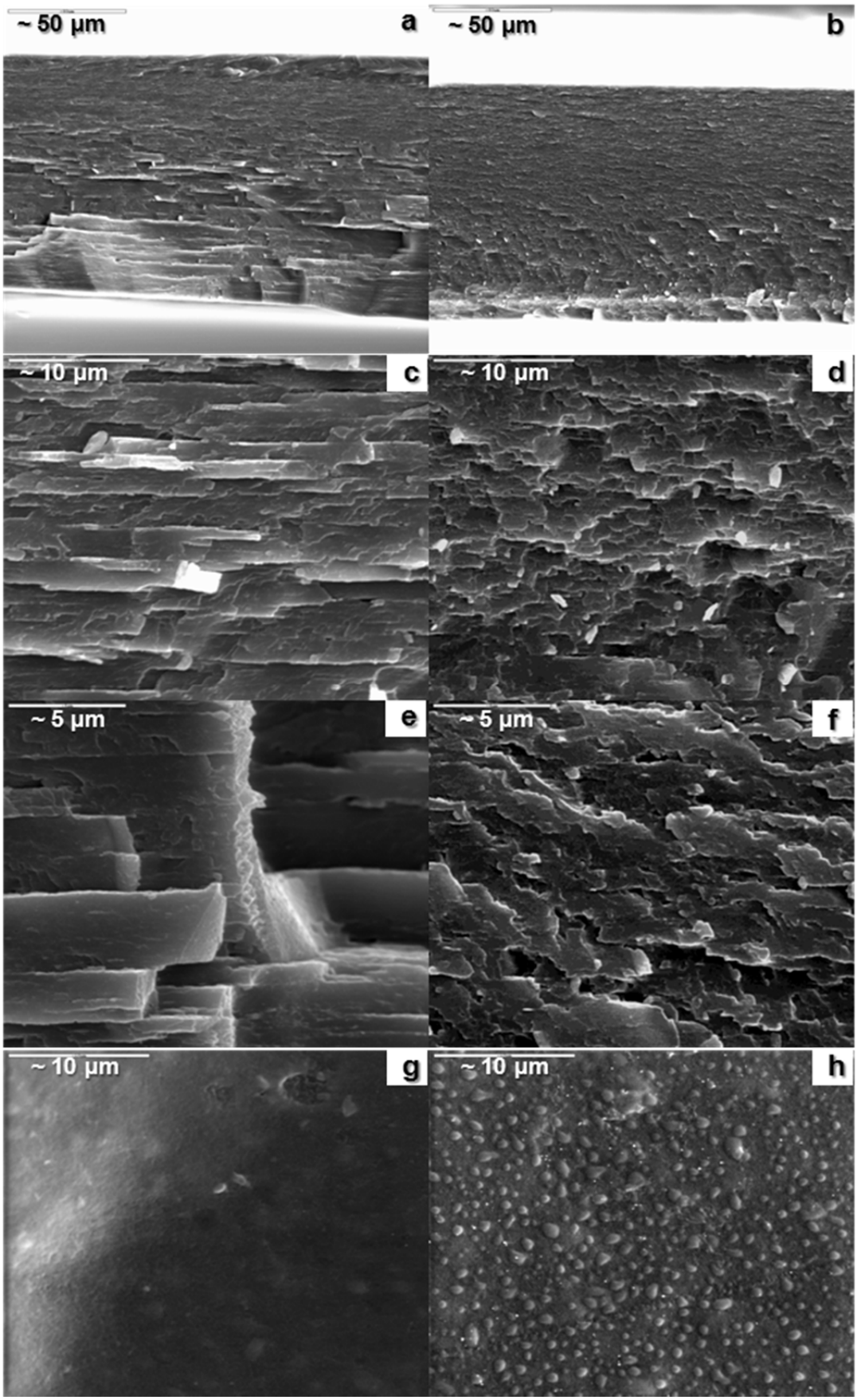

Figure 5. Low Temperature-Scanning Electron Microscopy images of alkaline-F (a, c and e) and acidic-F (b, d and f) cross sections and alkaline-F (g) and acidic-F (h) surfaces.

In figure 3a and b, FT-IR spectroscopic patterns of alkaline and acidic-F after four months are shown. The Amide A and Amide B band resulted in an increase of the wavenumbers (Amide A: 3275.91 cm^{-1} alkaline-F-4 and 3277.03 cm^{-1}acidic-F-4, Amide B: 3073.81 cm^{-1} alkaline-F-4 and 3074.39 cm^{-1}acidic-F-4) that might indicate a hydrogen bonding reduction leading to a water release and, consequently, a possible developing of protein-protein cross-linking. In the second derivative (Fig 3b), a band intensity decrease was evident, suggesting less free α and β structures, hence more aggregation after four months, being more accused in acidic-F-4.

With regard to film solubility, table 4 shows how alkaline and acidic-F-4 increased their values after four months (180 g/kg at pH10 and 80 g/kg at pH3) ($P \leq 0.05$), becoming less water resistant at alkaline conditions. This increment could be the result of a certain degree of film matrix disruption after storage. Acidic-F released higher amount of proteins than alkaline-F; while at pH 3 film protein losses were 20 g/kg lower than four months ago, pH10 film released 10 g/kg more protein after four months of storage (Table 4). Figure 5 illustrates the electrophoregram of the protein release in water. Alkaline-F presented the lowest band intensity, but compared to four months ago, the protein loss was slightly higher, observing some intensity in the actin band and below. Acidic-F almost did not show band intensity above LMM and released proteinic material such as actin and those of lower molecular weight. During film storage time, a more rigid polymer structure was formed and partially hydrolyzed proteins, in the acidic case, underwent more aggregation, forming strong interactions of myofibrillar proteins, as Artharn, Prodpran, and Benjakul (2009) suggested. Leerahawong, Tanaka, Okazaki, and Osako (2012) observed that, after 30 days of storage, the decrease in electrophoretic upper bands, such as MHC, may be caused by the protein polymerization and aggregation via Maillard reaction with plasticizers. The cross-linked proteins were insoluble, whereas almost all the glycerol was released (Orliac, Rouilly, Silvestre, & Rigal, 2002); which might suggest an increment of strong protein interactions and plasticizer migration in acidic-F.

As Table 4 shows, alkaline and acidic-F 4 presented no significant differences in *TS* and *Y* ($P \leq 0.05$). Compared to four months ago, alkaline-F preserved their resistance and elastic modulus, whereas acidic-F significantly improved both tensile strength and Young's modulus, reaching similar values to alkaline-F. The *EAB* was stable in acidic-F after four months. During the storage time, alkaline-F almost lost its elongation capacity, which might be due to an excessive protein aggregation.

Regarding DSC, aging produced different effects (Fig. 2) on films protein: 72.6 ± 1.4 ºC and 0.38 ± 0.02 J/g_{dm} in alkaline-F4, with not significantly reduced temperature and enthalpy of denaturation, and consistent not significantly different Tg of 67.4 ± 1.1 ºC; major changes were observed by a nearly complete vitrified acidic-F4 with a glass transition Tg ~69.4 ± 1.6 ºC. This may be attributed to a bigger protein-protein aggregation, consistently with FTIR results.

7.5. Conclusion

The present study emphasizes on concentrate acidic and alkaline solubilized myofibrillar proteins by iso-electric precipitation, due to their higher protein quality and stability. Acidic-C exhibited a significantly bigger protein-protection against degradation.

The unfolded proteins, after alkaline-C solubilization, led to more water and mechanical resistant films; although after 4 months of storage they lost their flexibility and water resistance, suggesting an excessive protein aggregation. Myofibrillar protein hydrolysis, in solubilized acidic-C, led to more plasticized film with poor properties, but protein aggregation suffered after four months resulted in a more resistant protein matrix partially improving its properties.

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VIII. Integral *Mastocarpus stellatus* use for antioxidant edible film development

Integral *Mastocarpus stellatus* use for antioxidant edible film development

Blanco-Pascual, N., Gómez-Guillén, M.C. and Montero, M.P. Integral *Mastocarpus stellatus* use for antioxidant edible film development. *Food Hydrocolloids.* Submitted 22nd April, 2013.

8.1. Abstract

Four sequential aqueous extracts (M1 to M4) were obtained from *Mastocarpus stellatus* at different temperatures. M1, extracted at 3 ºC overnight, showed higher extraction yield and antioxidant activity than M2, which was extracted at 45 ºC for 45 min. Extracts M3 and M4, obtained both at 91 ºC, were composed predominantly of a κ/ι-hybrid carrageenan, M4 containing higher protein proportion. Based on compositional and structural properties, four films were developed: FM3, FM4, FM3+M4 and FM3+M4+M1. FM4 was green coloured, more opaque, and water resistant, stronger and slightly less stretchable than FM3. The mixture of M3 and M4 for film development resulted in a film with intermediate characteristics. Addition of M1 to FM3+M4 provided mainly proteins and sulfated compounds (from carrageenan), resulting in a thicker and more water resistant film, with suitable mechanical properties in both tensile and puncture test, and much higher antioxidant activity.

8.2. Introduction

Seaweeds have been traditionally incorporated into Pacific and Asian foods for hundreds of years but they were not included in Western diets (Rinaudo, 2008). In recent decades there has been an increase in direct consumption of marine algae as food and also as components of functional products due to the fact that they are an excellent source of bioactive substances like sulfated-polysaccharides, peptides and polyphenols with biological activities, such as antioxidant (Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012).

Phycocolloids (e.g. carrageenan, agar and alginates) are special polysaccharides produced by several seaweed species, being carrageenan extracted from red seaweeds (Rhodophyceae) (Van De Velde, Peppelman, Rollema, & Tromp, 2001). Seaweeds also develop big diversity of antioxidant compounds such as carotenoids, phenols, minerals, sulfur compounds and vitamins (Tierney, Croft, & Hayes, 2010). Extraction of carrageenan is normally performed in water at temperatures near the boiling point for several hours and isolated by ethanolic precipitation (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009), being optional to introduce a previous extraction of possible active compounds.

Carrageenans are constituted by repeating water-soluble linear sulfated galactans, alternating 3-linked β-D-galactopyranose and 4-linked α-D-galactopyranose or 4-linked 3,6-anhydrogalactose that form the disaccharide repeating unit. Carrageenan are classified according to the presence of the 3,6-anhydrogalactose on the 4-linked residue and the position and number of sulfate groups (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009; Van de Velde, 2008). By sulfate content ascending order, natural carrageenan are composed of κ-carrageenan, ι-carrageenan, μ-carrageenan (biological precursor of κ-carrageenan), ν-carrageenan (biological precursor of ι-carrageenan) and λ-carrageenan (Gómez-Ordóñez & Rupérez, 2011; Van De Velde, Peppelman, Rollema, & Tromp, 2001). The rheological properties of the gelling carrageenans (κ and ι) are quite distinct: the κ-type forms gels that are hard, strong and brittle, whereas ι-carrageenan forms soft and weak gels (Van de Velde, 2008; Van De Velde, Peppelman, Rollema, & Tromp, 2001) glimpsing the same effect on carrageenan films (Hambleton, Perpiñan-Saiz, Fabra, Voilley, & Debeaufort, 2012; Han & Kim, 2008; Martins, Cerqueira, Bourbon, Pinheiro, Souza, & Vicente, 2012).

Carrageenan extraction conditions highly affect the final extract composition. For example, more than 2h of extraction at 95 ºC and pH 8 leads to a minimum amount of μ-precursor and maximum of κ-monomers (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006), and the lack of alkaline pretreatment might cause a lower ι-carrageenan extraction yield (Pereira & Mesquita, 2003).

Mastocarpus stellatus is one of the only carrageenophytes species currently harvested for industrial aims in the Atlantic coast but underutilized (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009), and κ-carrageenan's family is its main constituent (Gómez-Ordóñez & Rupérez, 2011), although previous studies have reported that, in fact, κ/ι-hybrid carrageenan is the principal structure extracted (Hilliou, Larotonda, Sereno, & Gonçalves, 2006). An integral *Mastocarpus* exploitation would be interesting in order to produce a more environmentally friendly material used as a renewable and biodegradable biopolymer for packaging applications. At the moment, films have been developed from different commercial carrageenan types (Han & Kim, 2008; Karbowiak, Hervet, Léger, Champion, Debeaufort, & Voilley, 2006), however, they do not stand out for having good antioxidant activity (Shojaee-Aliabadi, Hosseini, Mohammadifar, Mohammadi, Ghasemlou, Ojagh et al., 2013). So far, *Mastocarpus stellatus* edible films have never been developed, despite they would represent an interesting alternative application, due to the predominantly carrageenan composition and other active compounds whose concentration might be increased by exogenous addition.

The aim of this study is to develop edible active films from different *Mastocarpus stellatus* crude aqueous extracts, and to characterize their physical, structural and antioxidant properties.

8.3. Materials and methods

8.3.1. Seaweed sampling

Samples of *Mastocarpus stellatus* (M) were supplied by Porto-Muiños (Cerceda, A Coruña, Spain) and were collected in Galicia bay (A Coruña, Spain), washed several times with tap running water and air-dried at 50 ºC for 24-48 h in a ventilated oven. Seaweed samples were stored in sealed plastic bags at 2-4 ºC until analysis.

8.3.2. Seaweed extraction

Dried seaweeds were homogenized using an Osterizer blender (Oster, Aravaca, Madrid, Spain) with water in 1:15 (w:v) proportion and kept overnight at 3 ± 2 ºC. Seaweeds were then filtered to obtain extract M1. Water was added to the retentate in 1:20 (w:v) proportion, warmed up until 45 ºC for 45 min, and filtered to obtain extract M2. The second retentate was diluted in water 1:36 (w:v), warmed up at 91 ºC for 2 h and filtered to obtain extract M3. Third retentate was diluted in water 1:30 (w:v), warmed up at 91 ºC for 1.5 h and homogenized to obtain extract M4.

Extracts M1 and M2 were vacuum filtered (Whatman # 1) and freeze-dried. Extracts M3 and M4 were centrifuged at 3000 rpm for 5 min (Sorvall Evolution RC Centrifuge, Thermo Fisher Scientific Inc., Landsmeer, Netherlands). Supernatants were dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 65.0 ± 0.8 ºC and stored at room temperature.

8.3.3. Proximate analysis

Moisture and ash content of the raw material, M3 and M4 were determined according to official methods (A.O.A.C., 1995). Nitrogen content was determined using a combustion oven apparatus (Model FP-2000, Leco Corporation, St Joseph, MI, USA), according to Dumas (A.O.A.C., 2000), with a conversion factor of 6.25. Analysis was performed at least in triplicate, and results expressed as percentages. Carbohydrates content were estimated by difference, assuming a fat percentage content lower than 0.5% (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010).

8.3.4. Film preparation

Four film forming solutions were prepared: FM3, FM4, FM3+M4, FM3+M4+M1. Film-forming solutions were prepared by suspending 2% w/v dried extracts in distilled water (90 ºC) and homogenizing with ultra-turrax T25 basic (Ika-Werke GMBH &CO KG D-79219 Stanfer, Germany) at 17500-21500 rpm during 5 min. Glycerol (Panreac Química S.A. Montplet and Esteban S.A., Montcada i Reixac, Barcelona, Spain) was added at 30% (w/w) of the total dry matter content. The pH of the film-forming solutions (FS) were 6.5 ± 0.2 (portable pH-meter series 3 Star Orion with an electrode pH ROSS, Thermo Fisher Scientific Inc., Landsmeer, The Netherlands). M1 was added at 25% (w/w) of the dried seaweed extract content and was magnetically stirred during 5 min.

Film forming solutions were cast into petri dishes and dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 35.0 ± 0.8 ºC for 21-23 h. All films were conditioned at 58.0 ± 0.2% RH and 22 ± 1 ºC for 4 days prior to analysis.

8.3.5. Viscoelastic properties of film forming solutions (FS)

Dynamic viscoelastic 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 4º, gap 0.15 mm). A dynamic frequency sweep from 0.1 to 10 Hz took place at auto stress, at temperature of 5 ºC, and a target strain of 0.005%. The elastic modulus ($G´$; Pa) and viscous modulus ($G´´$; Pa) were plotted as functions of the frequency ramp. To characterize the frequency dependence of $G´$ over the limited frequency range, the following power law was used:

$$G´ = G_0´\omega^n$$

Where $G_0´$ is the energy stored and recovered per cycle of sinusoidal shear deformation at an angular frequency of 1 Hz, ω is the angular frequency and n is the power law exponent; which should exhibit an ideal elastic behaviour near-zero in gels. At least two determinations were performed for each sample. The experimental error was lower than 6% in all cases.

8.3.6. Thermal properties

Calorimetric analysis of extracts and films were performed using a differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated by running high purity indium (melting point, 156.4 ºC; melting enthalpy, 28.44 J/g). Samples of around 10-15 mg were weighed within ± 0.002 mg by an electronic balance (Model ME235S Sartorius, Goettingen,

Germany) and then tightly encapsulated in aluminium hermetic pans. An empty pan was used as reference. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 110 ºC at a heating rate of 10 ºC/min. Peak temperatures (T_{peak}, ºC) and enthalpies of conformational changes (ΔH) were measured at least in triplicate, the last data being normalized to dry matter content (J/g_{dm}) after desiccation of each particular capsule.

8.3.7. ATR-FTIR spectroscopy

Extract and film infrared spectra between 4000 and 650 cm^{-1} were recorded at least in triplicate using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA) as was described by Ojagh, Nunez-Flores, López-Caballero, Montero, and Gómez-Guillén (2011).

8.3.8. Antioxidant activities of freeze-dried extracts and films

The ferric reducing ability (FRAP) and the ABTS (2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assays were used to measure the antioxidant activity of both extracts (M1 and M2) and films (FM3, FM4, F-M3+M4 and F-M3+M4+M1), which were dissolved in distilled water. Extracts were homogenized in distilled water after heating up in thermostatic bath at their respective extraction temperature for 10 min. Films were shaken until being totally homogeneous and filtered (Whatman # 1) before the determination. The method used for the FRAP and ABTS assays were previously described by Gómez-Estaca, Montero, Fernández-Martín, and Gómez-Guillén (2009). Results were expressed as mg Fe^{2+} equivalents/mg of sample for FRAP and mg of Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of sample for ABTS, based on standard curves of $FeSO_4 7H_2O$ and vitamin C, respectively. All determinations were performed at least in triplicate and expressed in function of the wet sample weight.

8.3.9. Folin-reactive substances determination

Folin-reactive substances content was determined spectrophotometrically, in triplicate, using gallic acid as a standard according to a modified method of Slinkard and Singleton (1977) with the Folin Ciocalteu reagent. The absorbance of the resulting blue colour was measured at 765 nm (UV-1601, model CPS-240, Shimadzu, Kyoto, Japan). Folin-reactive substances content was expressed as mg gallic acid (GA) equivalent/g of sample.

8.3.10. Film determinations

8.3.10.1. Thickness

It was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 6-8 random locations in 15 films for each treatment as described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

8.3.10.2. Moisture content

It was determined at least in triplicate by drying samples of around 0.5 g at 105°C for 24 h, according to A.O.A.C. (1995). Water content was expressed as a percent of total weight.

8.3.10.3. Light absorption and transparency

The light barrier properties and transparency of the films were calculated at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 200 to 700 nm following the method described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

8.3.10.4. Colour

The colour parameters lightness (L^*), redness (a^*), and yellowness (b^*) were measured following the method described by Blanco-Pascual, Fernández-Martín, and Montero 2013).

8.3.10.5. Water vapour permeability (*WVP*)

It was determined at least in triplicate following the method described by Sobral, Menegalli, Hubinger, and Roques (2001) at room temperature and in a dissecator with distiller water.

8.3.10.6. Water solubility

Film solubility was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

8.3.10.7. Water resistance

Film water resistance was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

8.3.10.8. Mechanical properties

Tensile and puncture tests were run at least in triplicate using a texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) as was described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

8.3.11. Statistical analysis

Statistical tests were performed using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, Illinois, U.S.A.) for one-way analysis of variance. The variance homogeneity was made using the Levene test or, the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

8.4. Results and discussion

8.4.1. Yield and proximate composition of seaweed extracts

The four aqueous extracts M1, M2, M3 and M4 represented a yield of 7.98 ± 0.86, 4.48 ± 2.37, 23.18 ± 5.96 and 33.08 ± 8.3, respectively. Total extraction yield was 69.08 ± 2.85% (in dry basis), which was higher than in most studies, where only carrageenan in a yield range of 20-38% was isolated by ethanol-precipitation (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009; Pereira & Van De Velde, 2011). While M1 and M2, extracted at 3 ºC and 45 ºC, respectively, where considered mainly for their antioxidant

potential, M3 and M4 were concentrated biopolymer extracts in view of the high extraction temperature (91 ºC) used. M1 and M2 yields were much lower than the one previously reported by Jiménez-Escrig, Gómez-Ordoñez, and Rupérez (2012) for an aqueous extract obtained by using HCl 0.1M at room temperature (around 44%), probably due to the fact that our aqueous extraction did not involve any other enhancer reactive apart from water.

Proximate composition of M3 was 20.02 ± 0.96% moisture, 19.34 ± 0.11% ash, 4.95 ± 0.05% protein, and 55.13 ± 0.61% carbohydrate; while M4 presented 11.40 ±0 .54% moisture, 15.64 ± 0.27% ash, 12.1 ± 0.07% protein, and 60.40 ± 0.05% carbohydrate, which means more than two-fold protein content in M4 than in M3, and quite similar carbohydrate proportion. The relatively high ash content in both M3 and M4 might be associated to a concentration of Na, K, Ca and Mg, main macrominerals reported to be abundant in red algae (Rupérez, 2002; Uenishi, Fujita, Ishida, Fujii, Ohue, Kaji et al., 2010). Comparing these results with dried *Mastocarpus stellatus* proximate composition (17.71 ± 0.66% moisture, 16.65 ± 0.54% ash, 15.02 ± 0.53% protein, and 50.62 ± 0.59% carbohydrates), M3 presented slightly increased ash proportion, while most protein was recovered in M4. Both M3 and M4 extracts presented high carbohydrate proportion, attributed to the sequential 2-step carrageenan thermal extraction.

8.4.2. FTIR-ATR of carrageenan seaweed extracts

M3 and M4 IR-spectra are shown in Figure 1A. Both spectra presented a main absorption band at 1031 cm^{-1} in M3, slightly shifted to 1034 cm^{-1} in M4, and another band coinciding at 1155 cm^{-1} in both M3 and M4. These bands, attributed to the C-O stretching vibrations of pyranose compounds, are common to all polysaccharides, and revealed the predominant carbohydrate nature of both polymer extracts. Another characteristic broad band appearing at 1220 cm^{-1} in M3 and 1218 cm^{-1} in M4 corresponded to the abundance of esther sulfated groups, strongly suggesting carrageenan predominance. A number of bands in the frequency range between 930 and 800 cm^{-1} were also observed in both extracts, being characteristic of the type of carrageenan and the degree of sulfation. Thus, a strong band at 926 cm^{-1} in M3 and 928 cm^{-1} in M4, indicated the presence of 3,6-anhydro-D-galactose, typical in κ-carrageenan; another band at 844 cm^{-1} in M3 and 845 cm^{-1} in M4 (C-O-S vibration) was assigned to D-galactose-4-sulfate and attributed to both κ and ι-carrageenan, whereas a little feature appearing at 803 cm^{-1} indicated the presence of two sulfate ester groups on the anhydro-D-galactose residues, a characteristic of ι-carrageenan (sulfation on C2) (Gómez-Ordóñez & Rupérez, 2011; Pereira & Mesquita, 2003; Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003; Prado-Fernández, Rodríguez-Vázquez, Tojo, & Andrade, 2003). The concomitant presence of κ and ι-carrageenan features strongly suggested a major extraction of κ/ι-hybrid carrageenan in both sequential extractions (Gómez-Ordóñez & Rupérez, 2011; Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Van de Velde, 2008). All these IR-bands presented considerably higher intensities in M3 than in M4, which could be related to higher carrageenan concentration in M3, as well as other polysaccharides, such as neutral sugars and uronic acids, which might have been simultaneously extracted (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010). Moreover, most IR peaks in M3 were slightly shifted to lower wavenumbers as compared to M4 IR-peaks. This finding, together with a appreciable stronger absorbance at 1150-1100 cm^{-1} in M3, suggested an increased extraction of shorter polysaccharide chains with reduced ability to form intramolecular hydrogen bonds in the first carrageenan extract (M3) (Sun, Tao, Xie, Zhang, & Xu, 2010).

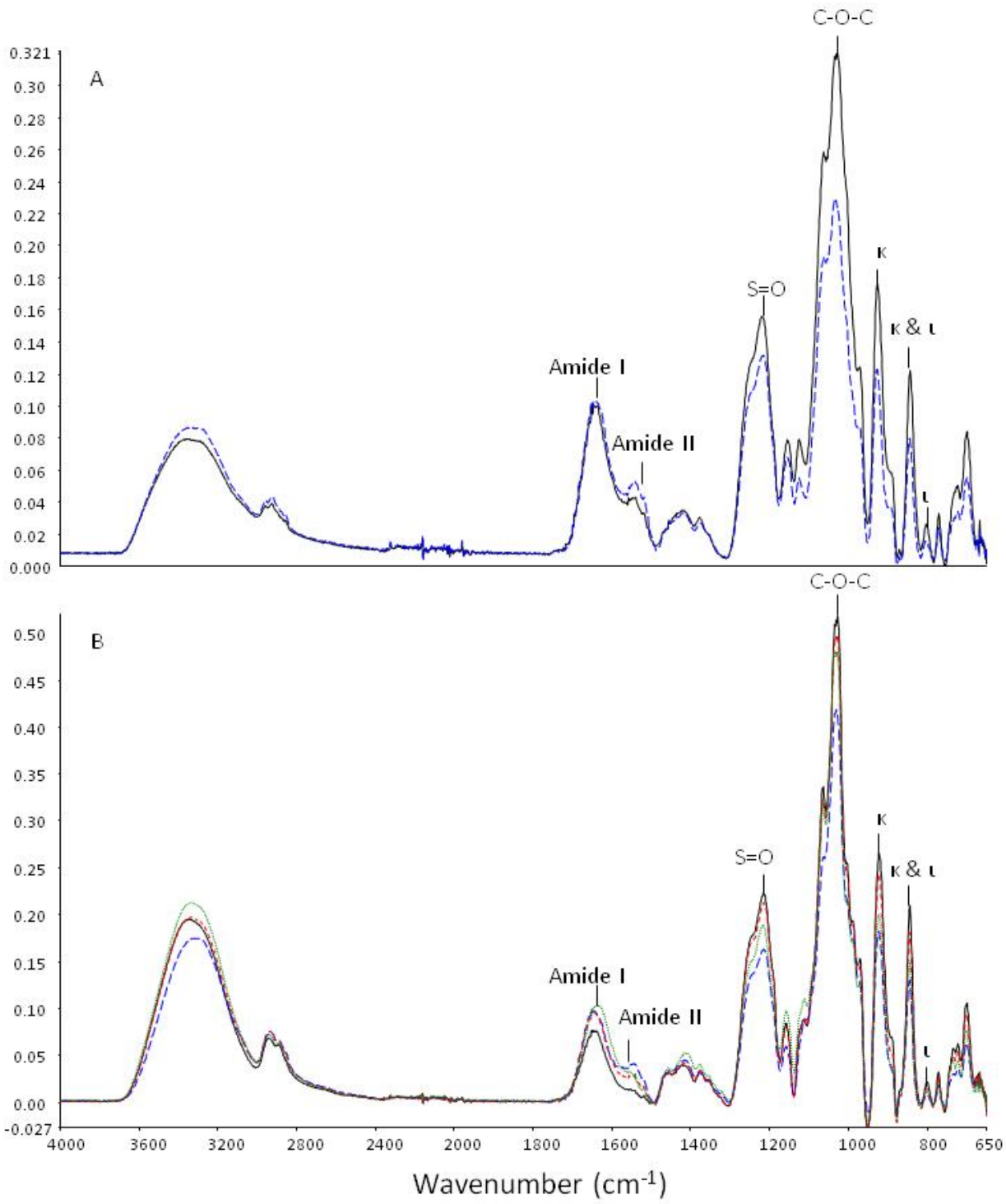

Figure 1. A. ATR-FTIR spectra of M3 and M4 (Black M3, Blue M4). B. ATR-FTIR spectra of FM3, FM4, FM3+M4 and FM3+M4+M1 (Black FM3, Blue FM4, Red FM3+M4, Green FM3+M4+M1).

No evidence of a broad band between 820 and 830 cm^{-1} was observed, indicating the absence of the highly sulfated λ-carrageenan (Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003). To enhance the spectral resolution, a second derivative spectrum was performed (data not shown). A little evidence at 871 cm^{-1} in both spectra suggested a vestigial presence of biological precursor monomers, i.e., μ-carrageenan (κ-carrageenan precursor) and ν-carrageenan (ι-carrageenan precursor) (Gómez-Ordóñez & Rupérez, 2011). Despite direct biopolymer extraction from *Mastocarpus stellatus* has previously been found to come at the expense of a rather high content (16-19% mole) of biological precursors (Souza,

Hilliou, Bastos, & Gonalves, 2011), Hilliou, Larotonda, Abreu, Ramos, Sereno, and Gonçalves (2006) showed minimum amount of μ-precursor after 2h of extraction at 95 ºC and pH 8, corresponding with a maximum in the κ-monomers relative content.

To obtain additional information in relation to the presence of each type of carrageenan and sulfated compounds, 805/845, 845/930 and 1240/930 cm^{-1} ratios were calculated (Fig. 1A). The 805/845 cm^{-1} ratio in M3 (0.23) was quite similar than in M4 (0.21), indicating that ι-carrageenan monomers proportion with respect to κ-carrageenan monomers did not differ to a great extent in both extracts (Pereira & Mesquita, 2003). The ratio 1240/930 cm^{-1} was considerably lower in M3 (0.89) than in M4 (1.07), which meant higher κ-carrageenan content compared to the μ-monomer precursor in M3. Thus it could be concluded that the κ-/ι-hybrid carrageenan, apparently extracted to a more extent in M3, presented similar ι-carrageenan proportion than in M4, but much less proportion of the μ-carrageenan precursor. In this connection, Hilliou, Larotonda, Abreu, Ramos, Sereno, and Gonçalves (2006) found that, unlike the ratio between κ-carrageenan and μ-carrageenan, the extraction time did not affect the ratio between κ-carrageenan and ι-carrageenan.

Two distinctive additional bands, located in the region ~1640 cm^{-1} and ~1545 cm^{-1} were assigned, respectively, to amide I and amide II bands (Fig. 1A), revealing the presence of proteins (Jebsen, Norici, Wagner, Palmucci, Giordano, & Wilhelm, 2012). Both bands showed higher intensity in M4 than in M3, in accordance with the higher protein content in the last sequential extraction M4. This protein material might correspond to residual phycobiliproteins, which are tetrapirrolinic linear proteins covalent bonded to cysteine residues (Theiss, Schmitt, Pieper, Nganou, Grehn, Vitali et al., 2011), mainly contributing to the amide I absorption band (Sühnel, Hermann, Dornberger, & Fritzsche, 1997).

8.4.3. DSC of seaweed extracts

DSC thermograms of dried *Mastocarpus stellatus* (M) and corresponding extracts M3 and M4 had the profiles shown in Figure 2A. Both M3 and M4 powder extracts depicted a main endothermic transition (rounded shaped) at similar ranges of 40 − 60 ºC, in line to the general pattern of κ-carrageenan gel systems (Nishinari & Watase, 1992), but with reduced signal due to the powder condition. Respective T_{peak} (ºC) were 53.53 ± 1.24 for M3 and 56.34 ± 0.79 for M4; while the corresponding ΔH (J/g_{dm}) were 0.78 ± 0.15 and 1.11 ± 0.07, respectively.

These mild endothermic events might represent disruption of helix aggregates and helix-coil transition (Hilliou, Larotonda, Sereno, & Gonçalves, 2006; Ridout, Garza, Brownsey, & Morris, 1996; Van De Velde, Antipova, Rollema, Burova, Grinberg, Pereira et al., 2005) resulting from the thermal induced breakage of weak physical cross-links and hydrogen-bonded interactions (Ramakrishnan & Prud'Homme, 2000).

The presumptive higher amount of shorter polysaccharide chains in M3, with reduced intramolecular cross-links, together with the lower proportion of highly sulfated biological precursor, might explain the decreased thermal transition temperature and total enthalpy in M3 as compared to M4 (Van De Velde, Rollema, Grinberg, Burova, Grinberg, & Hans Tromp, 2002). Moreover, the higher protein

presence in M4 might also result in an increase of both T_{peak} and ΔH, probably as a result of protein-carrageenan interactions (Baeza, Carp, Pérez, & Pilosof, 2002).

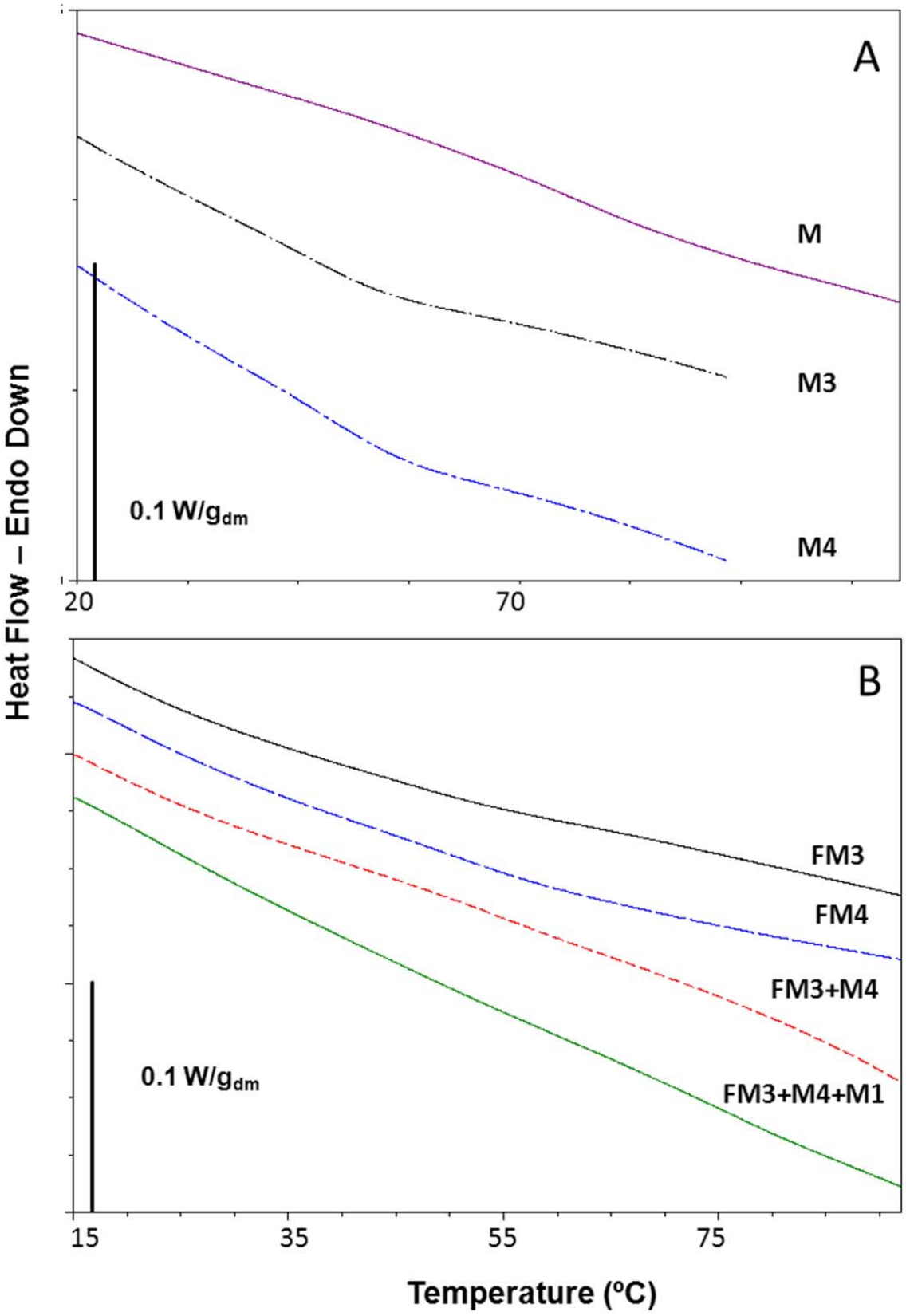

Figure 2. A. DSC of dried *Mastocarpus stellatus* (M) and extracts (M3 and M4) and B. DSC Films (FM3, FM4, FM3+M4 and FM3+M4+M1).

8.4.4. Rheology of film forming solutions

Film forming solutions were prepared from M3 (FSM3) and M4 (FSM4) biopolymer extracts, alone or in combination (FSM3+M4), to evaluate the effect of the integrated use of both extracts on film properties. In line with this approach, the first aqueous extract (M1) was incorporated to the blend (FSM3+M4+M1), in order to provide films with increased antioxidant capacity.

Figure 3 represents the mechanical spectra at 5 ºC of the different film forming solutions, in terms of elastic modulus (*G'*) and viscous modulus (*G''*) as a function of angular frequency. All samples showed a gel-like behaviour along the whole frequency range, as denoted by *G>G''* values, as previously reported for κ-carrageenan solutions at 0.5-1% concentration (Lafargue, Lourdin, & Doublier, 2007). The low setting temperature might have contributed to promote helices formation and aggregation in the film forming solution (Hossain, Miyanaga, Maeda, & Nemoto, 2001). The *G'* values were successfully modelled according to the power law (r^2=0.973-0.999), rendering power law exponents (*n'*) of 0.335 (FSM3), 0.360 (FSM4) and 0.344 (FSM3+M4). Moderate frequency dependence was observed in all cases, being highest in FSM4, coinciding with slightly lower *G'* values. The presumptive higher κ-carrageenan concentration in M3 and its lower relative content in the μ-monomer precursor could explain its slightly better gelling properties (Hilliou, Larotonda, Sereno, & Gonçalves, 2006). In addition, the higher protein content in M4 might also hinder the gel capacity, making the helical carrageenan aggregate more difficult to create (Andrade, Azevedo, Musampa, & Maia, 2010). Addition of M1 to the blended solution (FSM3+M4+M1) produced a sharp increase in *G'*, reducing considerably its frequency dependency (*n'*=0.1351). Different compounds in M1, which according to Jiménez-Escrig, Gómez-Ordoñez, and Rupérez (2012) would correspond greatly to polyphenols and sulfated polysaccharides, might promote interactions with the extracted κ-/ι-hybrid carrageenan, leading to a more stable and elastic gel network.

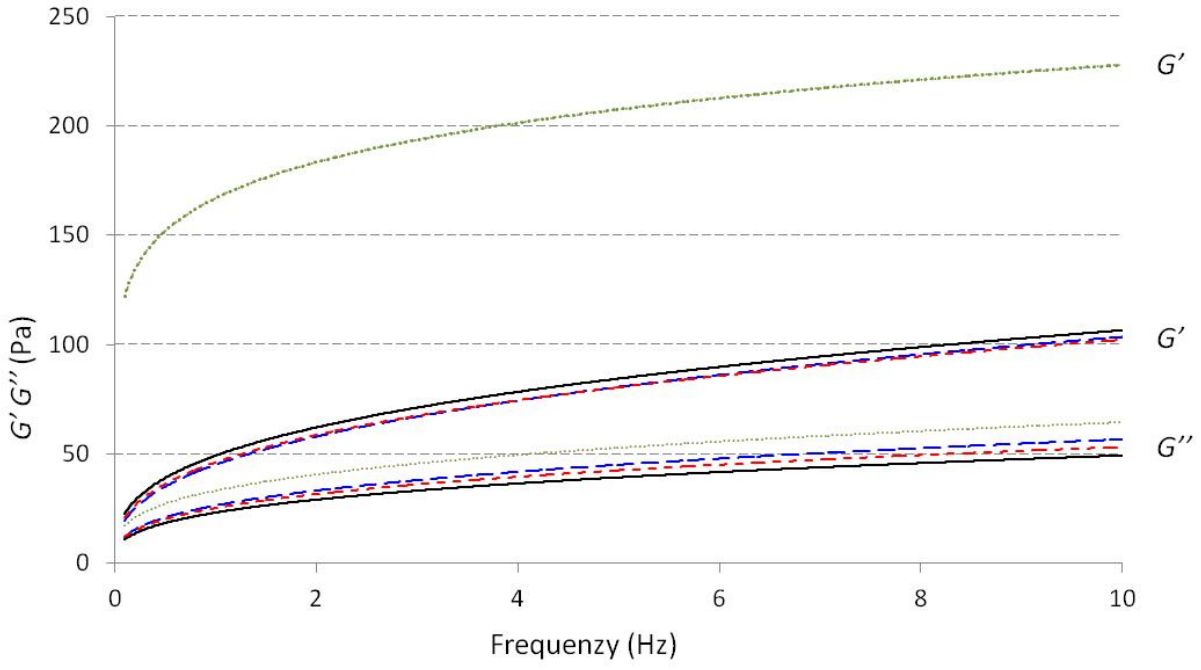

Figure 3. Elastic modulus (*G'*, Pa) and viscous modulus (*G''*, Pa) as a function of the angular frequency of film forming solution (FS) at 5 ºC. (Black FSM3, Blue FSM4, Red FSM3+M4, Green FSM3+M4+M1).

8.4.5. Film (F) properties

8.4.5.1. FTIR-ATR

The IR-spectra of the different films are shown in Figure 1B. All spectra resembled the profiles described above for both M3 and M4 extracts, with predominance of bands associated to the presence of polysaccharides (more specifically carrageenan), sulfate esters and proteins. The intensity of the band at ~1030 cm^{-1} was considerably increased in films as compared to the respective extracts, attributed to the glycerol linkage. In general, FM3+M4 film showed intermediate peak intensities and wavenumbers in most bands with respect to its FM3 and FM4 counterparts, in accordance to the presence of both extracts in the blended film.

Addition of M1 decreased peak intensities at ~1215 cm^{-1}, ~1030 cm^{-1}, ~923 cm^{-1}, ~845 cm^{-1} and ~800 cm^{-1}, in the FM3+M4+M1 film, as compared to the FM3+M4 film. The reduced intensity was not associated to a dilution effect, since M1 was not included at the expense of the M3+M4 proportion. In addition, an appreciable frequency up-shift was also observable at the sulfate ester band (from 1215 cm^{-1} to 1218 cm^{-1}) and slightly at the C-O stretching vibration of the pyranose ring (from 1031 cm^{-1} to 1032 cm^{-1}). All these IR-events strongly suggested that M1 compounds might interact with the κ-/ι-hybrid carrageenan, preferably at the sulfate ester groups. Interestingly, the ratio 1240/930 cm^{-1}, associated to the sulfation degree of the 3,6-anhydro-galactose, was highest in FM3+M4+M1 (0.91), denoting an appreciable carrageenan content in M1.

The addition of M1 induced an evident broadening of the amide I band in the FM3+M4+M1 film with respect to its FM3+M4 counterpart, revealing also the presence of proteins in M1. Red algae chloroplasts are known for containing chromophores termed phycobilins (O'Carra, Murphy, & Killilea, 1980), which are photosynthetic pigments thioether bonded to certain water soluble proteins named phycobiliproteins with sulfur-containing amino acids (Carra, Ó Heocha, & Carroll, 1964). Phycobiliproteins might have been firstly extracted in water (Cian, Martínez-Augustin, & Drago, 2012), as in the M1 extract, and lately collected in the last fraction (M4). The presence of these proteins in M1 might have also contributed to an overall increase of the sulfate ester groups in the most complex FM3+M4+M1 film.

The amide I band amplitude was lowest in FM3, in accordance to the low protein content in the M3 extract. FM3+M4+M1 amide I wavenumber exhibited an appreciable down-shift (1635 cm^{-1}) compared to FM3+M4 (1646 cm^{-1}), which might be related with increased hydrogen-bonding in FM3+M4+M1 structure, as a result of M1 addition.

FTIR results revealed that the M3+M4 mixture led to films with intermediate structural properties between FM3 and FM4 films, while the M1 addition resulted in a more sulfated film with higher protein content interacting with the κ-/ι-hybrid carrageenan via hydrogen bonds and sulfate ester linkages.

8.4.5.2. DSC

Typical DSC traces of the films (F) are shown in Figure 2B. A slight endothermic transition was evident with T_{peak} (ºC) temperatures of 51.84 ± 0.36 in FM3 and 51.74 ± 6.40 in FM4, and corresponding ΔH (J/g_{dm}) values were 0.25 ± 0.01 and 0.42 ± 0.04 respectively; which was consistent with the thermal behaviour of corresponding extracts (M3 and M4). Comparing FM3 and FM4, more energy was

necessary to melt FM4, suggesting a more resistant network, probably helped by protein-carrageenan interactions (Baeza, Carp, Pérez, & Pilosof, 2002). The lowering in the endothermic transitions in films with respect to the powder extracts might be due to the plasticizer effect of glycerol, lowering its crystallinity due to the newly created hydrogen bonds between carrageenan hydroxyl groups and glycerol (Ramakrishnan & Prud'Homme, 2000). Transition temperature T_{peak} in FM3+M4+M1 film increased considerably up to 80.07 ± 0.37 ºC, which might confirm further protein-carrageenan interactions reinforcing the film network.

8.4.5.3. Light barrier properties

Films exhibited in general low light transmission in the UV range (200-280 nm) (Figure 4), although in FM3 it increased slightly at 280 nm (5.26 ± 0.69%) resulting the less efficient UV barrier. In the visible range, as long as M4 relative content was higher, less transparent was the resulting film. The presence of M1 in the FM3+M4+M1 film rendered a light transmission profile quite similar as in the FM4 film, strongly suggesting the presence of pigments, likely bonded to proteins, in both M4 and M1 extracts. In FM4, FM3+M4 and FM3+M4+M1 two absorption plateaus were clearly defined in the ranges 400-450 nm and 600-750 nm, which might be associated with the presence of pigments, such as carotenoids and chlorophyll, absorbing at 400-450 nm (violet-blue-green colours), and phycoerythrin and phycocyanin at ~600 nm (red colour) (Sühnel, Hermann, Dornberger, & Fritzsche, 1997). As shown in table 1, FM3 was significantly (P≤0.05) the most transparent film, being evident that the transparency decreased when M4 proportion was higher in the film (FM4>FM4+M3) and also when M1 extra compounds were added (FM3+M4+M1) (P≤0.05).

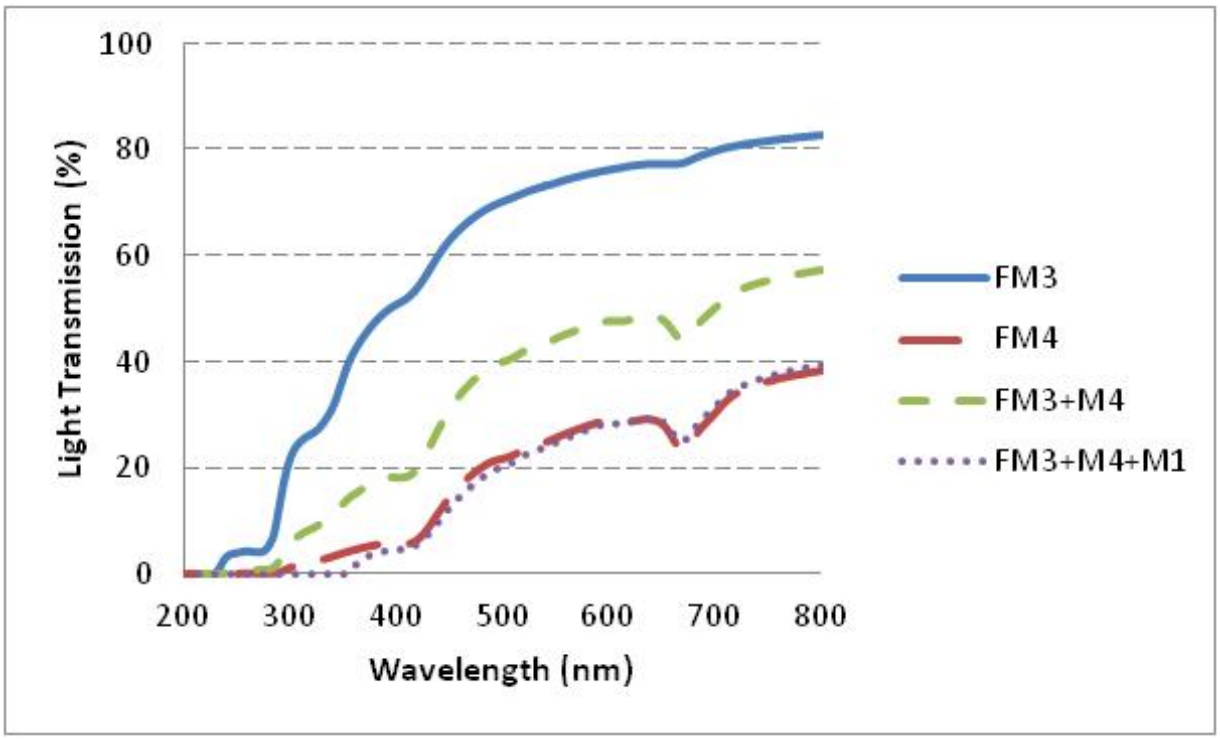

Figure 4. Light transmission (*T*, %) at several wavelengths (nm) of FM3, FM4, FM3+M4 and FM3+M4+M1. Results are the mean ± standard deviation.

*L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish) values are also shown in Table 1, where it is revealed that all films had low lightness (28-30) and slightly greenish tendency (-*a**). The presence of strongly red coloration in the M1 extract, seemed not greatly modify film greenish tendency in FM3+M4+M1 but conferred the highest yellowish coloration (+*b**).

Comparing these results with previous developed commercial κ-carrageenan films, neither films of this study transmitted as much light as those (around 30% in UV region and 80% in the visible region) nor were as transparent as them; having considerably lower lightness (88-95) and more red tendency (-0.26) (Rhim, 2012; Sánchez-García, Hilliou, & Lagaron, 2010; Shojaee-Aliabadi et al., 2013).

All these results were consistent with M1, M3 and M4 composition. FM3 was mainly composed of the most soluble components (predominantly carrageenan), showing film optical properties closer to the commercial carrageenan films (almost colourless and transparent). FM4 contained most of the seaweed compounds that remained after three extractions, including pigments and proteins, rendering more opaque films with greenish coloration. The high temperature used during the extraction procedure might have promoted a transition of the typical red coloration into a more greenish/yellowish one (Paull & Chen, 2008). On the other hand, addition of M1 to the M3+M4 mixture rendered films with increased coloration and opaqueness, comparable to those of FM4, as a result of the appreciable extraction of water-soluble light capturing molecules (phycobilins, chlorophylls and carotenoids) (Lin & Stekoll, 2011).

Film	*L**	*a**	*b**	Transparency
M3	30.70 ± 0.58 a	-0.8 ± 0.07 a	1.72 ± 0.19 a	2.71 ± 0.06 a
M4	28.90 ± 0.62 bc	-0.88 ± 0.10 a	4.35 ± 0.17 b	11 ± 0.13 b
M3+M4	29.45 ± 0.23 b	-1.05 ± 0.06 b	3.85 ± 0.34 c	6.68 ± 0.28 c
M3+M4+M1	28.43 ± 0.37 c	-0.60 ± 0.16 c	5.26 ± 0.21 d	11.74 ± 1.39 b

Table 1. L^*, a^*, b^* and Transparency ($-\log(T_{600}/x)$) of FM3, FM4, FM3+M4, and FM3+M4+M1.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films (P≤0.05).

8.4.5.4. Physical properties

The physical properties of films are shown in Table 2. Thickness values increased with M4 presence in the film formulation (FM3<FM3+4<FM4) (p≤0.05).

The addition of M1 to the FM3+M4 formula resulted in an extraordinary increase of the film thickness (88 μm) compared to the rest of the films (44-48 μm), coinciding with considerable higher (p≤0.05) moisture content (~20%). The greater protein content in M4 might cause a reorganization of the fibrous structure producing an increase in thickness. The incorporation of M1 extract extended the network, probably favouring matrix gelation and expanding its volume almost the double, which might be promoted by water entrapment due to certain M1-induced plasticizing effect.

8.4.5.4.1. Water barrier properties

The highest film water solubility (Table 2) was found in FM3 (P≤0.05), considerably decreasing in FM3+M4 and even more in FM4. FM3+M4+M1 solubility resulted in a similar range to FM3+M4 and FM4.

Both M4 high protein content and increased sulfated groups might promote protein-carrageenan interactions during the drying process, resulting in more aggregated networks. Charged polysaccharides such as carrageenan, with a strong electrolyte character due to their sulfated groups, can interact with other components such as proteins, minerals and plasticizers, affecting negatively their water barrier properties (Karbowiak, Debeaufort, Champion, & Voilley, 2006). Extra protein addition with M1, however, did not confer any significant improvement in water solubility, with respect to the FM3+M4 film.

	FM3	FM4	FM3+M4	FM3+M4+M1
Thickness (μm)	43.98 ± 6.11 a	48.15 ± 5.51 b	47.05 ± 5.25 ab	88.45 ± 13.80 c
Moisture (%)	14.21 ± 1.05 a	12.10 ± 0.91 b	13.53 ± 0.95 ab	20.08 ± 0.43 c
Film solubility (%)	59.24 ± 3.29 a	30.36 ± 2.59 b	37.26 ± 3.61 c	34.53 ± 1.52 bc
WVP ($x10^{-8}$ g m^{-1} s^{-1} Pa^{-1})	3.57 ± 0.05 a	3.62 ± 0.01 a	3.64 ± 0.08 a	7.25 ± 0.06 b
TS (MPa)	13.88 ± 0.39 a	16.16 ± 0.76 b	14.13 ± 0.18 a	15.24 ± 3.74 ab
EAB (%)	2.40 ± 0.1 a	1.28 ± 0.08 b	1.38 ± 0.31 b	4.11 ± 1 c
Y (MPa)	459.12 ± 35.77 a	552.63 ± 36.61 b	516.77 ± 23.58 ab	316.56 ± 3.44 c
F (N)	23.24 ± 3.17 a	13.65 ± 1.37 b	17.35 ± 1.35 c	28.6 ± 2.14 a
D (%)	16.07 ± 2.62 a	8.01 ± 0.55 b	10.47 ± 1.10 c	16.53 ± 0.45 a

Table 2. Thickness, moisture, film solubility, water vapour permeability (*WVP*), Tensile strength (*TS*), elongation at break (*EAB*), Young's modulus (*Y*), puncture force (*F*) and puncture deformation (*D*) of FM3, FM4, FM3+M4, and FM3+M4+M1.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films (P≤0.05).

No significant differences were found between FM3, FM3+M4 and FM4 water vapour permeability (*WVP*) (P≤0.05) (Table 2). Despite its thickness was higher, *WVP* significantly increased in FM3+M4+M1, probably related to its increased moisture-induced plasticization, which favoured the moisture transfer (Hambleton, Perpiñan-Saiz, Fabra, Voilley, & Debeaufort, 2012). Solubility and *WVP* of all studied films were higher than previous results obtained with commercial carrageenan films, where solubility values of ~26% (Shojaee-Aliabadi et al., 2013) and *WVP* between 0.55 and 20 x 10^{-10} g m^{-1} s^{-1} Pa^{-1} were reported (Karbowiak, Debeaufort, & Voilley, 2007; Martins, Cerqueira, Bourbon, Pinheiro, Souza, & Vicente, 2012; Rhim, 2012; Sánchez-García, Hilliou, & Lagaron, 2010; Shojaee-Aliabadi et al., 2013).

Water resistance test results are shown in figure 5. In the initial time, immediately after adding water, no apparent modification of the film surfaces occurred, whereas appreciable changes were observable at longer times (>2 min) as a result of film swelling and partial transfer of colored constituents to the water. FM3 was the less water resistant film, as denoted its highest elongation before breakage, which took place after ~8 min; in addition, it was also the only film that showed water filtration before breakage. The higher proteinaceous residual material in the film formulation, leading to a more aggregated network, contributed noticeably to higher water resistance of both FM4 and FM3+M4 films, avoiding the water filtration. The FM3+M4+M1 film was the most water resistant film, presenting breakage at around 32 min, with the lowest elongation (1.5 cm). Besides being considerably thicker, the more

aggregated conformation already seen in FSM3+M4+M1 rheological analysis of the corresponding film forming solution, might promote a reinforced film network.

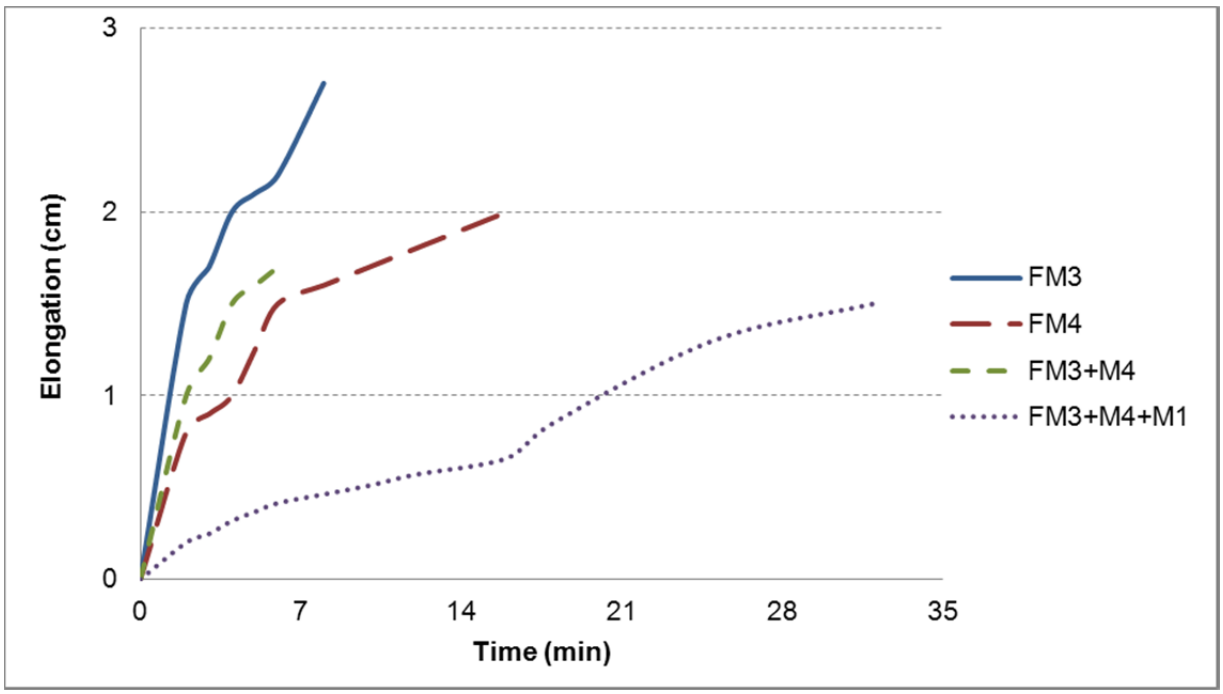

Figure 5. Water resistance: Elongation (cm) in time (min) until Breakage for FM3, FM4, FM3+M4 and FM3+M4+M1.

Water vapour permeability and water filtration could be an interesting way to assess subjacent phenomena of transport and diffusion through packaging and edible packaging films. Karbowiak, Hervet, Léger, Champion, Debeaufort, and Voilley (2006) already observed a huge increase of the diffusion of the small molecules in ι-carrageenan films containing more than 30% glycerol (w/w carrageenan) also showing an hydration and swelling effect.

8.4.5.4.2. Mechanical properties

FM4 showed significantly ($p \leq 0.05$) higher tensile strength (*TS*) and lower elongation at break (*EAB*) than its FM3 counterpart (Table 2), with FM3+M4 registering intermediate values, in good accordance to its blended nature providing intermediate compositional and structural properties. M1 addition did not induce any significant ($p > 0.05$) change in the tensile strength as compared to the FM3+M4 film, however, *EAB* was considerably increased, exhibiting the greatest values of all studied films. Such increase of the *EAB* in the more complex film could be strongly connected to its higher thickness and water content. *TS* and *EAB* values of all films were much lower than the results obtained in other studies made with commercial κ-carrageenan films (20-35 MPa, 15-37%, respectively) (Martins, Cerqueira, Bourbon, Pinheiro, Souza, & Vicente, 2012; Rhim, 2012; Shojaee-Aliabadi et al., 2013); and higher than results with commercial ι-carrageenan films (9 Mpa; 1.2%) (Hambleton, Perpiñan-Saiz, Fabra, Voilley, & Debeaufort, 2012). These differences could be largely attributed to the κ-/ι-hybrid nature of the extracted carrageenan used for film production, together with the presence of proteins and pigments, especially in FM4 and FM3+M4+M1 films (Han & Kim, 2008; Van de Velde, 2008).

As far as Young's modulus (*Y*) is concerned (Table 2), the highest (p≤0.05) stiffness also corresponded to FM4 film, decreasing with the M4 absence (FM3+M4>FM3). It is interesting to note that M1 addition led to an accused *Y*'s decrease. The higher sulfation degree in FM3+M4+M1, together with the presence of proteins, other small molecules and increased water content might promote an excessive number of cross-linking points in this film resulting in a reduced film stiffness.

Regarding puncture deformation (*D*) and force (*F*) no significant (p≤0.05) differences were observed between FM3 and FM3+M4+M1 (Table 2), which presented the highest values. On the contrary, FM4 showed the lowest (p≤0.05) values for both *D* and *F*, revealing a poorer puncture resistance, probably due to stronger junction zones caused by proteins covalently linked to the hybrid carrageenan, which might act as force concentrators in specific points making the material more prompt to breakage under delimited force fields.

Thus, it could be concluded that FM4 presented high *TS* whereas FM3 had higher perforation response. The most complex FM3+M4+M1 film resulted in as high puncture test values as FM3, but also increased more than 1.5 times the *EAB* values compared to FM3. The degree of sulfation, water-induced plasticization and protein-carrageenan interactions took an important role in determining the mechanical properties, which might differ depending on the type of test.

8.4.6. Antioxidant Activity and Folin-reactive substances

The antioxidant activity and Folin reactive substances of extracts (M1 and M2), as well as films (FM3, FM4, FM3+M4 and FM3+M4+M1) are shown in Table 3.

Sample	ABTS (mg vit C eq/g)	FRAP (mg Fe^{2+}/g)	Folin reactive substances (mg/g)
M1	45.30 ± 0.05 a	1.30 ± 0.01 a	28.53 ± 0.73 a
M2	44.54 ± 0.12 b	0.99 ± 0.002 b	22.10 ± 1.02 b
FM3	2.19 ± 0.11 a	1 ± 0.11 ab	6.64 ± 0.37 a
FM4	2.5 ± 0.06 b	0.90 ± 0.02 b	6.08 ± 0.4 a
FM3+M4	2.19 ± 0.11 a	1.07 ± 0.07 ac	7.75 ± 0.34 b
FM3+M4+M1	70.60 ± 0.47 c	1.16 ± 0.04 c	41.32 ± 3.19 c

Table 3. Antioxidant activity: ABTS, FRAP and Folin reactive substances of M3 and M4, FM3, FM4, FM3+M4 and FM3+M4+M1.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different Extract (E) or different film (F) (P≤0.05).

Both M1 and M2 aqueous extracts exhibited noticeable radical scavenging capacity in contrast to the FM3, FM4, FM3+M4 films, were values were considerably much lower. Regarding the ferric ion reducing capacity, it was almost negligible in both extracts and films, in accordance to previous work with another Rhodophyta species (*Chondrus crispus*) (Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012). It is noteworthy that the addition of M1 to the film significantly improved the ABTS activity, beyond the values of the M1 extract itself. M1 and M2 extracts contained an appreciable amount of Folin-reactive substances, which was slightly higher (p≤0.05) in the former, despite the lower extracting temperature. Although this technique is widely used to assess total phenol content, it should be taken into

consideration that other compounds present in the extract may react with the Folin reactive, including certain aromatic amino acids and sugars, which could also contribute as radical scavengers (Ikawa, Schaper, Dollard, & Sasner, 2003; Singleton, Orthofer, & Lamuela-Raventós, 1998; Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano et al., 1985). FM3+M4+M1 film considerably increased both its Folin-reactive substances content (5 times) and its free-radical scavenging capacity (30 times), mostly attributed to the presence of water soluble phycobilins and phycobiliproteins (Hirata, Tanaka, Ooike, Tsunomura, & Sakaguchi, 2000; Lin & Stekoll, 2011), given that phenolic content in red seaweeds is rather low (Holdt & Kraan, 2011). On the other hand, the higher sulfated content in M1 coming from the κ-carrageenan derivatives might also contribute to the FM3+M4+M1 higher antioxidant activity (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007; Yuan, Song, Zhang, Li, Li, & Gao, 2006).

8.5. Conclusion

Mastocarpus stellatus extracts could be a complementary source to take advantage of the main part of its ingredients for film development. M3 was adequate to develop transparent and flexible edible films (more similar to carrageenan films) while a subsequent extraction led to a higher protein content extract forming stronger junction zones during drying. The addition of M1 resulted in a more efficient plasticization and more mechanical and water resistant edible film, thanks to phycobilin attributes, which conferred antioxidant properties. This more complex film could be used to improve food preservation and as a possible component for functional food development.

8.6. References

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IX. Enzyme-assisted extraction of κ/ι-hybrid carrageenan from *Mastocarpus stellatus* for obtaining bioactive ingredients and their application for edible active films development

Enzyme-assisted extraction of κ/ι-hybrid carrageenan from *Mastocarpus stellatus* for obtaining bioactive ingredients and their application for edible active films development

Blanco-Pascual, N., Alemán, A., Gómez-Guillén, M.C. and Montero, M.P. Antioxidant and ACE-inhibitory Hydrolysates from *Mastocarpus stellatus* and their Use for Active Film Development. *Food & Function*. Submitted 2nd August, 2013.

9.1. Abstract

Two hydrolysates were obtained from dried *Mastocarpus stellatus* using alcalase. Phenolic content was partially removed from one of them. The phenolic-partially-removed hydrolysate (H) was found to be a potent ACE inhibitor. However, the phenolic-containing hydrolysate (Hp), showed a higher Folin-reactive substance content and antioxidant capacity (reducing power and radical scavenging capacity). Hp was therefore selected for the development of antioxidant Mastocarpus carrageenan-based films. F-Hp0 (without hydrolysate), F-Hp15 (with 15% hydrolysate) and F-Hp30 (with 30% hydrolysate) films were developed. κ/ι-hybrid carrageenan was the main film constituent and hydrolysate addition resulted in an increased sulfated proportion, higher protein content and higher number of hydrogen bonds. Therefore interactions between carrageenan helices, plasticizer and peptides in the film-forming solution were enhanced, especially in F-Hp15, and consequently the water vapour permeability (*WVP*) of the resulting film decreased. Nevertheless, F-Hp30 considerably improved transparency, UV/Vis light barrier, water resistance and elongation at break (*EAB*). Hp presence increased both puncture force (*F*) and puncture elongation (*E*), but not tensile strength (*TS*) or Young's modulus (*Y*). The addition of an increased concentration of hydrolysate to the films led to a considerable increase in the Folin-reactive substance content and the antioxidant activity, especially the radical scavenging capacity.

9.2. Introduction

Seaweeds have recently been included in Western diets as food and also as components of functional products because of their richness in polysaccharides, proteins (Fleurence, 1999), minerals and vitamins. Moreover, seaweeds are an excellent source of bioactive substances such as sulfated polysaccharides, peptides and polyphenols with biological activities, including antioxidant and antihypertensive properties (Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012; Plaza, Cifuentes, & Ibáñez, 2008; Sarmadi & Ismail, 2010; Tierney, Croft, & Hayes, 2010).

In the last decade, new marine bioprocess technologies have allowed the isolation of substances with antioxidant properties or bioactive peptides by enzymatic hydrolysis (Ngo, Wijesekara, Vo, Van Ta, & Kim, 2011). Seaweeds have proved to be a good source of peptides and polyphenols (Cian, Alaiz, Vioque, & Drago, 2013; Heo, Park, Lee, & Jeon, 2005; Senevirathne, Ahn, & Je, 2010). Red algae (Rhodophyta) are known to have a high protein content, mainly composed of bioactive phycobiliproteins (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011) and other wall proteins that might be more efficiently extracted by an enzyme-assisted treatment (Denis, Morancais, Gaudin, & Fleurence, 2009; Joubert & Fleurence, 2008).

Mastocarpus stellatus is one of the few carrageenophyte species on the Atlantic coast currently harvested for phycocolloid industry purposes, but it is still underutilized (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009).

Commercial carrageenan is commonly extracted at alkaline conditions (pH 7–9) at temperatures near boiling point (80–110 °C) for 3–4 h, providing yields of 20–40% (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Pereira, Critchley, Amado, & Ribeiro-Claro, 2009). However, high molecular weight carrageenan can also be extracted at mild temperatures (50 °C) for 1–5 h (Montolalu, Tashiro, Matsukawa, & Ogawa, 2008).

κ/ι-hybrid carrageenan has been reported to be the main biopolymer structure extracted from *M. stellatus* (Hilliou, Larotonda, Sereno, & Gonçalves, 2006), although other components, such as proteins, minerals and polyphenols, are also present in significant amounts (Sekar & Chandramohan, 2008). Mastocarpus enzymatic hydrolysis could produce both antihypertensive and antioxidant extracts, as previously reported with another species of the Rhodophyta phylum (Cian, Martínez-Augustin, & Drago, 2012; Wang, Jónsdóttir, Kristinsson, Hreggvidsson, Jónsson, Thorkelsson et al., 2010). Protein hydrolysates from different origins have been incorporated in the formulation of protein-based films to improve or confer bioactivity (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009; Salgado, Fernández, Drago, & Mauri, 2011).

Mastocarpus extraction could be maximized by first performing an enzymatic hydrolysis at mild temperatures and alkaline conditions followed by carrageenan precipitation and bioactive compound isolation.

The aim of the present study was to obtain two different potentially bioactive hydrolysates (antioxidant and antihypertensive) from dried *Mastocarpus stellatus* and to develop active Mastocarpus carrageenan-based films by adding the more antioxidant hydrolysate.

9.3. Materials and Methods

9.3.1. Seaweed sampling

Samples of *Mastocarpus stellatus* (M), kindly supplied by Porto-Muiños (Cerceda, A Coruña, Spain), were washed several times with running tap water and air-dried at 50 °C for 24–48 h in a ventilated oven. Seaweed samples were stored in sealed plastic bags at 2–4 °C for 1 week until use.

9.3.2. Unrefined biopolymer extraction

Dried seaweed was homogenized using an Osterizer blender (Oster, Aravaca, Madrid, Spain) with water in a 1:15 (w:v) proportion and kept for 12 h at 3 ± 2 °C. The seaweed was then filtered and subjected to two consecutive extractions in water at a 1:30 (w:v) proportion, at 91 °C for 2 h during the first step and 1.5 h during the second one. Each extract was centrifuged at 3000 rpm for 5 min (Sorvall Evolution RC Centrifuge, Thermo Fisher Scientific Inc., Landsmeer, The Netherlands) and blended. The supernatant was dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 65.0 ± 0.8 °C and this constituted the Mastocarpus biopolymer extract, which was stored at room temperature.

9.3.3. Seaweed hydrolysis

Dried seaweed was mixed with 4% distilled water (w/v) and subjected to enzymatic hydrolysis for 3 h, using alcalase 2.4L (EC 3.4.21.14, 2.64 AU/g, Sigma-Aldrich Inc., St. Louis, MO, USA) in optimal conditions for enzymatic activity (pH 8, 50 °C). The enzyme-substrate (seaweed) ratio was 1:20 (w:w) and the pH of the reaction was kept constant by addition of 1 N NaOH solution to the reaction medium using a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). The enzyme was inactivated by heating at 90 °C for 10 min. The hydrolysate was centrifuged at 7000 g for 15 min. The supernatant was subjected to two consecutive carrageenan extractions by precipitation with ethanol 1:3 (v/v) at 4 °C for 2

h. The precipitated carrageenan was dried at 65 ± 0.8 °C and weighed in order to evaluate extraction yields. The carrageenan-free liquid phase was centrifuged at 13000 g for 5 min. The supernatant was concentrated by rotary evaporation and was subsequently subjected to five organic extractions with ethyl acetate 1:5 (v/v), to remove most of the polyphenols and other compounds such as pigments. After decanting, the successive aqueous phases were concentrated by rotary evaporation. The concentrate was lyophilized, and this constituted the phenolic-partially-removed hydrolysate (H). The phenolic-containing hydrolysate (Hp) was obtained under the same conditions as described above, with the exception of the removal of polyphenol compounds with ethyl acetate. The Hp hydrolysate was selected for active film development.

9.3.4. Amino acid analysis of hydrolysates

The amino acid composition of the hydrolysates (H, Hp) was determined using a Biochrom 20 amino acid analyzer (Pharmacia, Barcelona, Spain) according to the method described by Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, Gómez-Guillén, and Montero (2011). The results were expressed as number of amino acid residues per 1000 residues.

9.3.5. Angiotensin-converting enzyme (ACE) inhibition of hydrolysates

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to determine ACE-inhibitory capacity of the hydrolysates (H, Hp), according to the method described by Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, Gómez-Guillén, and Montero (2011). The IC_{50} value was defined as the concentration of hydrolysate (μg/mL) required to inhibit 50% of ACE activity.

9.3.6. Film preparation

Three film-forming solutions were prepared to obtain the following films: F-Hp0 (without the addition of hydrolysate), F-Hp15 (with 15% hydrolysate) and F-Hp30 (with 30% hydrolysate). Film-forming solutions (FS) (2% w/v) were prepared from Mastocarpus biopolymer extract by adding hot distilled water (90 °C) and homogenizing with a T25 basic Ultra-Turrax (IKA-Werke GmbH & Co. KG, D-79219 Staufen, Germany) at 17500–21500 rpm for 5 min. Glycerol (Panreac Química S.A., Barcelona, Spain) was added at 10% (w/w) in relation to the seaweed extract content. The film-forming solutions were centrifuged at 3000 rpm for 3 min to remove air bubbles. Hp was then added at 15 and 30% (w/w) in relation to the seaweed extract content, and was magnetically stirred for 5 minutes. The film-forming solutions were cast into petri dishes and dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 35.0 ± 0.8 °C for 21 h. All the films were conditioned at 58.0 ± 0.2% RH and 22 ± 1 °C for 4 days prior to analysis.

9.3.7. Viscoelastic properties of film-forming solutions (FS)

A dynamic viscoelastic 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). A dynamic frequency sweep from 0.1 to 10 Hz took place at auto stress, at a temperature of 10 °C and a target strain of 0.005%. The elastic modulus (*G'*; Pa) and viscous modulus (*G''*; Pa) were plotted as functions of the frequency ramp. To characterize the frequency dependence of *G'* over the limited frequency range, the following power law was used:

$G' = G_0'\omega^n$

where G_0' is the energy stored and recovered per cycle of sinusoidal shear deformation at an angular frequency of 1 Hz, ω is the angular frequency and n is the power law exponent, which should exhibit an ideal elastic behaviour near zero in gels. At least two determinations were performed for each sample. The experimental error was less than 6% in all cases.

9.3.8. Viscosity

A viscosity test for film-forming solutions was performed at 25 °C in the cone-plate cell (cone angle 4°, gap = 150 mm) of the Bohlin rheometer at a constant shear rate of 0.5 s^{-1}. The results are averages of eight determinations and are expressed as Pa·s.

9.3.9. Thermal properties

Calorimetric analysis of extracts and films were performed using a differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated by running high-purity indium (melting point, 156.4 °C; melting enthalpy, 28.44 J/g). Samples of around 10–15 mg were tightly encapsulated in aluminium hermetic pans. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 180 °C at a heating rate of 10 °C/min. Peak temperatures (T_{peak}, °C) and enthalpies of conformational changes (ΔH) were measured at least in triplicate, the latter data being normalized to dry matter content (J/g_{dm}) after desiccation of each particular capsule.

9.3.10. ATR-FTIR spectroscopy

Extract and film infrared spectra between 4000 and 650 cm^{-1} were recorded at least in triplicate using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA), as described by Ojagh, Nunez-Flores, López-Caballero, Montero, and Gómez-Guillén (2011).

9.3.11. Antioxidant activities of hydrolysates and films

ABTS radical [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging capacity and FRAP (ferric reducing ability of plasma) were used to measure the antioxidant activity of the hydrolysates (H, Hp) and films (F-Hp0, F-Hp15, F-Hp30). Both hydrolysates and films were dissolved in distilled water and shaken until they were totally homogeneous. The film solutions were filtered through Whatman No. 1 paper. The method used for the FRAP and ABTS assays was previously described by Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, Gómez-Guillén, and Montero (2011). Results were expressed as μmoles Fe^{2+} equivalents/g for FRAP and mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g) for ABTS, based on standard curves of $FeSO_4 7H_2O$ and vitamin C, respectively. All determinations were performed at least in triplicate.

9.3.12. Folin-reactive substances content of hydrolysates and films

Total Folin-reactive substances content was determined according to a modified method by Slinkard and Singleton (1977) with the Folin–Ciocalteu reagent. An aliquot of 10 μL of sample was mixed with 750 μL of distilled water and oxidized with 50 μL of Folin–Ciocalteu reagent. The reaction was

neutralized with 150 µL of sodium carbonate solution and incubated for 2 h at room temperature. The absorbance of the resulting blue colour was measured at 765 nm (UV-1601, model CPS-240, Shimadzu, Kyoto, Japan). Results were expressed as mg gallic acid (GA) equivalent/g of sample. All determinations were performed at least in triplicate.

9.3.13. Film determinations

9.3.13.1. Thickness

The thickness was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 6–8 random locations in 15 films for each treatment as described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

9.3.13.2. Moisture content

The moisture content was determined according to A.O.A.C. (1995).

9.3.13.3. Protein content

The protein content was determined by a LECO FP-2000 nitrogen/protein analyzer (Leco Corp., St. Joseph, MI, USA), according to Dumas (A.O.A.C., 2005) and using a nitrogen-to-protein conversion factor of 6.25.

9.3.13.4. Light absorption and transparency

The light barrier properties and transparency of the films were calculated at least in triplicate using a UV-1601 spectrophotometer (model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 250 to 800 nm following the method described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

9.3.13.5. Colour

The colour parameters lightness (L^*), redness (a^*), and yellowness (b^*) were measured following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

9.3.13.6. Water vapour permeability (*WVP*)

The water vapour permeability (*WVP*) was determined at least in triplicate following the method described by Sobral, Menegalli, Hubinger, and Roques (2001) at room temperature and in a dissecator with distilled water.

9.3.13.7. Water solubility

Film solubility was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

9.3.13.8. Water resistance

Film water resistance was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

9.3.13.9. Mechanical properties

Tensile and puncture tests were run at least in triplicate using a TA.XT plus TA-XT2 texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA) as described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

9.3.14. Statistical analysis

Statistical tests were performed using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, Illinois, USA) for one-way analysis of variance. The variance homogeneity was evaluated using the Levene test, or the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

9.4. Results and Discussion

9.4.1. Extraction yield of seaweed hydrolysis

Carrageenan extraction yield was 28.65% (dry weight basis) and hydrolysate yields were 19.04% for H and 39.17% for Hp (dry weight basis); therefore total seaweed extraction yield by H and Hp hydrolysis was 47.69 and 67.82%, respectively. While H extraction resulted in a similar yield to another previously reported alcalase red seaweed hydrolysis, Hp was much higher than almost all the protease extracts tested (Wang et al., 2010).

Although carrageenan extraction was performed at a milder temperature (50 °C) than the conventional one (80 °C) (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006), these conditions could be suitable for its extraction, since the yields obtained were quite good. (Montolalu, Tashiro, Matsukawa, & Ogawa, 2008) indicated that the carrageenan obtained in extractions at 50 °C for long times (5 h) showed good gelling properties, being better than those obtained with shorter times. Therefore, enzymatic hydrolysis would allow concomitant extraction of bioactive compounds (hydrolysate) and carrageenan, improving the total yield and adding value to the seaweed extraction.

9.4.2. Mastocarpus protein hydrolysates

9.4.2.1. Protein content and amino acid composition

The protein content of marine algae varies greatly within species. Reports have shown that, in general, red seaweeds contain high levels of proteins (Harnedy & FitzGerald, 2011). The dried *Mastocarpus stellatus* contained 15.02 ± 0.53% of protein. Protein content was concentrated in the hydrolysates up to 37.86 ± 1.07% for H and 31.32 ± 0.96% for Hp.

The amino acid composition of H and Hp, expressed as residues per 1000 total amino acid residues, is shown in Table 1.

As expected, a similar amino acid profile was observed in both hydrolysates. Both H and Hp showed high contents of Ser, Gly, Ala, Asp and Glu, and relatively high contents of Leu, Thr, Val, Pro and Phe. The sum of the aspartic and glutamic acid contents was 192 residues/1000 residues and 198 residues/1000 residues for H and Hp, respectively. The high acidic amino acid content is typical of red seaweeds (Cian, Martínez-Augustin, & Drago, 2012). Nevertheless, some differences between the hydrolysates were noteworthy. Some amino acids (Ser, Thr, Arg, His) were concentrated in the more purified hydrolysate (H), owing to the removal of other amino acids, mainly hydrophobic residues (Ala, Val, Ile, Leu, Pro, Met). These hydrophobic amino acids might have been extracted during the ethyl acetate extraction, suggesting that some of them could be linked to the polyphenols extracted.

Amino acid	**Number of residues / 1000 residues**	
	H	Hp
Asp	102	105
Thr	73	66
Ser	153	92
Glu	90	93
Gly	112	116
Ala	104	120
Cys	5	7
Val	47	65
Met	16	19
Ile	34	40
Leu	72	88
Tyr	28	28
Phe	43	43
His	12	6
Lys	29	26
Arg	27	23
Pro	48	57
Hyp	0	0
Hyl	5	5

Table 1. Amino acid composition of Mastocarpus hydrolysates (H, Hp)

9.4.2.2. ACE-inhibitory capacity

Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of blood pressure and hypertension, because it catalyses the conversion of inactive angiotensin-I into angiotensin-II, a potent vasoconstrictor, and inactivates bradykinin, a potent vasodilator (Murray & FitzGerald, 2007).

The amount of Mastocarpus hydrolysate required to inhibit 50% of the ACE activity (IC_{50}) is shown in Table 2.

Both H and Hp showed a high ACE-inhibitory capacity. The phenolic-partially-removed hydrolysate (H) showed considerably higher ACE-inhibitory capacity (IC_{50} of 91 µg/mL) than the phenolic-containing hydrolysate (Hp), probably owing to H's higher peptide concentration (7%). Furthermore, some peptides of Hp might be interacting with polyphenols, therefore being less available for ACE binding. However, Jeon (2005) reported that, among seven flavourzyme enzymatic digestions of brown seaweed, the hydrolysate with the highest polyphenol content showed the highest ACE-inhibitory capacity. Moreover, some polyphenolic compounds have been shown to exert ACE-inhibitory activity (Pozo-Bayón, Alcaíde, Polo, & Pueyo, 2007). ACE-inhibitory capacity might also be influenced by small differences in the amino acid compositions of the hydrolysates. Peptide ACE-inhibitory activity could be strongly influenced by the presence of hydrophobic (aromatic or branched side chains) amino acid residues at the C-terminal positions (Murray & FitzGerald, 2007; Sato, Hosokawa, Yamaguchi, Nakano, Muramoto, Kahara et al., 2002). The hydrophilic–hydrophobic partitioning in the sequence is also a critical factor in the inhibitory activity (Sheih, Fang, & Wu, 2009). ACE inhibition is also highly dependent on the molecular weight of peptides, those that are very short and have low molecular weight being more active (Alemán and Martínez, 2013).

	IC_{50}* (µg/mL)
H	91.62 ± 2.44 a
Hp	148.32 ± 3.16 b

Table 2. ACE-inhibitory capacity of Mastocarpus hydrolysates. Different letters (a, b) indicate significant differences (p≤0.05). * IC_{50}: concentration (µg/mL) required to inhibit 50% of ACE activity.

The IC_{50} of the ACE-inhibitory capacity of the H hydrolysate was 17.5 times lower than the IC_{50} value of the alcalase hydrolysate derived from red algae *Porphyra yezoensis* (Qu, Ma, Pan, Luo, Wang, & He, 2010). The ACE-inhibitory capacity of algae hydrolysates has been reported in other works (He, Chen, Wu, Sun, Zhang, & Zhou, 2007; Sato et al., 2002; Suetsuna, 1998). Although, the use of a different method and its associated modifications to test ACE-inhibitory capacity makes direct comparison of IC_{50} values difficult (Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, Gómez-Guillén, & Montero, 2011)., H could be considered as a potent ACE-inhibitory hydrolysate.

9.4.2.3. Folin-reactive substances and antioxidant activity

Folin-reactive substances, ferric reducing power and ABTS radical scavenging ability of the hydrolysates are shown in Table 3. Both hydrolysates presented a noticeable amount of Folin-reactive substances. As was expected, considering the method of hydrolysate preparation, the Folin-reactive substances content was higher in Hp (phenolic-containing hydrolysate) than in H (phenolic-partially-removed hydrolysate). Some polyphenols, however, might not have been fully separated with ethyl acetate in the H hydrolysate. Moreover, although the Folin–Ciocalteu assay is a widely used method to

determine total phenolic content, additional substances can react with the Folin reagent, including sugars and proteins, and should be taken into account (Prior, Wu, & Schaich, 2005).

The hydrolysis process would allow an improved extraction of phenolic compounds as well as the release of low molecular weight peptides (Wang et al., 2010), which contribute to enhance the antioxidant properties. Hp showed higher antioxidant activity than H (1.4 times higher for reducing power and 2.7 times higher for ABTS radical scavenging), probably owing to a greater presence of phenolic compounds in Hp. The positive correlation between the polyphenolic content of algae and their antioxidant activity has been well documented (Athukorala, Kim, & Jeon, 2006; Karawita, Siriwardhana, Lee, Heo, Yeo, Lee et al., 2005; Kim, Shin, Lee, Park, Park, Yoon et al., 2009; Siriwardhana, Lee, Jeon, Kim, & Haw, 2003; Wang, Jonsdottir, & Ólafsdóttir, 2009).

Sample	ABTS (mg vit C eq/g)	FRAP (µmol Fe/g)	Folin reactive substances (mg/g)
H	35.95 ± 1.59 a	84.52 ± 1.38 a	36.02 ± 3.26 a
Hp	93.26 ± 2.55 b	106.19 ± 1.05 b	75.61 ± 0.56 b
F-Hp0	3.07 ± 0.18 a	4.54 ± 0.08 a	7.33 ± 0.34 a
F-Hp15	17.56 ± 0.90 b	11.77 ± 0.38 b	15.97 ± 1.46 b
F-Hp30	27.51 ± 0.83 c	13.75 ± 0.06 c	22.17 ± 0.36 c

Table 3. Antioxidant activity and Folin reactive substances of hydrolysates and films.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different hydrolysates (H) or different films (F) (P≤0.05).

On the other hand, the hydrolysate peptide fraction can also contribute to antioxidant activity. It is well known that biological activities of protein hydrolysates are related to the amino acid composition, sequence, molecular weight and peptide configuration. For example, phosphorylated serine and threonine are known to bind metals (Elias, Kellerby, & Decker, 2008), being more hydrophilic and reactive because of their hydroxyl group. Amino acids with non-polar aliphatic groups, such as alanine, leucine or proline, have high reactivity to hydrophobic PUFA radicals, while hydrogen donors such as aspartic and glutamic acids are able to quench unpaired electrons or radicals by supporting protons (Qian, Jung, & Kim, 2008). The abundance of these amino acids in the peptide sequences of hydrolysates could also be responsible for their antioxidant activity. As previously mentioned, the hydrophobic amino acid content was higher in Hp than in H, which might also have contributed to the higher antioxidant capacity (ABTS and FRAP) of Hp compared with H.

Various studies have been carried out to evaluate the antioxidant potential of marine algae hydrolysates (Ahn, Jeon, Kang, Shin, & Jung, 2004; Cian, Alaiz, Vioque, & Drago, 2013; Heo, Park, Lee, & Jeon, 2005; Je, Park, Kim, Park, Yoon, Kim et al., 2009; Park, Shahidi, & Jeon, 2004; Wang et al., 2010). However, to our knowledge, no reference has been made in previous studies to the antioxidant or ACE-inhibitory activity of Mastocarpus hydrolysates.

Given the Folin-reactive substances content and antioxidant activity results of the hydrolysates, both hydrolysates could be considered potential antioxidants. However, because of its greater potential, Hp was selected to develop active Mastocarpus films with antioxidant activity.

9.4.3. Development of active films

Increasing concentrations of hydrolysate (Hp) were added to Mastocarpus biopolymer film-forming solutions. Concentrations above 30% produced sticky, unmanageable films. For this reason, 30% was chosen as the maximum hydrolysate concentration that could be used for film development. A concentration of 15% hydrolysate was also chosen in order to maintain a balance between the film's physicochemical properties and the active properties that could be provided by the hydrolysates.

9.4.3.1. FTIR-ATR

Infrared spectra of F-Hp0, F-Hp15 and F-Hp30 films and Hp freeze-dried *Mastocarpus stellatus* hydrolysate were measured (Figure 1). Hp was analysed to assess its possible contribution to film structure. All film spectra showed a band at approximately 845 cm^{-1} (C-O-S vibration), assigned to D-galactose-4-sulfate (present in both κ- and ι-carrageenan), and a strong band at 924 cm^{-1}, which indicated the presence of 3,6-anhydro-D-galactose, a typical feature of κ-carrageenan (Gómez-Ordóñez & Rupérez, 2011; Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003).

The concomitant presence of κ- and ι-carrageenan features strongly suggested a greater extraction of κ/ι-hybrid carrageenan, as previously reported with *M. stellatus* (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Van de Velde, 2008). F-Hp0 and F-Hp15 spectra had similar band intensities, while F-Hp30 had the above-mentioned bands considerably reduced, attributed to the reduced carrageenan amount in this film formulation in comparison with Hp, which had a much lower IR intensity at these wavenumbers. Although high temperature (80–90 °C) is adequate for suitable carrageenan extraction, a certain amount of biopolymer might have been extracted during the hydrolysis carried out at 50 °C. In this regard, (Montolalu, Tashiro, Matsukawa, & Ogawa, 2008) reported an appreciable extraction yield of high molecular weight carrageenan at 50 °C in *Kappaphycus alvarezii*. The strong band at 1037 cm^{-1} in Hp confirmed the predominantly polysaccharide nature of the hydrolysate. Moreover, the greater absorption in the Hp IR-spectrum at wavenumbers between 1100 and 1150 cm^{-1} as compared to the films also suggested an increased proportion of shorter polysaccharide chains (Sun, Tao, Xie, Zhang, & Xu, 2010).

A small band at approximately 803 cm^{-1} in the film spectra indicated the presence of two sulfate ester groups on the anhydro-D-galactose residues (sulfation on C2), characteristic and distinctive of ι-carrageenan (Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003; Prado-Fernández, Rodríguez-Vázquez, Tojo, & Andrade, 2003). This feature, which was not found in the hydrolysate, was most prominent in F-Hp0, and became smaller with an increasing Hp amount in the film formulation (F-Hp0>F-Hp15>F-Hp30). Despite the presence of ι-carrageenan, the *M. stellatus* film spectra were quite similar to κ-carrageenan standards, as previously shown by Gómez-Ordóñez and Rupérez (2011).

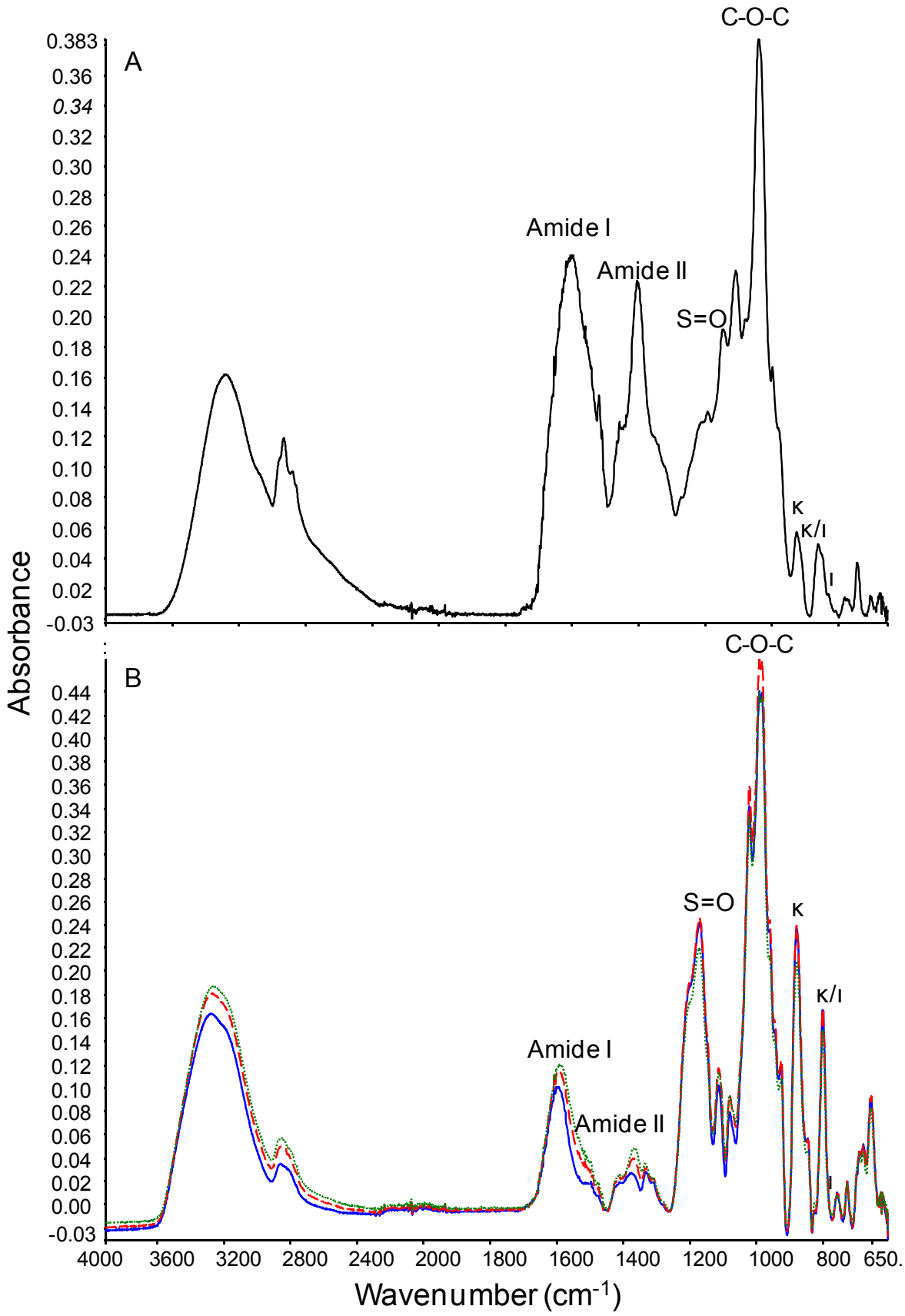

Figure 1. A. ATR-FTIR spectrum of Hp hydrolysate. B. ATR-FTIR spectra of F-Hp0 (continuous blue line), F-Hp15 (discontinuous red line) and F-Hp30 (dotted green line) films.

No evidence of a broad band between 820 and 830 cm^{-1} was found, indicating the absence of highly sulfated λ-carrageenan. The second derivative spectra of the films revealed trace evidence at 871 cm^{-1} of μ-carrageenan (κ-carrageenan precursor) and ν-carrageenan (ι-carrageenan precursor), which was not observable in Hp (data not shown).

The strong absorption bands at ~1216–1217 cm^{-1} (S = O), assigned to the presence of ester sulfate groups, were noticeably lower in F-Hp30, coinciding with the highest proportion of added hydrolysate. The 1217/924 cm^{-1} ratio, as a measure of relative total sulfate groups with respect to κ-carrageenan content, was much higher in Hp (2.6) than in any of the films studied (0.88 in F-Hp0, 0.91in F-Hp15 and 0.94 in F-Hp30), suggesting an additional source of sulfated compounds in Hp, probably phycobiliproteins with sulfur-containing amino acids (Carra, Ó Heocha, & Carroll, 1964). In this respect, Dumay, Clément, Morançais, and Fleurence (2013) observed that enzymatic digestion was an effective treatment for phycoerythrin extraction. Accordingly, the 1217/924 cm^{-1} ratio became higher as a result of the increase in the hydrolysate amount in the film formulation.

The IR-spectrum of Hp also revealed strong bands at 3277 cm^{-1}, 2929 cm^{-1} and 1600 cm^{-1} and a small shoulder at 1518 cm^{-1}, which could be assigned, respectively, to amide A, amide B, amide I and amide II of constituent proteins, most likely phycoerythrin and phycocyanin (Smith & Alberte, 1994). Comparison of the films showed that as the added hydrolysate percentage increased the amide I amplitude became more evident, and it exhibited a wavenumber down-shift to 1631 cm^{-1} in F-Hp30 as compared to 1639 cm^{-1} in F-Hp0 and F-Hp15, which denoted more hydrogen bonding in F-Hp30, attributed to the higher proportion of shorter peptides. In addition, the reduced band intensity of the ester sulfate group in F-Hp30 and the slight frequency up-shift from 1216.1 cm^{-1} in F-Hp0 and F-Hp15 to 1217.5 in F-Hp30 could be indicative of appreciable carrageenan-peptide interactions in the film with the highest amount of added hydrolysate.

9.4.3.2. DSC

DSC thermograms of the freeze-dried *Mastocarpus stellatus* hydrolysate, Hp, and the F-Hp0, F-Hp15 and F-Hp30 films are shown in Figure 2. The hydrolysate showed two main endothermic peak temperatures, T_{peak} (°C), at 86.62 ± 6.22 and 130.16 ± 0.63, and corresponding ΔH (J/g_{dm}) values of 3.51 ± 0.72 and 0.48 ± 0.05, respectively, which might correspond to phycoerythrin and phycocyanin fragments (Mishra, Shrivastav, & Mishra, 2008). Temperatures were higher than those where protein normally features, probably because hydrolysis might shift maximal peak temperature towards higher temperatures (Briones-Martínez, Juárez-Juárez, Oliver-Salvador, & Cortés-Vázquez, 1997; Chronakis, 2001; Nishinari & Watase, 1992). Low enthalpies also suggested the presence of hydrolysis products stabilized by different amounts of hydrogen bonds and hydrophobic interactions. Another endothermic transition in Hp with T_{peak} (°C) at 50.75 ± 0.66 and ΔH (J/g_{dm}) 0.39 ± 0.05 was evidence of the κ-carrageenan constituent (Nishinari & Watase, 1992).

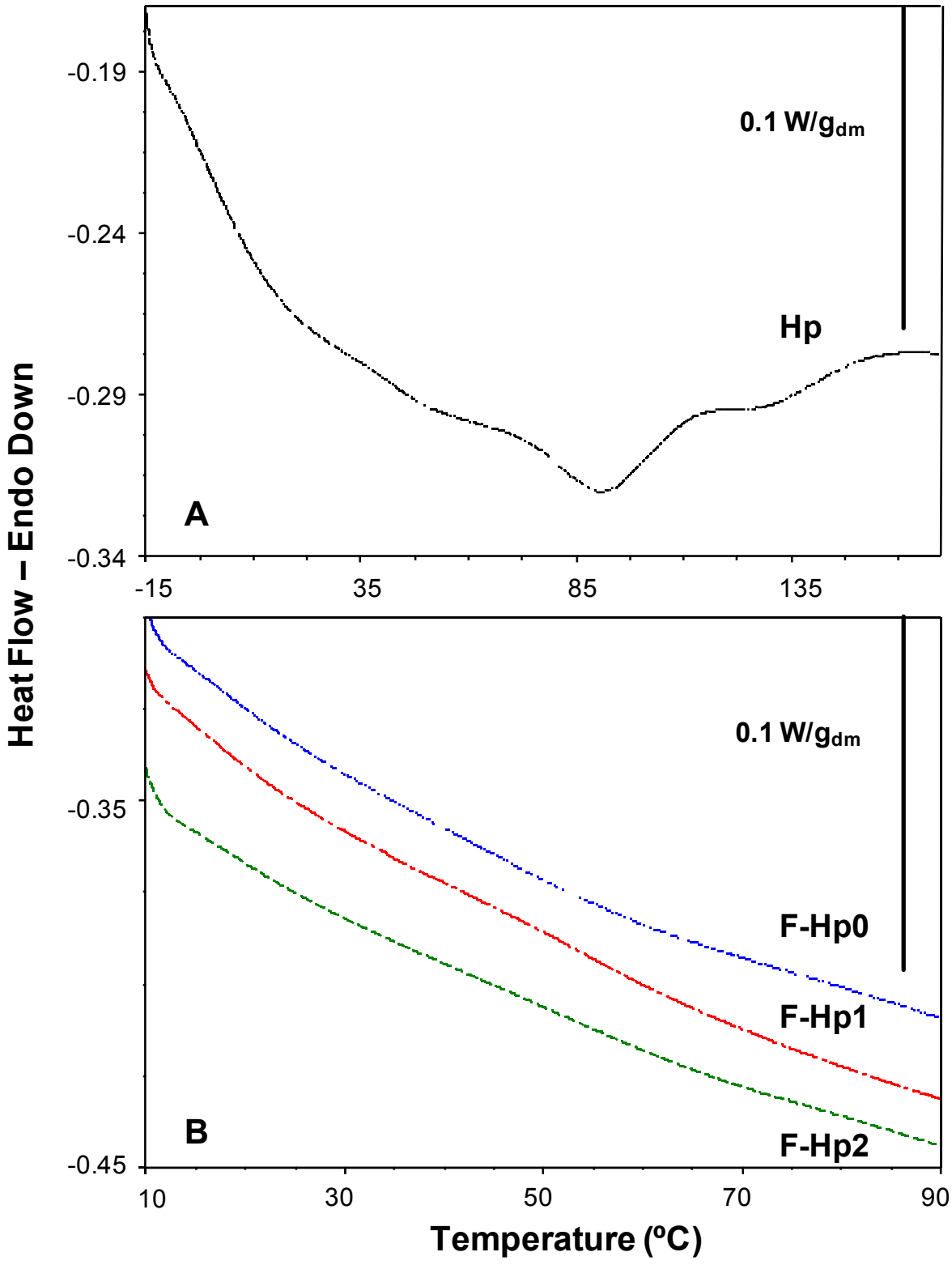

Figure 2. A. DSC of Hp hydrolysate and B. DSC of F-Hp0, F-Hp15 and F-Hp30.

DSC thermograms of F-Hp0, F-Hp15 and F-Hp30 are shown in Figure 2B. Slight endothermic transitions were hardly observable, with T_{peak} values (°C) of 57.73 ± 0.20 in F-Hp0, 61.07 ± 2.14 in F-Hp15 and 64.65 ± 1.86 in F-Hp30, and ΔH (J/g$_{dm}$) of 0.41 ± 0.02, 0.19 ± 0.01 and 0.11 ± 0.04 respectively, indicative of the helix-to-coil transition suffered by the κ/ι-carrageenan as a result of the breakage of weak physical cross-links (Hossain, Miyanaga, Maeda, & Nemoto, 2001). Thermal

transitions, however, were not as sharp as those in a pure carrageenan curve (Iijima, Hatakeyama, Takahashi, & Hatakeyama, 2007; Matsuo, Tanaka, & Ma, 2002). The increased protein content in films with added hydrolysate might promote carrageenan-peptide interactions (Baeza, Carp, Pérez, & Pilosof, 2002), which could explain the increase in T_{peak} temperatures. However, the enthalpy reduction suggested a hydrolysate-induced plasticizing effect in the films as a result of increasing the free water and chain mobility.

9.4.3.3. Rheology

Figure 3 shows the mechanical spectra of the film-forming solutions at 10 °C, in terms of elastic modulus (*G′*) and viscous modulus (*G″*) as a function of angular frequency. The F-Hp0 solution, which had the lowest G′ values, was the only one with a crossover point where *G′* = *G″*. At frequencies below 2 Hz, the F-Hp0 solution was characterized by a dominant viscous behaviour (*G′*<*G″*), which turned into a gel-like behaviour at higher frequencies; thus it could be classified as a concentrated solution constituting an entanglement network. In the absence of KCl, solutions of κ-carrageenan cooled down to 9 °C have been shown to adopt helical structures, which did not aggregate to form self-supporting gels (Núñez-Santiago, Tecante, Garnier, & Doublier, 2011).

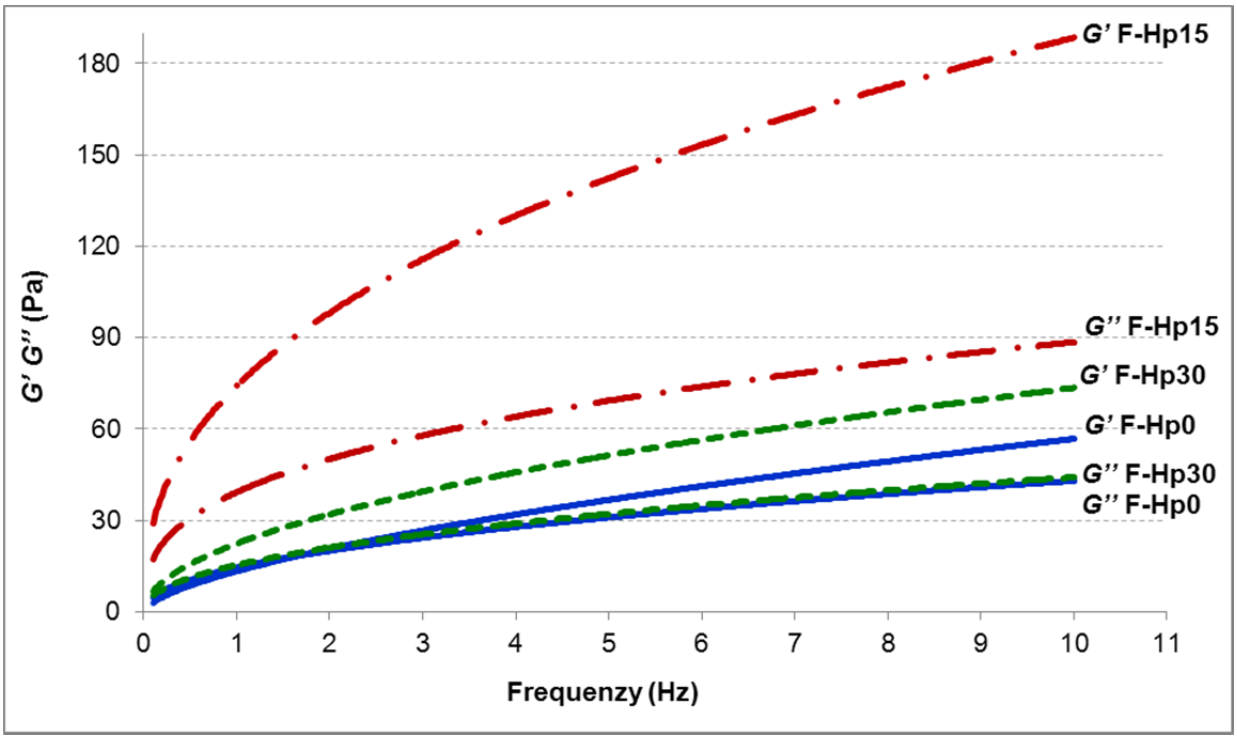

Figure 3. Elastic modulus (*G′*, Pa) and viscous modulus (*G″*, Pa) as a function of the angular frequency of F-Hp0, F-Hp15 and F-Hp30 film-forming solutions at 10 °C.

In contrast, the F-Hp15 and F-Hp30 solutions showed a typical gel-like behaviour denoted by *G′*>*G″* values within the whole frequency range, as previously reported in other studies on carrageenan (Lafargue, Lourdin, & Doublier, 2007). The *G′* values were successfully modelled according to the power law (r^2~0.99) in all three cases. The hydrolysate addition to the film-forming solution at 15% concentration caused a remarkable increase in *G′*, much higher than with 30%. The rheological behaviour of the F-Hp15

solution showed lower frequency dependence of *G'* than the F-Hp30 and F-Hp0 solutions, as deduced from the lowest power law exponent value (n') (0.63 in F-Hp0, 0.40 in F-Hp15, 0.52 in F-Hp30). Hp concentrations higher than 30% conferred sticky, unmanageable properties to the films. All these findings suggest that the hydrolysate added at the appropriate concentration led to stronger gels with increased structural stability, probably due to hydrolysate components (mainly peptides and phenolic compounds) favouring aggregation of carrageenan helices to form a three-dimensional network. Interactions between carrageenan and proteins have previously been shown to produce much stronger gels than single carrageenan gels (Baeza, Carp, Pérez, & Pilosof, 2002). Similarly, the ability of polyphenols to interact with polysaccharides forming complexes has been well documented (Le Bourvellec & Renard, 2012). Nevertheless, the higher hydrolysate amount in the F-Hp30 solution considerably reduced the gel-forming capacity with respect to the F-Hp15 solution, with the helical aggregates probably having more difficulty in being created as a result of a carrageenan-dilution effect (Andrade, Azevedo, Musampa, & Maia, 2010).

The apparent viscosity of the film-forming solutions, measured at 25 °C and shear rate of 0.5 s^{-1}, was considerably higher in the F-Hp15 solution (14.89 ± 0.53 Pa·s) than in the F-Hp0 and F-Hp30 solutions (3.47 ± 0.01 Pa·s and 4.72 ± 0.15 Pa·s, respectively), strongly suggesting effective interactions at the right concentration between carrageenan and other compounds naturally present in the hydrolysate, presumably peptides and phenolic compounds.

9.4.3.4. Light barrier properties

Colour parameters, *L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish), are shown in Table 4. All the films were quite similar, having low lightness (28–29) and slightly greenish and yellowish tendencies. The F-Hp30 film exhibited the highest (P≤0.05) lightness and greenish colouration, and lowest (P≤0.05) yellowish tendency. Changes in *L*a*b** values, however, did not correlate with increasing amounts of added hydrolysate in the film, which could be due to a different degree of interactions between protein pigments and carrageenan. Comparing these results with previously developed commercial κ-carrageenan films, the present *M. stellatus* films presented considerably lower lightness and more red tendency, owing to the concomitant extraction of non-carrageenan compounds (Rhim, 2012; Sánchez-García, Hilliou, & Lagaron, 2010; Shojaee-Aliabadi, Hosseini, Mohammadifar, Mohammadi, Ghasemlou, Ojagh et al., 2013).

Film	*L**	*a**	*b**	Transparency
F-Hp0	28.65 ± 0. 29 a	-0.57 ± 0.03 a	4.57 ± 0. 11 a	7.14 ± 0.24 a
F-Hp15	27.99 ± 0.08 b	-0.30 ± 0.05 b	4.72 ± 0. 04 b	6.61 ± 0.30 ab
F-Hp30	29.25 ± 0.05 c	-0.70 ± 0.03 c	4.36 ± 0.04 c	6.12 ± 0.12 b

Table 4. L^*, a^*, b^* and Transparency ($-\log(T_{600}/x)$) of F-Hp0, F-Hp15 and F-Hp30.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films (P≤0.05).

In general, the films exhibited low light transmission in the UV range (250–300 nm) (0–1.12%) (Figure 4), as compared to commercial κ-carrageenan films (Sánchez-García, Hilliou, & Lagarón, 2010), with F-Hp0 providing the least efficient UV barrier. Two absorption peaks were defined in all the films in

the ranges 400–450 nm and 600–700 nm, which might be associated with the presence of pigments, such as carotenoids and chlorophyll, which absorb at 400–450 (violet-blue-green colours), and phycoerythrin and phycocyanin at 600 nm (red colour) (Sühnel, Hermann, Dornberger, & Fritzsche, 1997). In the visible range, the light transmission was significantly (P≤0.05) lower in F-Hp30, especially in the wavelength range between 350 and 700 nm, which might be largely due to the increase in thickness associated with the hydrolysate addition, as Table 5 shows. The hydrolysate contained small molecules (mainly peptides and oligosaccharides) that might have interfered in carrageenan helix aggregation during the film drying process. This interference might have caused a plasticizing effect with an increase in free volume that would have resulted in thicker films.

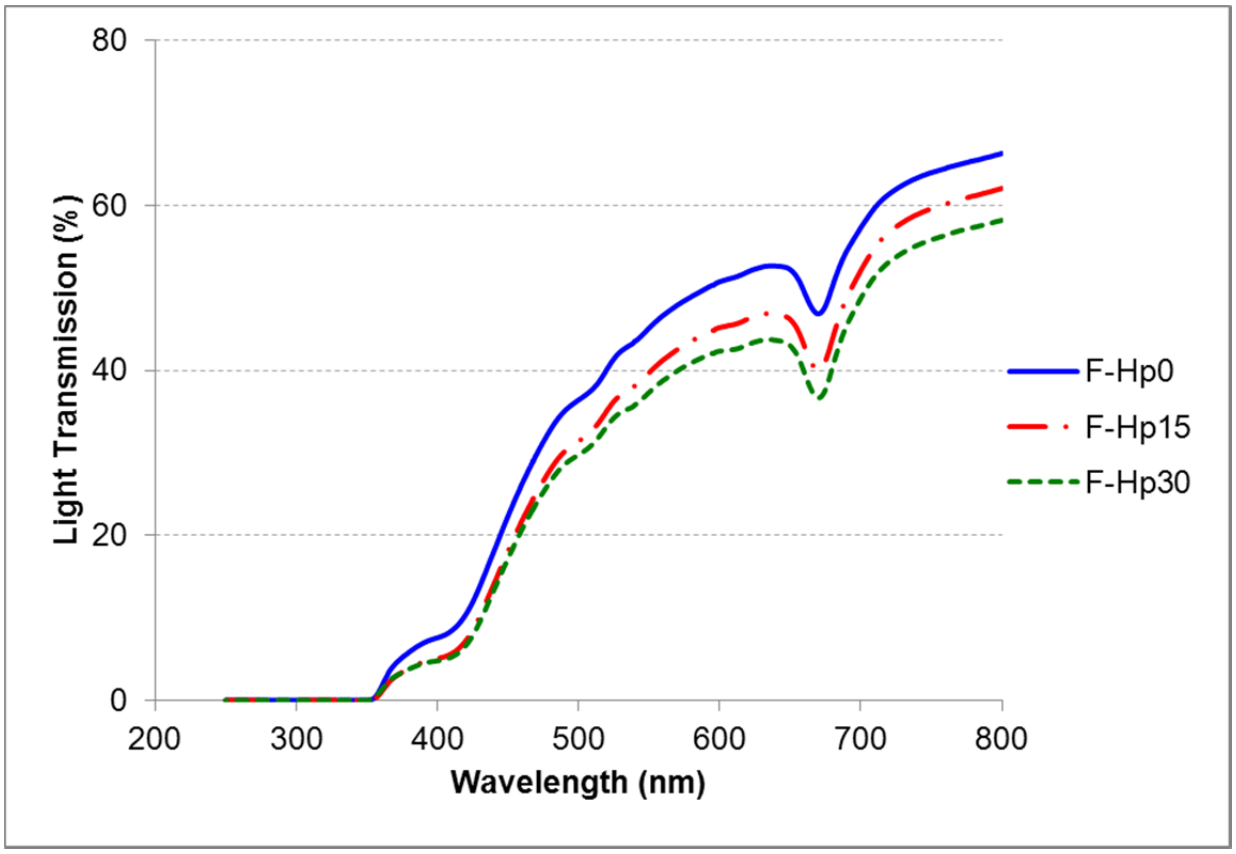

Figure 4. Light transmission (*T*, %) at various wavelengths (250–800 nm) of F-Hp0, F-Hp15 and F-Hp30.

9.4.3.5. Physicochemical properties

Slight variations in moisture content were observed among the three film formulations (Table 5), with F-Hp30 showing slightly higher values, which could be related to its increased thickness. The protein content in the films increased significantly (P≤0.05) with the addition of increasing amounts of hydrolysate (Table 5).

9.4.3.5.1. Water barrier

No significant differences in film water solubility were found in *M. stellatus* films with either 15 or 30% added hydrolysate (Table 5). A similar finding was reported earlier in gelatine films incorporating different percentages of gelatine hydrolysate (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, &

Montero, 2009). Although the solubility values were not high, the films totally lost their original structure, becoming a very viscous solution with gelling tendency at low temperatures. Solubility was similar to previous results obtained in commercial carrageenan films (Shojaee-Aliabadi et al., 2013).

	F-Hp0	F-Hp15	F-Hp30
Thickness (μm)	51.82 ± 3.34 a	56.05 ± 3.65 a	68.36 ± 4.04 b
Moisture (%)	13.46 ± 0.28 a	12. 13 ± 0. 14 b	14.59 ± 2.75 ab
Protein content (%)	8.90 ± 0.06 a	10.98 ± 0.21 b	13.05 ± 0.24 c
Film solubility (%)	20.97 ± 4.5 a	25.77 ± 3.64 a	22.16 ± 2.95 a
WVP (x10^{-8} g m^{-1} s^{-1} Pa^{-1})	3. 78 ± 0. 17 a	3. 20 ± 0. 12 b	4.04 ± 0. 30 a
TS (MPa)	59.94 ± 2.27 a	51.37 ± 3.75 b	41.63 ± 2.95 c
EAB (%)	0.95 ± 0.11 a	1.59 ± 0.09 b	2.47 ± 0.24 c
Y (MPa)	1797 ± 61 a	1347 ± 74 b	1054 ± 45 c
F (N)	23.47 ± 1.08 a	26.36 ± 2.14 b	30.38 ± 2.27 b
D (%)	7.73 ± 0.47 a	8.61 ± 0.53 a	12.24 ± 1 b

Table 5. Thickness, moisture, protein content, film solubility, water vapour permeability (*WVP*), tensile strength (*TS*), elongation at break (*EAB*), Young's modulus (*Y*), puncture force (*F*) and puncture deformation (*D*) of F-Hp0, F-Hp15 and F-Hp30
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films (P≤0.05).

No significant (P≤0.05) differences were found between F-Hp0 and F-Hp30 water vapour permeability (*WVP*) (Table 5). In contrast, F-Hp15 had the lowest permeability, probably owing to effective carrageenan-protein interactions, as previously commented. Despite the greater thickness of F-Hp30, *WVP* was not reduced by adding 30% hydrolysate. The extra protein and plasticizer effect caused by Hp addition may have resulted in a less dense network (Gontard, Guilbert, & Cuq, 1993). The present films were more water vapour permeable than previously reported commercial carrageenan films (Karbowiak, Debeaufort, & Voilley, 2007; Martins, Cerqueira, Bourbon, Pinheiro, Souza, & Vicente, 2012; Rhim, 2012; Sánchez-García, Hilliou, & Lagaron, 2010; Shojaee-Aliabadi et al., 2013).

Water resistance test results are shown in Figure 5. Noticeable differences among samples were observed after 10 minutes. Although every film elongated up to 2 cm, F-Hp0 showed a faster elongation speed (3.2 cm/h) than F-Hp15 and F-Hp30 (~2.9 cm/h). The hydrolysate addition led to a significantly higher breakage resistance in F-Hp30 in comparison with F-Hp0 and F-Hp15, probably related to the higher carrageenan peptide interactions. The films did not show any water filtration before breakage time.

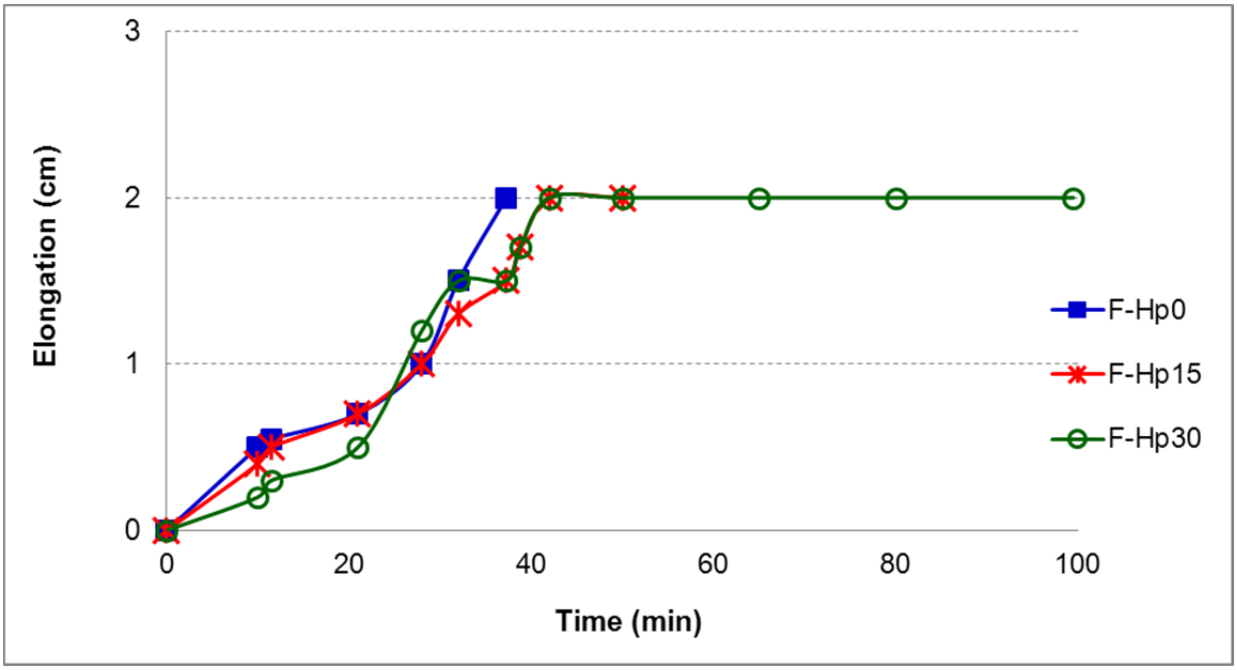

Figure 5. Water resistance: Elongation (cm) in time (min) until breakage for F-Hp0, F-Hp15, and F-Hp30.

9.4.3.5.2. Mechanical properties

F-Hp0 had the significantly highest (P≤0.05) tensile strength (*TS*) (Table 5), which was lowest in F-Hp30. The opposite behaviour was found regarding the elongation at break (*EAB*) values, confirming the hydrolysate-induced plasticizing effect in the film. The *TS* and *EAB* values in the three *M. stellatus* films studied were, respectively, higher and lower than the results reported with commercial κ-carrageenan or ι-carrageenan films (Martins, Cerqueira, Bourbon, Pinheiro, Souza, & Vicente, 2012;12; Shojaee-Aliabadi et al., 2013), suggesting a reinforcement effect caused by the presence of other non-carrageenan components. As far as Young's modulus (*Y*) is concerned (Table 5), the highest stiffness also corresponded to F-Hp0 (P≤0.05), decreasing with increasing amount of Hp. The small molecules (mainly peptides and oligosaccharides) that form part of the hydrolysate have been proved to act as film plasticizers by preventing carrageenan helix associations and increasing the molecular mobility of polymer chains, which in the case of F-Hp30 was favoured by the increased water plasticizing effect. Salgado, Fernández, Drago, and Mauri (2011) also observed a reduction in *TS* and *Y* and an increase in *EAB* in protein films with added hydrolysate, which, in view of the lack of film moisture increase, was attributed to interferences in protein cross-linking caused by peptides interacting with the polymer matrix.

There were no significant differences in puncture force (*F*) between F-Hp0 and F-Hp15 (P≤0.05) (Table 5), whereas F-Hp30 had higher values. Puncture deformation (*D*) was significantly higher (P≤0.05) in F-Hp30, with no differences between F-Hp0 and F-Hp15. It is worth noting that the films with the highest hydrolysate amount had better resistance to the puncture test, contrary to what happened with the tensile test, although similar behaviour was found regarding puncture deformation and elongation at break. The slight increase in puncture force could be related to the above-mentioned carrageenan-hydrolysate compound interactions in F-Hp30, but the considerable plasticizing effect exerted by the hydrolysate and the high water content was the main factor determining the film's tensile properties.

9.4.4. Antioxidant activity and Folin-reactive substances of the films

Folin reactive substances, ferric reducing power and ABTS radical scavenging capacity of the films are shown in Table 3. Films without algae hydrolysates (F-Hp0) contained Folin reactive substances and exhibited some antioxidant activity measured by both FRAP and ABTS assays. The incorporation of increasing concentrations of Hp significantly increased the Folin-reactive substances content (3-fold increase in F-Hp30), as well as FRAP and ABTS values (3- and 9-fold increase, respectively, in F-Hp30).

The antioxidant properties of squid gelatine films (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009) and sunflower films (Salgado, Fernández, Drago, & Mauri, 2011) were also improved by the addition of hydrolysates from squid gelatine and bovine plasma, respectively, but the antioxidant activity increase reported was much lower than in the present work.

9.5. Conclusion

Mastocarpus stellatus hydrolysis could be a complementary way to extract bioactive components in addition to carrageenan. From the two hydrolysates obtained, the more purified peptide hydrolysate (phenolic content partially removed) showed the highest ACE-inhibitory capacity, while keeping peptides and polyphenols together resulted in a more antioxidant hydrolysate.

Mastocarpus stellatus hydrolysate can be used as an active ingredient for film development. The addition of Hp led to more efficient plasticization, a higher proportion of sulfated compounds content and increased film antioxidant activity.

Depending on the hydrolysate proportion, film properties were affected differently: 15% Hp addition decreased film water vapour permeability, while 30% Hp improved film optical properties, breakage water resistance and film puncture test response.

In view of the results obtained, these films might be used as a possible ingredient for functional packaging development for food susceptible to oxidation.

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X. Antioxidant film development from unrefined extracts of brown seaweeds *Laminaria digitata* and *Ascophyllum nodosum*

Antioxidant film development from unrefined extracts of brown seaweeds *Laminaria digitata* and *Ascophyllum nodosum*

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10.1. Abstract

Film-forming alginate-rich extracts from brown seaweeds *Laminaria digitata* (L) and *Ascophyllum nodosum* (A) were obtained using Na_2CO_3 (Lc, Ac) or NaOH (Lh, Ah) at different temperatures (21, 50 and 75 °C) and after different acid pretreatments (H_2SO_4 and HCl). L extractions were more protein efficient than A extractions. NaOH extractions were more carbohydrate efficient, while Na_2CO_3 extractions presented almost two-fold ash content. The higher proportion of preserved guluronic units extracted with NaOH led to more efficiently cross-linked films; whereas the higher amount of degraded and dispersed uronic acids obtained in Na_2CO_3 extractions favoured film compound interactions with glycerol. Lh film was the most compact film, the least water vapour permeable and the most mechanical and water resistant film, while Ac resulted in the most transparent, water soluble and plasticized film. A-films presented higher antioxidant activity than L-films, as measured by ABTS, FRAP and Folin reactive substances, especially when the extraction was carried out using NaOH (Ah).

10.2. Introduction

Many types of brown seaweeds (Phaeophyceae) are available on the Spanish coasts, such as *Laminaria digitata* and *Ascophyllum nodosum*, both of which contain polysaccharides such as laminaran, fucoidan and alginate. Alginates are normally present in algae cell walls as insoluble calcium or magnesium salts, being responsible for their strong, flexible tissue. Industrial alginate extraction is basically divided into a first acid pretreatment to convert insoluble alginate salts into alginic acid, followed by an alkaline extraction to convert insoluble alginic acid into soluble sodium alginate, which passes into the aqueous phase; further alginate isolation requires solid/liquid separation, precipitation and drying. Depending on the species, the extraction can take several hours and repetitions (Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009), and it has been demonstrated that after 2 h alginates start depolymerizing (Vauchel, Arhaliass, Legrand, Kaas, & Baron, 2008).

A small number of works have focused their study on optimizing alginate extraction (Arvizu-Higuera, Hernández-Carmona, & Rodríguez-Montesinos, 2002; Hernández-Carmona, McHugh, & López-Gutiérrez, 1999; Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009), most of them dealing with pre-extraction steps or drying conditions (Arvizu-Higuera, Hernández-Carmona, & Rodríguez-Montesinos, 1997; Hernández-Carmona, McHugh, Arvizu-Higuera, & Rodríguez-Montesinos, 1998); however, the effect of using different sodium sources for the sodium alginate salt formation has not been studied. Sodium alginate is commonly used as a thickening and gelling food ingredient (Mabeau & Fleurence, 1993; Rioux, Turgeon, & Beaulieu, 2007a), while other polysaccharides, such as laminaran, fucoidan and alginic acid, are more interesting for their potential biological activities (Jiménez-Escrig, Gómez-Ordóñez, & Rupérez, 2011).

Alginate is a complex mixture of oligo-polymers mainly consisting of polymannuronic acid and polyguluronic acid (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007). While mannuronic acid presents a β (1-4) linkage with a flexible linear conformation, guluronic acid forms an α (1-4) linkage, introducing a steric hindrance around the carboxyl group and resulting in rigid, folded structural conformations which provide molecular chain stiffness. An efficient integral brown seaweed extraction would be interesting to produce an environmentally friendly unrefined biopolymer-rich extract

for various industrial applications, such as food packaging improvement material. Due to their linear structure and high molecular weight, alginates can form strong films and adequate fibrous structures in solid state (Rinaudo, 2008), being considered a good filmogenic material (Hambleton, Perpiñan-Saiz, Fabra, Voilley, & Debeaufort, 2012; Nakamura, Nishimura, Hatakeyama, & Hatakeyama, 1995; Siddaramaiah, Swamy, Ramaraj, & Lee, 2008); however, they do not stand out for having good antioxidant activity (Norajit, Kim, & Ryu, 2010; Salmieri & Lacroix, 2006).

Seaweed extracts are normally rich in polyphenolic compounds and carotenoids (Cardozo, Guaratini, Barros, Falcão, Tonon, Lopes et al., 2007), which have been reported to have antioxidant properties (Gupta & Abu-Ghannam, 2011), but there is hardly any study of their current use in food products.

The aim of this study is to prepare optimal crude extracts that enable edible active film development from brown seaweeds *Laminaria digitata* and *Ascophyllum nodosum*, and to characterize their physicochemical, structural and antioxidant properties.

10.3. Materials and methods

10.3.1. Seaweed sampling

Samples of *Laminaria digitata* (L) and *Ascophyllum nodosum* (A), kindly supplied by Porto-Muiños (Cerceda, A Coruña, Spain), were collected in Galicia bay (A Coruña, Spain), washed several times with running tap water and air-dried at 50 °C for 24–48 h in a ventilated oven. The seaweed samples were stored in sealed plastic bags at 2–4 °C until use.

10.3.2. Seaweed extraction

Twelve different Laminaria and Ascophyllum extractions were carried out following different acid pretreatments (0.1–0.5 M H_2SO_4 and 0.1–0.2 M HCl), followed by different alkaline treatments (3–4% Na_2CO_3 and 4% NaOH) at different temperatures (21, 50 and 75 °C), as shown in Table 1. Dried seaweeds were homogenized in an Osterizer blender (Oster, Aravaca, Madrid, Spain) with the respective acid solution in a 1:10 (w/v) proportion and kept overnight at 3 ± 2 °C, then washed several times with running tap water until stable pH was reached (portable pH-meter series 3 Star Orion with a ROSS pH electrode, Thermo Fisher Scientific Inc., Landsmeer, The Netherlands). Sodium carbonate (C) extractions were carried out with magnetic stirring in Na_2CO_3 solutions (3% in Ascophyllum and 4% in Laminaria) in a 1:60 proportion (w/v) for 3 h, then homogenized in an Osterizer blender and centrifuged at 6000 rpm for 5 min (Sorvall Evolution RC Centrifuge, Thermo Fisher Scientific Inc., Landsmeer, The Netherlands). The supernatant was dialyzed overnight in 32/32 dialysis tubing (Visking membrane, MWCO 12–14000, regenerated cellulose, Medicell International Ltd, London, UK) at 3 ± 2 °C. Sodium hydroxide extractions were carried out with distilled water in a 1:60 proportion (w/v), stirring magnetically for 3 h in a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne, France) which maintained constant pH 10 by the addition of 4% NaOH, and then homogenized and centrifuged at 6000 rpm for 5 min.

The supernatants were dried in an oven for 3 days (FD 240 Binder, Tuttlingen, Germany) at 65.0 ± 0.8 °C to constitute the different algal extracts, which were stored at 21 °C until use (within one week).

The suitability of the different extractions was evaluated in terms of yield, film-forming capacity and preliminary film water response. Film-forming capacity was evaluated by the ease with which the films could be peeled off the Petri dish and their consistency when handled. For film water response, three 9 cm^2 pieces of each film were placed in distilled water for 30 min at 22 °C, to evaluate their subsequent consistency manually. Four Laminaria (L) and Ascophyllum (A) extracts with the highest yields and filmogenic capacity were selected for further film characterization, two prepared with Na_2CO_3 (Lc, Ac) and the other two with NaOH (Lh, Ah).

10.3.3. Proximate analysis

Moisture and ash content of the selected dried seaweed crude extracts were determined according to official methods (A.O.A.C., 1995). Nitrogen content was determined using a combustion oven apparatus (Model FP-2000, Leco Corporation, St Joseph, MI, USA), according to Dumas (A.O.A.C., 2000), with a conversion factor of 6.25. Each analysis was performed at least in triplicate, and results expressed as percentages. Carbohydrate content was estimated by difference, assuming a fat content lower than 2% (Dawczynski, Schubert, & Jahreis, 2007; Rioux, Turgeon, & Beaulieu, 2007a).

10.3.4. Film preparation

Film-forming solutions were prepared by suspending 1.5% w/v dried extracts in distilled water at the corresponding extraction temperature and homogenizing with a T25 basic Ultra-Turrax (IKA-Werke GmbH & Co. KG, D-79219 Staufen, Germany) at 17500–21500 rpm for 5 min. Glycerol was added at 30% (w/w) of the total dry matter content. The pH of the film-forming solutions was 10 ± 0.8. Film-forming solutions were cast into Petri dishes and dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 35.0 ± 0.8 °C for 21–23 h. All films were conditioned at 58.0 ± 0.2% RH and 22 ± 1 °C 4 days prior to analysis.

10.3.5. ATR-FTIR spectroscopy

Dried extract and film infrared spectra between 4000 and 650 cm^{-1} were recorded at least in triplicate using a Perkin-Elmer Spectrum 400 Infrared Spectrometer (Perkin-Elmer Inc., Waltham, MA, USA), as described by Ojagh, Nunez-Flores, López-Caballero, Montero, and Gómez-Guillén (2011).

10.3.6. Thermal properties

Calorimetric analysis of dried seaweeds, crude extracts and films was performed using a previously calibrated differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA). Samples of around 10–15 mg were weighed and encapsulated in aluminium hermetic pans. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 115 °C at a heating rate of 10 °C/min. Peak temperatures (T_{peak}, °C) and denaturation enthalpies (ΔH, by linear baseline integration) were measured at least in triplicate and normalized to dry matter content (J/g_{dm}). Glass transition temperatures, Tg (°C), were determined by the midpoint method, usually within ±1 °C.

10.3.7. Film determinations

10.3.7.1. Thickness

It was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 6–8 random locations in 15 films for each treatment, as described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

10.3.7.2. Moisture content

It was determined at least in triplicate by drying samples of around 0.5 g at 105 °C for 24 h, according to A.O.A.C. (1995). Water content was expressed as a percentage of total weight.

10.3.7.3. Light absorption and transparency

The light barrier properties and transparency of the films were calculated at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 200 to 800 nm, following the method described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

10.3.7.4. Colour

The colour parameters of lightness (L^*), redness (a^*) and yellowness (b^*) were measured following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

10.3.7.5. Water vapour permeability (*WVP*)

It was determined at least in triplicate following the method described by Sobral, Menegalli, Hubinger, and Roques (2001), at room temperature in a dissecator with distiller water.

10.3.7.6. Water solubility

Film water solubility was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

10.3.7.7. Water resistance

Film water resistance was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

10.3.7.8. Mechanical properties

Tensile and puncture tests were run at least in triplicate using a TA.XT plus TA-XT2 texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA), as described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

10.3.8. Antioxidant activities of films

The ferric reducing ability (FRAP) and the ABTS (2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assays were used to measure the antioxidant activity of the films, which were

dissolved in distilled water, shaken until totally homogeneous and filtered (Whatman No. 1). The water soluble fraction was used for the antioxidant activity determination. The methods used for the FRAP and ABTS assays were previously described by Gómez-Estaca, Montero, Fernández-Martín, and Gómez-Guillén (2009). Results were expressed as mg Fe^{2+} equivalents/mg of sample for FRAP and mg of Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of sample for ABTS, based on standard curves of $FeSO_47H_2O$ and vitamin C, respectively. All determinations were performed at least in triplicate and expressed in function of the wet sample weight.

10.3.9. Folin-reactive substances determination

Folin-reactive substances content was determined spectrophotometrically, in triplicate, using gallic acid as a standard, according to a modified method of Slinkard and Singleton (1977) with the Folin–Ciocalteu reagent. The absorbance of the resulting blue colour was measured at 765 nm (UV-1601, model CPS-240, Shimadzu, Kyoto, Japan). Folin-reactive substances content was expressed as mg gallic acid (GA) equivalent/g of sample.

10.3.10. Statistical analysis

Statistical tests were performed using the SPSS computer program (SPSS Statistical Software Inc., Chicago, Illinois, USA) for one-way analysis of variance. The variance homogeneity was evaluated using the Levene test, or the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

10.4. Results and discussion

10.4.1. Yield and film capacity of seaweed extracts

Yield, film-forming capacity and film water response results of the differently processed crude extracts are shown in Table 1. Based on the highest yield and best film-forming properties, four extracts were selected, two extracted with sodium carbonate, Lc (0.1 M H_2SO_4 at 75 °C) and Ac (0.1 M HCl at 21 °C), and two with sodium hydroxide, Lh (0.2 M HCl at 75 °C) and Ah (0.2 M HCl at 75 °C) (see Table 1).

Alginate extraction is normally carried out with alkaline Na_2CO_3 solutions (Istini, Ohno, & Kusunose, 1994) of varying concentrations, such as 3% concentration for Ascophyllum (Rioux, Turgeon, & Beaulieu, 2007a) and 4% for Laminaria (Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009). Preliminary results showed that carbonate salts formed in the aqueous phase crystallized during film drying, acting in detriment to visual appearance and mechanical properties. Consequently, a dialysis step to eliminate excess salt should be included in sodium carbonate extractions. To avoid this inconvenience, NaOH allowed an alginate alkaline extraction at a similar pH to the carbonate extraction but with a different sodium source, giving a good alternative to simplify the process and to make the film development easier.

Alkaline extractions in Laminaria have been carried out at mild temperatures (21 °C), to avoid alginate degradation (Vauchel, Kaas, Arhaliass, Baron, & Legrand, 2008), but Ascophyllum extractions

are normally carried out at 70 °C (Rioux, Turgeon, & Beaulieu, 2007b) and different temperatures have been tested in other brown seaweeds such as *Macrocystis pyrifera* (70, 80, 90 °C) (Hernández-Carmona, McHugh, & López-Gutiérrez, 1999). On the other hand, for film development, partial alginate degradation might be useful to favour matrix inter-connections; therefore temperatures of 21, 50 and 75 °C were tested in this study.

Sea weed	Acid pretreatment		Alkaline treatment			Yield (%)	Filmogenic Capacity		Extraction selected
	Acid	Concentration (M)	Alkali	Concentration (%)	Temperature (ºC)		Film forming ability	Film water response*	
L	H_2SO_4	0.1	Na_2CO_3	4	21	69.04 ± 11.75	+	+	
L	H_2SO_4	0.1	Na_2CO_3	4	50	66.9 ± 13.44	++	+	
L	**H_2SO_4**	**0.1**	**Na_2CO_3**	**4**	**75**	**78.02 ± 16.81**	**++**	**+**	**Lc**
L	H_2SO_4	0.5	Na_2CO_3	4	21	62 ± 2.55	+	+	
L	H_2SO_4	0.5	Na_2CO_3	4	50	67.6 ± 7.64	++	+	
L	H_2SO_4	0.5	Na_2CO_3	4	75	74.5 ± 9.76	++	+	
L	HCl	0.1	NaOH	4	21	28.65 ± 0.92	+	+	
L	HCl	0.1	NaOH	4	50	32.65 ± 2.76	+	+	
L	HCl	0.1	NaOH	4	75	34.4 ± 1.55	+	+	
L	HCl	0.2	NaOH	4	21	38.87 ± 1.6	+	+	
L	HCl	0.2	NaOH	4	50	40.38 ± 1.41	+	+	
L	**HCl**	**0.2**	**NaOH**	**4**	**75**	**43.75 ± 0.74**	**+**	**+**	**Lh**
A	**HCl**	**0.1**	**Na_2CO_3**	**3**	**21**	**42.77 ± 6.62**	**+**	**++**	**Ac**
A	HCl	0.1	Na_2CO_3	3	50	35.64 ± 0.06	-	-	
A	HCl	0.1	Na_2CO_3	3	75	32.42 ± 3.08	-	-	
A	HCl	0.2	Na_2CO_3	3	21	39.26 ± 5.53	+	+	
A	HCl	0.2	Na_2CO_3	3	50	38.66 ± 2.06	-	-	
A	HCl	0.2	Na_2CO_3	3	75	51.93 ± 7.81	-	-	
A	HCl	0.1	NaOH	4	21	16.54 ± 1.95	+	+	
A	HCl	0.1	NaOH	4	50	31.88 ± 0.96	++	+	
A	HCl	0.1	NaOH	4	75	41.23 ± 0.81	++	++	
A	HCl	0.2	NaOH	4	21	29.82 ± 0.25	+	+	
A	HCl	0.2	NaOH	4	50	39.75 ± 0.21	++	+	
A	**HCl**	**0.2**	**NaOH**	**4**	**75**	**47.43 ± 0.95**	**++**	**++**	**Ah**

Table 1. Yield and Filmogenic capacity of Laminaria and Ascophyllum extractions.

Yield results are the mean ± standard deviation. Film formig ability was measured by -: no capacity, +: moderate capacity and ++: good capacity. Film water response was measured by -: no film, +: medium solubility and ++: lower solubility.

* Lower solubility was considered better result.

As shown in Table 1, increasing temperatures improved Na_2CO_3 extractions in Laminaria; however, Ascophyllum extracts totally lost their film-forming capacity at temperatures higher than 21 °C. The use of NaOH did not improve L extraction yield compared with those made with Na_2CO_3, but NaOH Ascophyllum extracts provided similar yields to those obtained with carbonate and provided much better film-forming properties.

Acid pretreatment has been carried out with 0.5 M H_2SO_4 in Laminaria (Vauchel, Arhaliass, Legrand, Kaas, & Baron, 2008), and with 0.1 M HCl in other brown seaweed species such as *Macrocystis pyrifera* (Hernández-Carmona, McHugh, Arvizu-Higuera, & Rodríguez-Montesinos, 1998). In an attempt to find milder acid conditions for L extraction, 0.1 M instead of 0.5 M H_2SO_4 pretreatment was found to give extractions with similar yields and filmogenic capacity. Furthermore, two HCl pretreatments were evaluated in both L and A extractions at different concentrations (0.1 and 0.2 M).

No HCl pretreatment showed yields as high as those obtained with sulfuric acid. While the carbonate Ascophyllum extraction yield was not affected by pretreatment HCl concentration, 0.2 M HCl resulted in the highest yields in sodium hydroxide extractions.

Yields of the extractions selected in the present work were much higher than yields of isolated alginate extracts obtained by 0.5 N sulfuric acid pretreatment and 4% sodium carbonate at 21 °C (~38%) (Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009), or by 0.2 N sulfuric acid and 1–2% sodium carbonate at 21 °C (20–34%) (Istini, Ohno & Kusunose, 1994). The higher extraction yields were attributed to unrefined extractions containing other components apart from alginates, such as laminaran, fucoidan, proteins and carotenoids (Cardozo et al., 2007).

10.4.2. Proximate composition of dried seaweeds and extracts

Proximate composition of the dried seaweeds and crude extracts is shown in Table 2.

Sample	Moisture (%)	Protein (%)	Ashes (%)	Carbohydrates (%)*
L	10.03 ± 0.15 a	9.2 ± 0.12 a	24.14 ± 0.45 a	56.63
A	17.55 ± 0.05 b	8.45 ± 0.91 a	15.44 ± 0.18 b	58.56
Lc	17.44 ± 0.4 a	5.47 ± 0.25 a	46.68 ± 0.99 a	30.41
Ac	16.46 ± 0.05 b	3.57 ± 0.05 b	42.05±0.9 b	37.92
Lh	16.78 ± 0.09b	9.20 ± 0.12 c	27.90 ± 0.8 c	46.12
Ah	16.96 ± 0.5 b	4.65 ±0. 01d	20.53 ± 0.06 d	57.86

Table 2. Moisture, protein, ashes and carbohydrate content (% dry weight) in Laminaria (L) and Ascophyllum (A) dried seaweeds and selected Lc, Ac, Lh and Ah extracts.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different dried seaweeds and seaweed extracts (P≤0.05).
*Determined by difference.

Both Laminaria and Ascophyllum presented similar carbohydrate (≈ 57%) and protein (≈ 8.5%) contents. Considering negligible the possible contribution of fat, the relatively lower ash proportion in A

could largely be accounted for by the higher moisture content, probably due to a different hygroscopic nature. Sodium hydroxide extractions were noticeably more carbohydrate efficient than sodium carbonate extractions, with Ah extraction showing significantly higher carbohydrate content than Lh. In contrast, Laminaria extractions were more protein efficient than Ascophyllum extractions, and sodium hydroxide treatment also extracted a higher protein amount than sodium carbonate. Interestingly, the sodium hydroxide extracts had considerably lower ash content than the sodium carbonate extracts, even though the latter were subjected to dialysis for excess salt removal. Thus the effect of alkaline treatment seems to predominate over the effect of algal species.

10.4.3. FTIR-ATR of brown seaweed extracts and respective films

Infrared spectra of Lc, Ac, Lh and Ah extracts and films are shown in Figures 1A and 1B, respectively. All spectra presented medium to strong IR absorption bands at 1200–970 cm^{-1}, which are mainly due to C-C and C-O pyranoid ring stretching and C-O-C glycosidic bond stretching common to all seaweed polysaccharide standards (Gómez-Ordóñez & Rupérez, 2011).

In general terms, the spectra presented peak resemblances by type of alkaline extraction. While the sodium hydroxide extractions showed high similarity between the two seaweed species, the carbonate sodium extractions differed more, probably owing to differences in the acid pretreatment. The strength of the sulfuric acid pretreatment might have caused partial degradation of the target polysaccharides (Hahn, Lang, Ulber, & Muffler, 2012).

The crude extract spectra had two strong bands, at ~1600 cm^{-1} and ~1400 cm^{-1}, attributed to the asymmetric and symmetric carboxylate group stretching vibrations ($–COO^–$) on the polymer backbone, which would confirm the presence of soluble alginate; in particular, the carbonyl group shifted as carboxylate anion at 1600 cm^{-1} (Gómez-Ordóñez & Rupérez, 2011; Paşcalau, Popescu, Popescu, Dudescu, Borodi, Dinescu et al., 2012). In general, NaOH extract peaks were more intense than sodium carbonate extract peaks, confirming the higher carbohydrate content reported earlier in the proximate composition section.

A small shoulder around 1730 cm^{-1} corresponded to the carbonyl group shifted as carboxylic acid ester (C=O) and might indicate a minimal presence of free alginic acid remaining from the extraction procedure or even possible crude fucoidans (García-Ríos, Ríos-Leal, Robledo, & Freile-Pelegrin, 2012; Gómez-Ordóñez & Rupérez, 2011).

Another characteristic broad band appearing at 1220–1260 cm^{-1} corresponded to the presence of sulfate ester groups (S=O), indicating the presence of fucoidan and sulfated polysaccharides as previously reported in brown seaweeds (Jiao, Yu, Zhang, & Ewart, 2011). Sodium hydroxide Ascophyllum extraction (Ah) presented the highest sulfated groups content. Apparently, there were no sulfated groups present in the Na_2CO_3 extractions, which might indicate that sulfated polysaccharides were less efficiently extracted. Fucoidans are normally extracted in acidic solution (0.01 M HCl) (Rioux, Turgeon, & Beaulieu, 2007a) at temperatures ranging from 70 to 100 °C for several hours (Hahn, Lang, Ulber, & Muffler, 2012).

Differences in the presence of sulfate groups could be due to a less efficient extraction or to partial fucoidan degradation (Hahn, Lang, Ulber, & Muffler, 2012; Pomin, Valente, Pereira, & Mourão, 2005).

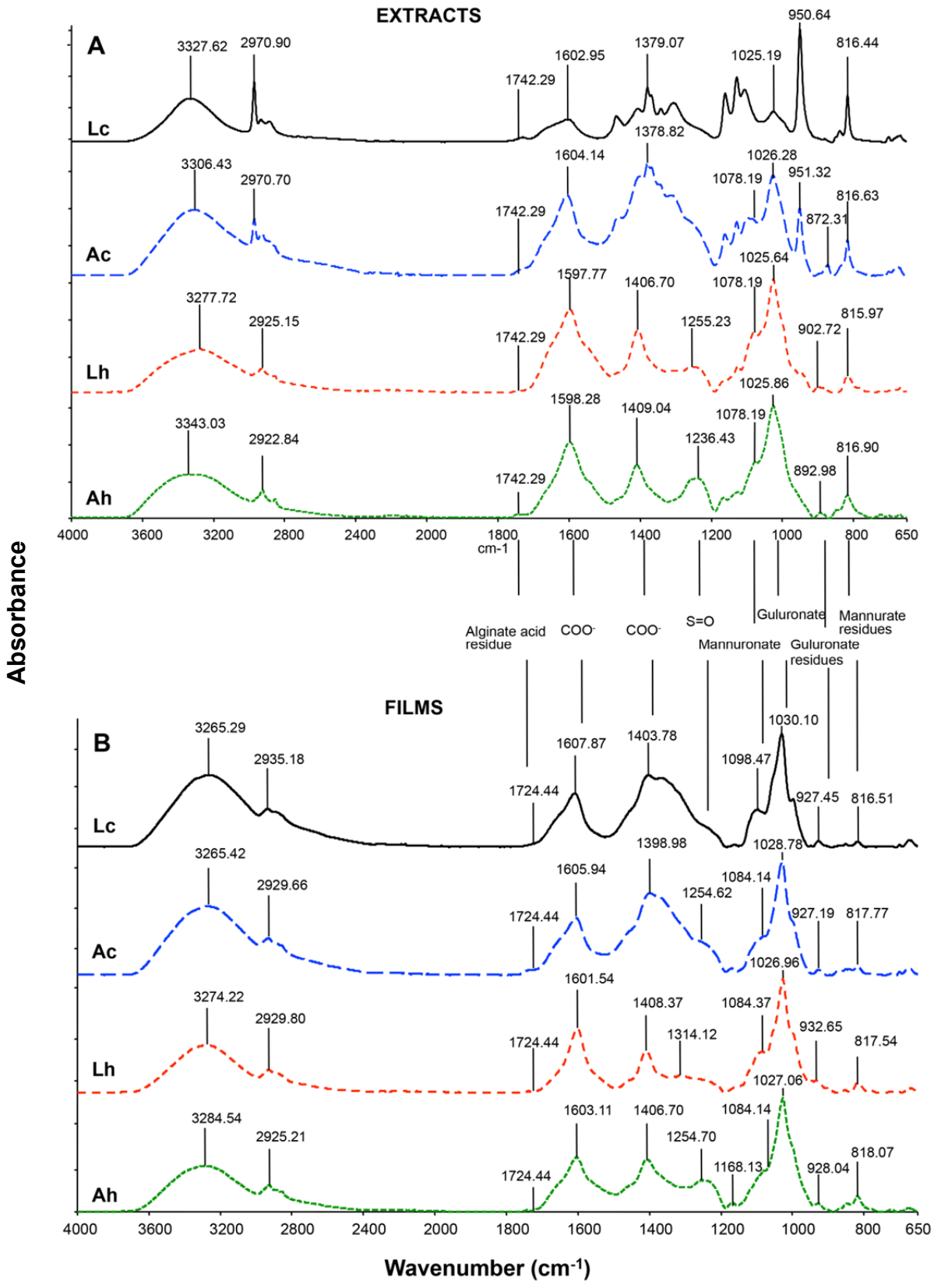

Figure 1. A. ATR-FTIR spectra of Lc, Ac, Lh and Ah extracts. B. ATR-FTIR spectra of Lc, Ac, Lh and Ah films.

A number of bands in the frequency range between 1130 and 800 cm^{-1} were also observed in the extracts. Some of those bands might correspond to guluronic and mannuronic units. The weak band at 1124–1128 cm^{-1} and the larger band at 1025 cm^{-1} may be assigned to C-O and C-C stretching vibrations of the pyranose ring, which might indicate the presence of mannuronic units and guluronic units respectively (Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003). IR spectroscopy is useful for quantitative estimation of the mannuronic to guluronic acid (M/G) ratio in brown seaweeds (Gómez-Ordóñez & Rupérez, 2011). The physical properties of the alginate chains in aqueous medium depend not only on the M/G (1125/1024 cm^{-1}) ratio (Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003) but also on the distribution of M and G units in the chain (Rinaudo, 2008). M/G ratios <1, as in Ac (0.54), Lh (0.25) and Ah (0.24), indicate a large proportion of guluronic acid, which has the ability to form strong junctions; however, ratios >1, as in Lc (2.07), are indicative of a lower guluronic proportion, which might result in softer, more elastic structures (Gómez-Ordóñez & Rupérez, 2011). NaOH treatment led to a predominance of guluronic unit extraction, while the Na_2CO_3-Laminaria extraction (Lc) had the lowest guluronic units proportion and highest mannuronic units' proportion.

The band at ~950 cm^{-1} is assigned to the C-O stretching vibration of uronic residues (mannuronic and guluronic acid residues) and the ~815 cm^{-1} band is specific to mannuronic acid residues that do not form part of the units (Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003). The sodium carbonate extracts had evident peaks, while the sodium hydroxide extracts showed almost no presence of uronic acid residues.

It could be concluded that the sodium hydroxide extractions recovered a higher proportion of preserved alginate units, while the sodium carbonate extractions recovered a higher amount of dispersed uronic acids, especially in the case of the Lc extraction, which would indicate a more degraded polysaccharide recovery.

With regard to the films, the corresponding spectra differ mainly depending on the extraction type rather than on the seaweed species. Unlike the extracts, the M/G ratio was <1 in all films, with almost the same values presented by the Na_2CO_3 films (Lc = 0.33 and Ac = 0.34) and the NaOH films (Lh and Ah = 0.36), which would indicate that film development resulted in an improved structure reorganization. The 1124–1128 cm^{-1} band (proportion of mannuronic units) was more acute in both Laminaria films (Lc and Lh), which might positively affect the film elasticity.

Shifts of the ~1400 cm^{-1} band to lower wavenumbers together with shifts of ~1600 cm^{-1} to higher wavenumbers are normally associated with a reduction of $–COO^-$ group involvement in film cross-linking (Paşcalau et al., 2012). Wavenumber shifts in the Na_2CO_3 films (Lc: 1403.78 cm^{-1} and 1607.87 cm^{-1}, Ac: 1398.98 cm^{-1} and 1605.94 cm^{-1}) compared with the NaOH films (Lh 1408.37 and 1601.54 cm^{-1}, Ah 1406.7 and 1603.11 cm^{-1}) indicated that the Na_2CO_3 extracts resulted in less cross-linked films than the NaOH extracts. On the other hand, hydrogen bonding has a strong influence on –OH stretching, probably being influenced by plasticizer interactions. The peak due to OH-guluronate bending (1025 cm^{-1}) can shift to higher wavenumbers, indicating a possible weakening of hydrogen bonds (Siddaramaiah, Swamy, Ramaraj, & Lee, 2008), as occurred in the Na_2CO_3 films (Lc 1030 cm^{-1} and Ac 1028.78 cm^{-1}). Weaker

interactions shown in the Na_2CO_3 films might also be attributed to the lower carbohydrate proportion and the higher ash content in the corresponding parent extracts.

10.4.4. DSC of dried seaweeds, extracts and films

DSC thermograms of dried *Laminaria digitata* (L) and *Ascophyllum nodosum* (A) and corresponding extracts Lc, Ac, Lh and Ah are shown in Figures 2A and 2B, respectively.

Both L and A had two main endothermic transitions, corresponding to the melting of different amounts of free and bound water. The first L and A melting peaks appeared at respective T_{peak} values (°C) of 67.66 ± 1.8 and 52.11 ± 3.36 and corresponding ΔH (J/g_{dm}) of 1.24 ± 0.13 and 0.11 ± 0.04. A second melting transition was observed at T_{peak} values (°C) of 95.93 ± 0.98 and 82.24 ± 2.93 and corresponding ΔH (J/g_{dm}) of 14.05 ± 1.33 and 8.46 ± 2.08 for L and A, respectively.

Because brown seaweed is rich in alginate, two different kinds of endothermic transition were expected: one corresponding to weakly bound water, which starts to be released at around 40 °C, and a second one related to the water trapped in hydrogen bonding with the algae components at around 90–120 °C (bound to –OH, dipole-dipole interactions) (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007).

Higher melting temperatures and energies in dried Laminaria than in Ascophyllum denoted different physical hindrances for bound water, which might be due to higher molecular weight structures responsible for stiffness and strength or a larger amount of well-organized guluronic units (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007; Russo, Malinconico, & Santagata, 2007).

Regarding the extracts, the respective similar weakly bound water melting temperature T_{peak} values (°C) were 46.13 ± 2.21 for Lc, 47.26 ± 1.47 for Ac and 50.86 ± 1 for Lh with a corresponding ΔH (J/g_{dm}) of 0.31 ± 0.17, 0.59 ± 0.03 and 0.41 ± 0.17. The Ah extract showed hardly any endothermic transition related to the weakly bound water, coinciding with the lowest ash proportion and highest carbohydrate content. The lower T_{peak} in the Na_2CO_3 extracts might be related to a shorter average chain length and higher fraction of mannuronic acid, as reported earlier in the FTIR results, retaining water less strongly (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007).

The extracts had a glass transition temperature around 75–85 °C. The Tg (°C) of each sample was 76.0 ± 7.1 in Lc, 76.9 ± 13.29 in Ac, 84.07 ± 4.13 in Lh and 85.04 ± 0.21 in Ah. Sodium alginate is an amorphous polymer with an irregular structure that does not crystallize and it is known to have a Tg around 80 °C (Siddaramaiah, Swamy, Ramaraj, & Lee, 2008). Glass transitions might be caused by inter- and intramolecular hydrogen bonding formed by the sodium alginate hydroxyl and carboxylate groups (Siddaramaiah, Swamy, Ramaraj, & Lee, 2008), being stronger in the sodium hydroxide extracts. The proportion and structural properties of the polymer segments contained by each extract determine their thermal behaviour. A higher concentration of carboxylate segments would result in higher Tg temperatures, while a higher concentration of hydroxyl segments would lead to lower Tg values (Siddaramaiah, Swamy, Ramaraj, & Lee, 2008). As reported earlier in the FTIR results, the strength of the NaOH extract structures might be caused by the higher guluronic proportion, while the higher

proportion of dispersed uronic acids recovered in the Na_2CO_3 extracts might have resulted in a weaker structure.

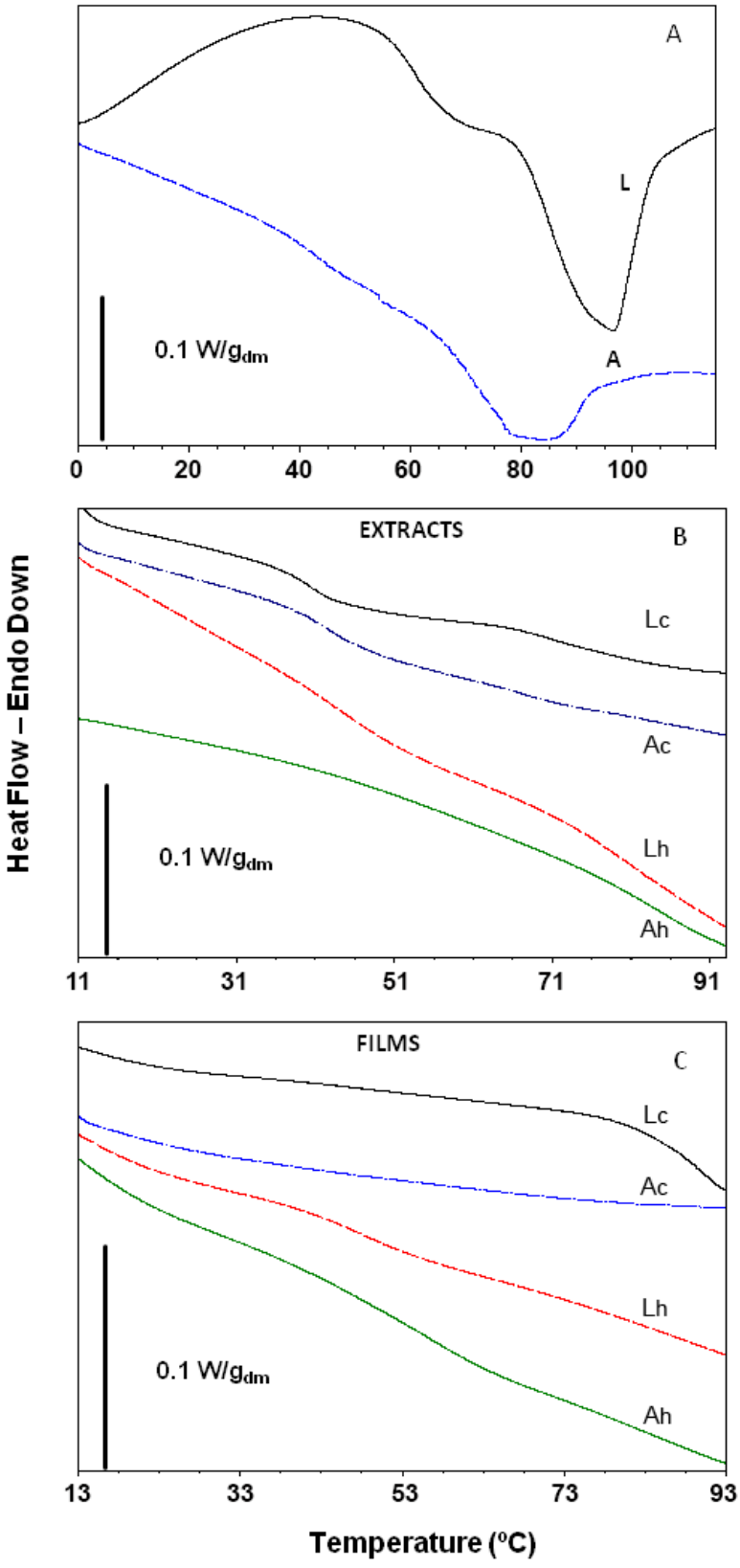

Figure 2. A. DSC of dried *Laminaria digitata* (L) and *Ascophyllum nodosum* (A); **B.** DSC of Lc, Ac, Lh and Ah extracts and **C.** DSC of Lc, Ac, Lh and Ah films.

DSC traces of Lc, Ac, Lh and Ah films are shown in Figure 2C. The sodium carbonate films did not show any thermal transition between 0 and 100 °C. In contrast, the sodium hydroxide films had a T_{peak} (°C) at 54.76 ± 0.86 and 63.29 ± 0.82 for Lh and Ah, respectively, with a ΔH (J/g_{dm}) of 0.22 ± 0.01 and 0.35 ± 0.03, which would correspond to weakly bound water, probably owing to water-glycerol interactions. The apparent lack of weakly bound water in the Na_2CO_3 films, as deduced by the absence of melting temperature (T_{peak}), might be caused by a preferential interaction between glycerol and alginate, expelling water from the structure (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007). Like the Na_2CO_3 films, the NaOH films did not show any glass transition within the temperature range studied, which might be caused by the presence of glycerol. Films made of neat alginates have been reported to be essentially amorphous, and the addition of plasticizer seems to increase that behaviour, probably dropping the glass transition temperatures to a lower range (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007).

10.4.5. Film properties

10.4.5.1. Light barrier properties

In general, the films exhibited low light transmission in the UV range (200–280 nm) (Figure 3), providing an efficient UV barrier. In the visible range, there was almost no light transmission until 400 nm. Film Ac was significantly (P≤0.05) the most transparent (Table 3). Film Ah had the lowest light transmission, increasing markedly in the 700–800 nm range, while Lc transmitted around 15% of light in the 400–800 nm range.

All the films had a light absorption peak at around 650–700 nm, strongly suggesting the presence of red-brown pigments (Bricaud, Babin, Morel, & Claustre, 1995; Gildenhoff, Herz, Gundermann, Büchel, & Wachtveitl, 2010), probably bonded to proteins (Gildenhoff, Amarie, Gundermann, Beer, Büchel, & Wachtveitl, 2010). Wavelengths proximate to 600 nm would be more reddish and close to 700 nm would be more brownish (Bricaud, Babin, Morel, & Claustre, 1995).

While the Laminaria films had one peak at around 675 nm, the Ascophyllum films showed two peaks, one at 675 and another one at around 650 nm. The principal absorption bands of chlorophyll a and b are found at 675 nm and 646 nm, and 437 nm and 470 nm, respectively. The spectral contributions of carotenoids are found at 486 and 525 nm (Goss, Wilhelm, & Garab, 2000). Fucoxanthin is the reddish most characteristic pigment (carotenoid) extracted from the chloroplast of brown seaweeds and typically absorbs at 675 nm, but there could be other pigments, such as β-carotene, chlorophyll a and pheophytin (de Quirós, Frecha-Ferreiro, Vidal-Pérez, & López-Hernández, 2010). Both fucoxanthin and chlorophyll are bound to some proteins, forming complexes in thylakoids and acting as a light harvesting system (Gildenhoff, Amarie, Gundermann, Beer, Büchel, & Wachtveitl, 2010; Wang, Wang, Zhang, & Tseng, 2005). In particular, the Lh film had a strong, sharp absorption peak at 675 nm, suggesting a higher fucoxanthin proportion which might be bonded to proteins. Proximate analysis confirmed a higher proportion of proteins extracted in Lh, compared with the other extracts.

The films from all the unrefined extractions contained most of the residual seaweed compounds, including pigments and proteins, producing more opaque films with a more red-brown colouring

compared with films developed with commercial alginates (Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013; Wang, Liu, Holmes, Kerry, & Kerry, 2007; Yoo & Krochta, 2011).

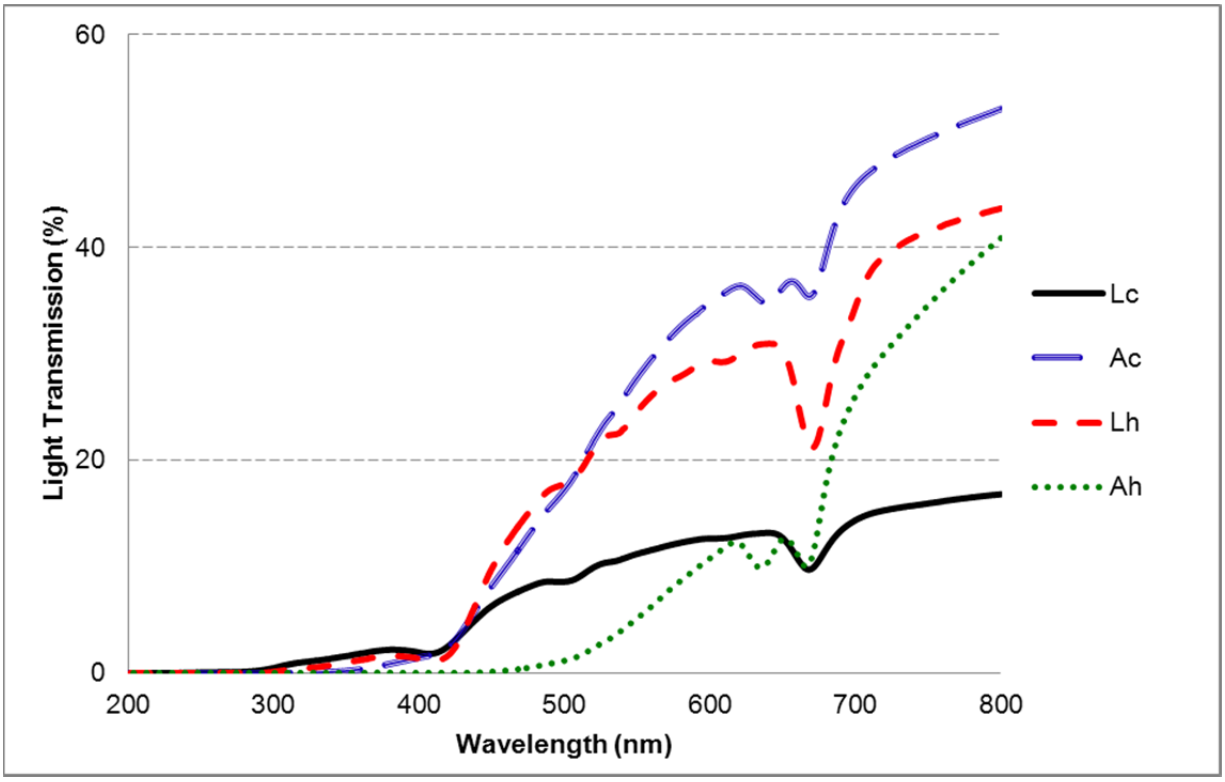

Figure 3. Light transmission (*T*, %) at 200–800 wavelength range (nm) of Lc, Ac, Lh and Ah films.

*L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish) values are given in Table 3, which shows that all the films had low lightness (24–28). The Laminaria films had a slightly higher yellowness tendency (+ *b**), while a reddish (+*a**) tendency was more marked in the Ascophyllum films. The L film yellowish-brown tendency might be caused by a higher presence of fucoxanthin (Ferraces-Casais, Lage-Yusty, de Quirós, & López-Hernández, 2012).

Film	*L**	*a**	*b**	Transparency
Lc	28.07 ± 0.3 a	-0.03 ± 0.07 a	5.99 ± 0.29 a	17.12 ± 0.71 a
Ac	24.04 ± 0.33 b	1.78 ± 0.06 b	2.52 ± 0.16 b	8.39 ± 0.76 b
Lh	28.47 ± 0.21 a	0.24 ± 0.05 c	4.34 ± 0.14 c	11.96 ± 0.44 c
Ah	25.15 ± 0.43 c	0.82 ± 0.36 d	-0.47 ± 0.63 d	16.36 ± 0.76 a

Table 3. L^*, a^*, b^* and Transparency ($-\log(T_{600}/x)$) of Lc, Ac, Lh and Ah films. Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films (P≤0.05).

10.4.5.2. Physical properties

The physical properties of the films are shown in Table 4. Thickness values were similar in Lc, Ac and Ah (52–50 μm), being lower (P≤0.05) in Lh. The lower thickness in Lh would be indicative of a denser, compact film network. This higher compacting might be the result of better cross-linking between alginate and proteins. Moisture content was considerably higher (P≤0.05) in the Na_2CO_3 films than in the

NaOH films, probably as a result of the greater ash content in the parent extracts. No significant (P≤0.05) differences in moisture content were found depending on seaweed species.

Film	Lc	Ac	Lh	Ah
Thickness (μm)	52.42 ± 3.6 a	55.09 ± 3.74 a	44.74 ± 3.46 b	59.75 ± 6.04 a
Moisture (%)	43.93 ± 0.89 a	40.83 ± 0.54 a	15.64 ± 0.14 b	16.39 ± 0.90 b
Film solubility (%)	79.23 ± 0.91 a	85.27 ± 2.49 b	74.42 ± 0.76 c	70.33 ± 2.37 c
WVP ($x10^{-8}$ g m^{-1} s^{-1} Pa^{-1})	4.90 ± 0.07 a	4.64 ± 0.08 a	1.66 ± 0.32 b	2.62 ± 0.16 c
TS (MPa)	6.01 ± 1.58 a	1.50 ± 0.19 b	40.72 ± 4.05 c	9.13 ± 1.98 a
EAB (%)	1.20 ± 0.35 a	3.49 ± 0.32 b	0.91 ± 0.37 a	2.16 ± 0.81 b
Y (MPa)	102.65 ± 3.16 a	5.99 ± 0.51 b	1081.93 ± 67.37 c	159.56 ± 42.84 a
F (N)	4.92 ± 0.85 a	10.22 ± 0.87 b	23.46 ± 1.11 c	11.24 ± 3.09 b
D (%)	7.28 ± 0.63 a	62.58 ± 1.63 b	7.61 ± 0.67 a	13.90 ± 1.96 c

Table 4. Thickness, moisture, film solubility, water vapour permeability (*WVP*), tensile strength (*TS*), elongation at break (*EAB*), Young's modulus (*Y*), puncture force (*F*) and puncture deformation (*D*) of Lc, Ac, Lh and Ah films.
Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films (P≤0.05).

10.4.5.2.1. Water barrier properties

Film water solubility was lower (P≤0.05) in the NaOH films, regardless of the seaweed species (Table 4). Of the Na_2CO_3 films, Ac was the more soluble. Glycerol more efficiently linked to uronic units in the Na_2CO_3 films, together with their higher mineral content, probably led to weaker films; in contrast, the enhanced polymer cross-linking in the NaOH films led to a reduction of solubility. Water solubility values were slightly higher than results obtained with commercial alginate films with cation complexation (Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013), but the presence of other seaweed compounds such as proteins, pigments and minerals might result in a less soluble film compared with those previously developed with sodium alginate without cation complexation (Abdollahi, Alboofetileh, Rezaei, & Behrooz, 2013; Zactiti & Kieckbusch, 2006).

The sodium hydroxide films were also more impermeable to water vapour (*WVP*) (P≤0.05) than the films made with sodium carbonate extracts (Table 4). As in the case of the solubility test, film structure determined the *WVP* results. Film Lh showed the highest barrier to water vapour, probably owing to the more compact cross-linked film, while the weaker matrix in the Na_2CO_3 films made water vapour transfer easier. The *WVP* values of all the films studied were higher than previous results obtained with commercial alginate films (0.2–5 x 10^{-9} g m^{-1} s^{-1} Pa^{-1}) (Benavides, Villalobos-Carvajal, & Reyes, 2012; Tapia, Rojas-Graü, Rodríguez, Ramírez, Carmona, & Martin-Belloso, 2007), which might be caused by the residual presence of hydrophilic seaweed compounds and uncross-linked fractions.

The water resistance test results are shown in Figure 4. Film Lh stood out from the rest for being by far the most water resistant, with breakage at 106.83 ± 30.05 min, no water filtration and the highest

film elongation (2 cm) through time. Although Lc film breakage (at 7.12 ± 0.71 min) and elongation (0.6 ± 0.02 cm) were much lower than the values for Lh, it was considerably more water resistant than both Ascophyllum films (up to 0.28 ± 0.05 min). The higher proportion of mannuronic units found in Laminaria films together with the higher extract protein proportion may have positively affected the wet-induced film elongation.

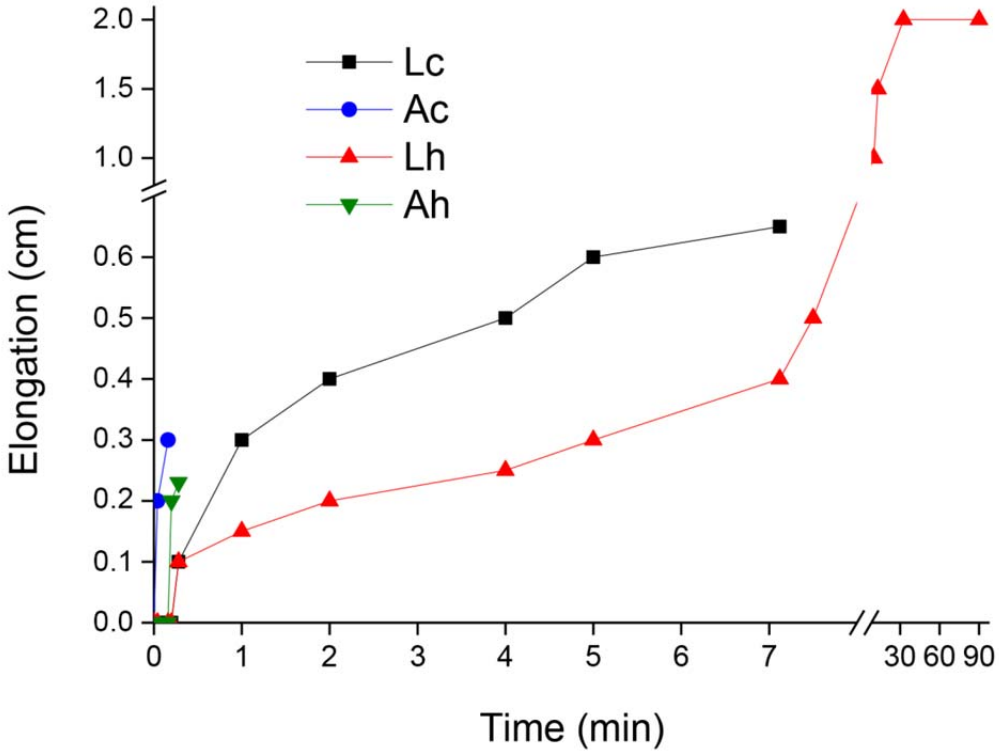

Figure 4. Water resistance: Elongation (cm) in time (min) until breakage of Lc, Ac, Lh and Ah films.

10.4.5.2.2. Mechanical properties

Tensile and puncture test results are shown in Table 4. Tensile strength (*TS*), elastic modulus (*Y*) and puncture force (*F*) values were significantly (P≤0.05) higher in the NaOH films than in the Na_2CO_3 films, especially in the case of Lh, which showed noticeably higher *TS*, *Y* and *F* values. The increased mechanical resistance of Lh film was in accordance with the more compact matrix and high degree of cross-linking, probably associated with the higher protein content promoting polymer-polymer interactions. *TS* values for Lh were even higher than results obtained for films made with cation complexation (Pereira, Carvalho, Vaz, Gil, Menses, & Bártolo, 2013) or with higher sodium alginate concentrations (Su Cha, Choi, Chinman, & Park, 2002; Yoo & Krochta, 2011). The *Y* in both Laminaria and Ascophyllum films was in the same range as values previously reported for 1–4% commercial alginate films (Abdollahi, Alboofetile, Razaei, & Behrooz, 2013; Yoo & Krochta, 2011), but much lower than those reported by other authors (Avella, Pace, Inmirzi, Impallomeni, Malinconico, & Santagata, 2007; Paşcalau et al., 2012).

Elongation at break (*EAB*) and puncture deformation (*D*) values were slightly (P≤0.05) higher in A-films than in L-films, which could be connected with more efficient plasticization. *EAB* values of all the films studied were in the same range as results obtained with 1–1.5% commercial sodium alginate films (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007; Paşcalau et al., 2012), lower than others developed with higher alginate concentrations (Su Cha, Choi, Chinnan, & Park, 2002; Yoo & Krochta, 2011) and much lower than films made by adding $CaCl_2$ for cation complexation (Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013).

10.4.5.3. Antioxidant activity and Folin-reactive substances

The antioxidant activity and Folin reactive substances of the films are shown in Table 5. The Ascophyllum films exhibited noticeable radical scavenging capacity in contrast to the Laminaria films, for which values were much lower (P≤0.05). In this connection, Laminaria has previously been reported to present antioxidant capacity, but this might be greatly affected by processing and storage conditions (Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001). The ferric ion reducing capacity was almost negligible in L-films, in accordance with (Norajit, Kim, & Ryu, 2010), who did not find any ferric ion reducing activity in commercial alginate films. The presence of carotenoids, in particular fucoxanthin, with reported antioxidant capacity (Le Tutour, Benslimane, Gouleau, Gouygou, Saadan, & Quemeneur, 1998), might also have contributed to the radical scavenging capacity of the films.

Film	ABTS (mg vit C eq/g)	FRAP (mg Fe^{2+}/g)	Folin reactive substances (mg/g)
Lc	9.72 ± 0.19 a	0.7 ± 0.09 a	3.37 ± 0.24 a
Ac	14.02 ± 0.20 b	2.56 ± 0.06 b	10.23 ± 0.98 b
Lh	4.20 ± 0.51 c	1.74 ± 0.21 c	35.76 ± 0.67 c
Ah	20.36 ± 0.90 d	4.11 ± 0.51 d	44.02 ± 1.26 d

Table 5. Antioxidant activity: ABTS, FRAP and Folin reactive substances of Lc, Ac, Lh and Ah films. Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the film (F) (P≤0.05).

The sodium hydroxide films contained a higher (P≤0.05) amount of Folin-reactive substances than the Na_2CO_3 films, especially the Ah film, which exhibited the highest ABTS and FRAP values. Although this technique is widely used to assess total phenol content, it should be taken into consideration that other compounds present in the extract may react with the Folin reactive, including certain aromatic amino acids and sugars, which could also contribute as radical scavengers (Ikawa, Schaper, Dollard, & Sasner, 2003; Singleton, Orthofer, & Lamuela-Raventós, 1998; Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano et al., 1985). Antioxidant activity of seaweed polysaccharide components may depend on various factors, such as sulfation level, molecular weight and sugar residue composition (Jiménez-Escrig, Gómez-Ordóñez, & Rupérez, 2011). Film Ah showed the highest sulfation level by FTIR, which might be caused by a higher proportion of sulfated polysaccharides in the original extract, mainly fucoidan, which are also known to have remarkable antioxidant activity (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

10.5. Conclusion

Laminaria digitata and *Ascophyllum nodosum* unrefined extracts would be complementary sources from which to take advantage of their main ingredients for film development, producing films with suitable mechanical properties without any cation complexation. The type of alkaline treatment determined the main differences with respect to the film water barrier properties, while seaweed species had some importance regarding the mechanical properties. Sodium carbonate treatment resulted in extracts with good plasticizing capacity while sodium hydroxide extraction led to polymer chains with improved cross-linking capacity.

Although Lc gave the highest extraction yield, film developed with this extract did not stand out in any physicochemical test. Ac was adequate to develop transparent, flexible edible films, while the higher protein content in the Lh extract led to films with increased cross-linking, lower water vapour permeability and much higher mechanical strength. Thanks to pigments and the higher presence of sulfated polysaccharides, the Ah extract resulted in films with the highest antioxidant properties, which could be used to improve food preservation or to design functional foods.

10.6. References

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XI. Peptide microencapsulation by core-shell printing technology for edible film application

Peptide microencapsulation by core-shell printing technology for edible film application

Blanco-Pascual, N., Koldeweij, R.B.J., Stevens, R.S.A., Montero, M.P., Gómez-Guillén, M.C. & Ten Cate, A.T. Peptide microencapsulation by core-shell printing technology for edible film application. *Food and Bioprocess Technology: An International Journal*. Submitted 5th August, 2013.

11.1. Abstract

This paper presents a new microencapsulation methodology for incorporation of functional ingredients in edible films. Core-shell microcapsules filled with demineralized water (C) or 1% peptide solution (Cp) were prepared using the microencapsulation printer technology. Shell material, composed of a stearic acid:carnauba wax mixture (75:25) represented around 10% of the capsule weight, corresponding to a shell material:peptide ratio of 13.3:1 on dry basis. C capsules were more spherical and homogeneous than Cp ones. Cp's more irregular morphology would explain the slightly higher size of d90 (126 μm) compared to C (122.50 μm). Microcapsules were more stable at pH 5 and 7 (<30% peptide released in 3 hours) than at pH 2 and 9.2 (40-50% in 3 hours). A procedure for homogeneous microcapsule inclusion in hydrophilic *Laminaria digitata* edible films was developed, without losing microcapsule integrity either in the filmogenic solution or during the drying process. Films with added microcapsules were stronger and more deformable, more opaque, more water soluble but less permeable to water vapour and less resistant to perforation.

11.2. Introduction

Microcapsules have been widely developed in food industry and used as carriers of different substances for a range of applications, such as core material protection or controlled delivery systems (Gibbs, Kermasha, Alli, & Mulligan, 1999). The most commonly used encapsulation techniques are emulsification, coacervation, spray drying, spray cooling, freeze drying, fluid bed coating and extrusion technologies, liposome and cyclodextrin encapsulation (de Vos, Faas, Spasojevic, & Sikkema, 2010; Gibbs, Kermasha, Alli, & Mulligan, 1999). A relatively new approach for the preparation of well-defined core-shell microcapsules is the TNO encapsulation printer (Houben, 2012), which has never before been reported to be used in food applications.

Core-shell microcapsules have the advantage that they allow high payloads and well-defined release characteristics, in contrast to matrix-type microcapsules as, for instance, prepared by spray-drying. However, core-shell capsules are often more difficult to produce. Encapsulation printing is a suitable technique to prepare high quality monodisperse core-shell microcapsules. Compared to, for instance, coacervation methods, this technique presents advantages such as the continuity and the mildness of the process, and the wide range of materials that can be processed (aqueous, oils/waxes, polymers, solutions, dispersions).

There are many natural and synthetic polymers used for microcapsule preparation, and there are a number of advantages related to lipid materials for their use as matrix agents, like their biocompatibility, biodegradability, ability to entrap a wide range of water soluble and insoluble compounds and the fact that they are also fairly economic (Bhoyar, Morani, Biyani, Umekar, Mahure, & Amgaonkar, 2011). Carnauba wax is a thermoplastic solid obtained from the carnauba plant tree, consisting of a complex mixture of high molecular weight esters of acids and hydroxyacids that combined with stearic fatty acid forms water resistant structures at room temperature which melt at elevated temperatures (over 69.6 ºC for stearic acid and 82-86 ºC for carnauba wax). Carnauba wax/stearic acid combination has shown good results in previous different types of encapsulation studies (Balducci, Colombo, Corace, Cavallari, Rodríguez,

Buttini, et al., 2011; Fini, Cavallari, Rabasco Álvarez, & Rodríguez, 2011) being potentially suitable for the microencapsulation printer process due to its matrix characteristics.

Protein encapsulation by organic matter has been previously stated to preserve the activity of protein material both in dry and wet basis (Gibbs, Kermasha, Alli, & Mulligan, 1999; Tomaszewski, Schwarzenbach, & Sander, 2011). The development of a lipid-based microencapsulation method is an interesting approach to preserve potentially active ingredients, such as peptides or polyphenols, or even enhance their efficacy (Goodwin, Simerska, & Toth, 2012) for different food applications, while masking undesired properties such as the typically bitter peptide flavour (Sun-Waterhouse & Wadhwa, 2013) and excessive plasticizer effect (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009).

Renewable and biodegradable biopolymers have been widely investigated as edible film materials (Krochta & DeMulderJohnston, 1997). Polymers derived from underused natural resources, such as seaweeds, offer the greatest opportunities since their environmental compatibility is assured. Full extraction of *Laminaria digitata* seaweed may advantageously use the distinct functional characteristics of each film-forming ingredient like alginates, proteins and minerals. Proteins and polysaccharides normally form films with good mechanical properties but poor water barriers, because of their hydrophilicity, and lipid-nature microcapsule incorporation might improve their qualities. Although polysaccharides have been previously used for encapsulating lipid phases or emulsions and related applications (Balducci, et al., 2011), no information exists in the literature on the film-forming consequences of microcapsule addition with alginate-based *Laminaria digitata* extract as film principal matrix component.

The aim of this paper was (i) to develop a new methodology for microencapsulation of bioactive peptides, (ii) to characterize the physical properties and stability of the microcapsules, (iii) to investigate the inclusion of microcapsules in edible films and (iv) to characterize the structural and physicochemical properties of the resulting films.

11.3. Materials and methods

11.3.1. Materials

Analytical grade stearic acid and carnauba wax were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, Netherlands) and glycerol and polysorbate 80 (Tween 80) were obtained from Panreac Química S.A. (Montplet and Esteban S.A., Montcada i Reixac, Barcelona, Spain). Peptides were the hydrolyzed fraction 1 kDa extracted from *Dosidicus gigas* tunics.

11.3.2. Viscosity

The viscosity of the materials used for the encapsulation process was measured using an Anton Paar MCR 301 rheometer with concentric cylinder geometry. Samples were placed in the rheometer, equilibrated at each temperature (35 ºC for demineralized water and 1% peptide solution, 105 ºC for shell material) for 2 min.

11.3.3. Surface tension and contact angle measurements

The surface tension of the materials used for the encapsulating process was measured using a Krüss contact angle measuring system G10 (Etten-Leur, The Netherlands). Surface energy of liquid droplets was calculated using the static sessile drop method.

Surface hydrophobicity and wettability of the shell material in the filmogenic solution were evaluated from contact angle measurements (static sessile drop method) using a Krüss contact angle measuring system DSA100. A droplet (6.04 ± 0.02 µl) of *Laminaria digitata* filmogenic solution at 21 ± 1 ºC (1.04 ± 0.03 g/mL) was deposited on a thin smooth layer of shell material with a precision syringe. The method is based on the image processing and curve fitting for contact angle measurement from a theoretical meridian drop profile, measuring contact angle between the baseline of the drop and the tangent at the drop boundary. The contact angle was measured on both sides of the drop and averaged (θ).

11.3.4. Encapsulation printing technology

A custom-built set-up was used for microencapsulation printing experiments, consisting of: a heated reservoir for the core fluid connected to a high pressure pump; a heated piezo-driven print-head with a 30 µm diameter nozzle; a heated reservoir for liquid shell material, connected to a pump system and a splash-plate type nozzle, in which a jet of fluid shell material impinges on a splash plate, resulting in a thin, fluid curtain of shell material (Fig. 1).

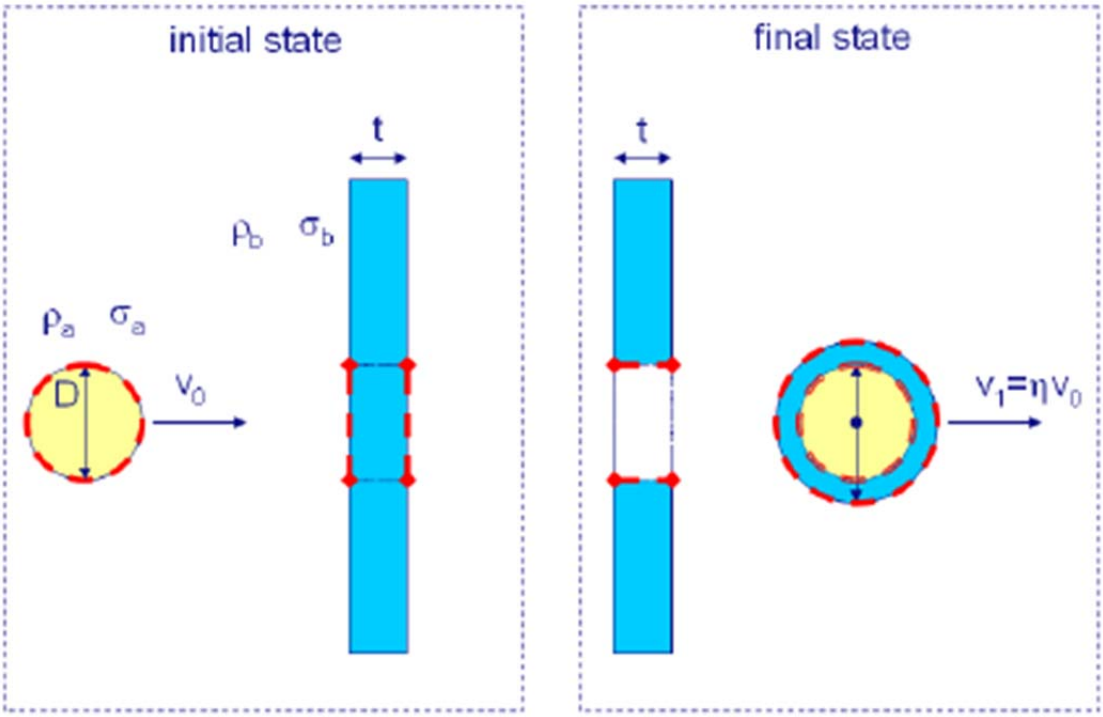

Figure 1. Schematic of the core material droplet generated by the inkjet technology before and after being printed through the screen made of shell material (Encapsulation of micron sized droplets, RBJ Koldeweij, MSc thesis, University of Twente 2010).

Demineralized water and 1% peptide solution were used as core fluids to prepare C and Cp microcapsules, respectively. A stearic acid and carnauba wax mixture (75:25) was used as shell material. Core fluids were printed at 35 °C, at a flow rate of 0.75 mL/min and a frequency of 20 kHz, producing core droplets of 106 µm. The shell material was processed at 105 °C and a flow rate of 29.6 mL/min to produce a thin liquid film over a splash plate at 85 ºC. Densities of materials were 0.81 ± 0.03 g/mL for shell material at 105 ºC, 0.97 ± 0.02 g/mL for demineralized water at 35 ºC and 1.04 ± 0.02 g/mL for 1% peptide solution at 35 ºC.

11.3.5. Seaweed unrefined extraction

Dried *Laminaria digitata* seaweed (Porto-Muiños, Cerceda, A Coruña, Spain) was homogenized using an Osterizer blender (Oster, Aravaca, Madrid, Spain) with 0.1 M H_2SO_4 solution in 1:10 (w:v) proportion and kept for 12 h at 3 ± 2 ºC and washed several times with running tap water until stabilizing pH (portable pH-meter series 3 Star Orion with an electrode pH ROSS, thermo Fisher Scientific Inc., Landsmeer, The Netherlands). Seaweed extraction was carried out with magnetic stirring in 4% Na_2CO_3 solution at 1:60 (w:v) proportion during 3 h, then homogenized in an Osterizer blender and centrifuged at 6000 rpm for 5 min (Sorvall Evolution RC Centrifuge, Thermo Fisher Scientific Inc., Landsmeer, The Netherlands). In order to remove excess of carbonate salts, the supernatant was dialyzed overnight in 32/32 dialysis tubing (Visking membrane MWCO-12-14000 regenerated cellulose, Medicell International Ltd, London, U.K.) at 3 ± 2 ºC. The dialysed supernatant was dried in an oven for 2 days (FD 240 Binder, Tuttlingen, Germany) at 65.0 ± 0.8 ºC and stored at 21 ºC until use.

11.3.6. Film preparation

Film-forming solutions were prepared by suspending 3% w/v dry *Laminaria digitata* unrefined extract in distilled water and homogenizing with magnetic stirring during 15 min at 75 ºC. Glycerol was added at 0.9% proportion (w/v) as plasticizer. Surfactant polysorbate 80 was added at 0.1% (w/v) to help in microcapsules dispersion and entrapment during drying process. The pH of the film-forming solutions (FS) was 10 ± 0.4. Film forming solutions were cast into Petri dishes and microcapsules were added at 1% (w/v). TMTP09030 Isopore polycarbonate membranes (Millipore, Billerica, U.S.A.) were placed over the filmogenic solution and dishes were dried in an oven (FD 240 Binder, Tuttlingen, Germany) at 35.0 ± 0.8 ºC for 7 h. The polycarbonate membranes were removed from the dried films before conditioning at 58.0 ± 0.2% RH and 22 ± 1 ºC for 4 days, prior to analysis. Three types of films were obtained: films with water-filled capsules (F-C), with peptide-filled capsules (F-Cp) and control films without capsules (F).

11.3.7. Differential Scanning Calorimetry (DSC)

Shell material percentage of freshly prepared microcapsules was calculated measuring each enthalpy proportion (ΔH, by linear baseline integration) with respect to the shell material melting enthalpy reference, using a Discovery Series differential scanning calorimeter (DSC) (TA Instruments, New Castle, DE, USA) previously calibrated.

Calorimetric analysis of F, F-C and F-Cp films were performed using a DSC model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated.

For all DSC experiments, samples of around 10-15 mg were weighed and sealed in aluminium hermetic pans. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 115 ºC at a heating rate of 10 ºC/min. Peak temperatures (T_{peak}, ºC) and melting enthalpies were measured at least in triplicate and normalized to dry matter content (J/g_{dm}) for film structure analysis.

11.3.8. Laser scattering method

Particle diameter of the microcapsules was measured using laser scattering analysis (Mastersizer, Malvern Instruments Ltd, Malvern, UK) with a small volume of isopropyl alcohol microcapsule dispersion homogenized in a dispersion unit controller. All the measurements were performed at room temperature with a refractive index of 1.45 and expressed as volume % of microcapsules.

11.3.9. Peptide encapsulation efficiency and stability

The peptide entrapment in the microcapsules was determined after total release of the capsule content in distilled water at 95 ºC during 20 min, in order to melt the lipid shell material. Then, the solution was cooled down to room temperature to precipitate out the lipids and filtered through 0.45 µm pore size filters.

Peptide concentration was determined at 280 nm with the Synergy HT Multi-Mode Microplate Reader and the UV-spectrophotometer Gen5™ BioTek's microplate data collection and analysis software (BioTek Instruments, Inc., Winooski, Vermont, USA). Demineralized water microcapsules results were taken into account as reference due to their small interference in the results caused by a slight release of matrix material (half-order release rate) (Shahidi & Han, 1993). Determinations were carried out in triplicate and the mean value of peptide released was calculated using a standard calibration curve made with increasing concentrations of peptide solution.

Peptide encapsulation efficiency was calculated by using the following formula:

Encapsulation efficiency = Peptide entrapped/Theoretical peptide content X 100.

For pH stability studies, microcapsules were placed into different buffer solutions, at pH 2.6, 5 and 7 (citrate-phosphate buffer) and pH 9.2 (carbonate-bicarbonate buffer) and were filtered through 0.45 µm pore size filters after 10 min, 1 hour and 3 hours respectively. All samples were evaluated in triplicate.

For film process stability studies, microcapsules were placed either into distilled water (pH 7) or into pH10 aqueous solution (0.012% Na_2CO_3) at 35 ºC for 30 min, 1, 3, 5, 7, 9, 11 and 24 hours, and were filtered through 0.45 µm pore size filters prior to peptide concentration analysis. All determinations were evaluated in triplicate.

11.3.10. Microcapsule and Film morphology

Optical microscopy using a Zeiss AxioImager M1m microscope with Epiplan objectives, 100W Halogen illumination source and AxioCam MRc 5 camera (Zeiss, Sliedrecht, The Netherlands) was used to measure microcapsule diameter and morphology.

Low temperature scanning electron microscopy (LowT-SEM) (Oxford CT1500 Cryosample Preparation Unit, Oxford Instruments, Oxford, England) was used to examine microcapsules and representative film surface and cross sections. Samples were mounted with an optical coherence tomography (OCT compound Gurr ®) and mechanically fixed onto the specimen holder and cryo-fractured after mounted as described by Gómez-Guillén, Ihl, Bifani, Silva, and Montero (2007).

Panoramic films pictures were taken with a Canon EOS 550d, MP-E 65mm optical lens at 5:1 amplification (Canon España, Alcobendas, Madrid, Spain).

11.3.11. Film characterization

11.3.11.1. Thickness

Film thickness of F, F-C and F-Cp was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 6-8 random locations in 15 films for each treatment as described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

11.3.11.2. Moisture content

Moisture content of films F, F-C and F-Cp was determined at least in triplicate by drying samples of around 0.5 g at 105 °C for 24 h, according to A.O.A.C. (1995). Water content was expressed as a percentage of total weight.

11.3.11.3. Optical properties

The light absorption and transparency of films F, F-C and F-Cp were calculated at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 200 to 800 nm following the method described by Pérez-Mateos, Montero, and Gómez-Guillén (2009).

The colour parameters of lightness (L^*), redness (a^*), and yellowness (b^*) were measured following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

11.3.11.4. Water barrier properties

Water vapour permeability (*WVP*) of films F, F-C and F-Cp was determined at least in triplicate following the method described by Sobral, Menegalli, Hubinger, and Roques (2001) at room temperature and in a dessicator with distilled water.

Film solubility was measured at least in triplicate following the method described by Blanco-Pascual, Fernández-Martín, and Montero (2013).

11.3.11.5. Mechanical properties

Tensile and puncture tests of films F, F-C and F-Cp were run at least in triplicate using a texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) as was described by Blanco-Pascual, Fernández-Martín, and Montero (2013) but with tensile test samples of 70 x 20 mm.

11.3.12. Statistical analysis

One-way analysis of variance was performed using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, Illinois, U.S.A.). The variance homogeneity was made using the Levene test or, the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

11.4. Results and discussion

11.4.1. Encapsulation printing

Both the viscosity and the surface tension of the starting materials are important parameters in the encapsulation printing process. For the selected nozzle size of 30 µm and flow rate of 0.75 mL/min, the viscosity limit for the core liquids was around 40 mPa·s in order to be processable through the inkjet nozzle at reasonable pressure. Shear-rate dependent viscosities of the core fluids were measured at the intended printing temperature (35 °C). 1% peptide solution and demineralized water remained fluid with low, shear-independent viscosity values of 0.75 – 0.82 mPa·s up to shear rates of 100 s^{-1} for 1% peptide solution and 1000 s^{-1} for demineralized water. Maximum viscosity values at 4000 s^{-1} were 1.7 mPa·s for demineralized water and 7 mPa·s for 1% peptide solution. Shell material viscosity was also low (7 – 7.6 mPa·s) and almost shear-independent at the intended used temperature, which allows formation of a thin liquid curtain to be used for encapsulation. Since in general viscosity values did not increase too much at high shear rates, both shell and core materials were ideal for the encapsulation printing process.

Surface tension values were 32.70 ± 0.13 mN/m for 1% peptide solution (35 ºC), 70.20 ± 0.15 mN/m for demineralised water (35 ºC) and 35.63 ± 0.18 mN/m for the shell material (105 ºC). Even at this low peptide concentration, surface tension of demineralized water and peptide solution were significantly different, indicating that the peptides act as surfactants, which could affect the process and result in different encapsulation efficiency.

Using the microencapsulation printer, core-shell microcapsules C and Cp were prepared, containing demineralized water or 1% peptide solution, respectively, inside a lipid shell.

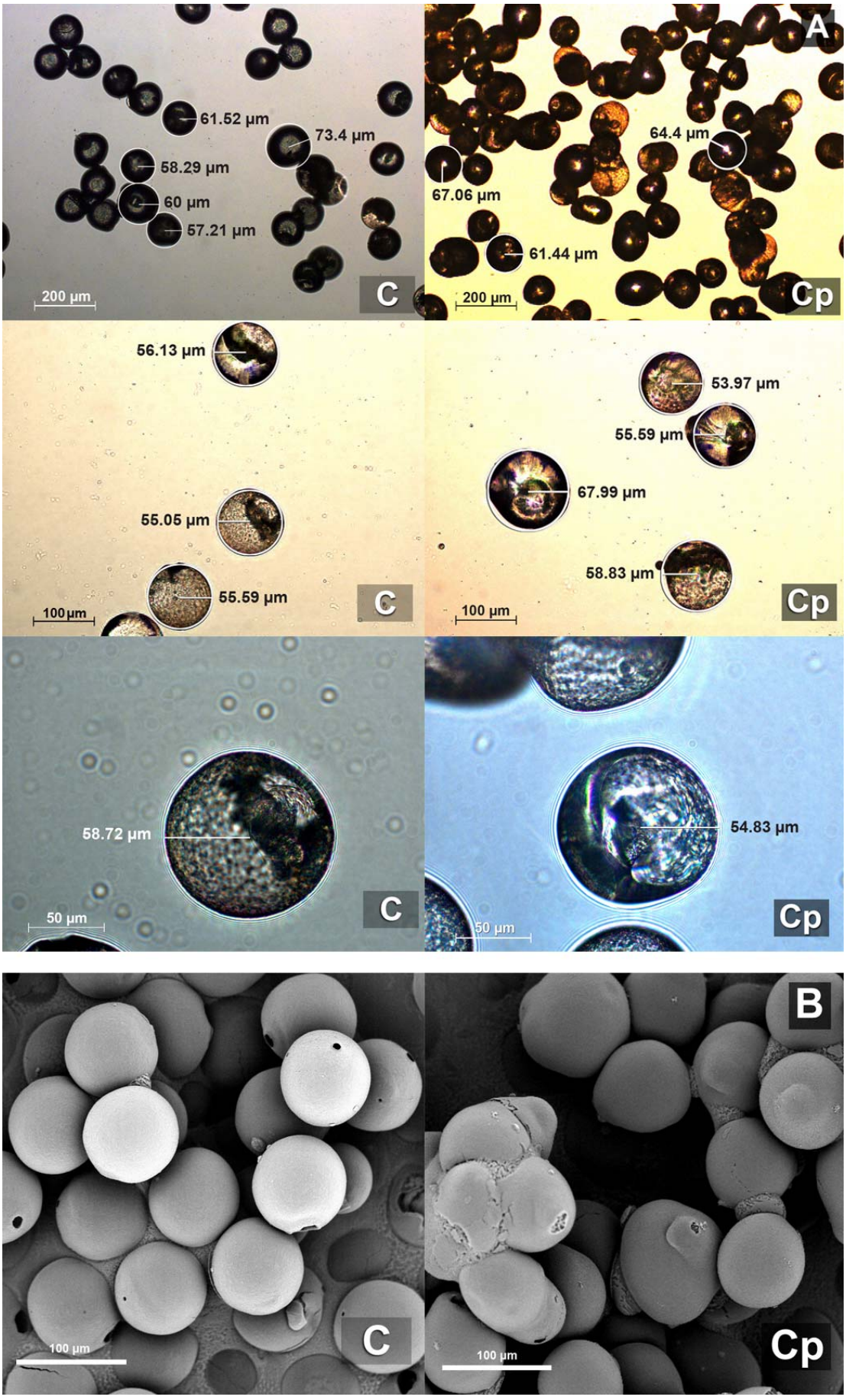

Figure 2. Optical microscopy (A) images and Low Temperature-Scanning Electron Microscopy (B) of C and Cp microcapsules.

11.4.2. Microcapsule characterization

Regarding the microcapsules morphology, at the optical microscope (Fig. 2A), microcapsule size was covered between 110 and 140 μm of diameter. The smallest microcapsules in this range were the expected result, in agreement with the targeted core droplet size of 106 μm, whereas bigger microcapsules might be caused by the collision and coalescence of two core droplets before or during encapsulation. C capsules were somewhat more uniform and spherical than Cp capsules. Overall, the observed capsules did not have cracking or porosity, which suggested high encapsulated materials integrity. There was no microcapsule aggregation, which is one of the main problems in encapsulation techniques, probably due to the carnauba wax presence (Joseph & Venkataram, 1995).

At the Low Temperature-Scanning Electron Microscopy (Fig. 2B), Cp microcapsule shapes were sometimes more oval-shaped and with a single protrusion, peptide nets were visible in the surface of some of them, revealing that the microcapsules were not as smooth as C. A little percentage of both C and Cp presented a little circular hole in one of the apices, which probably corresponded to an inefficient closure of the shell material during the encapsulation process, explaining the peptide material presence outside of Cp capsules. Cp irregularities might be caused by a slight interaction between peptides and stearic acid from the shell material. Stearic acid has shown to attract peptides and proteins from aqueous phases (Zadmard & Schrader, 2004). A previous LT-SEM study showed smooth and regular carnauba wax microcapsules of similar size and stearic acid microcapsules with fracture lines and irregularities on the surface (Fini, Cavallari, Rabasco Álvarez, & Rodríguez, 2011), which might suggest that a possible crystallization of the stearic acid domains, promoted by peptide interactions, could be the main responsible of microcapsule irregularities.

Differential Scanning Calorimetry was used to evaluate the percentage of shell material in the obtained capsules, based on differences in melting enthalpies (Fig. 3A). Shell material, C and Cp capsules depicted a main sharp endothermic transition at T_{peak} (ºC) of 74.21 ± 0.2, 65.65 ± 0.14 and 64.80 ± 0.04 respectively. Results showed that the corresponding ΔH (J/g) was 226.7 ± 0.71 for the shell material (75% stearic acid + 25% carnauba wax), 19.9 ± 6.3 for the capsules containing demineralised water (C) and 22.4 ± 4.7 for capsules with 1% peptide solution (Cp). Shell material represented 8.8 ± 3.0% and 9.9 ± 2.1% of the total microcapsule weight in C and Cp microcapsules, respectively.

Peptide encapsulation efficiency was 84.7 ± 3.4%. Encapsulation efficiency in microcapsules is very important in order to study the effectiveness of the process. The obtained peptide content was slightly lower than the targeted concentration, which might be caused by different reasons such as, a lower final peptide concentration in the printed droplet due to minor losses during sample process into the encapsulation printer; a minor loss of the core material's boundary layer suffered from the impact point between the droplet and the screen shell material; or the partial peptide-lipid shell material bonding, which would have not allowed total peptide release. The use of carnauba wax for lipid-based encapsulation materials development has also previously shown more than 80% of encapsulation efficiency (Bhoyar, Morani, Biyani, Umekar, Mahure, & Amgaonkar, 2011). Taking into account both the percentage of shell material and the efficiency of encapsulated peptide on dry basis, it could be concluded that the ratio of shell material to peptide was 13.3:1.

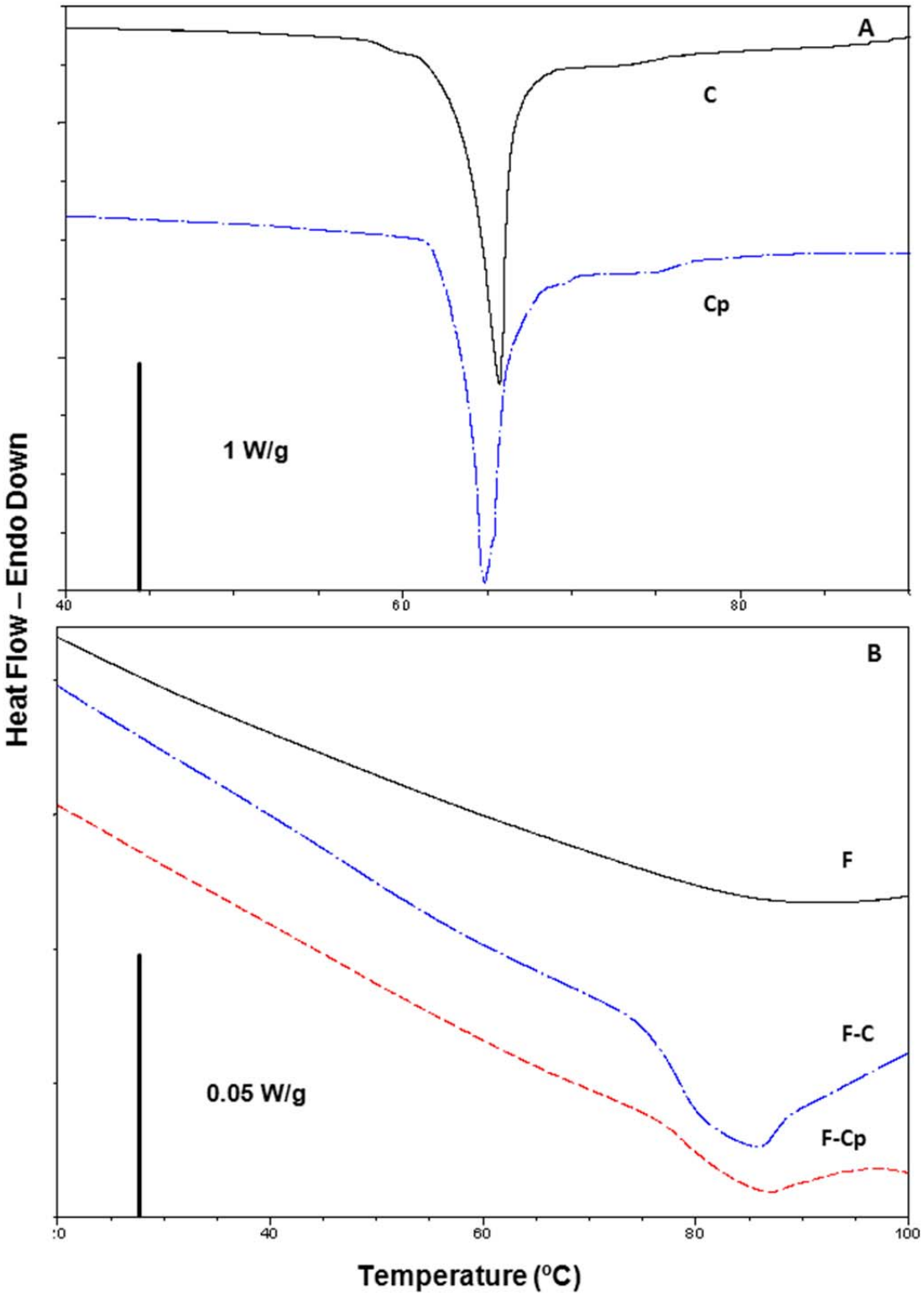

Figure 3. A. DSC of demineralized water microcapsules (C) and 1% peptide microcapsules (Cp). B. DSC films (F, F-C and F-Cp).

Laser scattering analysis (Fig. 4) revealed, with an error lower than 5%, that C had a diameter of d90 = 122.50 µm, d50 = 76.82 µm and d10 = 28.06 µm, while Cp had a diameter of d90 = 125.83 µm, d50 = 77.63 µm and d10 = 30.11 µm. The amount of particles with size lower than 30 µm, not observed in microscopic analysis, might be particles of shell material collected from the collision of the encapsulated material with the shell material and/or fragments derived from the sample handling.

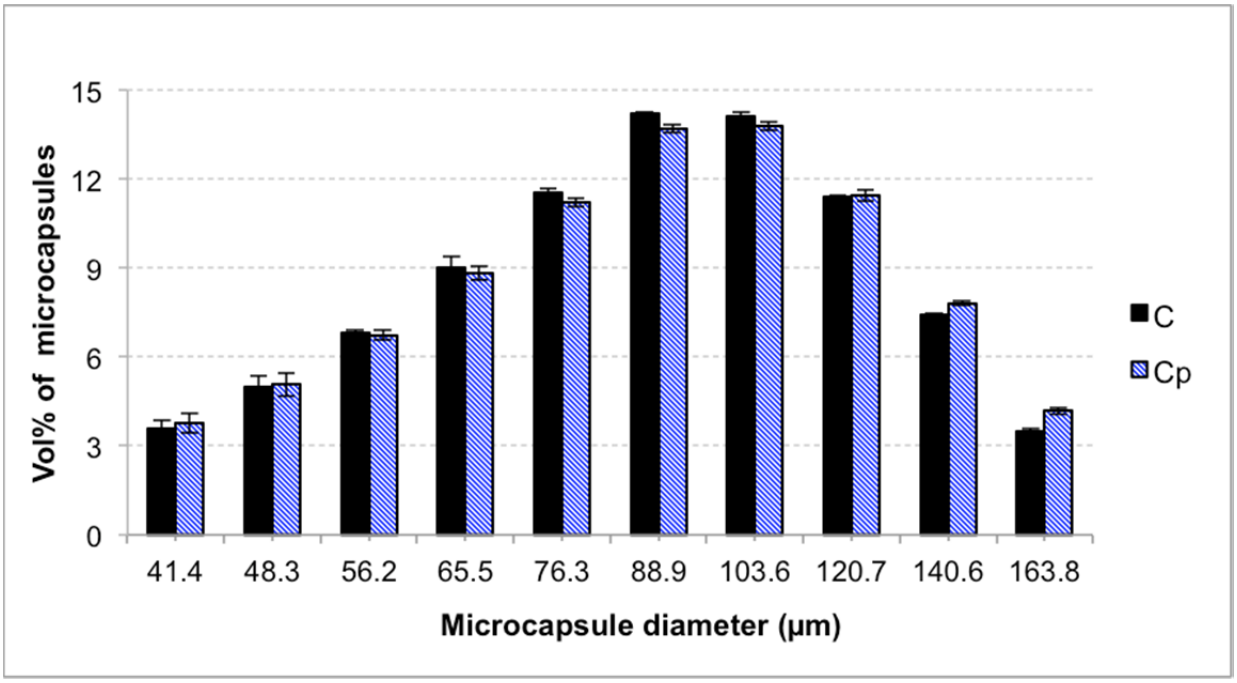

Figure 4. Microcapsule diameter of C and Cp as determined by laser scattering.

11.4.3. Microcapsule stability at different pH

Microcapsules are largely meant for incorporation of active ingredients in food products (Mellema, Van Benthum, Boer, Von Harras, & Visser, 2006). Stability of microcapsules in food systems can be very different depending on a number of environmental factors, including temperature, pH, moisture content, etc. This study, performed at room temperature (21 ± 1 ºC), was focussed on the stability of microcapsules subjected to pH variations in aqueous solutions, as a model system mimicking a film forming solution or even high-moisture food products. A high stability would increase the versatility for use of microcapsules in different food systems and processes, including edible films development.

Microcapsule stability profile (Fig. 5) showed that there was an early peptide amount (<10%) released, which according to Shahidi and Han (1993) could probably be due to residual process inefficiency or some microcapsule breakage. Microcapsules were significantly affected by the environmental pH. Peptide release at pH 2.6 and pH 9.2 was considerably higher than at milder pHs, representing around 40% of the total peptide content within the first hour. Further release up to 50% after 3 hours was observed at pH 9.2. Microcapsules at pH 5 showed more delayed and progressive release in water, up to around 20% after 3 hours, which might lead to a controlled release model. In this case, no peptides were released during the first hour. At pH 7, peptide release after 3 hours was in the same range as at pH 5, but most of the peptide content was released within the first hour.

Carnauba wax coated microcapsules were already reported to be insoluble in water, with release data lower than 30% during 7 hours (Raghuvanshi, Tripathi, Jayaswal, & Singh, 1992). Thus, the presence of carnauba wax contributes to slowing down the diffusion of active ingredients through the encapsulation material (Shahidi & Han, 1993), although in combination with the stearic acid it normally results in a more porous matrix (Fini, Cavallari, Rabasco Álvarez, & Rodríguez, 2011).

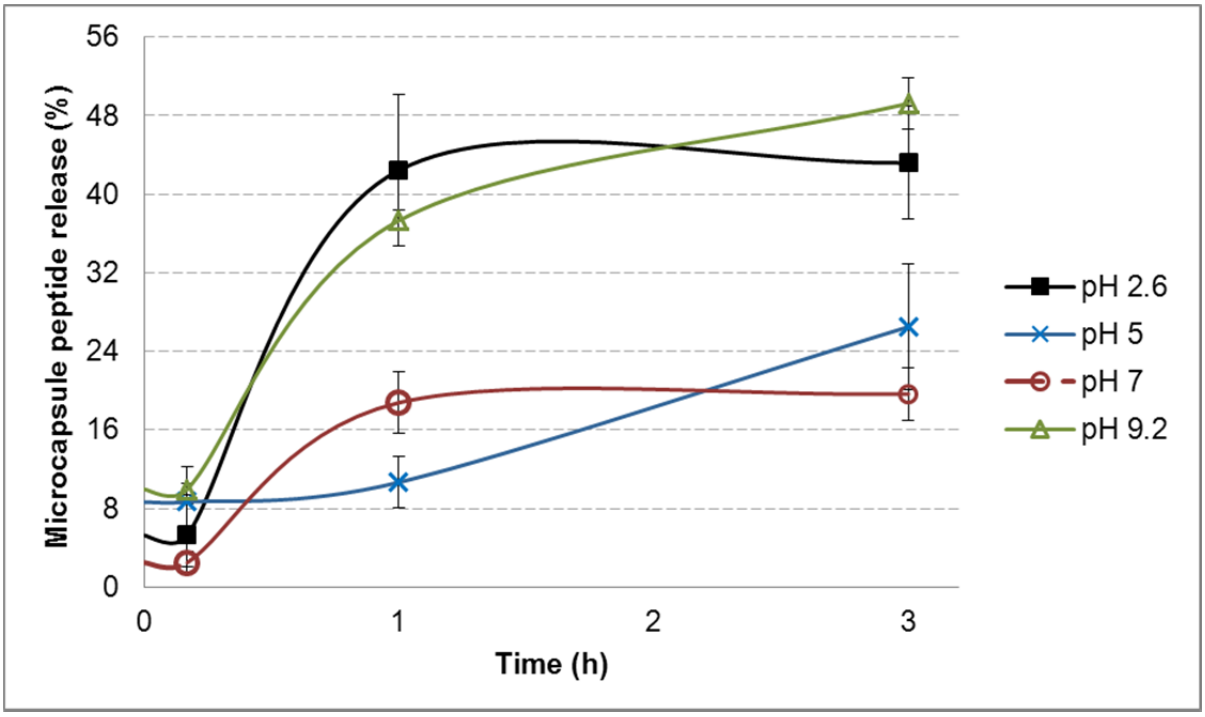

Figure 5. Microcapsule peptide stability at different pH during the first 3 hours at 21 ºC.

11.4.4. Microcapsule stability in film forming conditions

One possible food application of microcapsules is their addition to edible films. Free peptides can be added in the formulation of edible films, but they normally produce a negative rheological effect due to their plasticizing effect (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009). Excessive peptide interactions with matrix components during film preparation can be avoided by peptide microencapsulation, prior to the addition to the filmogenic solution. In the present study, the unrefined seaweed extract was obtained under relatively strong alkaline conditions, therefore, the resulting alginate-based film forming solution showed optimal performance at pH 10.

To check microcapsule stability in water and in the film forming alkaline solution, kinetics of peptide release were measured both in water at pH 7 and pH 10 at 35 ºC for 9 hours, which is the temperature and maximum time needed for film drying (Fig. 6). No significant differences were observed, showing an early ~30% release in the first 30 min and being stable thereafter, which was enough to cover the complete drying process. After around 6 hours the film dehydration process was completed, ensuring around 70% of peptides immobilized in the microcapsules in the resulting film. Compared to the pH stability results, temperatures higher than 21 ºC increased the release rate through the encapsulation matrix, probably due to weakening of the shell material at elevated temperatures and the resulting increased peptide diffusion (Shahidi & Han, 1993; Stojaković, Bugarski, & Rajić, 2012). Comparing microcapsule stability at pH 7 after 3 h (Figs. 5 and 6), the peptide release was significantly lower at 21 ºC (~20%) than at 35 ºC (~35%).

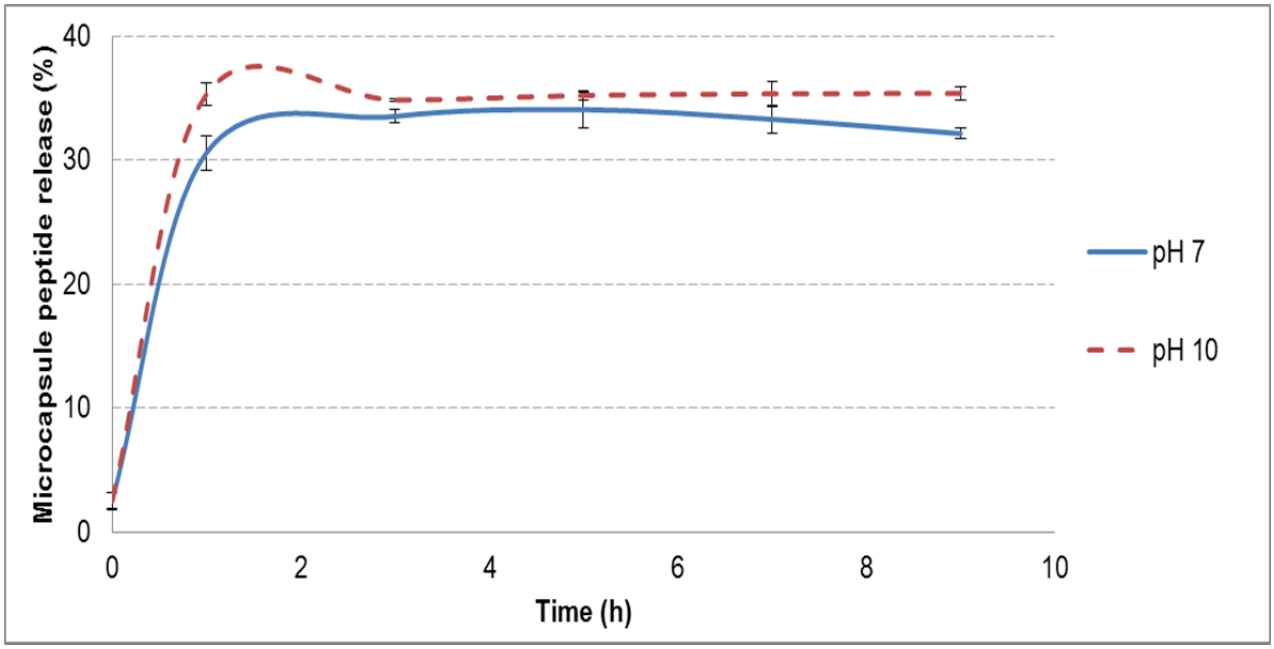

Figure 6. Microcapsule peptide stability in distilled water pH 7 and pH 10 at 35 ºC during 9 h.

11.4.5. Film preparation

In order to know the relative cohesive and adhesive molecular forces within the filmogenic solution and the added microcapsules, the contact angle (θ) of a droplet of the biopolymeric solution over the shell material was measured, and found to be 100.3 ± 0.9º at 21 ºC.

The contact angle indicates how hydrophobic the shell material is, where 180º represents absolutely no wetting and smaller contact angles imply increasing hydrophilic surface and higher tendency to wetting. According to Karbowiak, Debeaufort, Champion, and Voilley (2006), contact angle values θ>65º suggest a predominant hydrophobic system indicating very low surface affinity between the filmogenic solution and the lipid microcapsule surface, which resulted in poor wetting and aggregation of microcapsules, negatively affecting the film forming process.

More homogeneous distribution of microcapsules in the filmogenic solution was helped by the addition of polysorbate 80 as surfactant; however, strong microcapsule aggregation still occurred during the film drying process at 35 ºC. Considering that microcapsules tended to float or migrate to the surface of the biopolymer solution, a porous polycarbonate membrane was placed over the solution to immobilize the well dispersed microcapsules in the presence of the surfactant, avoiding aggregation during film drying.

Three different sets of films were prepared: F-C, containing water-filled microcapsules C; F-Cp, containing peptide-filled microcapsules Cp, and F, reference films without microcapsules.

Although the behaviour of C and Cp microcapsules when included into the filmogenic solution was essentially the same, slight differences in microcapsules distribution were perceptible in F-C and F-Cp films, as shown in Figure 7. Panoramic pictures showed that microcapsules in F-C were more visible at the film surface, while F-Cp microcapsules were more integrated inside the matrix, indicating possible differences in microcapsule density or in the degree of interaction with the film matrix components.

Interestingly, addition of both types of microcapsules did not significantly modify the film thickness (~141 μm) with respect to the film without microcapsules (139 μm). Moisture content was significantly higher ($P \leq 0.05$) in F (37.1 ± 0.9%), while no significant differences were found between F-C (31.0 ± 2.1%) and F-Cp (31.8 ± 2.1%), attributed to the hydrophobicity of the microcapsule lipid shell material.

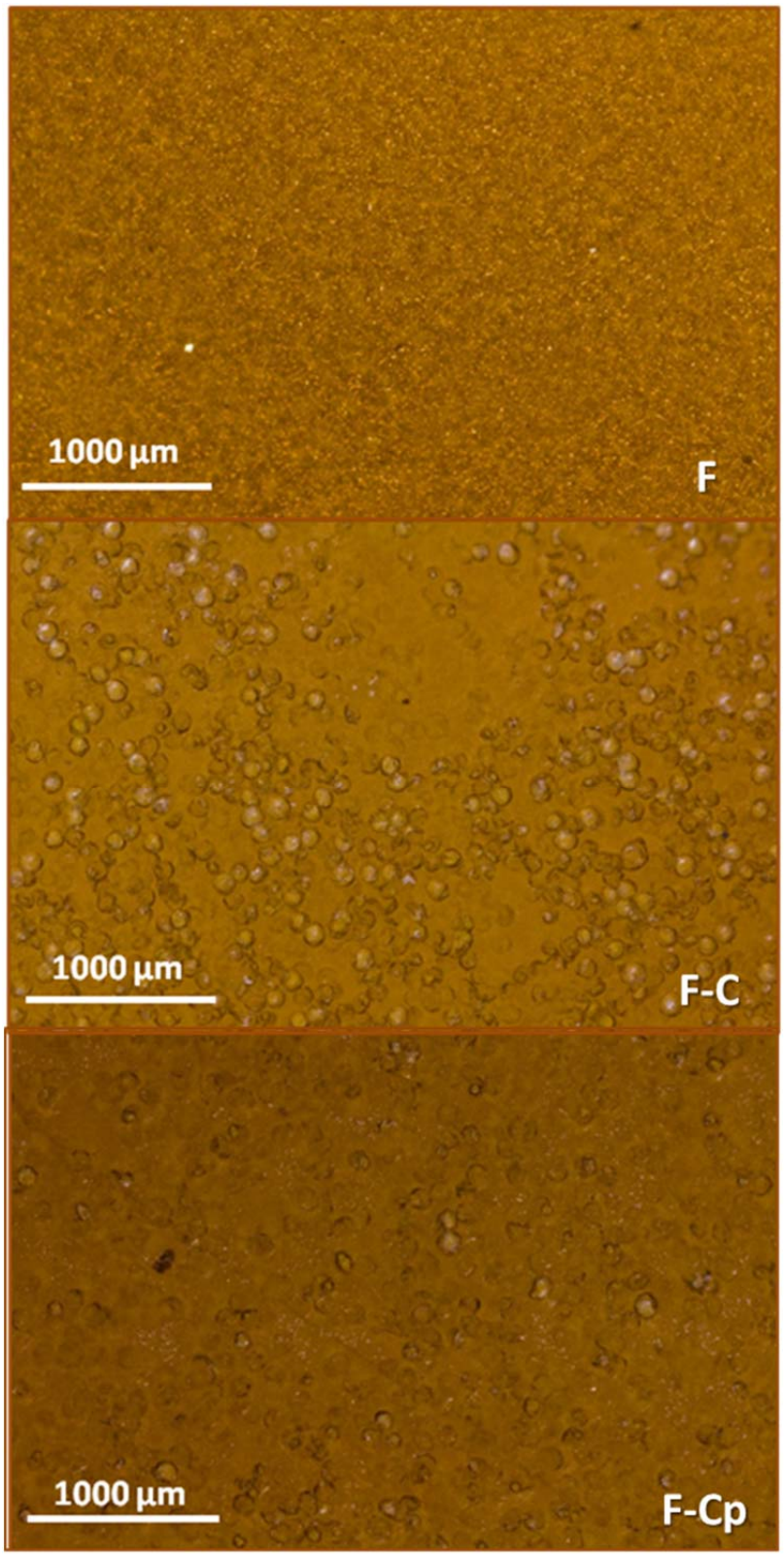

Figure 7. Panoramic photographic surface images of F, F-C and F-Cp films

11.4.6. Film properties

11.4.6.1. Thermal properties

DSC thermograms of F, F-C and F-Cp films are shown in Figure 3B. Microcapsule endothermic transitions were observed in F-C and F-Cp films as an endothermic peak at 82.88 ± 0.47 and 86.87 ± 0.28 respectively, with the corresponding ΔH (J/g_{dm}) of 0.88 ± 0.08 and 1.23 ± 0.26. Higher melting temperatures were needed to melt the shell material suggesting that microcapsules were efficiently entrapped into the film matrix. Alginates from F-C and F-Cp films might have interacted with the microcapsule components, probably the stearic acid with more capacity to form interactions. Apparently, Laminaria film matrix protected more efficiently Cp than C capsules, which was registered by higher enthalpy values. The 30% of peptides released during F-Cp film process might have increased film interactions between filmogenic compounds and peptides resulting in a stronger matrix than F-C. Films did not show any glass transition within the temperature range studied, which might be caused by the presence of glycerol. Films made of neat alginates have been reported to be essentially amorphous, with an irregular structure that does not crystallize (Siddaramaiah, Swamy, Ramaraj, & Lee, 2008), and the addition of plasticizer seems to increase that behaviour, probably dropping the glass transition temperatures to a lower range (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007)

11.4.6.2. Low-temperature scanning electron microscopy of F-Cp film

Low-temperature scanning electron microscopy (LT-SEM) cross-sections images of the film containing the Cp microcapsules are shown in Figure 8.

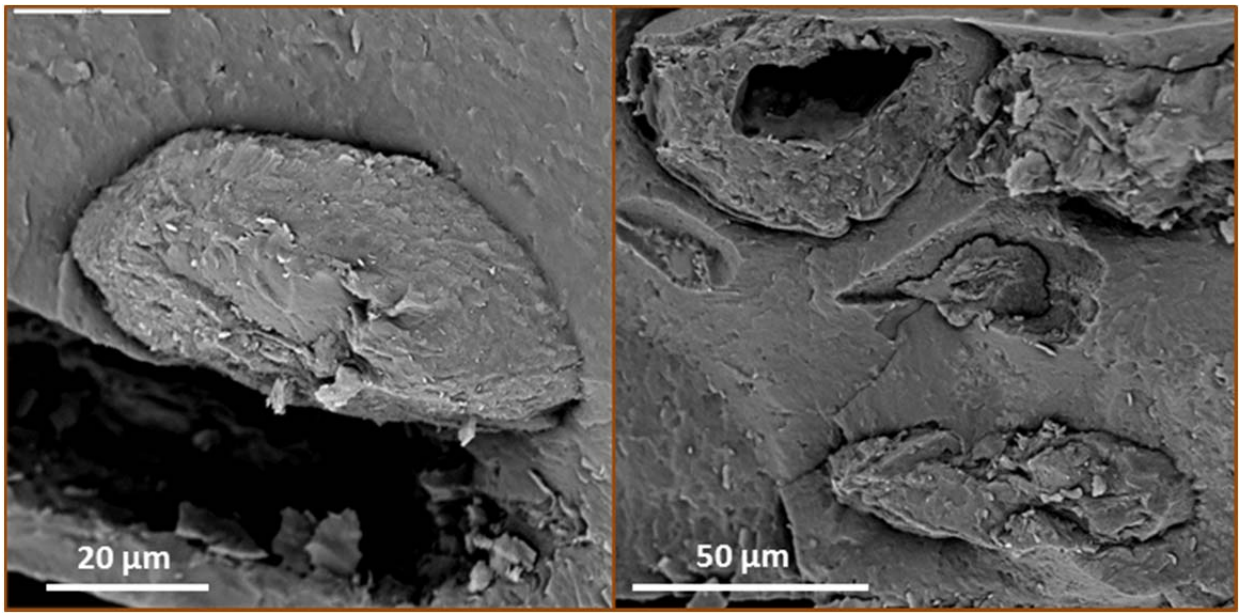

Figure 8. Low Temperature-Scanning Electron Microscopy images of F-Cp cross section.

Microcapsules were clearly distinguishable throughout the whole biopolymeric matrix, which exhibited a greatly dense and compact appearance. Capsules mostly retained their structural integrity, but not their spherical morphology. The cross sectional image shows an empty cavity within the microcapsule, suggesting that the peptide content tends to be disposed inside the wall of the capsule. The inclusion of microcapsules in the films caused a noticeable matrix disruption, in some cases leaving

large gaps between the biopolymer and the microcapsules, probably favoured by the strong hydrophobic nature of the shell material. Despite the fact that microcapsules led to strong discontinuities in the film microstructure, the compact nature of the alginate-based matrix was strong enough to support them without being fractured.

11.4.6.3. Water barrier properties

The film water solubility slightly increased (P≤0.05) with microcapsule incorporation in both F-C and F-Cp (Table 1). The lipid nature of the microcapsule would be expected to reduce film solubility; however microcapsules caused noticeable disruption in the film structure, leading to increased solubility. These effect was contrary to other studies, where lipid content was homogeneously mixed with the principal matrix component during the film forming solution process (Arcan & Yemenicioğlu, 2013). Solubility values were much higher than results obtained with commercial alginate films with cation complexation (~5%) (Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013), but similar to those developed with sodium alginate without cation complexation (~100%) (Abdollahi, Alboofetileh, Rezaei, & Behrooz, 2013; Zactiti & Kieckbusch, 2006). In contrast, F-C and F-Cp showed a significant reduction (P≤0.05) in the water vapour permeability (*WVP*); as compared to the F film (Table 1), which was largely attributed to the hydrophobic nature of the capsule lipid shell material. More specifically, the wax constituent has been previously reported to be effective at reducing the *WVP* of casein films added with paraffin wax (Sohail, Wang, Biswas, & Oh, 2006) or sodium caseinate-alginate films with beeswax (Fabra, Talens, & Chiralt, 2008). *WVP* of all studied films were considerably higher than previous results obtained with commercial alginate films (Benavides, Villalobos-Carvajal, & Reyes, 2012; Tapia, Rojas-Graü, Rodríguez, Ramírez, Carmona, & Martin-Belloso, 2007), which might be caused by the presence of hydrophilic compounds in the unrefined alginate seaweed extract.

Film	F	F-C	F-Cp
Solubility (%)	86.0 ± 1.7 a	94.8 ± 2.9 b	92.9 ± 1.9 b
WVP (x10^{-7} g m^{-1} s^{-1} Pa^{-1})	1.80 ± 0.02 a	0.80 ± 0.0 b	0.89 ± 0.3 b
TS (MPa)	6.6 ± 2.1 a	10.8 ± 0.9 b	10.1 ± 0.8 b
EAB (%)	1.31 ± 0.74 a	2.87 ± 0.4 b	3.23 ± 0.2 b
Y (MPa)	88 ± 25 a	183 ± 10 b	185 ± 14 b
F (N)	13.2 ± 2.3 a	8.61 ± 1.5 b	7.44 ± 1.4 b
D (%)	23.1 ± 4.0 a	10.9 ± 1.2 b	9.33 ± 1.5 b
*L**	25.1 ± 0.5 a	25.1 ± 1.1 ab	26.6 ± 0.8 b
*a**	3.52 ± 0.2 a	3.27 ± 0.5 a	3.75 ± 0.6 a
*b**	4.54 ± 0.5 a	4.57 ± 0.8 a	5.92 ± 1.0 a
Transparency	6.99 ± 0.27 a	8.42 ± 0.34 b	7.97 ± 0.28 b

Table 1. Solubility, water vapour permeability (*WVP*), tensile strength (*TS*), elongation at break (*EAB*), Young's modulus (*Y*), puncture force (*F*), puncture deformation (*D*), lightness (L^*), colour values a^*, b^* and transparency (− log (T_{600}/x)) of F, F-C and F-Cp films.
Results are the mean ± standard deviation. Different letters (a, b, c) in the same row indicate significant differences among the different films (P≤0.05).

11.4.6.4. Mechanical properties

F significantly showed the lowest tensile strength (*TS*), elongation at break (*EAB*) and elastic Young's modulus (*Y*) (p≤0.05) (Table 1), in good accordance to its poorer cross-linked structure. Microcapsule addition considerably increased tensile strength, elongation at break and Young's modulus. Fabra, Talens, and Chiralt (2008) showed that beeswax addition to sodium caseinate-alginate films improved both elastic modulus and tensile strength but, contrary to our results, reduced elongation at break. *EAB* and *TS* values of all studied films were lower than previous films developed with 2-4% commercial alginate (*EAB* of 17-30%) (*TS* of 12-27 MPa) (Su Cha, Choi, Chinnan, & Park, 2002) and also after adding $CaCl_2$ for cation complexation (~15% and ~45 MPa) (Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013).

Regarding puncture deformation (*D*) and force (*F*), microcapsule inclusion in both F-C and F-Cp led to a significant decrease in both *D* and *F* values (p≤0.05) (Table 1), probably as a result of film matrix disruptions caused by the microcapsules. As in tensile test, no significant differences were found between F-C and F-Cp. Previous results obtained with 1-1.5% commercial alginate films were in the same *F* range (13-22 N) and *D* range (25-60 ± 3.04%) as F film (Wang, Jia, Ruan, & Qin, 2007).

Thus, it could be concluded that microcapsules incorporation improved film mechanical tensile response due to the lipid microcapsule strength effect, while negatively affected to the puncture test film behaviour. The microcapsule matrix disruption that negatively affected film solubility might have also been responsible of the puncture breakage. While tensile strength test gives us more general mechanical film information, puncture test concentrates the force in one specific little area, being more affected by the discontinuous structure and poor cohesion between the film matrix and the microcapsules.

11.4.6.5. Colour properties

As shown in Table 1, film transparency is significantly (P≤0.05) higher in F, while no significant differences were found between F-C and F-Cp (P≤0.05). *L** (lightness), *a** (reddish/greenish) and *b** (yellowish/bluish) values are also shown in Table 1, where it is revealed that all films had low lightness (25-27). *a** and *b** did not show significant differences with or without entrapped microcapsules (P≤0.05). Fucoxanthin presence might be responsible for the yellowish-brown film tendency (Ferraces-Casais, Lage-Yusty, de Quirós, & López-Hernández, 2012).

In general, films were less transparent than those previously obtained with isolated commercial alginate, due to their intense colouration and the presence of the microcapsules; but constituted a better light absorption barrier (Pereira, Carvalho, Vaz, Gil, Mendes, & Bártolo, 2013; Yoo & Krochta, 2011). Films contained most of the seaweed compounds that remained in Laminaria extract, rendering more opaque films with more red-brown colouration; resulting in low lightness and low yellowness tendency compared to films developed with commercial alginates (Wang, Liu, Holmes, Kerry, & Kerry, 2007).

11.5. Conclusion

Printing of hydrophobic stearic acid/carnauba core-shell microcapsules resulted in an efficient process for active peptide protection. Microcapsule stability and peptide release rate depended on the environmental pH and temperature, giving the possibility of different applications depending on the final

food system. Microcapsule inclusion in hydrophilic *Laminaria digitata* edible film matrix resulted in a discontinuous film with improved tensile properties and water vapour permeability but higher film solubility and opacity and poorer puncture response. *Laminaria digitata* network, with microcapsules embedded in it, could be used for active and edible film applications, as a carrier for a wide range of molecules apart from peptides.

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XII. General discussion

12.1. Introduction

In the present work, two different raw materials were utilized for polymeric extraction: *Dosidicus gigas* muscle waste and seaweeds. Among all the seaweed species, one Rhodophyta (*Mastocarpus stellatus*) and two Phaeophytas (*Laminaria digitata* and *Ascophyllum nodosum*) were selected.

Integral extractions were carried out from the different raw materials. Proteins were recovered from the muscle of Dosidicus discarded during the skinning process, while unrefined extraction procedures were tested in seaweeds to extract more components. Whereas *Dosidicus gigas* extracts were mainly of protein nature, seaweed extracts had high polysaccharide content together with some proteins, pigments and minerals. All extracts were used for the development of edible films, and said films were compared in terms of their physicochemical and structural properties.

Mild aqueous extractions and enzymatic hydrolysis were performed in order to recover different active compounds. Antioxidant aqueous extracts rich in proteins, pigments and polyphenols were obtained from *Mastocarpus stellatus,* and antioxidant and antihypertensive hydrolysates rich in peptides were obtained from *Dosidicus gigas* tunics and from *Mastocarpus stellatus*.

Seaweed aqueous extracts and hydrolysates were directly added to Mastocarpus film-forming solutions and the resulting film activity and physicochemical properties were evaluated. However, active compounds encapsulation is often performed to protect them during processing. In this regard, a new encapsulation procedure was chosen to evaluate their integrity after the processing and their potential applications in food products, and so, Dosidicus hydrolysate was microencapsulated within lipid material (stearic acid + carnauba wax). Microcapsules were included in Laminaria film forming solutions to study their possible use as microcapsule carriers.

The general discussion will be divided in four sections in order to provide a clearer approach:

1. Development of protein films from muscle waste adhered to *Dosidicus gigas* tunics.
2. Development of polysaccharide films from seaweed extracts.
3. Study of the main characteristics of different films.
4. Potential film applications in the food industry.

12.2. Development of protein films from muscle waste adhered to *Dosidicus gigas* mantle

Total muscle proteins recovered by different solubilization methods (S-extracts), and myofibrillar proteins concentrated by a further isoelectric precipitation (C-extracts), have shown a good filmogenic capacity, providing the industry with six different viable possibilities of protein recovery that could be extrapolated to other seafood species (Fig. 1).

Extracts/Films	Description	
water-S	Muscle protein solubilized in water	total muscle
salt-S	Muscle protein solubilized in salt	
alkaline-S	Muscle protein solubilized at pH10	
acidic-S	Muscle protein solubilized at pH3	
alkaline-C	Muscle protein solubilized at pH10 and concentrated by isoelectric precipitation	concentrate
acidic-C	Muscle protein solubilized at pH3 and concentrated by isoelectric precipitation	
alkaline-C4	Films stored for 4 months	
acidic-C4	Films stored for 4 months	

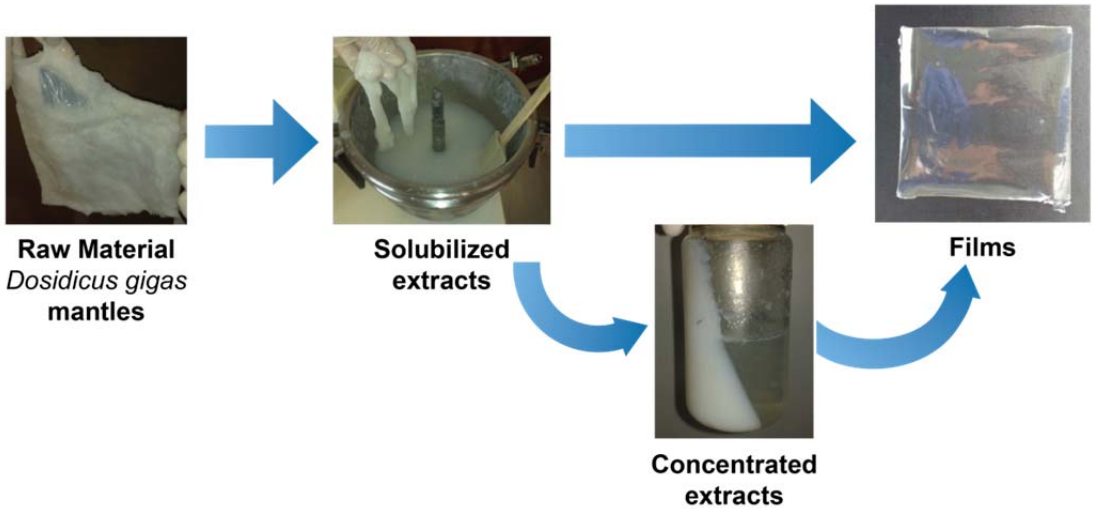

Figure 1. Muscle protein extraction for film development.

12.2.1. Solubilized extracts for film development

12.2.1.1. Solubilized extracts (S-extracts)

Regarding the water, salt, alkaline and acidic muscle protein solubilization (S-samples), unlike traditional protein recovery methods (Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009; Sánchez-Alonso, Careche, & Borderías, 2007), 100% of the recovered material was used for the development of films (including both the myofibrillar and the sarcoplasmic fraction, and non-protein nitrogen compounds). Both alkaline-S and acidic-S extracts resulted in a higher proportion of soluble protein.

Among the different types of sarcoplasmic proteins, it has been claimed that some proteins such as endogenous transglutaminase (TGase) play a more important role than others in bond formation, by catalyzing the cross-linking of myosin heavy chain molecules and probably other compounds under low-temperature settings, therefore resulting in more elastic systems. The cephalopod intrinsic calcium

content might activate TGase, especially during the film preparation phase at 4 ± 0.5 ºC for 21-23 h (Jodral-Segado, Navarro-Alarcón, López-Ga de la Serrana, & López-Martínez, 2003; Martín-Sánchez, Navarro, Pérez-Álvarez, & Kuri, 2009). Extreme pH values like those used in the present study, pH 3 (acidic) and pH 10 (alkaline), induce major protein conformational changes, probably having an adverse effect on the TGase activity (Jafarpour & Gorczyca, 2012). Indeed, DSC thermograms showed higher myofibrillar protein preservation in water and salt solubilized extracts, while alkaline conditions led to a higher denaturation and acidic conditions resulted in protein hydrolysis.

12.2.1.2. Films from solubilized extracts (S-films)

Water S-films were stiff and showed a good water barrier, but both water and salt solubilization led to less transparent films with no microbiological stability (exceeding the recommended limits of total viable bacterial count in fishery products >5 log CFU/g). All these films are edible and, as any food product, are subjected to microbial contamination. Due to this fact, the addition of conservative additives would be necessary to make them suitable for food or packaging applications. Most of the previous studies about edible films do not make shelf-life determinations, but it might probably be as short as for water or salt S-films. For this reason, alkaline and acidic S-films resistance to contamination is of great interest regarding edible/packaging applications.

Both alkaline-S and acidic-S films showed food grade microbiological results and their physicochemical properties are described in the following sections. Moreover, avoiding the use of salts for protein recovery prevented the formation of crystal deposits during film development and their negative consequences on the film properties.

Apart from myofibrillar and sarcoplasmic proteins, Dosidicus protein solubilization comprised all the intrinsic physiological ammonium chloride (NH_4Cl), endogenous NH_3, octopine, low molecular weight polypeptides and organic acids (Marquez-Rios, Moran-Palacio, Lugo-Sánchez, Ocano-Higuera, & Pacheco-Aguilar, 2007), which were responsible for the film off-flavour that could make them unsuitable for consumption. Nevertheless, both alkaline and acidic films might be used for packaging purposes even though they should be subjected to migration studies in the future.

12.2.2. Concentrated extracts for film development

12.2.2.1. Concentrated extracts (C-extracts)

In order to minimize the content of undesirable compounds, such as NH_3, Dosidicus muscle protein solubilized under acidic and alkaline conditions was concentrated by isoelectric precipitation (4.8) (C-extracts). Despite the protein recovery method had been refined, not only myofibrillar proteins were recovered but also a sarcoplasmic fraction, as shown by electrophoretic patterns (Fig. 2). Other authors have also reported some remaining sarcoplasmic fraction in protein concentrates (Jafarpour & Gorczyca, 2012).

12.2.2.2. Concentrated C-extracts *vs.* Solubilized S-extracts

Alkaline and acidic pH solubilization treatments resulted in different protein structure and conformation (Jafarpour & Gorczyca, 2012). Isoelectric precipitation did not modify the protein degradation pattern obtained by pH solubilization. While with the alkaline treatment, myosin (MHC), paramyosin (P) or actin (A) did not show signs of degradation, the acidic extraction showed a significant hydrolysis with a band density reduction at high molecular weights and the appearance of new bands below the 50 kDa regions. These low molecular weight bands might correspond to peptide fractions formed during protein degradation by endogenous acidic proteases (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008; Choi & Park, 2002), or by the acidic treatment itself.

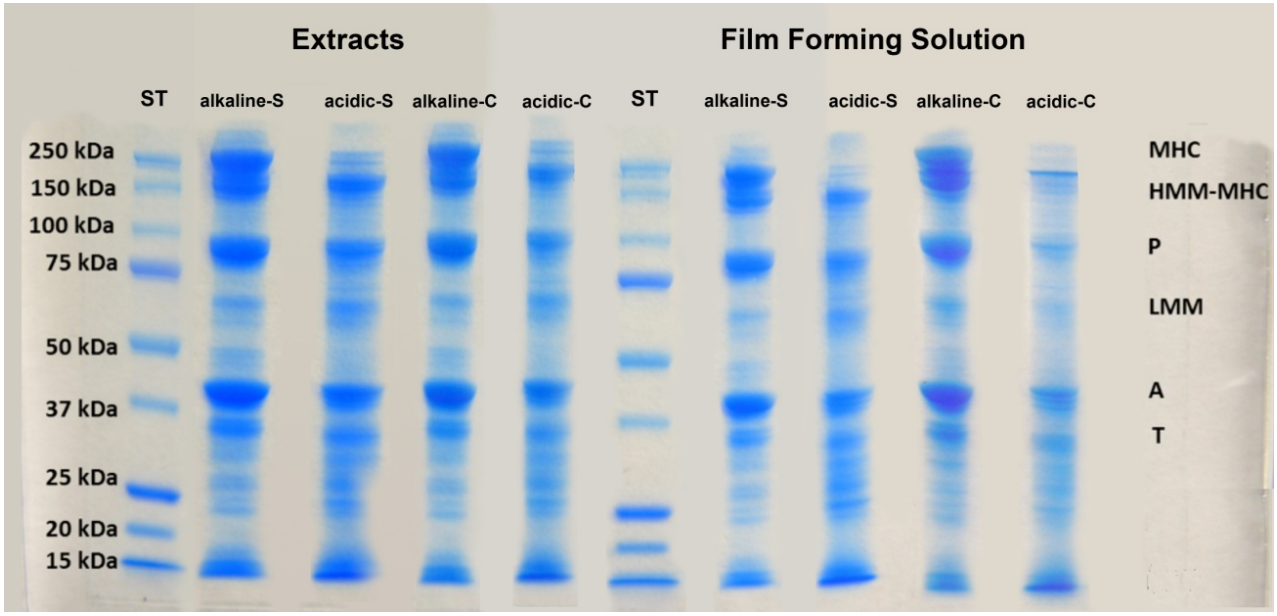

Figure 2. SDS-PAGE patterns under reducing conditions of the corresponding solubilized (S) and concentrated (C) extracts and film forming solutions, under alkaline and acidic conditions, respectively.

Comparing DSC thermograms of both S and C extracts, it was observed that isoelectric precipitation performed immediately after alkaline and acidic solubilization contributed to protein preservation, exhibiting under acid-pH-shift processing a higher protection against degradation, as has been reported by other authors (Totosaus, Montejano, Salazar, & Guerrero, 2002) (Fig. 3).

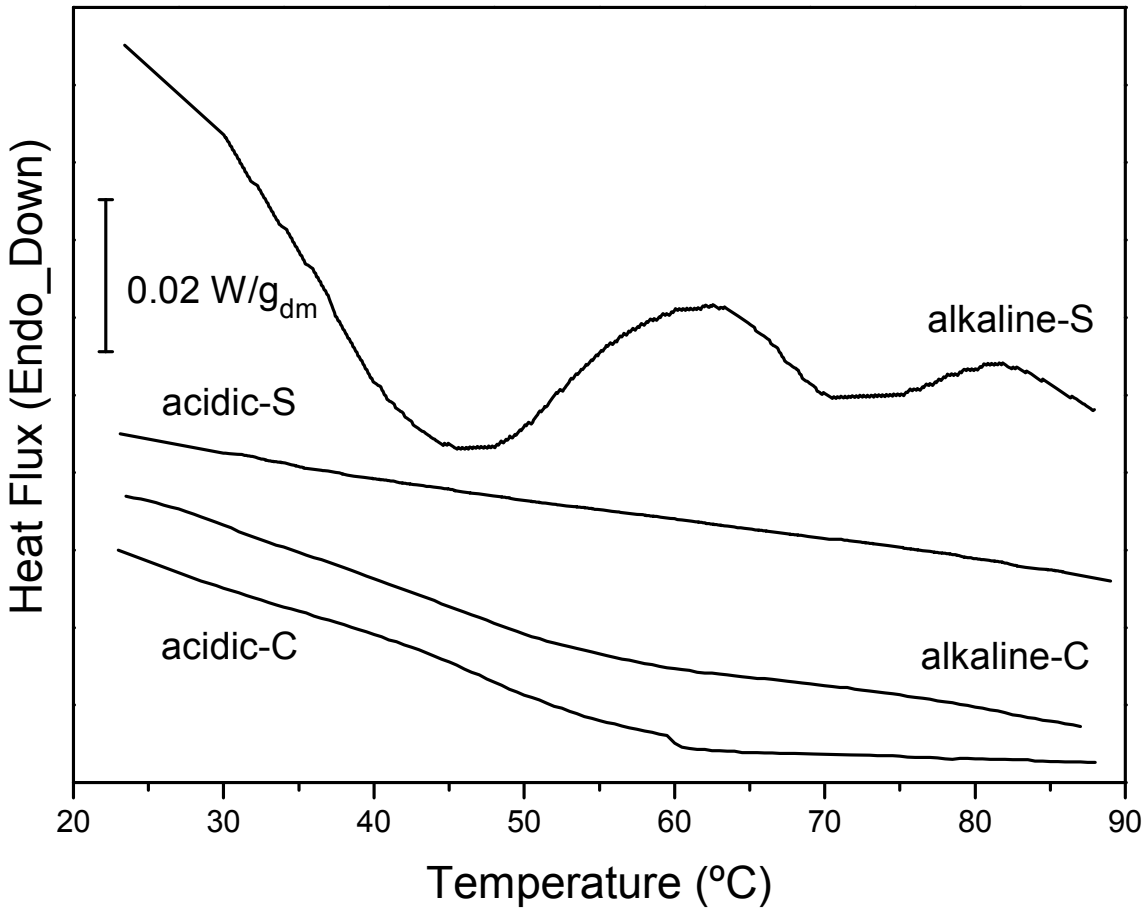

Figure 3. DSC of alkaline and acidic solubilized (S) and concentrated (C) extracts.

Moreover, concentration included a higher proportion of soluble protein than S-extracts and levelled alkaline and acidic values (Fig. 4), showing extraction yields similar to those obtained in previous studies (Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009). This higher proportion was probably caused by the lack of other insoluble compounds in C-extracts.

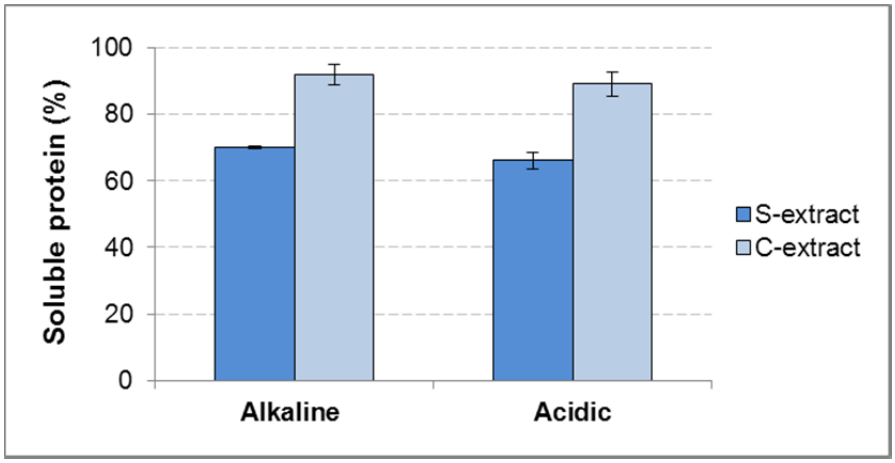

Figure 4. Water soluble protein in solubilized (S) and concentrated (C) extracts under alkaline and acidic conditions.

12.2.2.3. C-films and S-films comparison

FTIR Amide I region of the films spectra (1600-1700 cm^{-1}) confirmed that pH affected the secondary protein structure, and consequently, hydrophobic and hydrogen bonds would be formed differently in the films. Alkaline films were formed by proteins with a more preserved structure than acidic films, independently of whether the extracts were concentrated or not. Alkaline conditions led to a loss of the secondary structure that was reflected in the disappearance of the α-helical structure, which favoured a more organized film structure as was shown by the β-structures intramolecular aggregation. Acidic hydrolysis resulted in a higher hydrated and plasticized film with a reduction of the non-bonded peptide groups caused by a more extensive hydrogen bonding between protein fragments and glycerol (Chunli, Stading, Wellner, Parker, Noel, Mills, et al., 2006).

Films developed with alkaline extracts (S and C) presented more intramolecular aggregation, with more protein functional groups available to form covalent bonds (protein-protein cross-linking), which resulted in matrixes with a higher mechanical strength and water resistance (Figs. 5 and 6).

Acidic films showed a higher number of hydrogen bonds, with possible extra interactions between short protein fragments and plasticizers, which resulted in a higher matrix hydration (Chunli, et al., 2006), hence poor water and mechanical properties.

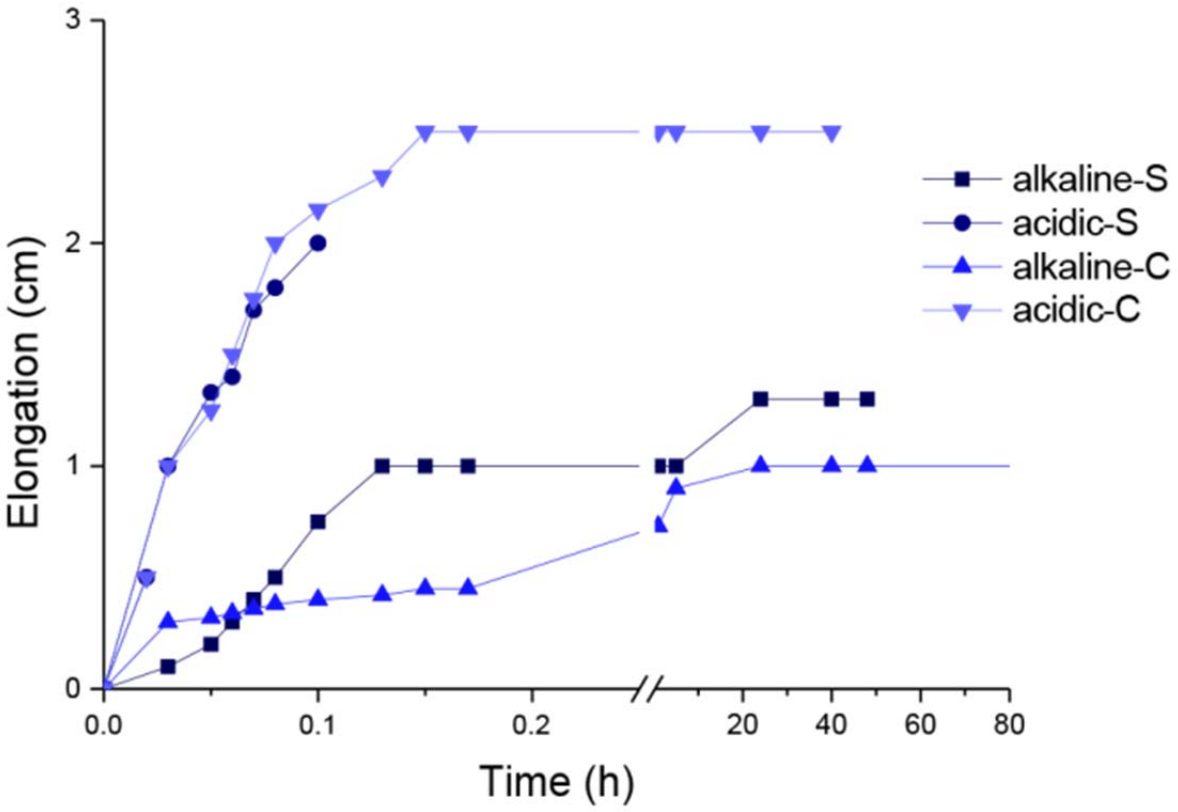

Figure 5. Water resistance to breakage of alkaline and acidic solubilized (S) and concentrated (C) films. *Alkaline-C film was unbreakable.

As shown in figures 4, 5 and 6, protein concentrates resulted in films of a higher quality. This high quality was clearly evident in terms of water resistance and mechanical strength. SDS-PAGE protein

patterns under alkaline and acidic conditions were quite similar for solubilized and concentrated proteins. Concentrates contained mainly myofibrillar proteins, and therefore an improvement in their resulting film characteristics was expected; for example, water resistance was extremely better in films from concentrates. The isoelectric precipitation resulted in concentrates with a high myofibrillar protein proportion, which might explain the improvement of the corresponding film mechanical strength and water resistance (Artharn, Benjakul, & Prodpran, 2008). By pH isoelectric precipitation, endogenous TGase was recovered in the concentrates, being more stable than in alkaline and acidic S-extracts (Jafarpour & Gorczyca, 2012). The alkaline method resulted in more accessible proteins, susceptible to be good substrates for the TGase enzyme and available to form other protein-protein interactions (Pérez-Mateos, Amato, & Lanier, 2004). These interactions might have determined an improved matrix conformation during film preparation.

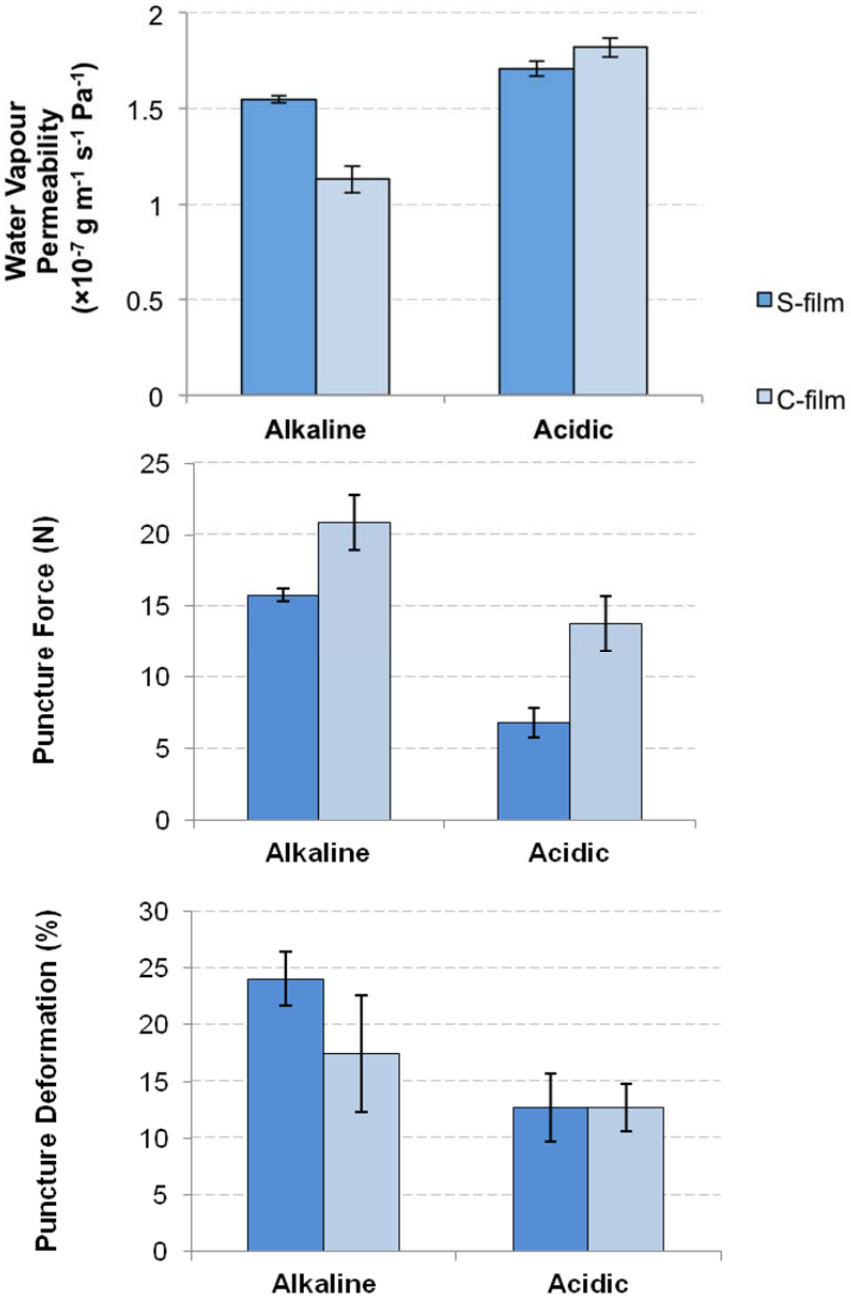

Figure 6. Water vapour permeability (*WVP*), puncture force (F) and puncture deformation (D) of alkaline and acidic solubilized and concentrated films.

12.2.3. Film storage stability

By myofibrillar protein isoelectric precipitation, the films were expected not only to improve their physical properties, but also their stability over time. Alkaline and acidic C-films were tested after four months (4) to evaluate the changes suffered by maturing under controlled conditions of humidity (~58%) and temperature (21 ºC).

One of the most remarkable improvements observed by using myofibrillar concentrates (C), instead of total protein solubilized extracts (S), was the reduction of the solubility of the film. After four months of storage, C-film matrixes suffered protein aggregation, which resulted in an increase in solubility, being more significant in alkaline-C films. Protein release was also lower in C-films than in S-films, but the acidic treatment still resulted in a higher protein release than the alkaline one. This might be caused by the weaker matrix formed by bonded hydrolyzed proteins, plasticizers and water, with an increased free volume (Fig. 7) (Cuq, Gontard, Cuq, & Guilbert, 1997).

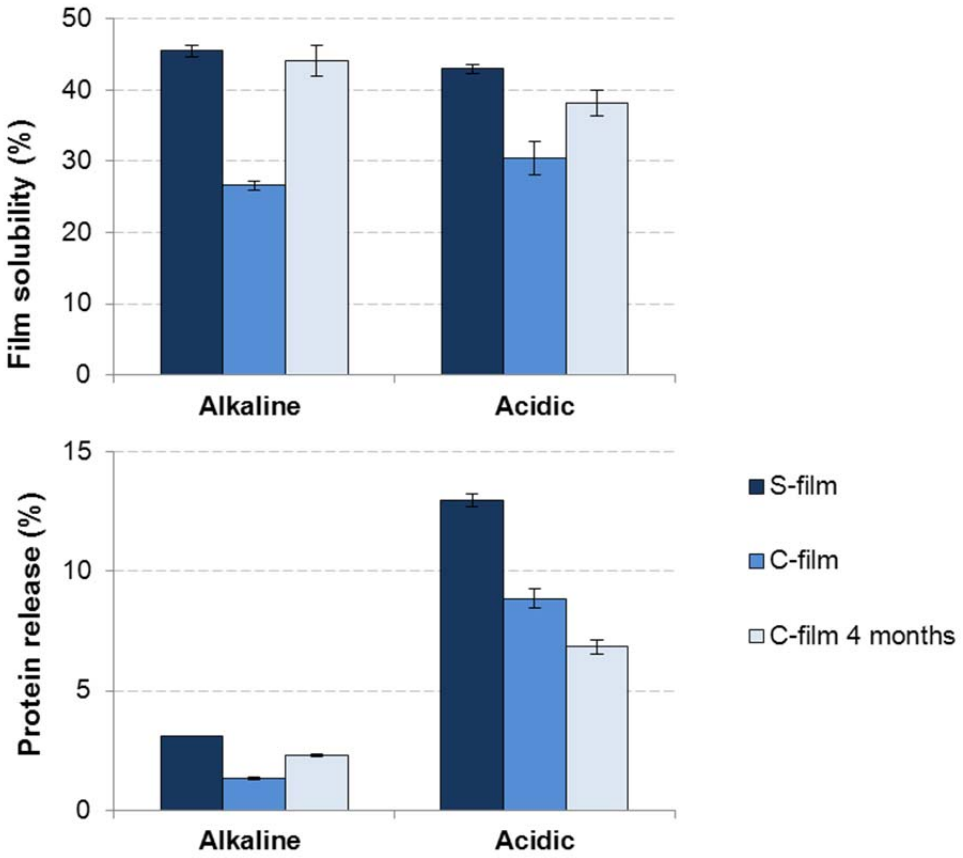

Figure 7. Film solubility and protein release of alkaline and acidic solubilized, concentrated and concentrated after 4 months of storage films.

The electrophoretic pattern of the proteins released, by both S and C films, is shown in figure 8. Water-S and alkaline-S films released a lower protein content than both acidic S and C films. Alkaline-S film presented faint bands in the actin (45 kDa), tropomyosin (30-35 kDa) and light chains of myosin (LMM) (17 kDa) regions. Those bands were hardly visible in the alkaline-C film, being only noticeable a very faint band at 17 kDa. Acidic films released more protein material, including a large amount of tropomyosin and even actin and paramyosin. This protein release was more intense in the acidic-S than

in the acidic-C film. After four months of storage (4), although alkaline-C film protein release was still low, it increased together with film solubility. On the contrary, the acidic-C film reduced the amount of proteins released in water.

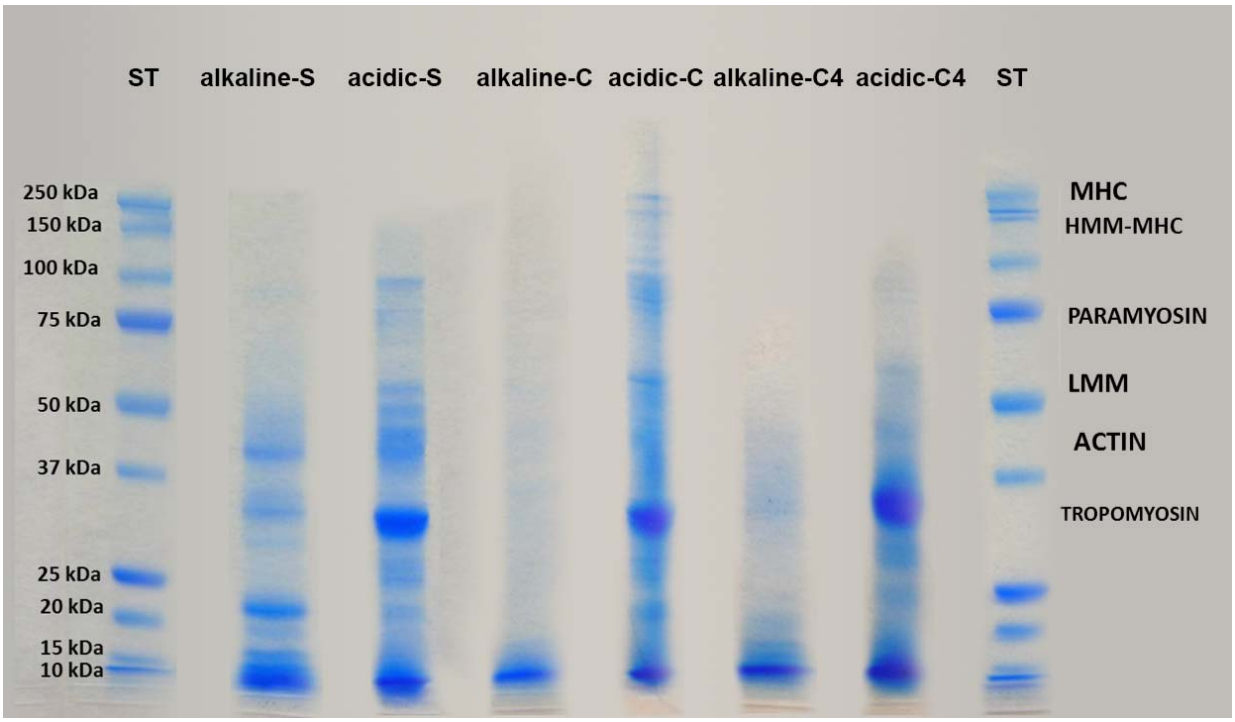

Figure 8. SDS-PAGE patterns under reducing conditions of the corresponding soluble fraction of alkaline-S, acidic-S, alkaline-C, acidic-C, alkaline-C4 and acidic-C4 films.

Regarding the mechanical properties (Figure 9), the formulation with C-extracts did not result in films with an increased tensile strength (*TS*) or elongation at break (*EAB*), properties that were shown to be even slightly lower than those obtained for films made with S-extracts. Nevertheless, Young's elastic modulus (*Y*) values were considerably higher in alkaline-C and acidic-C films. Apparently, a higher myofibrillar protein concentration mainly affected the film stiffness. After the storage time, the alkaline-C4 film maintained stable both the *TS* and *Y,* but had lost almost all its flexibility (*EAB*). On the contrary, the acidic-C4 film acquired more tensile strength and stiffness after four months, and its *EAB* was stable.

The acidic-C film probably suffered a protein polymerization and aggregation via Maillard reaction with the plasticizers, which might suggest an increase in protein-protein cross-linking (probably through covalent bonds) (Artharn, Prodpran, & Benjakul, 2009; Leerahawong, Tanaka, Okazaki, & Osako, 2012), together with a hydrogen bonding reduction accompanied by migration of the plasticizer to the surface, and therefore a higher plasticizer release in water (Orliac, Rouilly, Silvestre, & Rigal, 2002). Despite the plasticizer migration, the film flexibility was not damaged, probably because the improvement in the film structure compensated for the plasticizer loss, making the film stronger and more malleable.

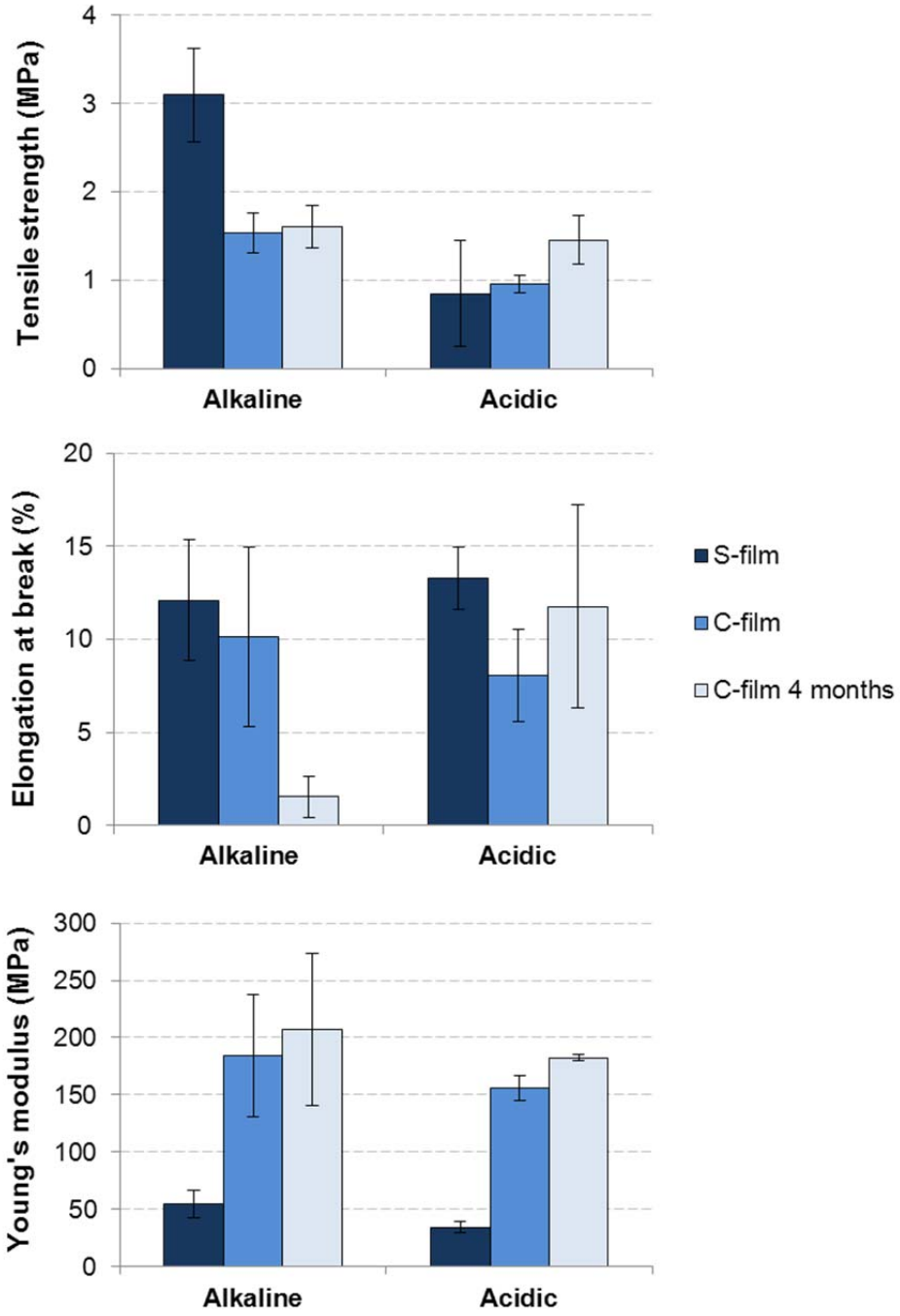

Figure 9. Tensile strength, elongation at break and Young's elastic modulus of alkaline and acidic solubilized, concentrated and concentrated after 4 months of storage films.

On the other hand, the increase in solubility of the alkaline-C film might result from a partial matrix disruption over the storage time. It is known that part of the sarcoplasmic content remained in the protein concentrates, allowing the retention of Dosidicus endogenous proteases. It has been observed that cephalopod mantle contains proteases that can be active both under acidic and alkaline conditions (Ayensa, An, Gómez-Guillén, Montero, & Borderías, 1999). While acidic proteases might have been boosted during the acidic-C film drying phase, the alkaline conditions might have induced a slow and progressive alkaline protease activity throughout the storage time, resulting in a partial film hydrolysis and consequently, in a loss of flexibility (Gómez Guillén, Hernández-Andrés, Montero García, & Pérez Mateos, 2006).

12.2.4. Conclusion

Since the traditional method to make *surimi* cannot be applied to *Dosidicus gigas* muscle, alternative manufacture methods have been developed with salt addition (Sánchez-Alonso, Careche, & Borderías, 2007) and acidic conditions (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008), but never obtaining a high gel forming ability due to the low protein functionality (De la Fuente-Betancourt, García-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sánchez, 2009). The alkaline protein extraction carried out in the present study has proved to confer high solubility to the proteins, and has been scarcely studied in cephalopods (Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009) although it would provide the industry with novel possibilities. Total solubilized and concentrated extracts showed a good functionality for film development.

Total solubilized extracts (water, salt, alkaline and acidic) were very suitable raw materials for the development of films. Both alkaline and acidic films were good edible alternatives for different food applications, such as packaging. Isoelectric precipitation improved, in general terms, the physical characteristics of the films. Alkaline conditions resulted in transparent films showing mechanical strength and water resistance, with partial protein denaturation, which might be suitable for short-term applications; while acidic conditions exhibited a remarkably higher structure protection over time, being a good option for long-term applications despite their significant hydrolysis.

Another option to improve film stability would be the incorporation of active compounds, and therefore it was studied as will be shown in the following results.

12.3. Development of polysaccharide films from seaweed extracts

Different red and brown seaweed unrefined extractions were tested to obtain suitable polymeric materials for novel food applications, such as the development of film-coatings.

On the one hand, sequential crude aqueous extractions were designed in *Mastocarpus stellatus* to obtain both active extracts by mild conditions, and polymeric extracts by traditional high temperature procedures. On the other hand, new alkaline treatments, for both *Laminaria digitata* and *Ascophyllum stellatus* extractions, were compared with the traditional ways of alginate isolation commonly used to obtain commercial colloids (Figs. 10 and 11).

In order to preserve the bioactivity of certain seaweed compounds, and to try to obtain an integral extraction, *Mastocarpus stellatus* enzymatic hydrolysis was also performed. The active extracts that emerged during the hydrolysis might be used to improve the quality of different food products. Specifically in this study, the consequences of direct addition of both hydrolysates and aqueous extracts to *Mastocarpus stellatus* film forming solutions were tested and compared with the intrinsic film activity.

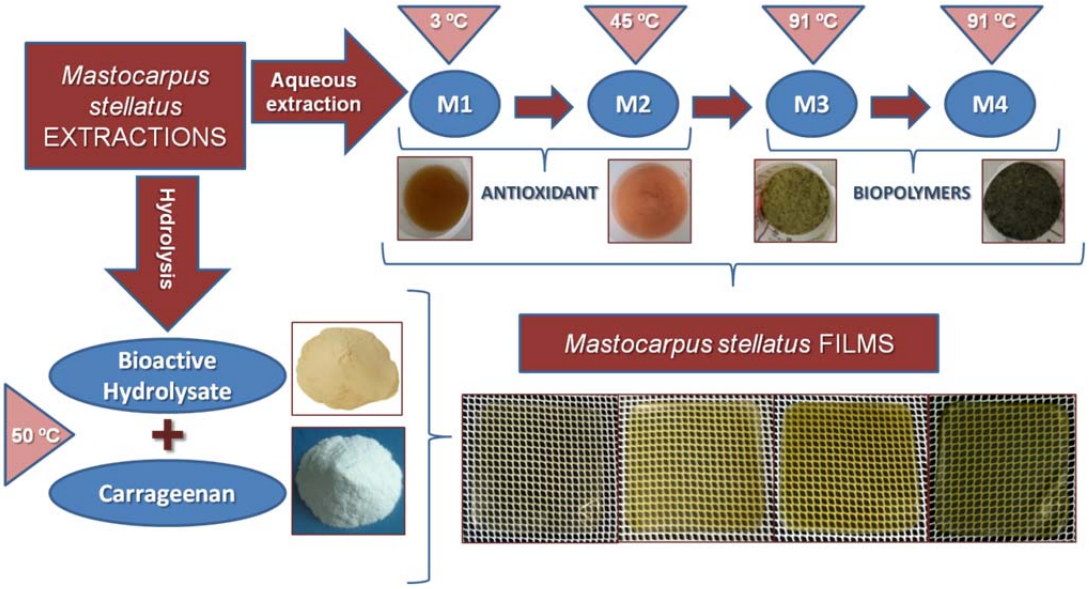

Figure 10. *Mastocarpus stellatus* extractions for film development.

In view of the possible mechanical damage derived from the direct addition of active compounds to edible films (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009), a new microencapsulation procedure was tested. Films with embedded microcapsules were developed as a new bioactive compound carrier system.

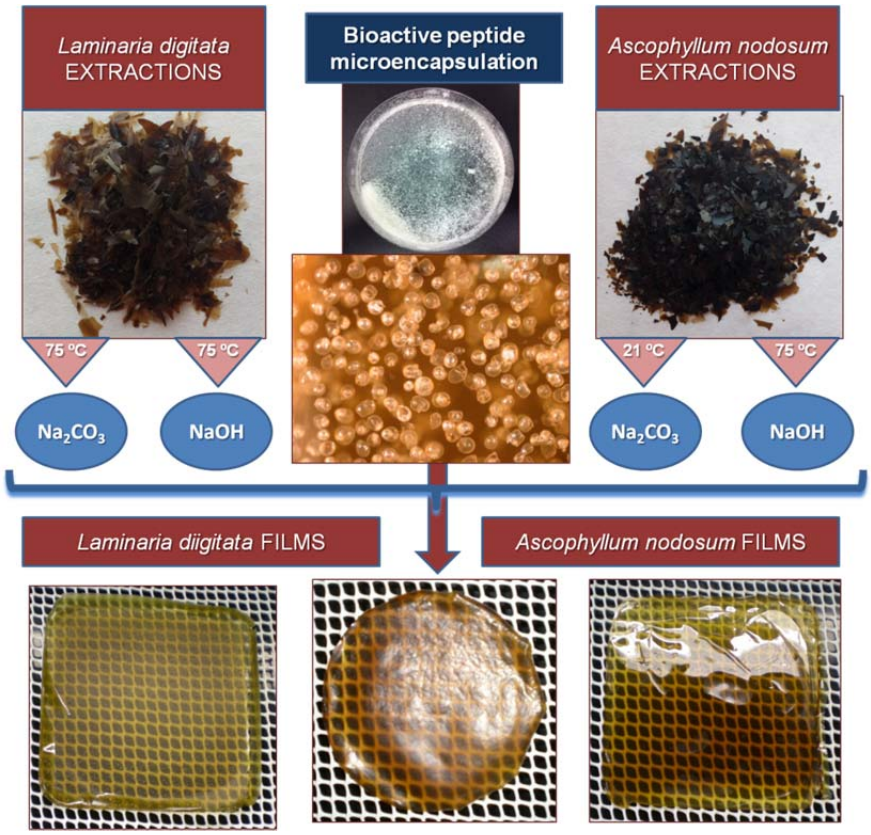

Figure 11. *Laminaria digitata* and *Ascophyllum nodosum* extractions for film development.

12.3.1. Seaweed extracts

There are not many studies about integral seaweed extraction (Sousa, Martins, Larotonda, Hilliou, Goncalves, & Sereno, 2008) for film development. Most studies are specifically focussed on phycocolloid isolation, carrageenan in red species (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006) and alginates in brown algae (Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009). Moreover, many studies about improving the phycocolloid extraction efficiency have been carried out (Hernández-Carmona, McHugh, Arvizu-Higuera, & Rodríguez-Montesinos, 1998; Montolalu, Tashiro, Matsukawa, & Ogawa, 2008). The extraction of bioactive compounds from seaweeds has also been studied (Holdt & Kraan, 2011; Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012; Tierney, Croft, & Hayes, 2010). The present work provides the industry with the possibility of extracting both active and polymeric materials in a continuous sequential process (Table 1).

It is interesting to note that the crude polymeric material, apart from being useful for its phycocolloid functionality, contained other interesting seaweed compounds such as proteins, pigments and minerals, which in traditional isolation procedures are usually discarded.

Extracts/Films	Description	
M1	First antioxidant aqueous extraction (4 ºC)	
M2	Second antioxidant aqueous extraction (45 ºC)	
M3	First carrageenan-rich extraction (91 ºC)	*Mastocarpus stellatus*
M4	Second carrageenan-rich extraction (91 ºC)	
H	Phenolic-partially removed hydrolysate (50 ºC)	Hydrolysate
Hp	Phenolic-containing hydrolysate (50 ºC)	*Mastocarpus stellatus*
Lc	Alginate-rich sodium carbonate extraction (75 ºC)	
Lh	Alginate-rich sodium hydroxide extraction (75 ºC)	*Laminaria digitata*
Ac	Alginate-rich sodium carbonate extraction (21 ºC)	
Ah	Alginate-rich sodium hydroxide extraction (75 ºC)	*Ascophyllum nodosum*

Table 1. Seaweed extracts.

12.3.1.1. Red seaweed extracts: *Mastocarpus stellatus*

Due to the fact that carrageenan extraction requires temperatures near to the boiling point to be boosted (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009), it might adversely affect the activity of other seaweed compounds (Kauffman, Kneivel, & Watschke, 2007), thus it is that two different continuous extraction procedures were developed:

1. Sequential aqueous extractions, where active compounds were firstly obtained under aqueous mild conditions and the rest of the compounds were extracted straightaway together with the carrageenan.

2. Extraction of carrageenan and active compounds assisted by hydrolysis: Enzymatic hydrolysis under alkaline conditions and moderate temperatures to favour the extraction, where

hydrolysates rich in active compounds were extracted simultaneously with carrageenan and subsequently separated by phycocolloid precipitation.

12.3.1.1.1. *Mastocarpus stellatus* biopolymeric extracts

FTIR results showed that native phycocolloids extracted by the aqueous process mainly contained κ/ι-hybrid carrageenan. A previous study about the nature of the carrageenan extracted from *Mastocarpus stellatus,* showed the predominant presence of the same hybrid (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009). Two different biopolymeric extracts were obtained in order to have two different raw materials susceptible to be used for different applications, that is, to develop different edible films.

M3 (3rd aqueous extract at 91 ºC for 1.5 h) presented characteristics quite similar to a pure carrageenan extract, since it contained the first soluble fraction remaining after mild aqueous extractions (mainly minerals), together with the highest proportion of soluble carrageenan and other polysaccharides.

M4 (4th aqueous extract at 91 ºC for 2 h) contained carrageenan in combination with the rest of the homogenized seaweed compounds that were not discarded by precipitation. Proteins, which are known to be abundant in red seaweeds (Fleurence, 1999), were mainly recovered in this extract, together with carrageenan and other polysaccharides.

M3 soluble fraction resulted in a less sulfated extract, with a lower amount of carrageenan precursors and also with a higher proportion of shorter soluble polysaccharide chains that resulted in a weaker cross-linked material (Van De Velde, Rollema, Grinberg, Burova, Grinberg, & Hans Tromp, 2002); while M4 protein content strengthened the extract through interaction with the carrageenan (Baeza, Carp, Pérez, & Pilosof, 2002).

12.3.1.1.2. *Mastocarpus stellatus* active extracts

Aqueous extraction did not involve any other chemical reactive to promote the extraction procedure apart from water, providing an easier and cheaper industrial application. M1 (1st aqueous extract) extraction at low temperatures (3 ºC) resulted in higher extraction yields and antioxidant activity than M2 (2nd aqueous extract) extraction under moderate temperatures (45 ºC) for short periods of time (45 min).

Although M1 and M2 active extracts will never reach the extraction yield values obtained in M3 and M4 biopolymer extractions, they represent an efficient antioxidant compound recovery phase during the integral seaweed extraction process (Fig. 12).

The extraction assisted by hydrolysis exhibited very different yields depending on whether there was a partial extraction of polyphenols (phenolic-partially removed hydrolysate H) or not (phenolic-containing hydrolysate Hp) (Figure 13). Other compounds apart from polyphenols (such as fat, pigments or even hydrophobic amino acids) were partially lost during the ethyl acetate extraction performed with H sample.

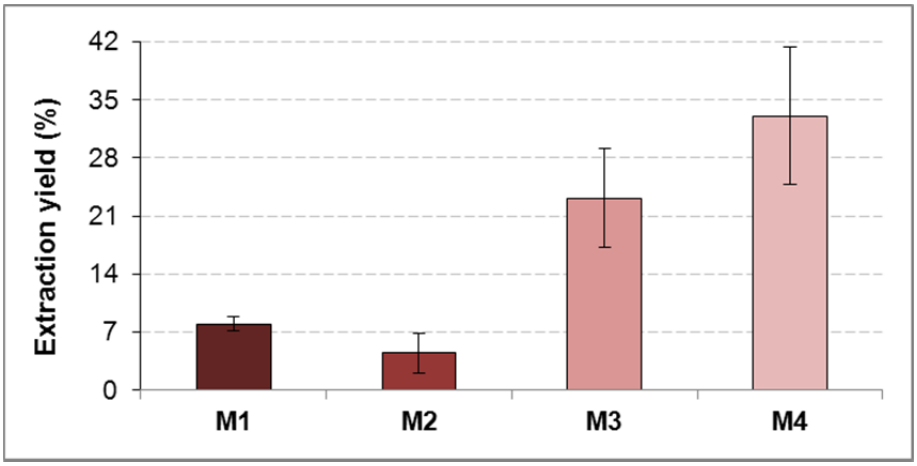

Figure 12. Extraction yield values of the different aqueous extracts consecutively obtained from dried *Mastocarpus stellatus.*

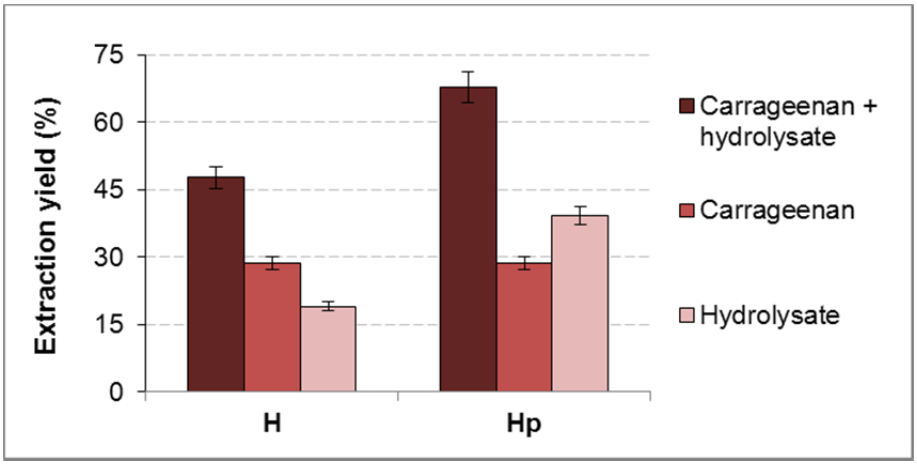

Figure 13. Extraction yield values of the integral *Mastocarpus stellatus* extraction (carrageenan fraction + hydrolysate) for the two different hydrolysis: phenolic-partially removed hydrolysate (H) and phenolic-containing hydrolysate (Hp).

The carrageenan fraction extracted from the enzymatic hydrolysis was considered to have characteristics similar to commercial carrageenan (Montolalu, Tashiro, Matsukawa, & Ogawa, 2008), presenting even the same extraction yield as the typical industrial process (28.65%) (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Pereira, Critchley, Amado, & Ribeiro-Claro, 2009). The present study was focussed on analysing whether the hydrolysis was useful or not for bioactive compounds extraction; hence no more studies regarding the carrageenan were performed. Nevertheless, in future works, it would be interesting to evaluate its physicochemical characteristics. According to Montolalu, Tashiro, Matsukawa, and Ogawa (2008), carrageenan with good functional gelling properties can be extracted at temperatures similar to those required for the hydrolysis (~50 ºC).

Antihypertensive and antioxidant activities were dependent on whether polyphenols were partially removed or not during the extraction procedure, obtaining the phenolic-partially removed (H) and phenolic-containing (Hp) *Mastocarpus stellatus* hydrolysates. Once polyphenols were removed, H

contained a higher hydrolyzed protein concentration, and therefore showed a higher antihypertensive capacity than Hp.

The antihypertensive activity of Mastocarpus hydrolysate had never been reported before, and its ACE inhibitory capacity resulted to be much higher than that reported for other red algae alcalase hydrolysates (He, Chen, Wu, Sun, Zhang, & Zhou, 2007; Qu, Ma, Pan, Luo, Wang, & He, 2010; Sato, Hosokawa, Yamaguchi, Nakano, Muramoto, Kahara, et al., 2002).

The polyphenol content in Hp resulted evident when looking at its antioxidant activity and amount of Folin reactive substances (Figure 14), which were much higher than in H. H radical scavenging ability was slightly lower than that of M1 and M2 aqueous extracts. The aqueous extracts were rich in polyphenols and soluble pigments bonded to proteins, while H mainly contained peptides and polyphenol residues. The polyphenol fraction extracted by alcalase hydrolysis (Hp) had an extremely higher antioxidant activity than the fraction obtained in the aqueous extracts (M1 and M2).

The results obtained from the *Mastocarpus stellatus* hydrolysis confirmed the previous assumptions about the possibility to enhance the extraction of bioactive compounds by enzymatic treatments (Cian, Alaiz, Vioque, & Drago, 2012; Heo, Park, Lee, & Jeon, 2005; Wang, Jónsdóttir, Kristinsson, Hreggvidsson, Jónsson, Thorkelsson, et al., 2010) and thus obtain both antihypertensive and antioxidant extracts.–The use of Mastocarpus hydrolysate as an ingredient for the development of functional food products might be interesting.

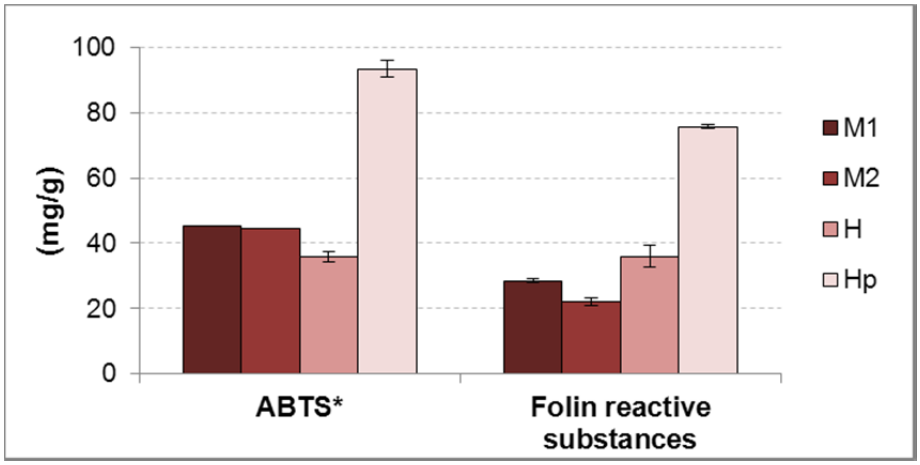

Figure 14. ABTS radical scavenging activity and Folin reactive substances content (mg/g) of M1 and M2 aqueous extracts, and H and Hp hydrolysates.
*mg vit C Eq/g film

Moderate temperatures (~50 ºC) have shown to be suitable conditions for the extraction of high molecular weight carrageenan from other red seaweed species (Montolalu, Tashiro, Matsukawa, & Ogawa, 2008), suggesting that during M2 aqueous extraction at 45 ºC and, specifically during Hp hydrolysis at 50 ºC, a certain amount of biopolymer was extracted (Nishinari & Watase, 1992). However, even at 4 ºC (M1) a little fraction of carrageenan content was also noticeable in its FTIR spectrum.

The water soluble sulfur-containing amino acids of the phycobiliproteins (Carra, Ó Heocha, & Carroll, 1964; O'Carra, Murphy, & Killilea, 1980), bonded to the phycobilin pigments (phycoerythrin and phycocyanin) (Lin & Stekoll, 2011), might have been first extracted in M1 (Cian, Martínez-Augustin, & Drago, 2012), and more effectively extracted by enzymatic digestion in Hp (Dumay, Clément, Morançais, & Fleurence, 2013). DSC results suggested a protein content in both M1 and Hp extracts (Mishra, Shrivastav, & Mishra, 2008), and FTIR spectra showed an increase in their sulfate content; this probably had an effect on the increase of the amount of Folin reactive substances, the radical scavenging activity and the reduction power.

The IR strong absorbance at 1600 and 1528 cm^{-1} for Hp indicated the more than likely presence of phycoerythrin and phycocyanin proteins (Smith & Alberte, 1994). The high 1217/924 cm^{-1} ratio assigned to an additional source of sulfate compounds also suggested the presence of phycobiliproteins and other sulfur-containing amino acids in Hp (Carra, Ó Heocha, & Carroll, 1964). The FTIR spectrum for Hp also showed a great absorption between 1100 and 1150 cm^{-1}, suggesting an increased proportion of shorter polysaccharide chains (Sun, Tao, Xie, Zhang, & Xu, 2010). Linear sulfated galactans can be cleaved by mild acid hydrolysis. A low temperature and acidic treatment could lead to oligosaccharide size and sulfation pattern modifications, whereas a strong acidic treatment might change the sulfation pattern and even destroy polysaccharide chains, resulting in lower molecular weight oligosaccharides that might have an improved bioactivity (Jiao, Yu, Zhang, & Ewart, 2011).

12.3.1.2. Brown seaweed extracts: *Laminaria digitata* and *Ascophyllum nodosum*

Brown seaweeds not only contain alginates, but also other polysaccharides such as laminaran and fucoidan, proteins, pigments and minerals; which might be interesting to extract as well. Unlike other authors, this study was focussed on optimizing both the acidic pretreatment (less strong acidic conditions) and a new alkaline treatment (NaOH at pH 10) in order to obtain unrefined extracts with high yields, complex compositions and a good filmogenic capacity (Arvizu-Higuera, Hernández-Carmona, & Rodríguez-Montesinos, 1997; Cardozo, Guaratini, Barros, Falcão, Tonon, Lopes, et al., 2007; Hernández-Carmona, McHugh, & López-Gutiérrez, 1999).

12.3.1.2.1. *Laminaria digitata* and *Ascophyllum nodosum* biopolymeric extracts

The new NaOH alkaline treatment approach allowed for an alginate extraction at a pH similar to that of the traditional sodium carbonate treatment (as shown in the FTIR spectra). In order to obtain a high yield (between 40-50%) together with a good filmogenic capacity, sodium hydroxide extractions required a strong acidic pretreatment and high treatment temperature (75 ºC).

In sodium hydroxide extractions, as shown in the proximate analysis, a higher proportion of carbohydrates were extracted and a considerable reduction of the salt content was achieved, as became clear from the observation of their ash content. Moreover, FTIR spectra confirmed that sulfated polysaccharides such as fucoidans were efficiently extracted with sodium hydroxide, whereas their presence was not registered when using sodium carbonate. Therefore, sodium hydroxide extraction would provide the industry with a good alternative to simplify the alginate extraction process and to obtain a good quality biopolymeric material for edible technological applications, such as film coating development.

Extraction procedures were also selected based in the purpose of softening as much as possible the acidic pretreatments. Despite hydrochloric acid pretreatments resulted in lower extraction yields than sulfuric acid, the latest caused a noticeable degradation of the targeted polysaccharides (Hahn, Lang, Ulber, & Muffler, 2012).

It was also seen that the partial alginate degradation, possibly caused by the high temperatures used during the alkaline treatment (Vauchel, Kaas, Arhaliass, Baron, & Legrand, 2008), might favour matrix interconnections.

FTIR results showed that sodium hydroxide extraction recovered a higher proportion of preserved alginate units, whereas sodium carbonate extractions recovered a higher amount of dispersed uronic acids. Sodium hydroxide alginates formed strong bonds with water molecules, while sodium carbonate shorter alginate chains resulted in weak interactions, as was evident from their respective DSC thermal transitions.

12.3.1.3. Seaweed extracts comparative

As observed in Figure 15, the integral Mastocarpus crude aqueous extraction yield was not significantly different from the extraction yield obtained with the phenolic-containing hydrolysate. Hence, both methods were good as an alternative option to the conventional red seaweed extractions (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006).

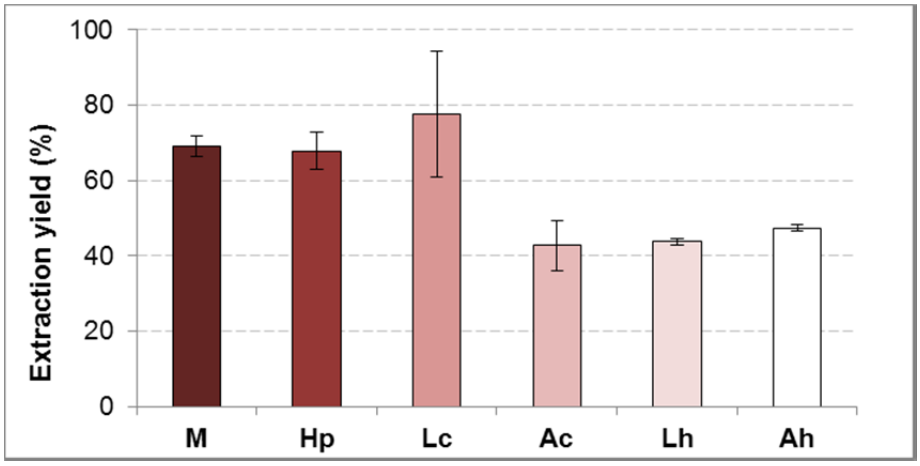

Figure 15. Extraction yields for Mastocarpus aqueous extraction (M = M1+M2+M3+M4), Mastocarpus phenolic-containing hydrolysate (Hp) extraction, sodium carbonate (c) and sodium hydroxide (h) extractions of Laminaria (L) and Ascophyllum (A).

Alkaline enzymatic hydrolysis under moderate temperatures might have provided conditions suitable for both bioactive compounds and carrageenan extractions, and resulted in good yields (Montolalu, Tashiro, Matsukawa, & Ogawa, 2008; Wang, et al., 2010), despite the temperatures were not as high as the ones used in traditional carrageenan extractions (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006).

The different composition observed in the proximate analysis for the original dried seaweeds partially determined the composition of the extracts (Figure 16), such as the highest protein content

observed in red seaweeds or, in the case of brown algae, a higher protein content in Laminaria than in Ascophyllum extracts.

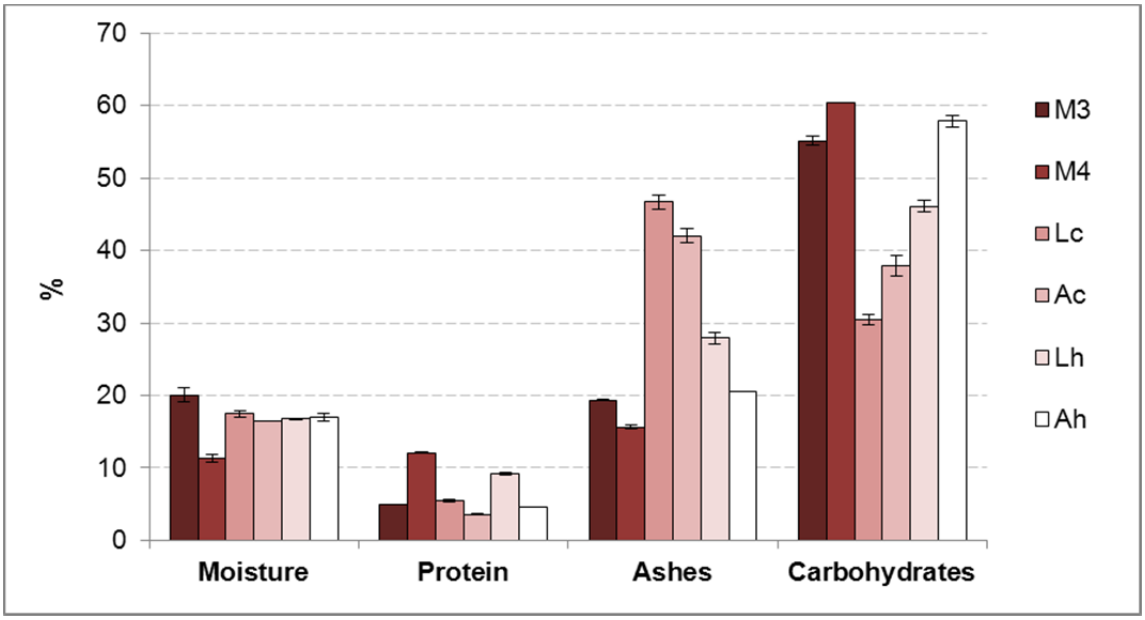

Figure 16. Proximate analysis of M3 and M4 Mastocarpus extracts, and of sodium carbonate (c) and sodium hydroxide (h) extracts of Laminaria (L) and Ascophyllum (A).

It is interesting to note that M4 and Lh were the most efficient protein extractions. Mastocarpus (M) sequential extraction and (Lh and Ah) sodium hydroxide extractions presented ash content similar to that of the original dried seaweeds, while sodium carbonate extraction provided an excessive amount of salt, probably derived from the alkaline treatment.

Mastocarpus extraction yields were much higher than those reported in previous studies about carrageenan (Pereira, Critchley, Amado, & Ribeiro-Claro, 2009) and alginate traditional extractions (Istini, Ohno, & Kusunose, 1994; Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009). However, brown seaweed sodium carbonate extraction achieved the same range of carbohydrate extraction as the traditional methods (20-38%), which are commonly used in the industry.

Red seaweed crude aqueous extractions and brown seaweed sodium hydroxide extractions were the most efficient procedures, showing the corresponding typical well preserved carrageenan and alginate FTIR spectra profiles (Fig. 17).

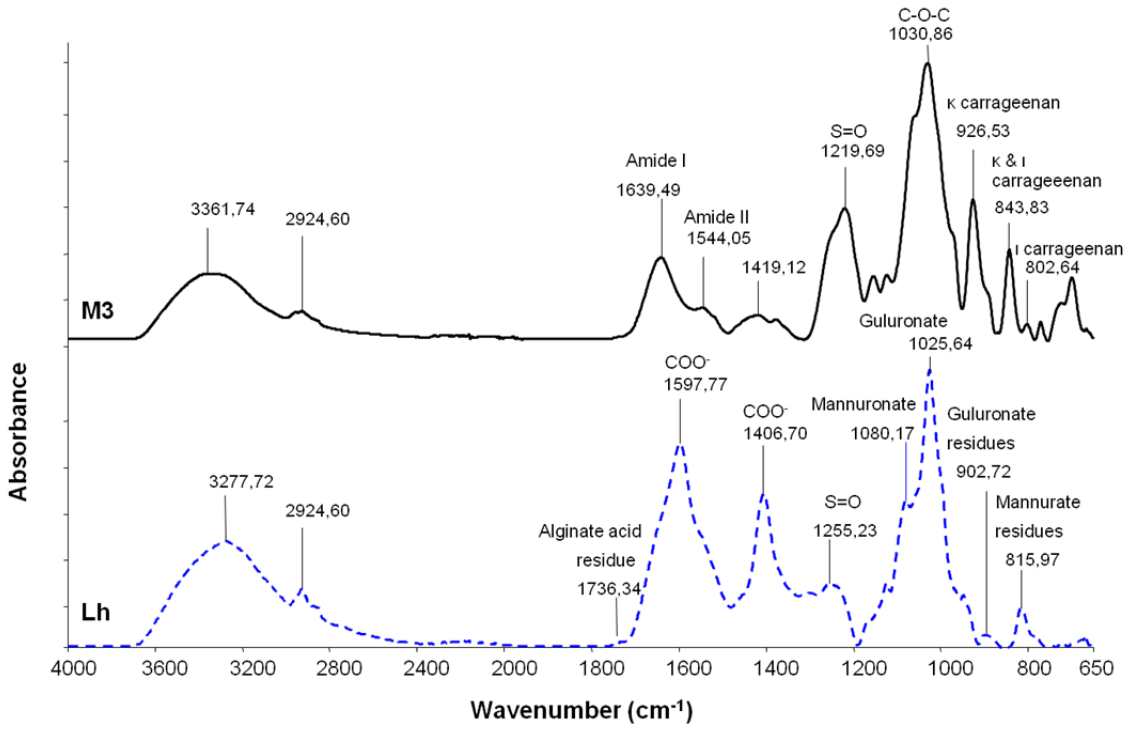

Figure 17. ATR-FTIR spectra of the typical κ/ι-hybrid carrageenan in Mastocarpus extract (M3) and the typical alginate profile in Laminaria sodium hydroxide extract (Lh).

12.3.2. Seaweed films

In order to obtain different edible films for various food applications, the filmogenic capacity of each seaweed extract was evaluated. In this regard, carrageenan or alginate cation complexation was not performed. The aim of this study was to produce a variety of edible films as novel food byproducts or food coatings, more than to produce extremely resistant films as plastic bags. Protein-polysaccharide mixtures are widely used in the *nouvelle cousin* due to the fact that they play an essential role in the microstructure of the final product. The film-coating materials proposed in the present study would offer the market a practical option to optimize the rational approaches to food design (Arboleya, Olabarrieta, Luis-Aduriz, Lasa, Vergara, Sanmartín, et al., 2008).

Furthermore, the combination of these extracts would open up some interesting possibilities for the production of a diversity of high quality and healthy edible films.

12.3.2.1. *Mastocarpus stellatus* films

12.3.2.1.1. Physicochemical properties of *Mastocarpus stellatus* films

Mastocarpus biopolymeric extracts were combined in different ways for the development of edible films. Three different films were obtained: M3, M4 and M3+M4 films. Since M3 extract was the sample with more similarities to commercial carrageenan, the same applied to the resulting films, being more transparent and flexible. Due to the M4 high protein content, the resulting films were stronger. M4 protein content also promoted a more opaque polysaccharide-protein matrix showing a higher mechanical strength and water resistance. Therefore, the mixture of M3 and M4 for film development led to M3+M4 films with intermediate physicochemical characteristics.

In order to improve the mechanical strength and water resistance of M3+M4 film, the glycerol content was reduced from 0.6% to 0.2% w/v, resulting in M3+M4-10 film (Cerqueira, Souza, Teixeira, & Vicente, 2012; Karbowiak, Hervet, Léger, Champion, Debeaufort, & Voilley, 2006). Although there are many studies about how glycerol content affects protein film properties, there are not so many regarding carrageenan films (Huber, 2009; Karbowiak, Hervet, Léger, Champion, Debeaufort, & Voilley, 2006; Moreira, Chenlo, Torres, Silva, Prieto, Sousa, et al., 2011).

Carrageenan films, developed without cation complexation, are known for having a low water resistance (Shojaee-Aliabadi, Hosseini, Mohammadifar, Mohammadi, Ghasemlou, Ojagh, et al., 2013). In this study, once water contacted Mastocarpus films, a matter-transferring flux was noticeable from the film to the water. During the water resistance test, M3 films even sustained some water filtration prior to film breakage.

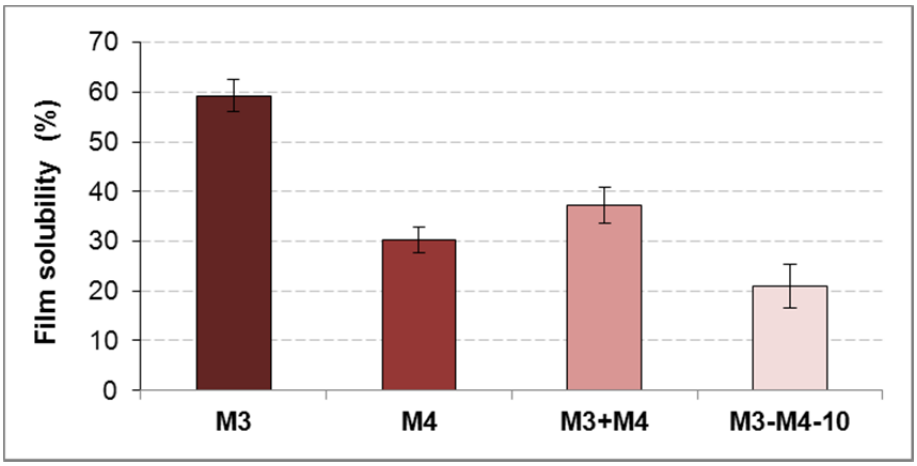

Figure 18. Film solubility of M3, M4, M3+M4 and M3+M4-10 films.

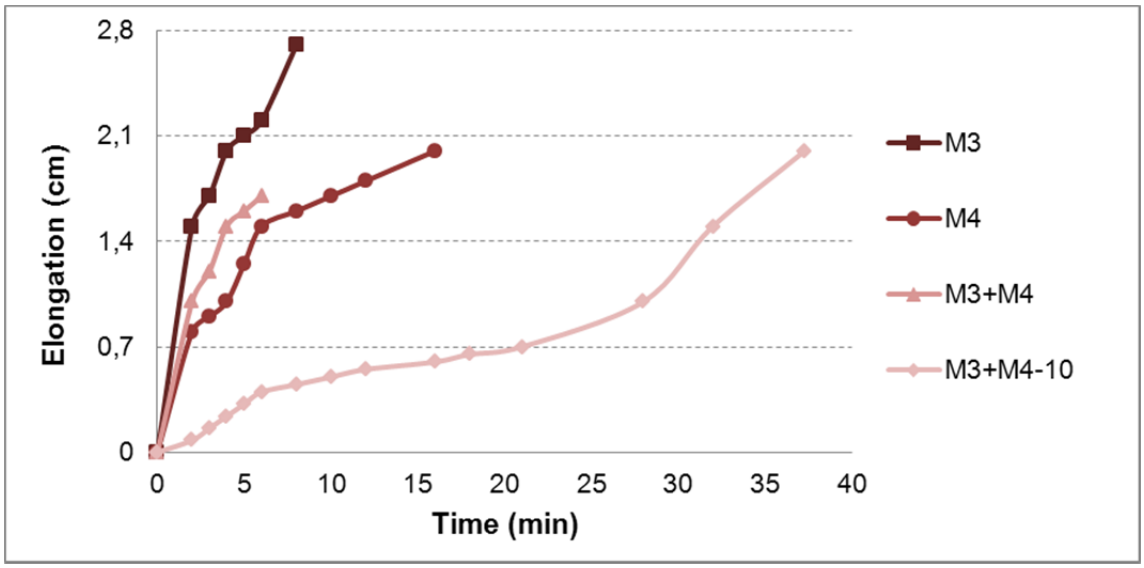

Figure 19. Water resistance of M3, M4, M3+M4 and M3+M4-10 films.

As expected, the low glycerol content in M3+M4-10 significantly improved the water barrier properties of the film. Both the film solubility and the breakage time were considerably reduced when water was poured over these films (Figs. 18 and 19), probably due to the hygroscopicity reduction caused

by the low glycerol proportion (Vieira, da Silva, dos Santos, & Beppu, 2011). However, the water vapour permeability was not significantly modified in any of the different Mastocarpus film combinations.

It is important to note that carrageenan films totally lost their integrity when they came into contact with water, forming a viscous swelling gel at room temperature, especially M3 films. Previous studies showed that ι-carrageenan films experienced a drastic increase in the diffusion of small molecules, hydration and swelling effect with glycerol proportions higher than 0.9% (Karbowiak, Hervet, Léger, Champion, Debeaufort, & Voilley, 2006).

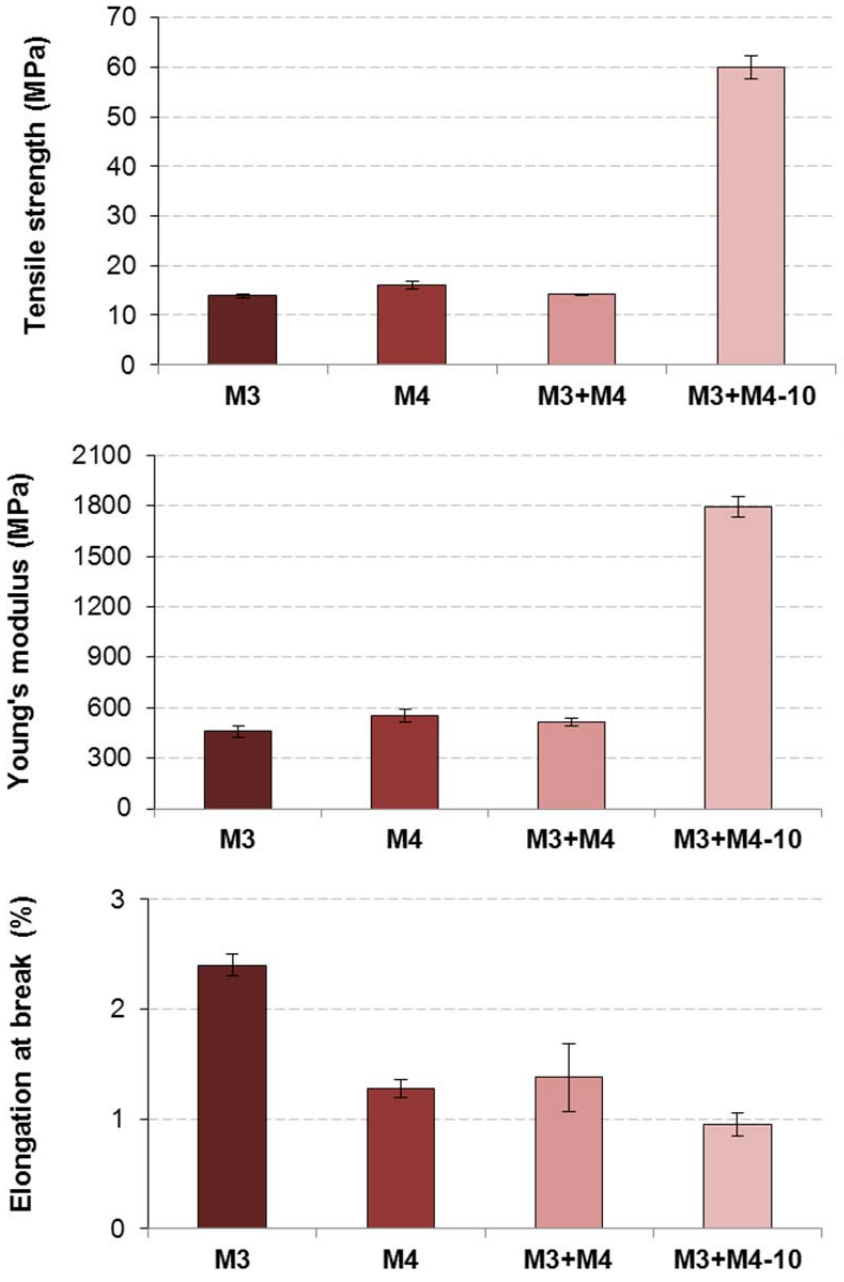

Figure 20. Tensile strength, Young's elastic modulus and elongation at break of M3, M4, M3+M4 and M3+M4-10 films.

The M3+M4-10 film also showed a significant improvement of both the tensile strength and the elastic modulus (Figure 20), resulting in a great film stiffness increase, which was even higher than the results obtained in studies with commercial carrageenan films (Rhim, 2012). The low plasticizer content probably promoted protein-carrageenan bonds instead of protein-glycerol and carrageenan-glycerol interactions.

M3 film showed the highest flexible response to water pressure and tensile strength (Figs. 19 and 20), exhibiting the highest elongation capacity in both water resistance and tensile tests. The reduction of the amount of plasticizer in M3+M4-10 film led to a reduction of the elongation at break. A reduction in film flexibility was also observed when the plasticizer content was reduced in protein films (Sobral, García, Habitante, & Monterrey-Quintero, 2004).

FTIR spectra revealed a higher proportion of more sulfated carrageenan in M4 extract, which might have boosted the formation of covalent bonds during the drying process and, together with the protein content, might have formed a strong protein-carrageenan matrix (Baeza, Carp, Pérez, & Pilosof, 2002). On the contrary, M3 shorter polysaccharide chains linked more efficiently to glycerol, and the lower sulfate content resulted in a physically weak cross-linked matrix (Van De Velde, Rollema, Grinberg, Burova, Grinberg, & Hans Tromp, 2002).

On the one hand, DSC thermograms showed that M3+M4-10 film resulted in the strongest structure due to the reduction in the plasticizer content, producing a film matrix with almost the same thermal behaviour as M4. On the other hand, M3 polysaccharide nature probably led to a more plasticized matrix, with a more gel-like structure, less resistant to water but malleable.

12.3.2.1.2. Antioxidant activity of *Mastocarpus stellatus* films

Commercial carrageenan films are not distinguished for having an outstanding antioxidant activity (Shojaee-Aliabadi, et al., 2013).

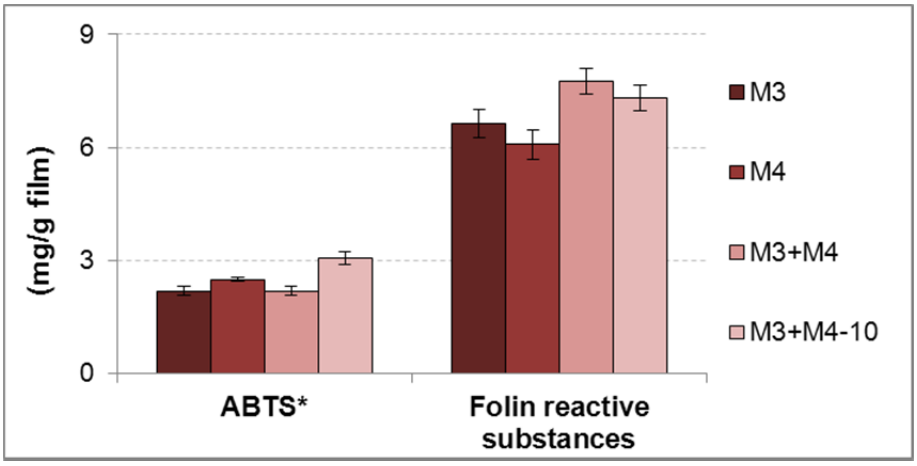

Figure 21. ABTS radical scavenging capacity and Folin reactive substances of M3, M4, M3+M4, and M3+M4-10 films.
*mg vit C Eq/g film

The sulfated content in κ-carrageenan derivatives might have contributed to the little ABTS radical scavenging capacity and the Folin reactive substances content in Mastocarpus films (Fig. 21) (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007). Free amino acids, other sugars such as neutral compounds and uronic acids could also react with the Folin reactive substances (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010; Singleton, Orthofer, & Lamuela-Raventós, 1998; Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, et al., 1985).

12.3.2.2. *Laminaria digitata* and *Ascophyllum nodosum* films

12.3.2.2.1. Physicochemical properties of *Laminaria digitata* and *Ascophyllum nodosum* films

Respective brown seaweed sodium carbonate and sodium hydroxide extracts from Laminaria and Ascophyllum were used to develop edible films, in order to compare how the extraction procedure and the species affect the filmogenic properties.

Alginate was the main component of the brown seaweed extracts, and since no cation complexation was carried out during the film development, films were mainly formed by a hydrogen-bonded porous matrix (Fig. 22). The pore size affected the film properties, leading to tolerable mechanical properties and a low water resistance.

The alkaline treatment determined the film water barrier properties, while the seaweed species affected the film mechanical properties. While water barrier properties seemed to be more influenced by the polymer chain status after the alkaline treatment, the mechanical properties were determined by the protein content.

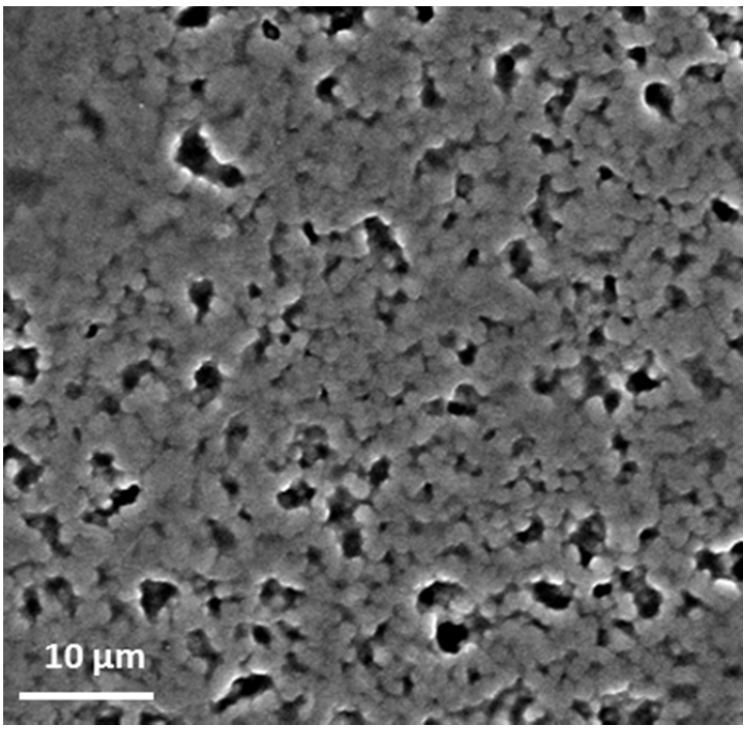

Figure 22. Low Temperature-Scanning Electron Microscopy surface image of a Laminaria sodium carbonate film (Lh).

Although the Laminaria sodium carbonate (Lc) extract resulted in the highest extraction yield, the films developed did not exhibit any outstanding physicochemical characteristic. Therefore, the Lc film thickness was tripled by increasing three times the solid content (Lc-3) in an attempt to improve its quality. Polysorbate 80 surfactant was also added to the Lc-3 composition, in a 0.1% (w/v) proportion, with the purpose of improving the interfacial tension between the film forming solution and the solids that might be added to change the physical characteristics of the film. Therefore, the way the surfactant might affect the film properties could also be analysed.

Sodium hydroxide films presented the lowest solubility and water vapour permeability values (Fig. 23), probably due to the higher presence of carbohydrates and to a higher proportion of guluronic units, as it was observed in the FTIR spectra. Guluronic units tended to form stronger bonds (Gómez-Ordóñez & Rupérez, 2011) and probably resulted in a more alginate-alginate and alginate-protein cross-linked matrix (Siddaramaiah, Swamy, Ramaraj, & Lee, 2008), as confirmed by the DSC results.

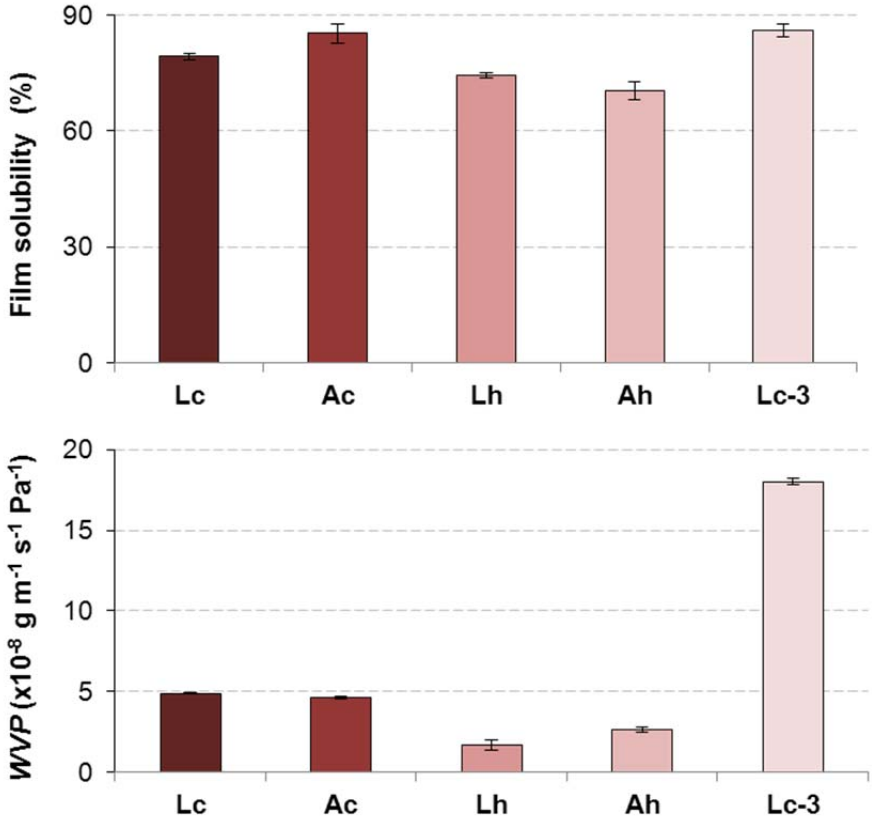

Figure 23. Film solubility and water vapour permeability (*WVP*) of Laminaria (L) and Ascophyllum (A) sodium carbonate (c) and sodium hydroxide (h) films and Laminaria sodium carbonate x3 (Lc-3) film.

On the contrary, sodium carbonate treatment led to a higher amount of uronic acid residues that might have linked more efficiently to glycerol and resulted in less alginate-alginate cross-linked films, as FTIR and DSC results revealed. This fact in combination with the high mineral content led to weaker interactions.

The high solids concentration in Lc-3 film, together with the presence of surfactant, adversely affected its water barrier properties, being more soluble than most of the films and much more permeable

to water vapour, probably due to the higher glycerol presence in combination with the surfactant. Polysorbate 80 is a non-ionic detergent derived from sorbitol and might increase the plasticizer effect.

Despite the higher protein content in Laminaria did not determine its solubility or *WVP*, it might be responsible for the water resistance of the film (Fig. 24). Laminaria films, compared to Ascophyllum ones, resisted minutes prior to breakage, especially Lh that resisted more than 1 hour. Comparing Laminaria films, the sodium hydroxide treatment provided the more compact conformation, resulting in a reinforced film network that improved both the water vapour permeability and the water resistance (Figs. 23 and 24). The Lh extract contained the highest proportion of protein and amount of well preserved alginate units.

Glycerol and polysorbate 80 increased the film elongation at break but, despite its higher thickness, Lc-3 resulted in a less water resistant film than Lc. This behaviour confirmed the increase in plasticity gained by the high plasticizer proportion and the surfactant addition.

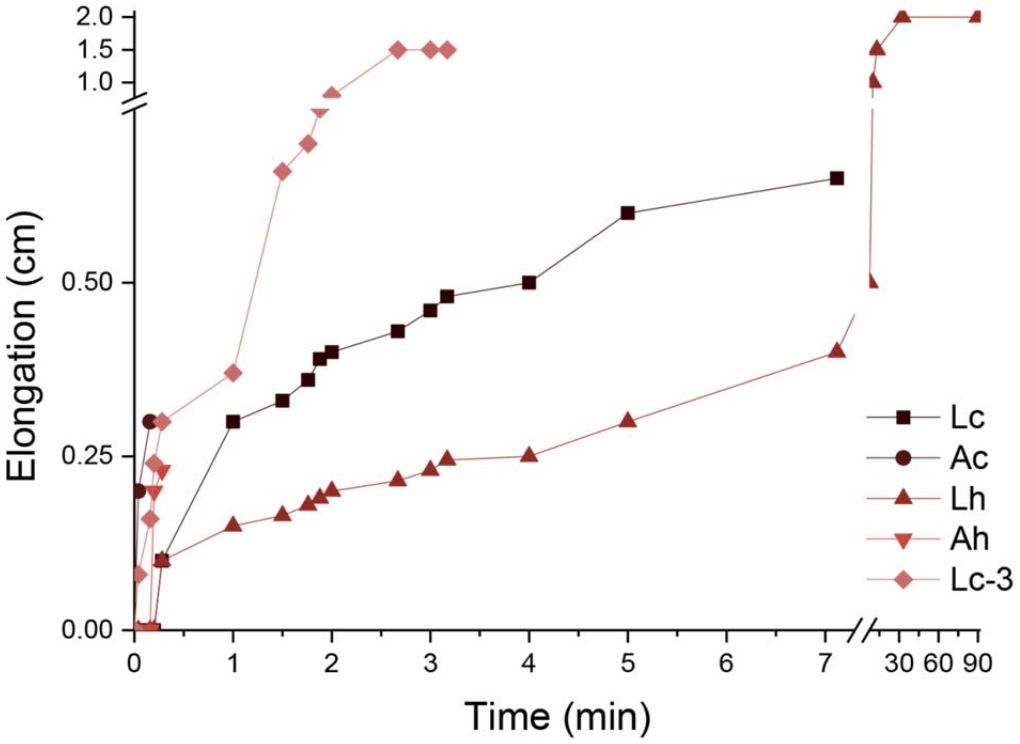

Figure 24. Water resistance of Lc, Ac, Lh, Ah and Lc-3 films.

Mechanical tests were differently affected by the extraction treatment carried out according to the species. The tensile strength and elastic modulus values for the sodium hydroxide treatment were higher when comparing the corresponding species with the values obtained for the sodium carbonate treatment. Tensile strength and puncture force were higher with the sodium hydroxide treatment, reaching significantly higher values in the Lh film. Meanwhile, sodium carbonate extractions obtained higher elongation at break values, reaching in the Ac film significantly higher elongation values in both tensile and puncture tests (Figs. 25 and 26). The surfactant plasticizing effect increased the puncture deformation in Lc-3 film, but not the elongation at break in a tensile test.

The higher proportion of longer guluronate segments in Lh, together with the high protein content, led to the best organized association of molecular chains in the film matrix, resulting in the strongest film in both tensile and puncture tests. Sodium carbonate treatment led to a higher dispersed uronic acid proportion, which conferred a more efficient plasticizing effect to the Ac film, as shown by its elongation at break and puncture deformation values.

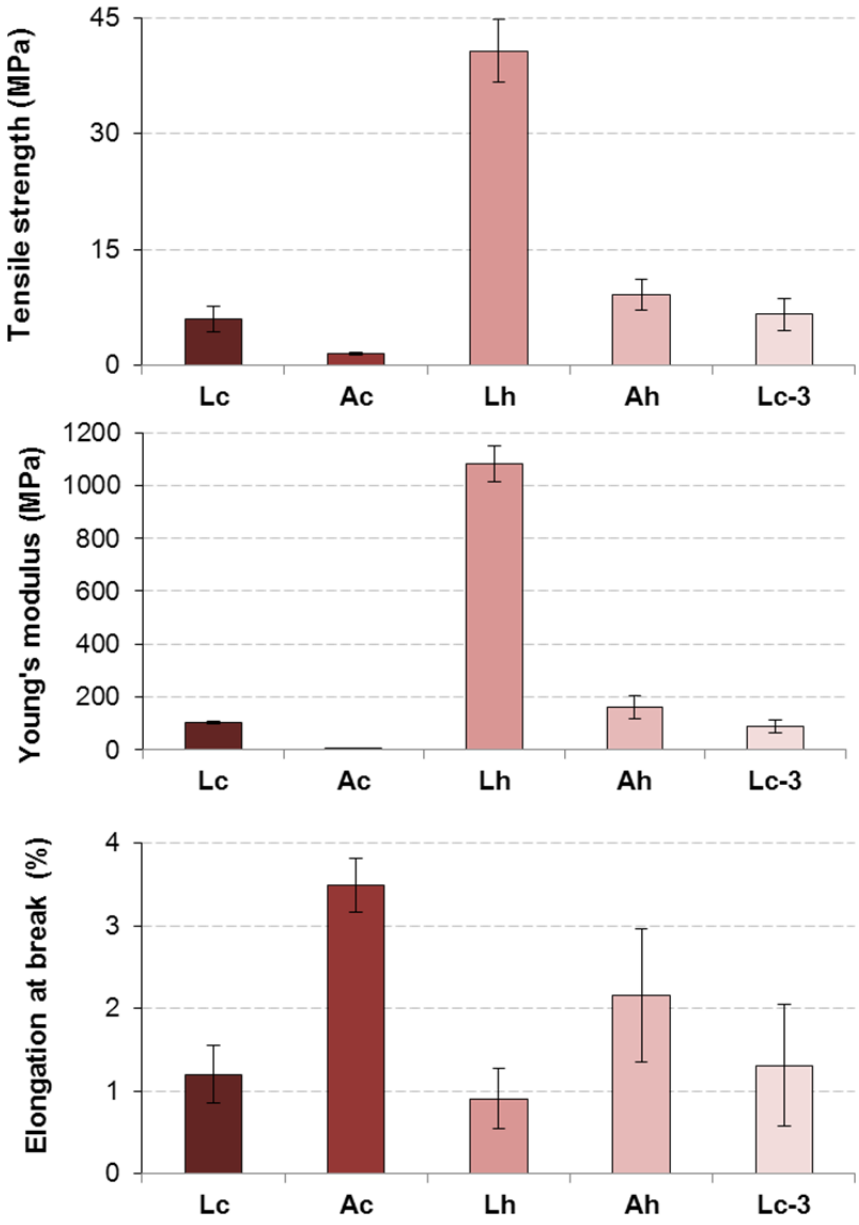

Figure 25. Tensile strength, Young's elastic modulus and elongation at break of Lc, Ac, Lh, Ah and Lc-3 films.

Nevertheless, the sodium carbonate extract richness in dispersed uronic acids, together with the higher alginate degradation experienced by the H_2SO_4 pretreatment, resulted in a poor mechanical strength. Lc-3 did not show an improvement in its tensile strength, but its puncture strength results were positively affected, showing a significant improvement in both the puncture force and deformation values as a result of the increase in film thickness.

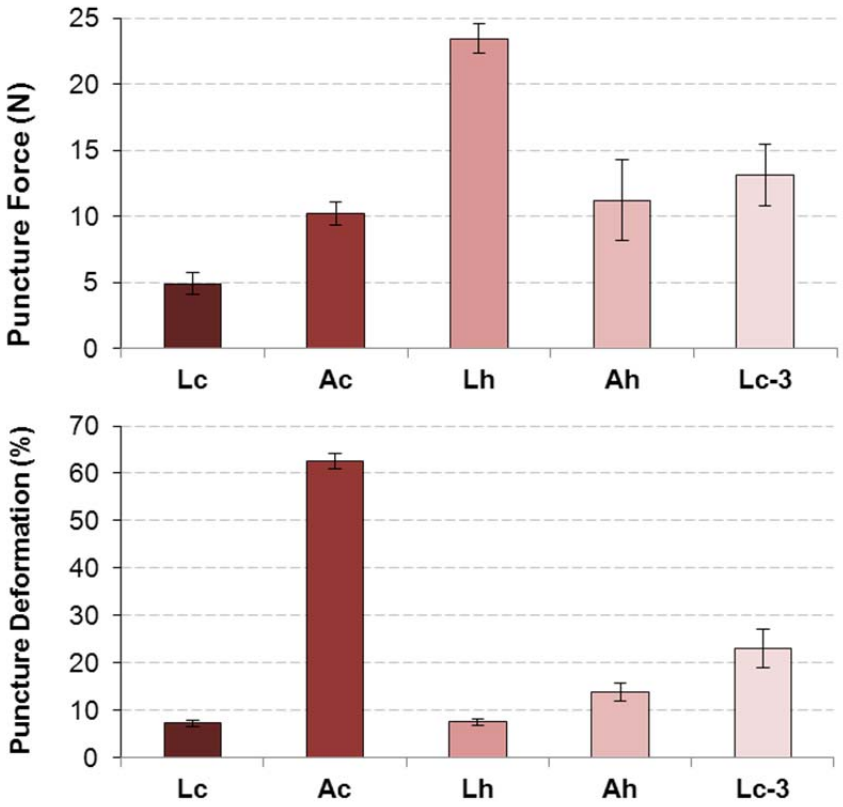

Figure 26. Puncture force and puncture deformation of Lc, Ac, Lh, Ah and Lc-3 films.

12.3.2.2.2. Antioxidant activity of *Laminaria digitata* and *Ascophyllum nodosum* films

The ABTS radical scavenging capacity of the films was more influenced by the seaweed species, while the Folin reactive substances content was determined by the alkaline treatment (Figure 27). Ascophyllum showed a higher antioxidant activity, although the effect was magnified by the sodium hydroxide treatment.

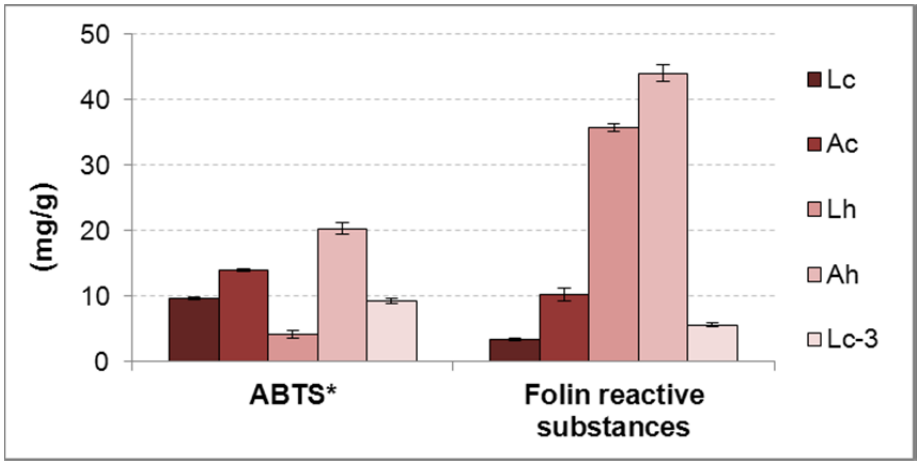

Figure 27. ABTS radical scavenging capacity and Folin reactive substances of Lc, Ac, Lh, Ah and Lc-3 films.

*mg vit C Eq/g film

Seaweed antioxidant activity is normally affected by the interaction of different factors such as sulfation level, molecular weight and sugar residue composition (Jiménez-Escrig, Gómez-Ordóñez, & Rupérez, 2011). However, alginates are not distinguished for having an outstanding antioxidant activity (Norajit, Kim, & Ryu, 2010), which might explain their low ABTS values.

Polyphenols have been reported to be one of the brown algae minor components (Rupérez & Saura-Calixto, 2001), suggesting that Folin reactive substances might largely correspond to other compounds. Another factor to take into account is that the antioxidant activity of the film might have also been adversely affected by the processing and storage conditions (Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001). Since carotenoids are known to act as antioxidants, the presence of fucoxanthin might have greatly contributed to the antioxidant activity detected (Le Tutour, Benslimane, Gouleau, Gouygou, Saadan, & Quemeneur, 1998). The high content in Folin reactive substances found in sodium hydroxide films could also correspond to other compounds such as aromatic amino acids and short-chain carbohydrates (Ikawa, Schaper, Dollard, & Sasner, 2003; Singleton, Orthofer, & Lamuela-Raventós, 1998; Smith, et al., 1985).

Moreover, the highest sulfation level of Ah films observed in their FTIR spectra might correspond to their highest fucoidan proportion, which is known for its antioxidant activity; therefore resulting in the highest ABTS radical scavenging capacity and Folin reactive substances content (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

Lc-3 films showed the same ABTS radical scavenging capacity as Lc, but polysorbate 80 might have interacted with the Folin reactive substances resulting in the detection of a slightly higher amount of Folin reactive substances.

12.3.2.3. Seaweed films comparative study

Red and brown seaweed biopolymeric extracts resulted in films of different polysaccharide nature. According to the origin of each extract, Mastocarpus films mainly contained carrageenan, while Laminaria and Ascophyllum films essentially contained alginates. All the films were developed without cation complexation.

Mastocarpus films were less water soluble than brown seaweed films; especially M3+M4-10, due to the low plasticizer content (Table 2). Brown seaweed sodium carbonate films were the most soluble, probably due to the more efficient plasticization boosted by the high content of dispersed uronic acids in both Lc and Ac films, the higher proportion of degraded alginates in the Lc film and the addition of surfactant to the Lc-3 film.

Regarding the water vapour permeability (*WVP*), brown seaweed sodium hydroxide extracts resulted in the most impermeable films, while sodium carbonate extracts led to the most permeable films, character that was especially increased when adding a surfactant to Lc-3 film (Table 2). The higher proportion of preserved alginate units recovered with the sodium hydroxide treatment resulted in a stronger and more efficiently cross-linked matrix than anyone obtained from any Mastocarpus carrageenan combination.

The solubility and *WVP* of red seaweed (Mastocarpus) films, developed with 30% glycerol, were higher in comparison with the results reported for commercial carrageenan films developed without cation complexation (Karbowiak, Debeaufort, & Voilley, 2007; Rhim, 2012; Shojaee-Aliabadi, et al., 2013). M3+M4-10 film, with 10% glycerol, presented the lowest solubility values among all the red and brown seaweed films assayed.

Despite brown seaweed films solubility was lower than that of films developed with commercial sodium alginate without cation complexation (Abdollahi, Alboofetileh, Rezaei, & Behrooz, 2013; Zactiti & Kieckbusch, 2006), the *WVP* was higher (Benavides, Villalobos-Carvajal, & Reyes, 2012; Tapia, Rojas-Graü, Rodríguez, Ramírez, Carmona, & Martin-Belloso, 2007).

Film	Film solubility (%)	*WVP* ($x10^{-8}$ g m^{-1} s^{-1} Pa^{-1})	Water resistance to breakage time (min)	Film elongation at breakage time (cm)
M3	59.24 ± 3.29	3.57 ± 0.05	9.33 ± 0.58	2.7 ± 0.02
M4	30.36 ± 2.59	3.62 ± 0.01	18 ± 6.08	2 ± 0.02
M3+M4	37.26 ± 3.61	3.64 ± 0.08	8 ± 3.46	1.7 ± 0.01
M3-M4-10	20.97 ± 4.5	3. 78 ± 0. 17	37.29 ± 10.48	2 ± 0.01
Lc	79.23 ± 0.91	4.90 ± 0.07	7.2 ± 0.71	0.6 ± 0.01
Ac	85.27 ± 2.49	4.64 ± 0.08	0.16 ± 0.08	0.3 ± 0.01
Lh	74.42 ± 0.76	1.66 ± 0.32	106.8 ± 30	2 ± 0.02
Ah	70.33 ± 2.37	2.62 ± 0.16	0.28 ± 0.05	0.2 ± 0.01
Lc-3	86.04 ± 1.66	18.0 ± 0.2	3.17 ± 0.76	1.5 ± 0.01

Table 2. Film solubility and water vapour permeability (*WVP*) of M3, M4, M3+M4, M3+M4-10, Lc, Ac, Lh, Ah and Lc-3 films.

In summary, the compounds extracted in combination with the phycocolloids during the seaweed unrefined extractions did not improve the water barrier properties in red seaweed films, or the *WVP* in brown seaweeds, but reduced the solubility of sodium alginate films.

The water resistance results (Table 2) were influenced by the protein content in both red and brown seaweeds, being more resistant those films developed with extracts rich in proteins (M4 and Laminaria). As observed for film solubility, the plasticizer content and the alginate units integrity highly affected the water resistance of the films, which was increased when the glycerol content was low (M3+M4-10 film) and preserved alginates formed more compact matrixes (Lh film). Except for Lh film, Mastocarpus films resisted more efficiently the water pressure, with a higher elongation response than brown seaweed films, and Ascophyllum films lost their integrity as soon as water contacted the matrix.

Broadly speaking, tensile test results (Table 3) confirmed that red seaweed films presented a higher mechanical strength than brown seaweeds, resulting in higher *TS* and *Y* values. As previous results have shown, the low plasticizer content in M3+M4-10 film and the highly cross-linked Lh film matrix made them the strongest films. Regarding *EAB* results, they depended on the polymer-glycerol

bonding capacity, being Ascophyllum and M3 films the most efficiently plasticized matrixes. The more carrageenan-like M3 film matrix and the uronic units structure in Ascophyllum films, linked more easily to glycerol, resulting in the most flexible films; while the rest of the films presented a poor plastic behaviour.

Film	*TS* (MPa)	*EAB* (%)	*Y* (MPa)
M3	13.88 ± 0.39	2.40 ± 0.1	459.12 ± 35.77
M4	16.16 ± 0.76	1.28 ± 0.08	552.63 ± 36.61
M3+M4	14.13 ± 0.18	1.38 ± 0.31	516.77 ± 23.58
M3-M4-10	59.94 ± 2.27	0.95 ± 0.11	1797.49 ± 61.28
Lc	6.01 ± 1.58	1.20 ± 0.35	102.65 ± 3.16
Ac	1.50 ± 0.19	3.49 ± 0.32	5.99 ± 0.51
Lh	40.72 ± 4.05	0.91 ± 0.37	1081.93 ± 67.37
Ah	9.13 ± 1.98	2.16 ± 0.81	159.56 ± 42.84
Lc-3	6.63 ± 2.12	1.31 ± 0.74	88.02 ± 24.77

Table 3. Tensile strength (*TS*), elongation at break (*EAB*) and Young's elastic modulus (*Y*) for M3, M4, M3+M4, M3+M4-10, Lc, Ac, Lh, Ah and Lc-3 films.

Red seaweeds tensile strength was lower than previous results obtained for films developed with commercial κ-carrageenan (Rhim, 2012; Shojaee-Aliabadi, et al., 2013), but higher than the results for commercial ι-carrageenan films (Hambleton, Perpiñan-Saiz, Fabra, Voilley, & Debeaufort, 2012), confirming a higher κ-units proportion in the κ/ι-hybrid carrageenan extracted. Apart from the strength of Lh films, the rest of the brown seaweed films presented lower *TS* values than commercial alginate films (Benavides, Villalobos-Carvajal, & Reyes, 2012). *EAB* and *Y* values were in general on the same range as previous results obtained for films developed with commercial alginates (Avella, Pace, Immirzi, Impallomeni, Malinconico, & Santagata, 2007; Yoo & Krochta, 2011).

In conclusion, the complex composition of the seaweed extracts resulted in red seaweed films with intermediate mechanical properties compared to the commercial carrageenan ones, while it did not significantly affect the mechanical properties of brown seaweed films compared to the commercial alginate ones.

Concerning the ABTS radical scavenging capacity and the Folin reactive substances content (Table 4), red seaweed films showed a lower radical scavenging ability than brown seaweeds, and Ascophyllum films presented the highest antioxidant activity. The Folin reactive substances content and the antioxidant activity vary with the species, but in general, brown seaweeds have higher free-radical scavenging properties than red seaweeds (Mohamed, Hashim, & Rahman, 2012).

Brown seaweed extraction procedures might have boosted Ascophyllum sulfated polysaccharides extraction, being decisive for their rheological and antioxidant properties (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007). Moreover, Ascophyllum alginate gels have been previously reported to show properties different from those typical of other brown seaweed alginates (Rioux, Turgeon, & Beaulieu, 2007; Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva,

Oliveira Rocha, & Leite, 2007), which might explain why their corresponding films resulted so mechanically different from Laminaria films.

Sample	ABTS (mg/g)	Folin reactive substances (mg/g)
M3	2.19 ± 0.11	6.64 ± 0.37
M4	2.5 ± 0.06	6.08 ± 0.4
M3+M4	2.19 ± 0.11	7.75 ± 0.34
M3+M4-10	3.07 ± 0.18	7.33 ± 0.34
Lc	9.72 ± 0.19	3.37 ± 0.24
Ac	14.02 ± 0.20	10.23 ± 0.98
Lh	4.20 ± 0.51	35.76 ± 0.67
Ah	20.36 ± 0.90	44.02 ± 1.26
Lc-3	9.22 ± 0.40	5.62 ± 0.3

Table 4. ABTS radical scavenging capacity and Folin reactive substances of M3, M4, M3+M4, M3+M4-10, Lc, Ac, Lh, Ah and Lc-3 films.

Brown seaweed sodium hydroxide extraction resulted in the highest amount of Folin reactive substances, which might be related to a more efficient extraction of different proteins, pigments and sugars (Ikawa, Schaper, Dollard, & Sasner, 2003; Singleton, Orthofer, & Lamuela-Raventós, 1998), and which, in turn, would have also improved film water vapour permeability.

In conclusion, Mastocarpus films did not show a significant improve in the antioxidant activity compared to commercial carrageenan films (Shojaee-Aliabadi, et al., 2013), while brown seaweed films, especially Ascophyllum, resulted in a significant improvement of the poor antioxidant activity showed by commercial alginate films (Norajit, Kim, & Ryu, 2010).

12.3.3. Seaweed active films

The direct addition of two Mastocarpus seaweed active extracts (aqueous extract and hydrolysate) to the film forming solution was tested for the development of Mastocarpus antioxidant films. On the other hand, Laminaria films were tested as carriers of lipid microcapsules filled with an active collagen hydrolysate extracted from *Dosidicus gigas* tunics.

In the case of *Mastocarpus stellatus* active film, the antioxidant activity of the film was measured in the first place, and secondly, the modification of the physicomechanical properties of the film was evaluated.

For the inclusion of microcapsules in *Laminaria digitata* films, the microencapsulation procedure was tested in the first place, and followed by the evaluation of both the microcapsules and the modification of the physicomechanical properties of the film due to their presence.

12.3.3.1. *Mastocarpus stellatus* active films

Two different types of bioactive extracts were selected to develop Mastocarpus antioxidant films, thus broadening the potential of Mastocarpus films for food applications.

12.3.3.1.1. Aqueous extract addition

Regarding M1 and M2 Mastocarpus aqueous extracts (see figure 14), M1 was selected for the development of antioxidant films, due to its high extraction yield, high ABTS radical scavenging capacity and Folin reactive substances content.

M1 was added at 25% (w/w) of the M3+M4 dried seaweed extract. M3+M4+M1 film improved 30 times the radical scavenging ability of M3+M4 film and 5 times the amount of Folin reactive substances detected (Fig. 28). Given the fact that red seaweeds phenolic content is rather low (Holdt & Kraan, 2011), the increase in ABTS and Folin values was mostly attributed to the presence of water soluble phycobilins (Lin & Stekoll, 2011). M1 extraction probably also included other compounds such as proteins and sugars that might have also contributed as radical scavengers (Ikawa, Schaper, Dollard, & Sasner, 2003; Singleton, Orthofer, & Lamuela-Raventos, 1999).

M3+M4+M1 film FTIR spectrum showed how M1 addition induced a clear broadening of the amide I band, which revealed M1 protein content. M1 addition also contributed to an overall increase in the interactions of the sulfate ester groups with the κ/ι-hybrid carrageenan matrix. Other authors have observed that extracts containing different compounds, like those possibly extracted in M1, could promote interactions between polyphenols and sulfated polysaccharides (Jiménez-Escrig, Gómez-Ordoñez, & Rupérez, 2012). These interactions might lead to a more stable and elastic film network. Rheology results confirmed this theory showing a sharp increase in M3+M4+M1 *G'*. FTIR spectra also showed a higher M3+M4+M1 sulfate content, probably related to the presence of phycobiliproteins in M1 , which contributed to its antioxidant activity (Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

Comparing M3+M4 with M3+M4+M1 (Figs. 28 and 29), the addition of M1 resulted in a film almost twice as thick, considerably less transparent, with the same water solubility, but two times more permeable to water vapour. Mechanical characteristics depended on the type of test performed: whereas tensile strength was not affected, M1 addition increased four times the elongation at break capacity and reduced the Young's elastic modulus. On the contrary, both the puncture force and the puncture deformation reached values closer to those of M3, which resulted to be the most commercial carrageenan-like film.

12.3.3.1.2. Hydrolysate addition

Since Mastocarpus Hp (phenolic-containing) hydrolysate showed a higher antioxidant activity than H (phenolic-partially removed) (see figure 14), and the purpose of the hydrolysate addition was to improve M3+M4 film antioxidant activity, Hp was selected to be added at 15 and 30% (w/w) in relation to the M3+M4 extracts content. In order to avoid an excessive plasticizing effect, the film with a reduced

glycerol content (M3+M4-10) was selected for the hydrolysate addition, resulting in M3+M4-10-Hp15 and M3+M4-10-Hp30 films respectively.

The incorporation of increasing concentrations of Hp significantly increased both the ABTS radical scavenging capacity and the Folin reactive substances content. Compared to the previous Mastocarpus film without active compounds, ABTS results showed a 5 times increase in the activity of M3+M4-10-Hp15 film and a 9 times increase for M3+M4-10-Hp30 film; whereas the Folin reactive substances were 2 times higher in the M3+M4-10-Hp15 film and 3 times higher in the M3+M4-10-Hp30 film.

FTIR spectra showed the reduction of the carrageenan proportion at the expense of Hp incorporation in the film, increasing the short peptide content and the hydrogen bonding and carrageenan-peptide interactions with increasing concentrations of Hp, and being especially noticeable in M3+M4-10-Hp30 films. DSC thermograms also suggested carrageenan-peptide interactions (Baeza, Carp, Pérez, & Pilosof, 2002) and a plasticizing effect induced by the increase in the hydrolysate addition, evidenced by a progressive melting temperature increase and enthalpy reduction.

As observed in the rheological analysis of the film forming solution, the hydrolysate addition in appropriate concentrations led to stronger gel properties, resulting in a more stable film structure by increasing the interactions between carrageenan helices, plasticizer and peptides. Therefore, M3+M4-10-Hp15 film increased the water vapour impermeability compared to M3+M4-10 and M3+M4-10-Hp30. The increase in the Hp concentration in the film did not affect the water solubility of the film, but it conferred more flexibility to the film and also improved its mechanical puncture strength (Figs. 28 and 29).

12.3.3.1.3. Comparative study of the addition of different extracts

The addition of different bioactive extracts to Mastocarpus film forming solutions resulted in a diverse film antioxidant activity response (Fig. 28). While M3+M4+M1 Folin reactive substances had a significantly higher free radical scavenging capacity than M3+M4 film, the M3+M4-10-Hp film activity depended on the amount of Hp added (30%>15%). It could be concluded that a significant amount of Mastocarpus active compounds were extracted in M1, and remained available in the Mastocarpus biopolymeric matrix, resulting in a remarkable increase in the film activity.

The hydrolysis-induced peptide release led to a pronounced increase in ABTS values and Folin reactive substances, however, the resulting films were not as active as with the M1 aqueous extract, probably due to an excessive interaction with the biopolymeric components.

When bioactive extracts were added to the film forming solutions, the resulting films experienced an increase in their thickness, which was higher with increasing amounts of hydrolysate and extremely high in M3+M4+M1, suggesting a better inclusion of the hydrolysate than of the M1 extract in the matrix. This less efficient matrix entrapment of M1 did not affect the film moisture values but resulted in the highest film water vapour permeability.

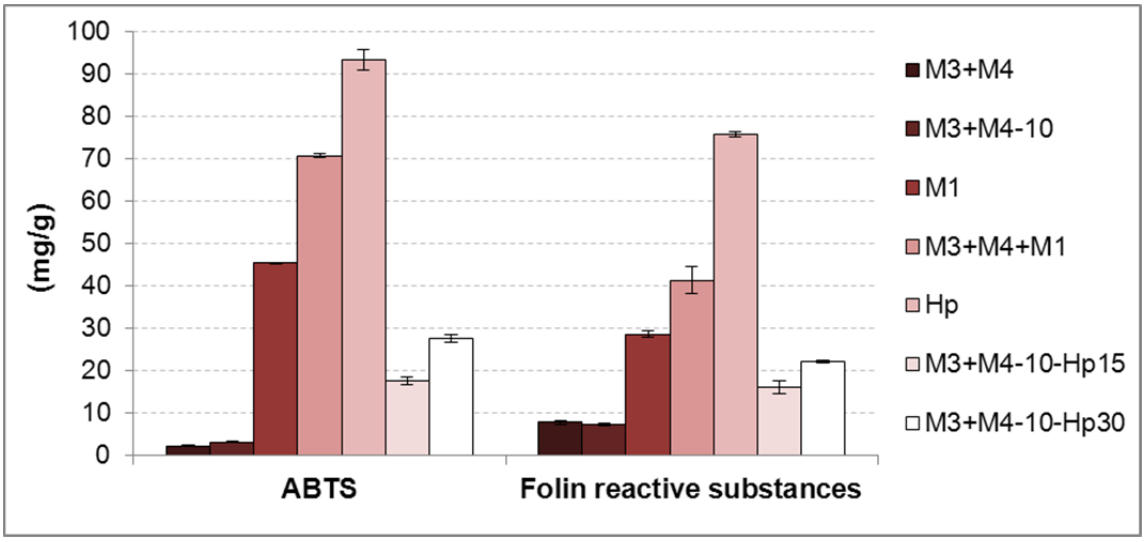

Figure 28. ABTS radical scavenging capacity and Folin reactive substances of M1 and Hp extracts, and M3+M4, M3+M4-10, M3+M4+M1, M3+M4-10-Hp15, and M3+M4-10-Hp30 films.

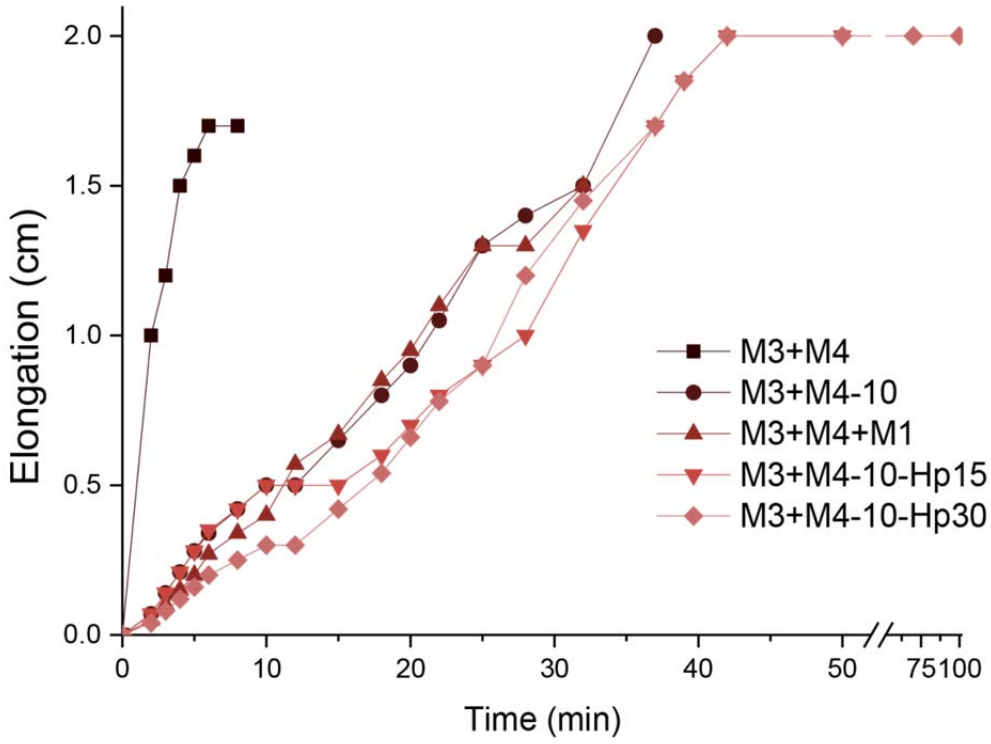

Figure 29. Water resistance for M3+M4, M3+M4-10, M3+M4+M1, M3+M4-10-Hp15, and M3+M4-10-Hp30 films.

M3+M4+M1 film extended the M3+M4 water resistance to film breakage time (Fig. 29), but both M3+M4 and M3+M4+M1 films lasted considerably less time and were elongated less centimetres than M3+M4-10, M3+M4-10-Hp15 and M3+M4-10-Hp30 films. This difference was probably caused by the low plasticizer content, but also proved that, although the hydrolysate addition can cause an adverse plasticizing effect (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009), the peptide

biopolymer matrix bonding (Baeza, Carp, Pérez, & Pilosof, 2002) resulted in an overall more water resistant film, being the M3+M4-10-Hp30 film highlighted due to its higher hydrolysate content.

Regarding the mechanical properties, in general, the addition of bioactive compounds weakened the film strength and the tensile stiffness, but considerably increased the film capacity to elongate prior to breakage, as it was also shown during the water resistance test. The tensile elongation response was two times higher in the M3+M4+M1 film than in any hydrolysate-incorporating film. Despite the detriment to the tensile strength and Young's modulus, the addition of bioactives to the films increased both the film puncture force and deformation (Fig. 30). M3+M4+M1 was also the most flexible film, and 30% was the optimal hydrolysate addition for obtaining the best puncture strength.

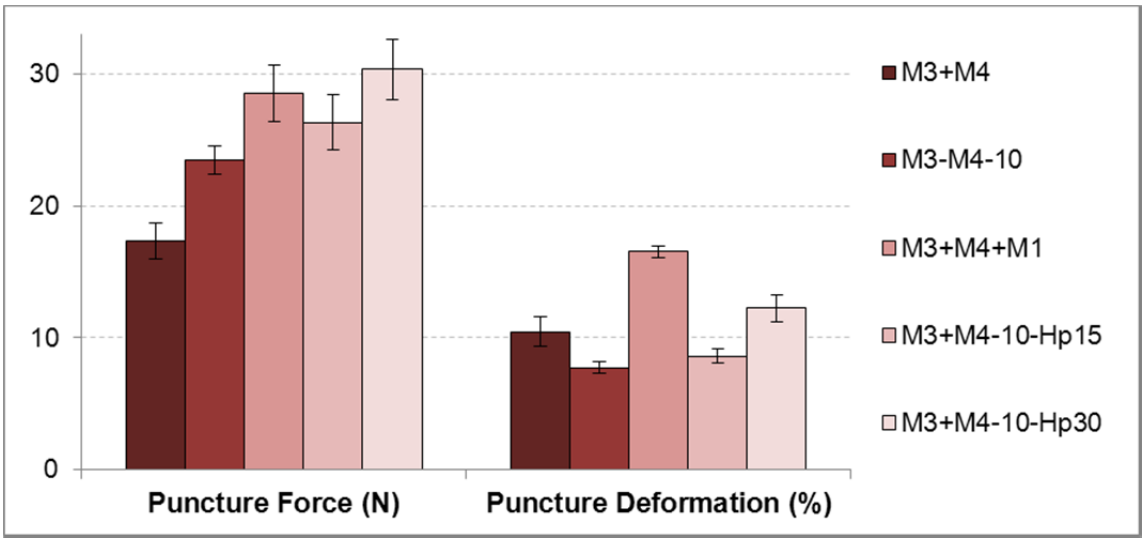

Figure 30. Puncture test results for M3+M4, M3+M4-10, M3+M4+M1, M3+M4-10-Hp15 and M3+M4-10-Hp30 films.

12.3.3.2. Microencapsulation of bioactive peptides

Dosidicus gigas tunic hydrolysis has been carried out in order to obtain bioactive peptides from its collagenous material. These hydrolysates have shown different activities such as antioxidant and antihypertensive. Direct hydrolysate addition to different food products, and specifically to film forming solutions, has reported to confer a bitter flavour (Sun-Waterhouse & Wadhwa, 2013) and excessive plasticization (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009), being the organic microencapsulation a good alternative to preserve the hydrolysate activity (Gibbs, Kermasha, Alli, & Mulligan, 1999) while avoiding the possible adverse consequences of the direct addition on both the sensory and physical properties.

In the present study, a <1kDa peptide fraction from *Dosidicus gigas* tunics hydrolysate, was used for microencapsulation.

12.3.3.2.1. Microencapsulation preliminary studies

Based on previous results, three different encapsulation coating materials were tested: i) an alginate rich extract (Lc), ii) a carrageenan rich extract (M3+M4 (50:50)), and iii) a lipid mixture composed

of stearic acid + carnauba wax (75:25). Due to their good filmogenic capacity, alginate and carrageenan have been widely used as encapsulation materials (Rinaudo, 2008), while lipid materials, as the carnauba wax/stearic acid combination, have shown good results in different drug encapsulation studies (Fini, Cavallari, Rabasco Álvarez, & Rodríguez, 2011), making them potentially suitable for food applications.

The inkjet printing microencapsulation technology (Houben, Rijfers, Brouwers, Eversdijk, & Van Bommel, 2009) provides the possibility to convert liquids into powders and separate core and shell flows, allowing a continuous mild process to obtain final products that are normally mono-dispersed core-shell microcapsules, but that can also be formed by matrix complexation.

Depending on the encapsulation coating material used, two different microencapsulation models were tested: i) gelation by cation complexation was performed to entrap the active hydrolysate inside the seaweed extract matrix capsule (Figure 31A), and ii) core peptide solution droplets were generated by the inkjet technology and encapsulated by a thin liquid film of shell material made of stearic acid + carnauba wax (Figure 31B).

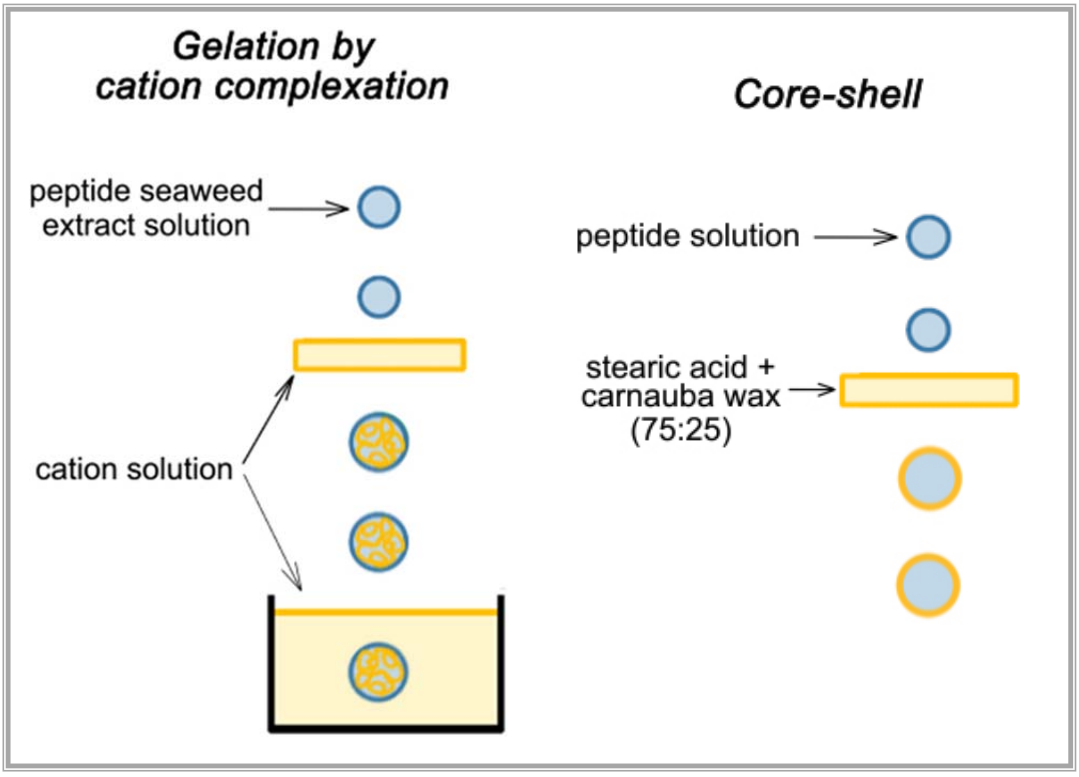

Figure 31. A. Gelation by cation complexation. **B.** Core-shell microencapsulation.

12.3.3.2.2. Gelation by cation complexation

Gelation was performed with 10% KCl and $CaCl_2$ solutions for the corresponding cation complexation with 1.5% Mastocarpus and Laminaria solutions, respectively. In order to accelerate cation complexation, peptide seaweed extract droplets were passed through a thin film and collected in a container with the same cation solution.

All raw materials were adjusted to the appropriate concentrations and temperatures that would result in suitable density values for being printed by the inkjet encapsulation technology (Table 5).

Sample	Density (g/mL)
1.5% *Mastocarpus stellatus* solution (21 ºC)	1.1 ± 0.03
1.5% *Laminaria digitata* solution (21 ºC)	1.02 ± 0.04
shell material: stearic acid/carnauba wax (105 ºC)	0.81 ± 0.03
core material: demineralized water (35 ºC)	0.97 ± 0.02
core material: 1% hydrolysate solution (35 ºC)	1.04 ± 0.02

Table 5. Density of potential raw materials to be used in the inkjet printing encapsulation technology.

One of the main limitations of the printing encapsulation technology is the sample viscosity. The viscosity of all potential raw materials was measured at increasing shear rates (Fig. 32).

Core and shell material processings are performed separately. While the shell material system tolerates much higher temperatures (<200 ºC) and suits better to high viscosities, the core inkjet process has more limitations (<80 ºC and viscosity dependent on the nozzle size). The viscosity of the lipid nature shell material was important in order to estimate the temperature to be used during its processing as shell material.

Since peptides (hydrolysate) would be present in the core material, high temperatures were undesirable during its processing (Kauffman, Kneivel, & Watschke, 2007). For comparison purposes, demineralized water was also microencapsulated. Demineralized water and 1% peptide solution core materials had low but stable viscosity values (<1mPa·s), being almost shear rate independent. However, seaweed materials showed much higher viscosity values. While 1.5% *Laminaria digitata* solution remained stable at increasing shear rates, with ~15 mPa·s values, 1.5% *Mastocarpus stellatus* solution had values of ~250 mPa·s at low shear rates that were brought down to ~125 mPa·s at around 100 s^{-1}.

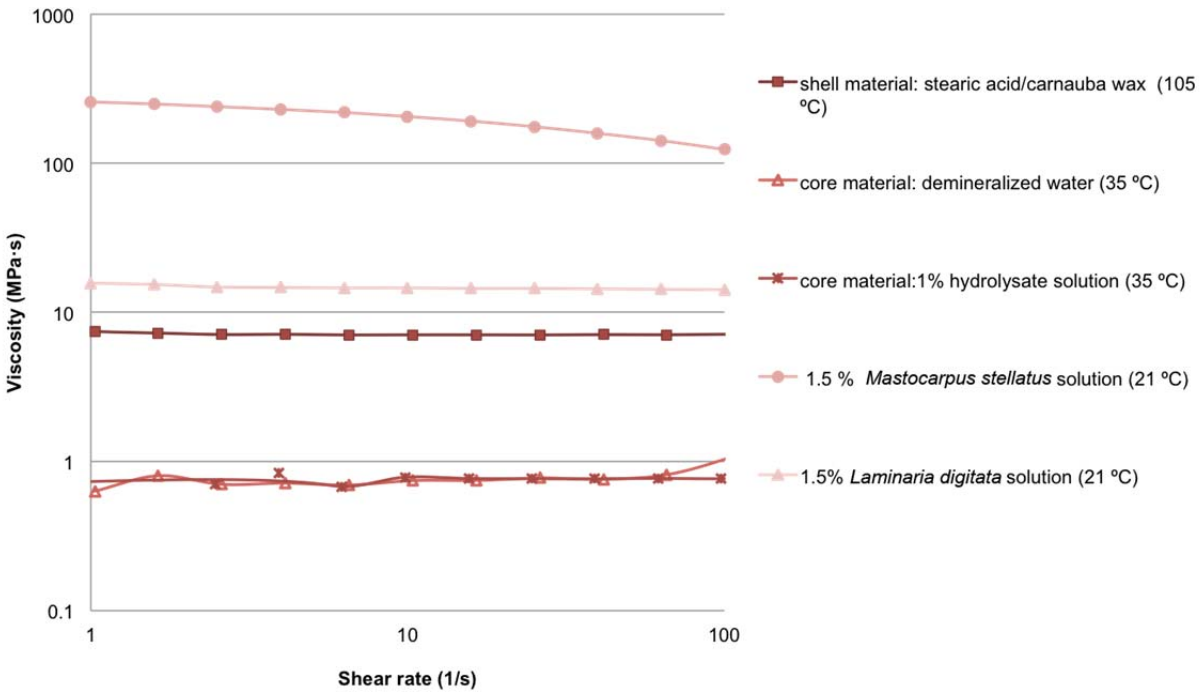

Figure 32. Shear rate dependent viscosities of the potential core and shell fluids for the encapsulation process.

Due to the high viscosity values of the seaweed solutions, printing encapsulation tests were performed with a lower dry matter content (1%) in an attempt to test their viability as encapsulation materials for gelation by cation complexation.

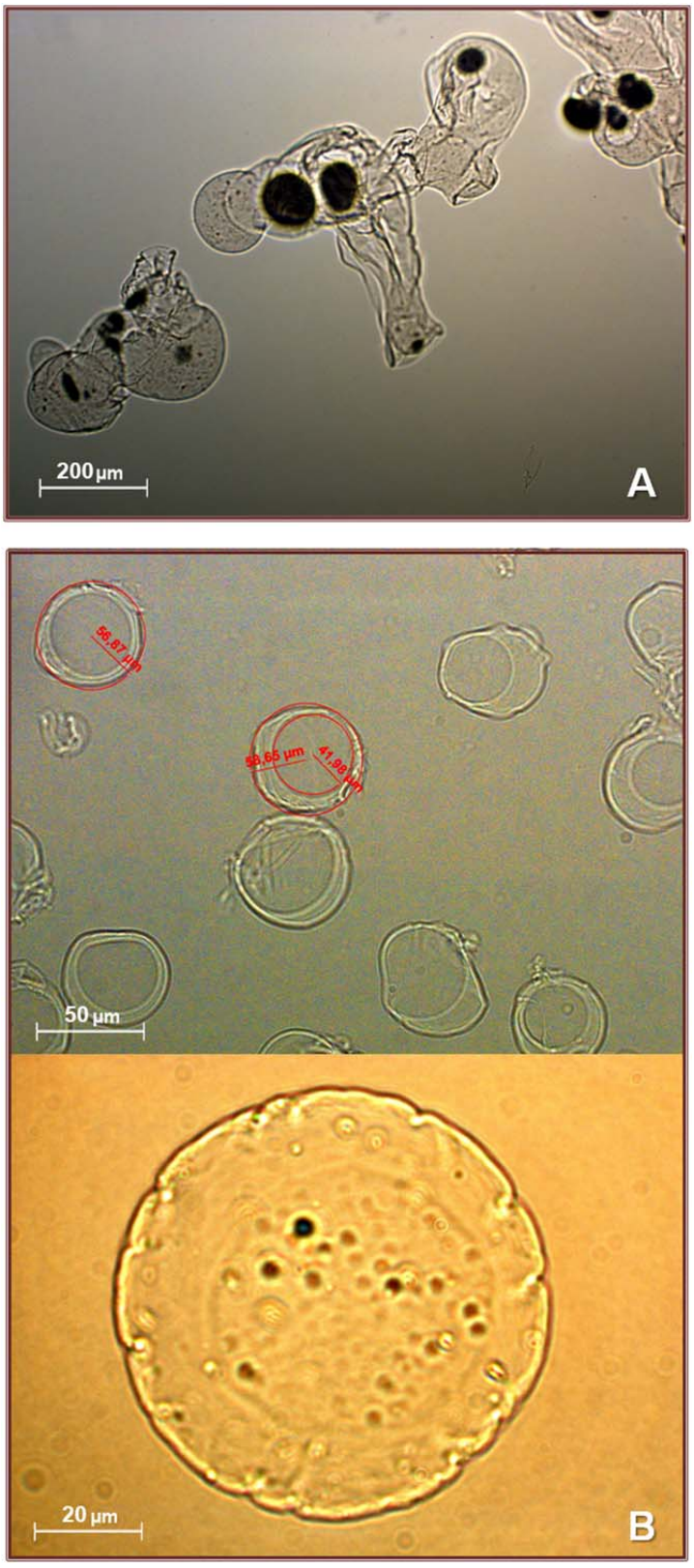

Figure 33. Microscopic pictures (Zeiss AxioImager M1m + AxioCam MRc 5) of **A.** 1% *Mastocarpus stellatus* microcapsules, and **B.** 1% *Laminaria digitata* microcapsules, obtained through gelation by cation complexation.

Despite having lowered the dry matter content in the Mastocarpus solution, it was still too viscous to be printed, even after an additional lowering of the solution concentration and an increase of its temperature (Fig. 33A). Moreover, carrageenan cation complexation was slower than it was expected to be, making the processing even more difficult. The high protein content in the Mastocarpus extract might have adversely affected the gelation process (Andrade, Azevedo, Musampa, & Maia, 2010), delaying the double helixes complexation (Wu & Imai, 2012).

On the other hand, the 1% Laminaria solution gelled forming spherical microcapsules of ~110 µm in diameter (Fig. 33B), but did not retain the peptides inside the matrix in an aqueous medium, releasing them during the first 3-4 hours (Fig. 34).

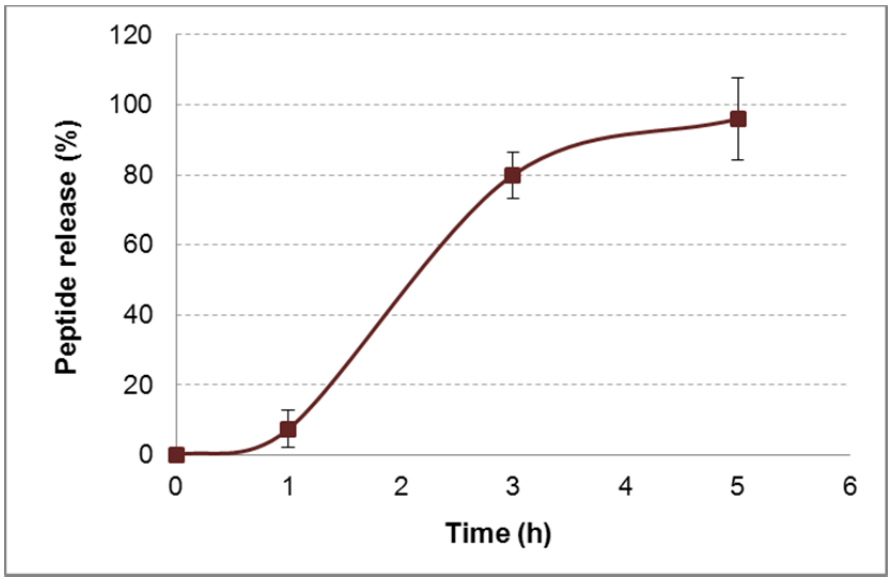

Figure 34. Peptide release over time of 1% *Laminaria digitata* microcapsules suspended in the 10% $CaCl_2$ collecting solution.

The alginate “egg-box” gel structure, as described by Grant (1973), resulted in a porous matrix that did not retain inside peptides smaller than 1kDa. It has been reported that alginate gels only retain molecules larger than 5000 kDa (Gibbs, Kermasha, Alli, & Mulligan, 1999)

12.3.3.2.3. Core-shell microencapsulation model

Due to the impossibility of both inkjet printing any Mastocarpus solution and retaining peptides inside a Laminaria matrix, seaweed extracts did not result in suitable raw materials for the microencapsulation of <1 kDa peptides. In this respect, the use of the core-shell model with lipid nature shell materials was proposed. Both core and shell materials had densities and viscosities suitable for the printing encapsulation technology, resulting in spherical microcapsules of 110-140 µm in diameter (Fig. 35A). Peptide solutions with concentrations higher than 1% (2, 5, and 20%) posed some difficulties during the process, resulting in sticky and non-homogeneous microcapsules (Fig. 35B).

Two different microcapsules were obtained, distilled water microcapsules (C) and 1% peptide solution microcapsules (Cp). Low Temperature-Scanning Electron Microscopy showed that 1% peptide microcapsules were more oval-shaped than capsules developed with distilled water, some of them

presented a single protrusion and a small percentage were not entirely closed during the encapsulation process (Fig. 36). Images suggested that microcapsule irregularities might have been caused by interactions between peptides and the stearic acid from the shell material. Stearic acid has shown to attract peptides and proteins from aqueous subphases (Zadmard & Schrader, 2004) and hydrophobic peptides can interact with stearic acid (Joseph & Nagaraj, 1988). However, both the hydrophobicity and the peptide charge can influence the affinity of the peptide-lipid interactions, showing the neutral peptides a deeper interaction than the charged ones (De Kroon, Soekarjo, De Gier, & De Kruijff, 1990).

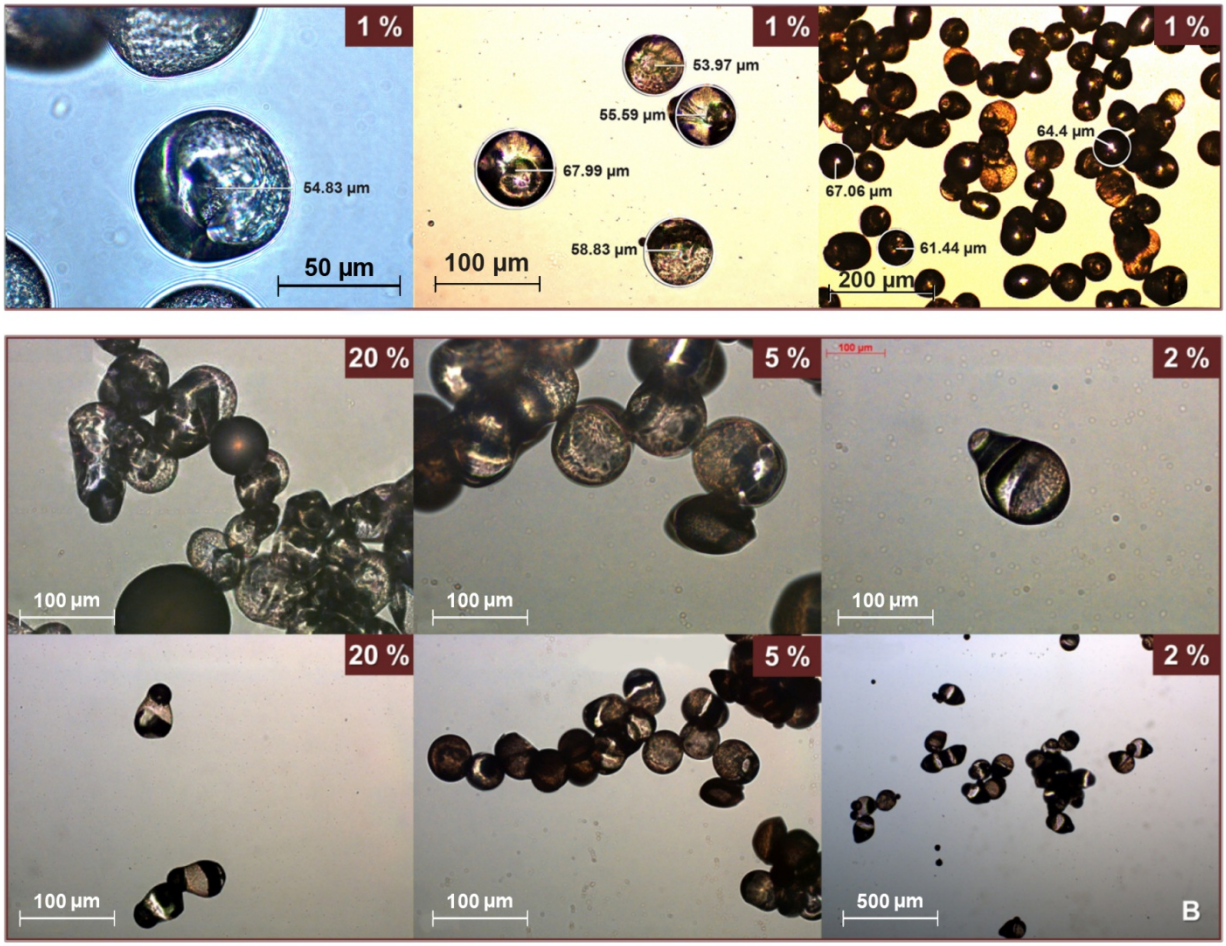

Figure 35. Microscopic pictures (Zeiss AxioImager M1m + AxioCam MRc 5) of **A.** 1% peptide core-shell microcapsules **B**. 2, 5 and 20% peptide core-shell microcapsules.

Despite the microcapsule slight irregularities, as shown by optical and low temperature electron microscopy, the peptide entrapment efficiency was 84.7 ± 3.4% and its stability varied depending on the environmental conditions. At pH 7, microcapsules presented only a first peptide release of ~20%, reaching the stability after 1 hour, and at pH 5 they exhibited a linear slow peptide release that might lead to a controlled release model. At pH 2.6 and 9.2 they released ~40% of the peptides after 1 hour, but while at pH 2.6 they reached the stability at said time point, at pH 9.2 they continued with a slow release over time. Depending on the purpose of the encapsulated peptides, pH modifications in the microcapsule carrier could be used to obtain the release model required (Fig. 37).

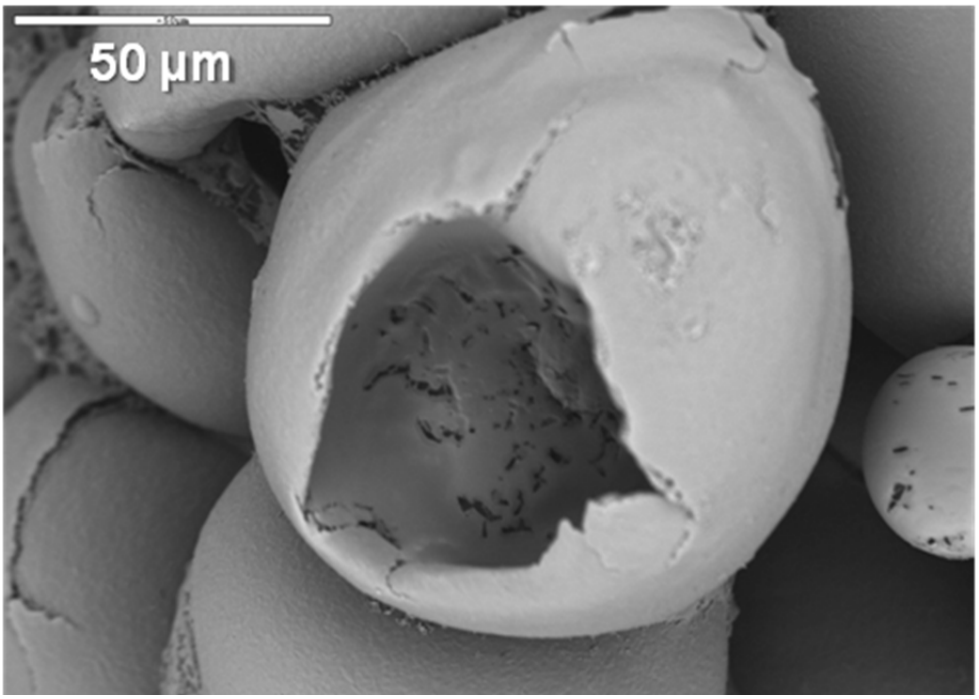

Figure 36. Low Temperature-Scanning Electron Microscopy picture (Oxford CT 1500 Cryo Sample Preparation Unit) of a 1% peptide microcapsule not entirely closed.

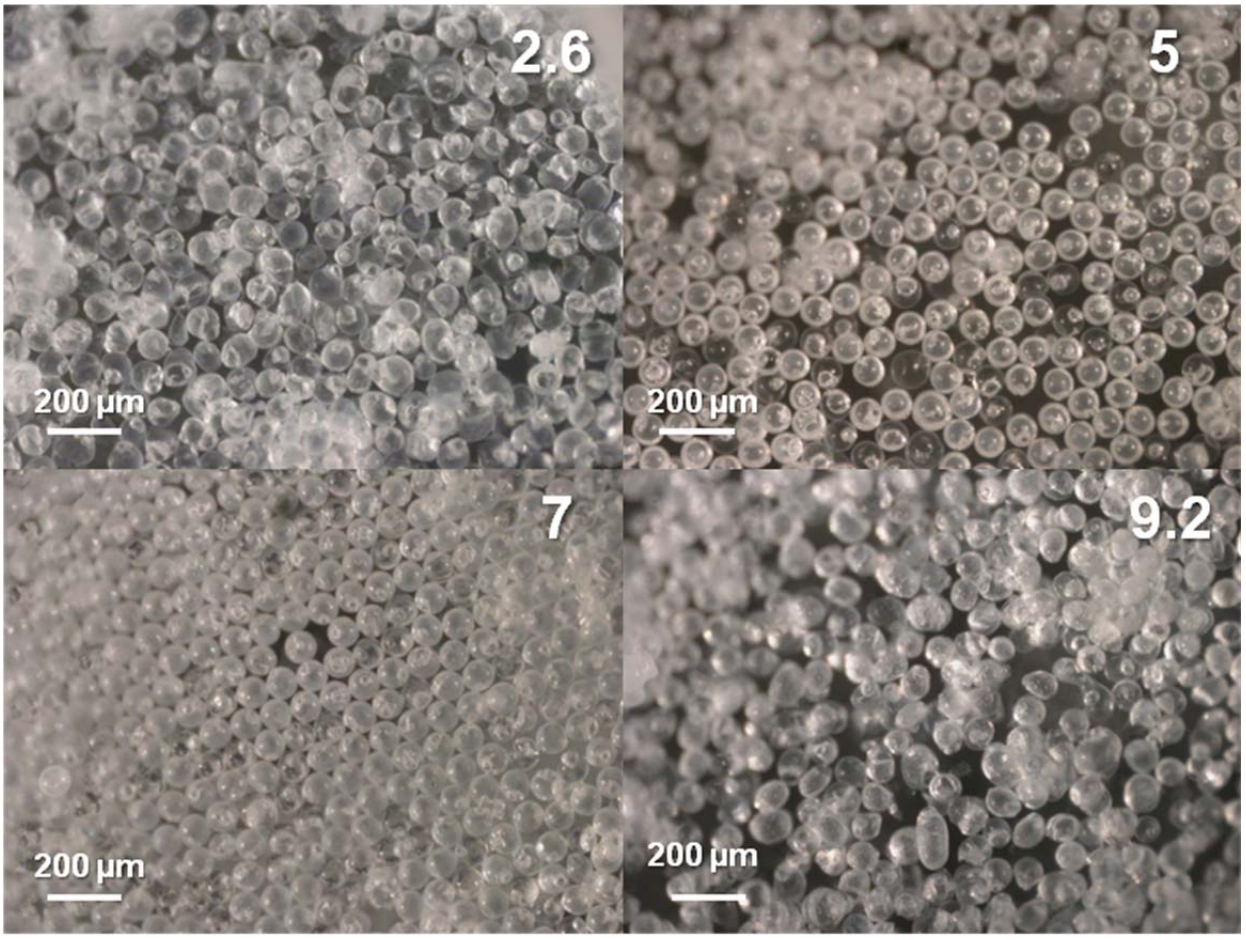

Figure 37. Amplified pictures (Stereomicroscope Stereo Zoom L2S8 APO. Leica Microsystems, Switzerland) of 1% peptide microcapsule release after 3 hours at pH 2.6, 5, 7 and 9.2.

12.3.3.3. Microcapsule incorporation in *Laminaria digitata* film

The diameter size of the microcapsules forced an increase in film thickness to promote their inclusion in the matrix. In order to obtain a microcapsule homogeneous dispersion in the film matrix, the addition of polysorbate 80 surfactant was carried out. Ultimately, the use of TMTP09030 Isopore polycarbonate membranes further improved the microcapsule dispersion during the film drying process.

The addition of microcapsules resulted in a discontinuous Lc-3 film with a lipid content that, in comparison with films without capsules, was stronger, with a higher stretching capacity, less permeable to water vapour but more water soluble, opaque and with a lower puncture strength.

Laminaria digitata hydrophilic films might be used as microcapsule carriers, contributing to the design of a peptide controlled-release system and to their preservation from the environmental conditions.

12.4. Study of the main characteristics of different films

Many different films have been developed in the present work. All of them were made of marine edible resources, and most of them turned out to be edible.

The appearance of edible films, including transparency and colour, is an important criterion in the selection of edible films, since the consumer prefers colourless transparent packaging materials. However, on occasion, consumers may also like translucent and coloured films.

Colour results (Table 6) revealed that both protein and polysaccharide films had a low lightness (L^*), being protein rich films slightly lighter. Protein rich films were more transparent than polysaccharide rich films; however, M3, having properties closer to commercial carrageenan films, resulted in the highest transparency among all the polysaccharide films obtained. The addition of hydrolysates to M3+M4-10 films, and the high glycerol content together with the presence of polysorbate 80 in Lc-3, contributed to increase film transparency. Among all the films, both solubilized (S) and concentrated (C) acidic protein films were the most transparent films.

Protein rich films were mainly colourless, which might have contributed to their higher transparency, while the yellowish tendency (+ b^*) of alkaline-S and acidic-C4 films was highlighted. The different protein state of alkaline-S and acidic-C films might have favoured Maillard reaction in both films but at a different rate. Alkaline conditions together with the drying temperature (45 ºC) might have boosted a fast protein glycation with sorbitol and glycerol during the development of muscle alkaline-S films, while the protein preservation in acidic-C films, together with the storage time in a suitable water activity environment (0.68), might have favoured the same Maillard reaction but at a slower rate during 4 months, resulting in an even higher increase in the yellowish tendency (Leerahawong, Tanaka, Okazaki, & Osako, 2012; Sanmartín, Arboleya, Villamiel, & Moreno, 2009).

Seaweed pigments were extracted together with polysaccharides, proteins and minerals, reducing film transparency and contributing to a specific colour in each case (Fig. 38).

All films constituted good UV barriers except for alkaline-C4 and M3 films (Fig. 39). As shown in previous results, the protein aggregation experienced during the storage time affected C-films in a

different way. Alkaline-C film suffered a matrix disruption (Anuchit Artharn, Prodpran, & Benjakul, 2009), such that it increased its opacity and failed to absorb the UV radiation. M3 extract resulted in films with properties closer to commercial carrageenan films (almost colourless and transparent) (Rhim, 2012) but that were not as good UV barrier as the rest of the polysaccharide rich films developed in this work.

Film	*L**	*a**	*b**	Transparency
Protein rich films				
water-S	34.44 ± 0.24	-1.00 ± 0.05	0.06 ± 0.01	3.48 ± 0.42
salt-S	35.98 ± 0.64	-1.15 ± 0.13	0.10 ± 0.1	3.40 ± 0.44
alkaline-S	34.23 ± 0.12	-1.24 ± 0.03	1.26 ± 0.07	1.14 ± 0.26
acidic-S	35.93 ± 0.87	-0.69 ± 0.03	-0.56 ± 0.09	0.73 ± 0.05
alkaline-C	34.73±0.27	-0.93±0.05	-0.51±0.01	0.95 ± 0.03
acidic-C	33.66±0.15	-0.93±0.04	0.07±0.11	0.6 ± 0.01
alkaline-C4	34.96±0.41	-0.85±0.15	0.84±0.23	1.16 ± 0.03
acidic-C4	33.35±0.06	-1.33±0.02	4.07±0.18	0.56 ± 0.08
Polysaccharide rich films				
M3	30.70 ± 0.58	-0.8 ± 0.07	1.72 ± 0.19	2.71 ± 0.06
M4	28.90 ± 0.62	-0.88 ± 0.10	4.35 ± 0.17	11 ± 0.13
M3+M4	29.45 ± 0.23	-1.05 ± 0.06	3.85 ± 0.34	6.68 ± 0.28
M3+M4+M1	28.43 ± 0.37	-0.60 ± 0.16	5.26 ± 0.21	11.74 ± 1.39
M3+M4-10	28.65 ± 0. 29	-0.57 ± 0.03	4.57 ± 0. 11	7.14 ± 0.24
M3+M4-10-Hp15	27.99 ± 0.08	-0.30 ± 0.05	4.72 ± 0. 04	6.61 ± 0.30
M3+M4-10-Hp30	29.25 ± 0.05	-0.70 ± 0.03	4.36 ± 0.04	6.12 ± 0.12
Lc	28.07 ± 0.3	-0.03 ± 0.07	5.99 ± 0.29	17.12 ± 0.71
Ac	24.04 ± 0.33	1.78 ± 0.06	2.52 ± 0.16	8.39 ± 0.76
Lh	28.47 ± 0.21	0.24 ± 0.05	4.34 ± 0.14	11.96 ± 0.44
Ah	25.15 ± 0.43	0.82 ± 0.36	-0.47 ± 0.63	16.36 ± 0.76
Lc-3	25.1 ± 0.5	3.52 ± 0.2	4.54 ± 0.5	6.99 ± 0.27
Microcapsule addition to polysaccharide rich films				
Lc-3-C	25.1 ± 1.1	3.27 ± 0.5	4.57 ± 0.8	8.42 ± 0.34
Lc-3-Cp	26.6 ± 0.8	3.75 ± 0.6	5.92 ± 1.0	7.97 ± 0.28

Table 6. L^*, a^*, b^* and Transparency ($-\log(T_{600}/x)$) of protein and polysaccharide rich films.

Red algae chloroplasts are known for containing chromophores called phycobilins (O'Carra, Murphy, & Killilea, 1980), which are photosynthetic pigments thioether-bonded to certain water soluble proteins named phycobiliproteins (Carra, Ó Heocha, & Carroll, 1964) that might have contributed to Mastocarpus films light barrier, depicting an absorption area at ~600 nm (red colour). Red seaweeds also contain other carotenoids and chlorophyll, responsible for the absorption at 400-450 nm (violet-blue-green colours) (Sühnel, Hermann, Dornberger, & Fritzsche, 1997). Nevertheless, M3 films did not present this light transmission profile and M1 addition resulted in the most opaque Mastocarpus film.

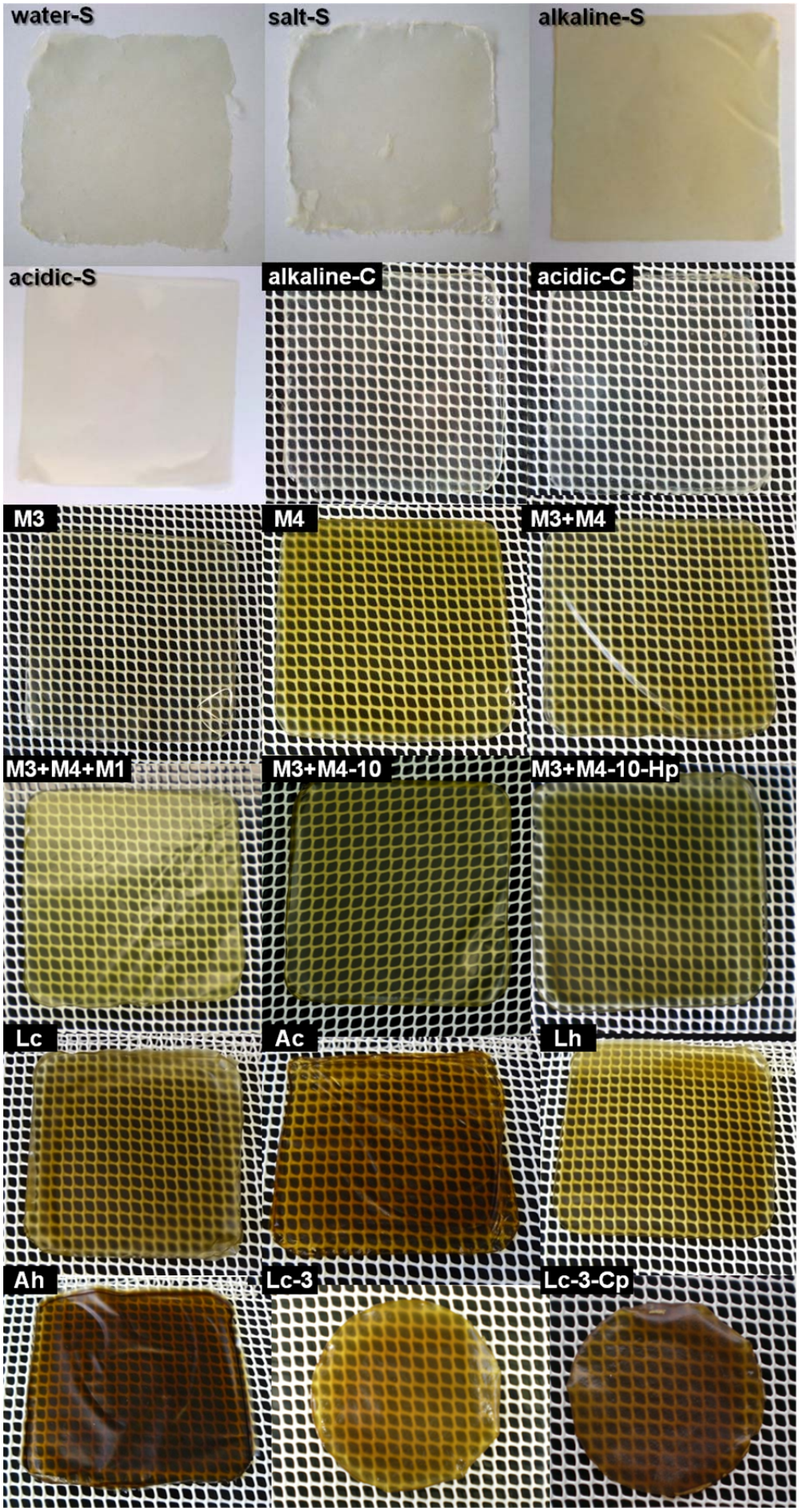

Figure 38. Pictures of protein and polysaccharide rich films.

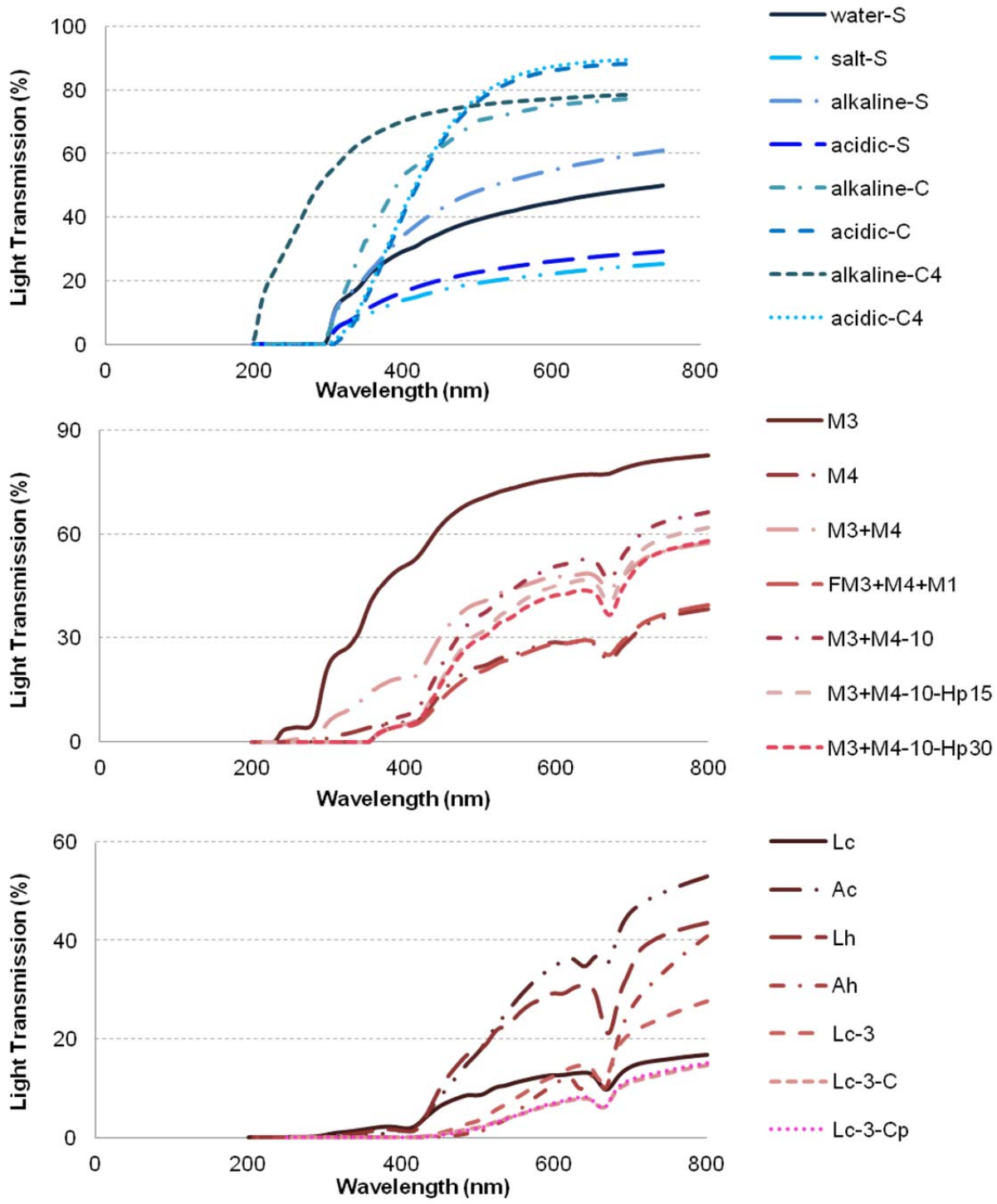

Figure 39. Light transmission at 200-800 wavelengths range of protein and polysaccharide rich films.

Brown seaweed films transmitted less light than the rest of the films, probably due to their strong red-brown pigments, which was quite evident from the absorption peak around 650-700 nm (Gildenhoff, Herz, Gundermann, Büchel, & Wachtveitl, 2010). Ascophyllum films presented a higher reddish tendency, closer to 600 nm, typical for chlorophyll and other carotenoids (Goss, Wilhelm, & Garab, 2000). Laminaria films depicted a main peak closer to the brown colour at 700 nm (Bricaud, Babin, Morel, & Claustre, 1995), specifically at 675 nm, corresponding to the carotenoid fucoxanthin that, in combination with

chlorophyll, binds to some proteins forming complexes in the thylakoid and acting as a light harvesting system (Gildenhoff, Amarie, Gundermann, Beer, Büchel, & Wachtveitl, 2010).

Film	Film solubility (%)	*TS* (MPa)	*EAB* (%)	*Y* (MPa)
Protein rich films				
water-S	40.7 ± 0.51	1.36 ± 0.32	1.82 ± 0.28	83.8 ± 17
salt-S	47.7 ± 0.38	0.97 ± 0.33	1.90 ± 0.89	51.0 ± 17
alkaline-S	45.5 ± 0.78	3.10 ± 0.53	12.1 ± 3.25	55.0 ± 12
acidic-S	42.9 ± 0.56	0.85 ± 0.60	13.3 ± 1.68	34.0 ± 4.9
alkaline-C	26.6 ± 0.63	1.54 ± 0.23	10.1 ± 4.82	184 ± 53
acidic-C	30.5 ± 2.34	0.96 ± 0.10	8.08 ± 2.47	156 ± 11
alkaline-C4	44.1 ± 2.17	1.61 ± 0.24	1.53 ± 1.08	207 ± 66
acidic-C4	38.2 ± 1.82	1.46 ± 0.27	11.7 ± 5.46	182 ± 2.9
Polysaccharide rich films				
M3	59.2 ± 3.29	13.9 ± 0.39	2.40 ± 0.1	459 ± 36
M4	30.4 ± 2.59	16.2 ± 0.76	1.28 ± 0.08	553 ± 37
M3+M4	37.3 ± 3.61	14.1 ± 0.18	1.38 ± 0.31	517 ± 24
M3+M4+M1	34.5 ± 1.52	15.2 ± 3.74	4.11 ± 1.0	317 ± 3.4
M3+M4-10	21.0 ± 4.5	60.0 ± 2.27	0.95 ± 0.11	1797 ± 61
M3+M4-10-Hp15	25.8 ± 3.64	51.4 ± 3.75	1.59 ± 0.09	1347 ± 74
M3+M4-10-Hp30	22.2 ± 2.95	41.6 ± 2.95	2.47 ± 0.24	1054 ± 45
Lc	79.2 ± 0.91	6.01 ± 1.58	1.20 ± 0.35	103 ± 3.2
Ac	85.3 ± 2.49	1.50 ± 0.19	3.49 ± 0.32	5.99 ± 0.5
Lh	74.4 ± 0.76	40.7 ± 4.05	0.91 ± 0.37	1082 ± 67
Ah	70.3 ± 2.37	9.13 ± 1.98	2.16 ± 0.81	160 ± 43
Lc-3	86.0 ± 1.7	6.6 ± 2.1	1.31 ± 0.74	88 ± 25
Microcapsule addition to polysaccharide rich films				
Lc-3-C	94.8 ± 2.9	10.8 ± 0.9	2.87 ± 0.4	183 ± 10
Lc-3-Cp	92.9 ± 1.9	10.1 ± 0.8	3.23 ± 0.2	185 ± 14

Table 7. Film solubility, tensile strength (*TS*), elongation at break (*EAB*) and Young's elastic modulus (*Y*) of protein and polysaccharide rich films.

Although Ascophyllum films presented a higher reddish tendency (+ *a**) than Laminaria, the 3 times increase in Lc thickness resulted in Lc-3 films with the highest reddish tendency, probably due to the dry matter increase. The inclusion of microcapsules did not interfere with film transparency but considerably reduced the full range of visible light transmitted, contributing favourably to food preservation from light.

In general, protein films were more transparent and colourless, while seaweed films presented the colour corresponding to the species of origin. Protein aggregation during film storage time was detrimental to the optical properties in *Dosidicus gigas* films. The direct inclusion of seaweed active compounds in the films, and the addition of microcapsules to the films improved the UV-visible light barrier properties of the films.

In general, edible protein and polysaccharide films show poor water barrier properties, but this study evidenced that the higher the protein content, the less water soluble and more water resistant were the films (Tables 7 and 8). Protein rich films were the least soluble and the most water resistant, specifically those elaborated under alkaline conditions and with the most purified protein concentrate, being alkaline-C film unbreakable and resistant to water filtration. On the other hand, polysaccharide films generally showed a higher tensile strength.

Among all polysaccharide rich films, red seaweed films presented better water barrier properties than brown seaweed samples. The high protein content in red algae evidenced the importance of the protein role in the formation of the film matrix.

Among all Mastocarpus films, those with bioactive extract addition presented better water barrier properties. Nevertheless, the films developed with the lowest glycerol content and with hydrolysate addition were the least soluble and the most water resistant. It is well known that glycerol adversely affects the water barrier properties of the films (Gontard, Guilbert, & Cuq, 1993), and that a higher protein content results in stronger matrixes (Gennadios, 2002).

Regarding the low water barrier of brown seaweed films, it is important to note two facts: i) despite Lh high solubility values, its water resistance to breakage time was as good as that of M3+M4-10-Hp30, and ii) the lipid nature of the microcapsules considerably improved the water vapour barrier of the films, but made them more susceptible to water solubility and less water resistant.

Nevertheless, protein rich films showed lower tensile strength (*TS*) and Young's elastic modulus (*Y*) (Table 7) than polysaccharide rich films. Mastocarpus films, developed with a low glycerol content and with hydrolysate addition, and Lh films, were the most outstanding for their strength and stiffness.

In general, protein rich films showed a higher elongation at break (*EAB*) capacity than polysaccharide rich films, being C-films the most flexible among all of them. Protein aggregation, caused by aging, adversely affected C-film flexibility, yet maintaining higher values in comparison with polysaccharide films.

Results showed that both the strength and stiffness were inversely related to the elongation of the films at the breakage point. Films with higher elongation values usually require a lower load to cause film breakage (Gennadios, Weller, & Testin, 1993).

In conclusion, protein rich films generally presented better water barrier and flexibility properties than polysaccharide rich films, while polysaccharide rich films were more resistant to mechanical stress.

12.5. Potential film industrial applications

The main techniques used to form edible coatings are spray systems and immersion procedures, such as dip coating; while solvent casting and extrusion processes are applied to form edible films. The main disadvantage of these techniques is the loss of quality, since there is no control over the shape, size and size distribution of the dispersed elements (additives, ingredients).

The leading technologies utilized for the production of edible films are similar to those used for thermoplastic structures, under different conditions for the solvent casting and the extrusion, but sharing the same principles.

Film	Film Elongation (cm)	Breakage time (min)	Filtration time (min)
Protein rich films			
water-S	1.3 ± 0.5	Unbreakable	300 ± 30
salt-S	1 ± 0.1	3000 ± 120	120 ± 18
alkaline-S	1.3 ± 0.1	2880 ± 60	10.2 ± 1.8
acidic-S	2 ± 0.2	5.4 ± 0.6	*
alkaline-C	1 ± 0.1	Unbreakable	*
acidic-C	2.5 ± 0.2	2400 ± 90	*
Polysaccharide rich films			
M3	2.7 ± 0.02	9.33 ± 0.58	8.33 ± 0.58
M4	2 ±0.02	18 ± 6.08	*
M3+M4	1.7 ±0.01	8 ± 3.46	*
M3+M4+M1	1.50 ± 0.01	32.33 ± 16.26	*
M3+M4-10	2 ± 0.01	37.29 ± 10.48	*
M3+M4-10-Hp15	2 ± 0.01	50.08 ± 14.18	*
M3+M4-10-Hp30	2 ± 0.01	99.5 ± 10.61	*
Lc	0.6 ± 0.02	7.12±0.71	*
Ac	0.3±0.01	0.16 ± 0.08	*
Lh	2 ± 0.2	106.83 ± 30.05	*
Ah	0.2±0.01	0.28±0.05	*
Lc-3	1.5 ± 0.01	3.17 ± 0.76	*
Microcapsule addition to polysaccharide rich films			
Lc-3-C	1 ± 0.01	3.5 ± 0.71	*
Lc-3-Cp	1.5 ± 0.01	1.88 ± 0.34	*

Table 8. Water resistance parameters: maximum film elongation, breakage time and filtration time of protein and polysaccharide rich films.
***** No filtration

Solvent casting is the most used technique to form hydrocolloid edible films, but edible films are usually produced by continuous film casting, mold casting or draw-down bar methods. The mold casting and the draw-down bar techniques are simple and inexpensive methods that can be used for the production of edible films at a laboratory scale, while the continuous film casting method (known as spread coating) can be accomplished by applying a wet film layer onto a belt conveyor and then passing it through a drying chamber (Fig. 40).

All the edible biopolymeric materials utilized in the experiments were easy to produce and handle since they required low temperatures and non-toxic solvents. Some formula adjustments were required in order to make them suitable for the “spread coating” procedure. Red seaweed film forming solutions needed temperatures lower than those commonly used during the film forming solution processing, and it

was also necessary to increase the viscosity of brown seaweed film forming solutions by increasing the dry content (Fig. 41).

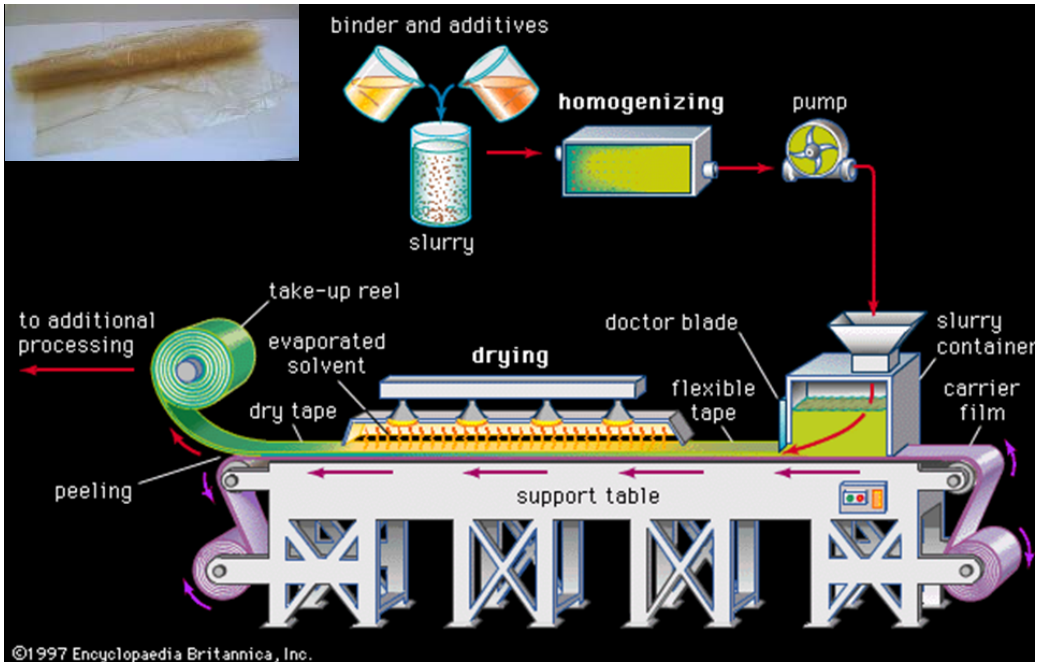

Figure 40. Continuous film casting scheme (spread coating).

All films developed in this study contained edible nutrients extracted from marine products and were susceptible of use in the final food products, either forming part of a packaging improvement as wrapping materials, or in the final serving presentation as stand-alone films. Moreover, they could be directly coated onto a food as thin layers, or formed into films without changing the original ingredients or the processing method.

Dosidicus gigas muscle solubilized protein films might be used as part of seafood products packaging. However, in water-S and salt-S films, the addition of a food preservative (for example potassium sorbate E 202) is recommended.

Alkaline and acidic concentrated proteins resulted in better quality films. Apart from the possibility of being part of an edible packaging, they could be used in the final presentation of different foods, like seafood products and vegetables, as edible film coatings protecting the contents. Due to the fact that nitrogenous compounds were removed during the concentration, films showed a less intense or even non-existent flavour, which would increase the number of possible applications.

Seaweed films would represent a different way of human algae consumption, reducing the strong algae flavour of the traditional commercial presentations, and making possible their commercialization in two different presentations: i) the powder form, for different cooking applications as a coating material; or ii) the film form, to apply over food products of different origin such as, vegetables, confectionery or seafood, forming part of the final serving presentation.

Apart from the wide range of culinary applications for seaweed films, the seaweed extraction procedures have allowed the possibility of conferring (technological and nutritional) functionality to these

products, so that they could be included in the growing health food products market. Moreover, procedures like hydrolysis have shown to improve seaweed compounds digestibility, increasing its nutritional value (Fleurence, 1999; Gupta & Abu-Ghannam, 2011).

Figure 41. Film developed by “spread coating”.

All the studied edible films were susceptible to heat-sealing. Water resistant films, such as alkaline-C and acidic-C, could be used in combination with food products with high moisture content; while less water resistant films could be combined with more fatty ingredients and dried food presentations. A broad spectrum of foods from different origins can be applied in the development of new food serving presentations with edible films, where the traditional Asian small bags or spring roll-like products represent possible uses (Fig. 40), but also films as salad containers, edible fish greased paper or as condiment cube coating material.

Using the seaweed extract powder as starting ingredient, many different ingredients and additives can be added to the formula to change its properties. Moreover, by changing the drying process different textures can be obtained, crispy as M4 films or gummy as M3 films.

In view of the results obtained, we should examine the possibility of developing new products by mixing concentrated protein and seaweed extracts in such a way that the weaknesses could be strengthened and new filmogenic textures could be developed.

Edible film coatings would play an important role in food preservation by: i) protecting the food from reacting with environmental factors, ii) reducing the transfer rate of the food constituents to the environment, iii) promoting in some cases an easier handling, and iv) exploring new flavour combinations or masking undesirable or strong tastes.

As for microcapsules, apart from the mentioned benefits attributed to edible film coatings, they specifically present more advantages, such as: i) the controlled release of the core material, ii) the core material dilution when it has to be used in small amounts, and iii) the possibility to be used in different food products apart from edible films (Shahidi & Han, 1993).

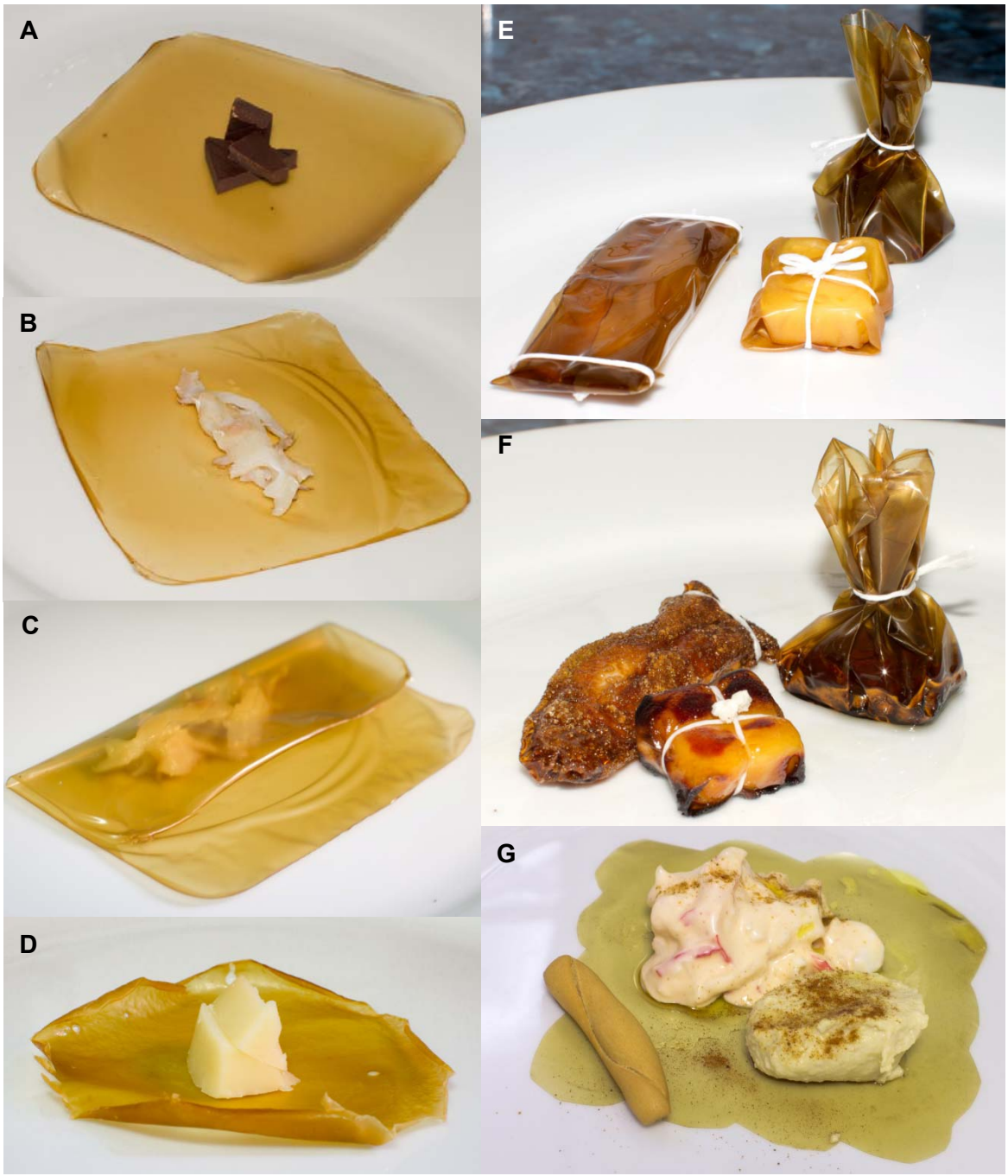

Figure 42. Different film applications in food products. A: Mastocarpus film with chocolate. B & C: Mastocarpus antioxidant film with smocked cod. D: Laminaria film with cheese. E: Different film crude coating presentations. F: Fried film coating presentations G: Serving presentation example.

Further organoleptic analysis and industrial scale studies are still ongoing in order to extend the applications of the developed film coatings and find consumer attractive combinations. This kind of food products offer new consumption trends for these materials, which are either considered industrial waste or underutilized species.

XIII. Conclusions

1. *Dosidicus gigas* muscle proteins, either as solubilized or concentrated extracts, present filmogenic properties, although each type shows different characteristics. The concentrated films highlights are their greater mechanical strength and water resistance. The acidic concentrated films are elastic and malleable, while the alkaline concentrated films are stronger and very water resistant.

2. The stability of the *Dosidicus gigas* acidic concentrated films is much higher than that obtained with alkaline concentrated films.

3. Extractions performed in several steps, from both red and brown seaweeds, result in a complex polymeric material with a filmogenic capacity similar to and, in some cases, improved in relation to that of the commercial carrageenan and alginate.

4. The aqueous active extracts and the hydrolysates obtained, with antioxidant and antihypertensive properties, can constitute in themselves food ingredients.

5. The integral Mastocarpus extraction allows the recovery of hybrid κ/ι-carrageenan and proteins, which results in stiffer and more water resistant films, due to the carrageenan-protein interactions; while films developed with more purified carrageenan extracts are more malleable and transparent. These differences in their properties allow more diverse applications.

6. The sodium hydroxide treatment of brown seaweeds represents a novel and alternative alginate extraction method, wherein the structure of the uronic acid units is more preserved. This favours inter-polymeric interactions, and consequently, a higher mechanical strength in the films, especially in *Laminaria digitata*. On the contrary, with the classic sodium carbonate extraction, smaller polymeric fractions are obtained, which produce a higher plasticizing effect, resulting in more malleable films, especially in *Ascophyllum nodosum*.

7. *Dosidicus gigas* protein films are more transparent, colourless, with a higher water barrier and more malleable; while seaweed polysaccharide films present the characteristic colour of the source species and show more tensile strength.

8. The development of films with active extracts (aqueous or hydrolysates) of *Mastocarpus stellatus* confers antioxidant properties, increases the light (UV/V) barrier properties, water resistance, and puncture strength, being these last physical properties mainly attributed to protein-carrageenan interactions.

9. The inkjet printing technology by the *core-shell* model, using stearic acid/carnauba wax as encapsulation materials, resulted in a very efficient process for the homogeneous microencapsulation of active peptides.

10. A procedure for the homogeneous inclusion of microcapsules in the films while preserving their integrity, either in the filmogenic solution or during the drying process, has been developed. The inclusion of microcapsules improves the tensile strength, the water vapour impermeability and the opacity of these films.

General conclusion

It is possible to develop films with highly varied properties and susceptible of many applications, at an industrial scale, using proteins recovered from *Dosidicus gigas* waste and red and brown seaweed extracts.

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XV. Resumen ampliado / Extended abstract [2]

[2]Este resumen ampliado se presenta en cumplimiento de las directrices de la normativa de desarrollo del Real Decreto 99/2011, de 28 de enero, que regula los estudios de doctorado en la Universidad Complutense de Madrid (UCM) (BOUC nº 14, de 21 de diciembre de 2012) y de acuerdo con las especificaciones establecidas por la Comisión de Doctorado de la UCM.

This extended abstract is included in fulfilment of the directives of the regulation of development of the Real Decreto 99/2011, de 28 de enero, which regulates the studies of doctorate at the Universidad Complutense de Madrid (UCM) (BOUC nº14, de 21 de diciembre de 2012) and in agreement with the specifications established by the Commission of Doctorate of the UCM.

15.1 Resumen ampliado

15.1.1. Introducción

En la actualidad, se está concediendo un creciente interés al desarrollo de diferentes estrategias para el uso sostenible de los recursos marinos (Caddy & Griffiths, 1995).

La industria procesadora del pescado, concretamente la de cefalópodos, genera grandes cantidades de residuos (hasta 50-75% de la captura) con alto contenido proteico, que podrían recuperarse para usos alternativos al desarrollo de fertilizantes y piensos animales (Shahidi, 1994; Cuclas, 1997), y así asegurar su valor añadido y minimizar la cantidad de desechos (Rustad, Storrø, & Slizyte, 2011). Durante la retirada de pieles del manto de *Dosidicus gigas*, entre un 30-40% del músculo se descarta, siendo posible la recuperación de estas proteínas para el desarrollo de nuevos co-productos (Campo-Deaño, Tovar, Jesús Pombo, Teresa Solas, & Javier Borderías, 2009).

Por otro lado, las costas atlánticas son ricas en especies de algas comestibles infrautilizadas, como por ejemplo *Mastocarpus stellatus* (alga roja), *Laminaria digitata* y *Ascophyllum nodosum* (algas pardas). Estas algas se han estudiado debido a su riqueza en carragenato y alginato, de alto interés industrial, y compuestos potencialmente bioactivos; pero sería interesante buscar métodos alternativos de procesamiento para el desarrollo de formas de consumo alternativas que potencien su uso en Occidente (Plaza, Cifuentes, & Ibáñez, 2008; Rinaudo, 2008).

El desarrollo de métodos de extracción más eficientes e integrales podría dar lugar a distintas materias primas con propiedades tecnológicas. En *D. gigas* se ha estudiado ampliamente la solubilización proteica en medios acuosos, salinos, ácidos y básicos, y su concentración mediante precipitación isoeléctrica (Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009; Sánchez-Alonso, Careche, & Borderías, 2007); mientras que en el caso de las algas, casi todos los estudios se han focalizado en la optimización de la extracción de carragenato y alginato en algas rojas y pardas, respectivamente (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009).

Además, en los últimos años, se han estudiado materias primas de diversos orígenes orgánicos, como alternativa a los materiales sintéticos, para el desarrollo de recubrimientos comestibles y/o biodegradables (Petersen, Væggemose Nielsen, Bertelsen, Lawther, Olsen, Nilsson, et al., 1999). Por un lado, se ha estudiado la capacidad filmogénica de algunas proteínas musculares como las recuperadas de los desechos de la industria procesadora de diferentes especies de pescado (Artharn, Benjakul, & Prodpran, 2008) y del cefalópodo *Todadores pacificus* (Leerahawong, Arii, Tanaka, & Osako, 2011); y por otro, la del carragenato y alginato extraídos tradicionalmente (Han & Kim, 2008; Nakamura, Nishimura, Hatakeyama, & Hatakeyama, 1995). Nunca antes se ha planteado maximizar la extracción de las proteínas musculares de *Dosidicus gigas* y todos los compuestos con potencial tecnológico que las algas contienen (proteínas, otros polisacáridos…), aparte de los ficocoloides, para el desarrollo de recubrimientos comestibles.

Por otro lado, se ha demostrado que, tanto los polisacáridos azufrados (carragenato en alga roja y fucano en alga parda), como los péptidos y polifenoles que contienen las algas, entre otros, pueden

mostrar actividad biológica, como por ejemplo antioxidante y antihipertensora (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011; Ngo, Wijesekara, Vo, Van Ta, & Kim, 2011). Debido a que esta actividad es, en ocasiones, susceptible a los cambios bruscos de pH y temperatura, sería interesante desarrollar métodos de extracción adecuados para su preservación, que a la vez fueran compatibles con ser combinados con las extracciones en condiciones más extremas de los materiales poliméricos. Por ejemplo, la combinación de extracciones acuosas y/o hidrólisis enzimática seguidas de una extracción de carragenato a altas temperaturas y cortos períodos de tiempo, sería una buena alternativa de tratamiento en *M. stellatus* (Herrero, Cifuentes, & Ibáñez, 2006; Wang, Jónsdóttir, Kristinsson, Hreggvidsson, Jónsson, Thorkelsson, et al., 2010).

Dado que las coberturas comestibles son menos estables que las sintéticas, la posibilidad de añadir compuestos activos en su formulación mejoraría la calidad y propiedades funcionales de las películas (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). Sin embargo, la adición directa de hidrolizados en recubrimientos comestibles puede ir en detrimento de sus propiedades filmogénicas (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009), y conferir además sabor amargo en algunas ocasiones (Sun-Waterhouse & Wadhwa, 2013), planteándose nuevos métodos de incorporación de péptidos activos, tales como la microencapsulación.

La microencapsulación es una tecnología que se ha comenzado a utilizar en la industria alimentaria como vehículo de compuestos, para protegerlos por diversos motivos, y en el caso de los ingredientes activos para resguardarlos de los elementos externos, ya sea durante el procesamiento del alimento o en el producto final (Gibbs, Kermasha, Alli, & Mulligan, 1999). Existen muchos tipos de encapsulación (coacervación, atomización, liposomas…), pero recientemente se ha aplicado la tecnología de inyección para la generación mediante impresión de un nuevo método de encapsulación (Houben, 2012), que produce de manera controlada y constante microcápsulas homogéneas según el modelo *core-shell*, que hasta ahora no se ha aplicado en alimentos. La microencapsulación por impresión podría ser una buena metodología de encapsulación de hidrolizados.

Teniendo todo esto en consideración, la obtención de distintos extractos poliméricos y activos de *D. gigas*, *M. stellatus*, *L. digitata* y *A. nodosum* y la microencapsulación, se plantean como nuevas oportunidades para desarrollar recubrimientos comestibles, con o sin actividad.

15.1.2. Objetivos

El objetivo de esta tesis fue el desarrollo de películas activas y comestibles de proteínas y polisacáridos, a partir de recursos de origen marino, tales como los desechos del procesado industrial de cefalópodos y las algas infrautilizadas.

Para este fin, se consideraron los siguientes objetivos parciales:

1. Valorización de materiales poliméricos recuperados de distintos recursos marinos, para la producción de proteínas musculares de desechos del procesado industrial de *Dosidicus gigas,* y extractos no refinados de carragenato y alginato a partir de algas rojas (*Mastocarpus stellatus*) y pardas (*Laminaria digitata* y *Ascophyllum nodosum*).

2. Desarrollo de películas a partir de los biopolímeros extraídos de productos marinos, y su caracterización físico-química para explorar distintas aplicaciones alimentarias.

3. Obtención y caracterización de extractos acuosos e hidrolizados con propiedades activas (antioxidante y antihipertensora), a partir de los desechos del procesamiento industrial de *D. gigas* y de *M. stellatus*, para su utilización como ingredientes funcionales.

4. Desarrollo de películas antioxidantes mediante la incorporación de compuestos activos en las matrices biopoliméricas.

5. Microencapsulación de péptidos activos extraídos de las túnicas de *D. gigas*, mediante el método *core-shell* utilizando la tecnología de impresión por inyección, caracterización de las microcápsulas y su incorporación en películas comestibles.

15.1.3. Resultados

Obtención de materias primas y desarrollo de películas a partir de músculo de *Dosidicus gigas*

Las proteínas de *D. gigas* se recuperaron mediante dos métodos: i) solubilizando en medio acuoso, salino, alcalino y ácido y ii) concentrando mediante precipitación isoeléctrica de las proteínas solubilizadas en medio alcalino y ácido. Mientras que solubilizando se consigue un uso integral de la materia prima, concentrando se restringe a las proteínas susceptibles de precipitar, lo cual garantizaría la eliminación de la mayoría de los compuestos nitrogenados responsables del sabor y olor característicos de esta especie. En los medios alcalino y ácido se obtuvieron los más altos rendimientos, y al concentrarlos se recuperaron proteínas estructuralmente mejor preservadas, y por lo tanto más funcionales.

Mientras que solubilizando en medio alcalino se recuperaron proteínas parcialmente desnaturalizadas, con la miosina desdoblada y signos de agregación intramolecular; solubilizando a pH ácido las proteínas se hidrolizaron, obteniendo un material polimérico de menor peso molecular. Concentrando mediante precipitación isoeléctrica se obtuvo una mayor estabilidad proteica de estos extractos, especialmente notable en proteínas solubilizadas en medio ácido.

Los extractos solubilizados y concentrados de músculo de *D. gigas* fueron buenas materias primas para el desarrollo de películas comestibles, con total manejabilidad, transparentes y con total absorción de luz ultravioleta. Las películas de extracto solubilizado en agua presentaron alta barrera al agua; si bien estas películas junto con las obtenidas solubilizando con sal, no fueron microbiológicamente estables ni presentaron tan buenas propiedades mecánicas como las elaboradas con extractos obtenidos a pH alcalino o ácido.

Los extractos solubilizados en pH alcalino dieron lugar a películas más resistentes al agua, a la tracción y perforación mecánica. Estas propiedades mejoraron mucho más con el concentrado alcalino, probablemente debido a un aumento de interacciones proteína-proteína. Los extractos solubilizados en pH ácido formaron películas con más interacciones proteína-plastificante y deformables. El concentrado

ácido disminuyó moderadamente esta afinidad por el agua, mejorando discretamente sus propiedades de barrera al agua.

Tras 4 meses de conservación en condiciones de temperatura y humedad controladas, las películas de concentrado alcalino y ácido sufrieron una agregación proteica que se tradujo en un aumento de solubilidad. Mientras que las películas alcalinas sufrieron una pérdida de interacciones proteína-proteína que se reflejó en una pérdida total de propiedades mecánicas y de barrera al agua, en condiciones ácidas las películas sufrieron una reorganización de la matriz que resultó en una ligera mejora de sus propiedades mecánicas y de retención de proteínas en la red polimérica al entrar en contacto con el agua. En general, las películas de concentrado ácido fueron más estables.

Obtención de materias primas y desarrollo de películas a partir de *Mastocarpus stellatus*

Con el objetivo de aprovechar al máximo todos sus componentes, *M. stellatus* se sometió a dos tipos de extracciones: i) Extracción acuosa escalonada y ii) Hidrólisis con precipitación de carragenato.

En la extracción acuosa escalonada se obtuvieron dos extractos poliméricos, ricos en el híbrido κ/ι-carragenato y en proteínas, con buena capacidad filmogénica: M3 y M4. M3 presentó características químicas más parecidas al carragenato comercial, mientras que M4 presentó un alto contenido en otros compuestos presentes en el alga tales como proteínas. En esta misma extracción, también se obtuvieron dos extractos antioxidantes: M1 y M2. M1 presentó mayores rendimientos de extracción y actividad antioxidante, por lo que se eligió como ingrediente en el desarrollo de películas antioxidantes.

En la hidrólisis se obtuvieron dos hidrolizados con actividad antioxidante y antihipertensora: H (con el contenido fenólico parcialmente descartado) y Hp (con contenido fenólico completo). Mientras que H presentó la mayor capacidad inhibidora de la enzima convertidora de angiotensina (ECA), Hp fue el hidrolizado con mayor capacidad antioxidante y mayor rendimiento de extracción. Hp se seleccionó para el desarrollo de películas antioxidantes, aunque obviamente cualquiera de los dos podrían constituir en sí mismos ingredientes alimentarios.

A partir de las dos extracciones ricas en polisacáridos, se desarrollaron dos películas con un comportamiento diferente. Mientras que M3 fue adecuada para elaborar películas transparentes y flexibles, M4 originó una matriz más resistente al agua y a la fuerza mecánica debido a la mayor proporción de uniones proteína-carragenato. La mezcla de M3 y M4 dio lugar a películas con propiedades intermedias; y la disminución de la proporción de plastificante aumentó considerablemente la resistencia al agua y mecánica de la película M3+M4.

M1 y Hp presentaron un alto contenido en compuestos azufrados, lo que podría ser debido a dos cosas: i) una pequeña fracción de carragenato extraído de manera colateral ii) la presencia de ficobiliproteínas ricas en aminoácidos azufrados (Carra, Ó Heocha, & Carroll, 1964) con actividad antioxidante (Hirata, Tanaka, Ooike, Tsunomura, & Sakaguchi, 2000; Lin & Stekoll, 2011). A pesar de que la actividad antioxidante de M1 fue menor que la de Hp, su incorporación en las películas dio lugar a la mayor actividad antioxidante. Este efecto probablemente se debió a una menor interacción entre los compuestos de M1 y los de la matriz polimérica. Los péptidos de Hp interaccionaron más eficientemente

con las hélices de carragenato y el plastificante de la película, disminuyendo su disponibilidad para reaccionar con agentes oxidantes.

La incorporación de M1 mejoró la resistencia al agua y mecánica de las películas. La adición de Hp tuvo un efecto plastificante en las películas que mejoraron su elongación por tracción; aunque dependiendo de la proporción de Hp añadida las propiedades filmogénicas se vieron afectadas de forma diferente. Así por ejemplo, la adición de un 15% de Hp, con respecto al contenido seco de la película, disminuyó su permeabilidad al vapor del agua, mientras que al añadir un 30% sus propiedades ópticas, resistencia al agua y respuesta a la perforación aumentaron.

Obtención de materias primas y desarrollo de películas a partir de *Laminaria digitata y Ascophyllum nodosum*

En las algas pardas *L. digitata* y *A. nodosum*, se analizaron diversos métodos de extracción, con el objetivo de suavizar al máximo las condiciones ácidas del pre-tratamiento y optimizar tanto la temperatura como el tipo de tratamiento alcalino. Estos extractos, además de contener alginato en mayor o menor medida degradado, contendrían la mayor parte del resto de compuestos de cada alga. Se diseñó una nueva extracción alcalina con hidróxido sódico y se comparó la tradicionalmente llevada a cabo con carbonato sódico para cada especie. De cada tipo de extracción, se eligieron aquellos extractos que presentaron mejores rendimientos y capacidad filmogénica.

Mientras que con NaOH se extrajeron más eficientemente los carbohidratos, con Na_2CO_3 los extractos presentaron el doble de contenido en cenizas. El tratamiento con NaOH recuperó mayor proporción de alginato no degradado con un alto contenido en unidades de gulurónico, lo cual facilitó la formación de interacciones poliméricas en la película. Sin embargo, la extracción con Na_2CO_3 recuperó un alto porcentaje de alginato degradado y unidades de ácidos urónicos dispersos, lo cual facilitó sus interacciones con el plastificante.

El tipo de tratamiento alcalino condicionó las diferencias entre películas como barrera al agua, mientras que la especie de alga influyó en sus propiedades mecánicas. El extracto obtenido con Na_2CO_3 de Ascophyllum dio lugar a películas transparentes y flexibles, mientras que el extracto obtenido con NaOH de Laminaria formó la película más fuerte y menos permeable al vapor de agua.

La presencia de pigmentos como la fucoxantina, y de polisacáridos sulfatados como los fucanos, confirió actividad antioxidante a las películas (Le Tutour, Benslimane, Gouleau, Gouygou, Saadan, & Quemeneur, 1998; Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

La película obtenida con el extracto de NaOH en Ascophyllum tuvo mayor capacidad antioxidante que el resto de películas desarrolladas a partir de algas pardas, probablemente debido a la presencia de una mayor proporción de compuestos sulfatados. Esta actividad destacó por ser parecida a la obtenida en Mastocarpus al añadir hidrolizado antioxidante en su fórmula.

Como técnica de microencapsulación de péptidos bioactivos, se estudió un nuevo método por inyección, mediante impresión en una solución de ácido esteárico y cera de carnauba, siguiendo el

modelo *core-shell*. Se obtuvo una eficiencia de encapsulación del 84.7 ± 3.4% con una ratio de material de encapsulación:péptido de 13,3:1, y las cápsulas fueron homogéneas con un diámetro de 110-140 µm.

Las microcápsulas mostraron diferente estabilidad dependiendo del pH y la temperatura del medio, siendo más estables a bajas temperaturas y pH 7. Para evitar la aglomeración y rotura de las cápsulas, durante el proceso de elaboración de películas de extracto en Na_2CO_3 de Laminaria, fue necesario desarrollar un método específico de inclusión, obteniendo películas más resistentes y deformables a la tracción mecánica.

Todas las películas desarrolladas en el presente trabajo podrían ser susceptibles de formar parte de la presentación final de productos alimenticios, ya sea como parte del envase o como ingrediente propio del diseño del alimento.

Los extractos solubilizados de *D. gigas,* dado su alto contenido en compuestos nitrogenados, mostraron un aroma a pescado característico, por lo que su aplicación quedaría restringida. El resto de extractos poliméricos podrían usarse de manera más versátil directamente en diversos tipos de comida, siendo posible la modificación de su fórmula para adecuarlo a las necesidades culinarias y abriendo la posibilidad de mezclar extractos ricos en proteínas con extractos ricos en polisacáridos para obtener propiedades intermedias, o quizá las mejores de ambos.

Las películas antioxidantes podrían emplearse en la elaboración de alimentos funcionales, como parte de la presentación final de un producto alimenticio, mejorando su conservación en el tiempo y sus propiedades nutritivas. Pero también se podrían aplicar desde el punto de vista tecnológico para prevenir de la oxidación durante el proceso culinario o durante la conservación en tiempos cortos, por ejemplo en productos destinados a servicios de *catering*, o incluso una conservación propiamente dicha en tiempos más prolongados. Además, el carácter hidrofílico de las películas desarrolladas las convierte en un buen medio para vehiculizar microcápsulas hidrofóbicas, ofreciendo al mercado de alimentos funcionales una nueva forma de incorporar compuestos activos a los alimentos.

15.1.4. Conclusiones

1. Las proteínas musculares de *Dosidicus gigas,* tanto en forma de extractos solubles como de concentrados, presentan propiedades filmogénicas, aunque muestran características diferentes. Las películas de concentrados destacan por su mayor resistencia mecánica y al agua. Las de concentrados ácidos son elásticas y deformables, mientras que las de concentrados alcalinos son más fuertes y muy resistentes al agua.

2. La estabilidad de las películas de *Dosidicus gigas* realizadas con concentrados ácidos es muy superior a la de las obtenidas con concentrados alcalinos.

3. Las extracciones en diversas etapas, tanto de algas rojas como pardas, dan lugar a un material polimérico complejo, con una capacidad filmogénica comparable, y en algún caso mejorada, con respecto al carragenato y alginato comerciales.

4. Los extractos activos acuosos y los hidrolizados obtenidos, con propiedades antioxidantes y antihipertensoras, pueden constituir en sí mismos ingredientes alimentarios.

5. La extracción integral en Mastocarpus permite obtener el híbrido κ/ι-carragenato y proteínas, lo que da lugar a películas más rígidas y más resistentes al agua debido a las interacciones carragenato-proteína; mientras que las películas formuladas con extractos más purificados de carragenato son más deformables y transparentes. Esta diferencia en sus propiedades permite diversificar sus aplicaciones.

6. El tratamiento con hidróxido sódico de algas pardas constituye un método alternativo y novedoso de extracción de alginato, donde las unidades de ácidos urónicos están estructuralmente más preservadas. Esto favorece una mayor interacción interpolimérica y, en consecuencia, mayor resistencia mecánica en las películas, en especial en *Laminaria digitata*. Por el contrario, en la extracción clásica con carbonato sódico, se obtienen fracciones poliméricas más pequeñas, que producen mayor efecto plastificante, lo cual se traduce en películas más deformables, en especial en *Ascophyllum nodosum.*

7. Las películas proteicas de *Dosidicus gigas* son más transparentes, incoloras, con mayor barrera al agua y más deformables; mientras que las películas de polisacáridos de algas son del color característico de la especie de origen y más resistentes a la tracción.

8. La formulación de películas con extractos activos (acuosos o hidrolizados) de *Mastocarpus stellatus* confiere propiedades antioxidantes y aumenta las propiedades de barrera a la luz (UV/V), resistencia al agua, fuerza y deformación a la perforación, atribuyéndose estas propiedades físicas principalmente a las interacciones proteína-carragenato.

9. El modelo *core-shell* mediante la técnica de impresión por inyección, utilizando como materiales de encapsulación el ácido esteárico/cera de carnauba, es un proceso muy eficiente para la microencapsulación homogénea de péptidos activos.

10. Se ha desarrollado un método para incorporar las microcápsulas de manera uniforme en las películas sin que pierdan su integridad, tanto en la solución filmogénica, como durante el secado. La inclusión de microcápsulas aumenta las propiedades de resistencia a la tracción, la impermeabilidad al vapor del agua y la opacidad de estas películas.

Conclusión general

Es posible desarrollar películas a nivel industrial con propiedades muy diversas, susceptibles de numerosas aplicaciones, a partir de proteínas procedentes de residuos de *Dosidicus gigas* y de extractos de algas rojas y pardas.

15.1.5. Aportaciones fundamentales de la tesis doctoral

Esta memoria propone métodos de valorización de residuos industriales y de recursos infrautilizados, con materiales ricos en proteínas y en polisacáridos. Algunos de estos métodos contemplan como alternativa la utilización integral de los recursos aumentado los rendimientos, la versatilidad de su uso y en ocasiones incluso su funcionalidad. Además, se han escogido procesos suaves y ecológicos. Por todo esto se estima que los extractos obtenidos en este trabajo son altamente competitivos a nivel industrial.

Los extractos poliméricos sirven de base como material filmogénico, dando lugar a películas con propiedades muy variadas, lo cual nos permite diversificar el diseño de productos y su aplicación.

Por primera vez, se estudia el desarrollo de películas como posible aplicación industrial de concentrados de músculo de *D. gigas*, abriendo nuevas posibilidades de mercado a un desecho tan abundante como los restos de músculo adheridos a las túnicas y pieles del manto, y encontrando una nueva aplicación a las proteínas concentradas tras su solubilización en medio alcalino.

Las extracciones desarrolladas para *M. stellatus* son fácilmente aplicables a escala industrial, abriendo la posibilidad de su uso en otras especies de algas rojas. Además, el presente trabajo propone un nuevo método de extracción con hidróxido sódico de algas pardas, igualmente fácil de aplicar a nivel industrial, con altos rendimientos y buenas propiedades tecnológicas.

Los extractos activos acuosos y los hidrolizados obtenidos, con propiedades antioxidantes y antihipertensoras, pueden constituir en sí mismos ingredientes alimentarios. Concretamente, los hidrolizados de algas obtenidos en este estudio mostraron una actividad antihipertensora mucho mayor que la de las obtenidas hasta el momento con otros extractos de alga referidos en la bibliografía.

Por primera vez, se ha estudiado y conseguido la aplicación alimentaria del modelo *core-shell* de encapsulación, encapsulando péptidos bioactivos mediante la técnica de impresión por inyección y utilizando como material de encapsulación ácido esteárico y cera de carnauba.

La incorporación de microcápsulas en las películas ha requerido el desarrollo de un método de inclusión uniforme que evite la pérdida de su integridad, tanto en la solución filmogénica, como durante el secado. Las películas protegen las cápsulas y ofrecen la posibilidad de utilizarse como ingrediente principal en el desarrollo de productos alimenticios funcionales, siendo posible el diseño de distintos modelos de liberación del contenido encapsulado.

Todos estos extractos y microcápsulas ofrecen la posibilidad de combinarse para el desarrollo de diferentes materiales de recubrimiento, según el tipo de producto final que se busque y su aplicación futura. En cuanto a estas nuevas posibilidades, varios estudios están actualmente en proceso, con la intención de que en un futuro cercano se pueda hablar de estas películas como una opción culinaria real.

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15.2. Extended abstract

15.2.1. Introduction

Nowadays, a growing concern is being raised around the development of different strategies for the sustainable use of marine resources (Caddy & Griffiths, 1995).

The fish processing industry, and, more specifically the cephalopod industry, generates a great amount of waste (up to 50-75% of the catch) with high protein contents, which could be reused for purposes other than the development of fertilizers and animal feeds (Shahidi, 1994; Cuclas, 1997), thus assuring its added value and minimizing the amount of residues (Rustad, Storrø, & Slizyte, 2011). During the skinning of *Dosidicus gigas* mantle, between 30-40% of the muscle is discarded, being the recovery of these proteins possible for the development of new byproducts (Campo-Deaño, Tovar, Jesús Pombo, Teresa Solas, & Javier Borderías, 2009).

On the other hand, Atlantic coasts are very rich in underutilized edible seaweed species, like for example, *Mastocarpus stellatus* (red algae), *Laminaria digitata* and *Ascophyllum nodosum* (brown algae). These seaweeds have been studied due to their high content in carrageenan and alginates, of high industrial interest, and potentially bioactive compounds; nevertheless, it would be interesting to find new processing methods for the development of alternative consumption forms which could expand their use in Western countries (Plaza, Cifuentes, & Ibáñez, 2008; Rinaudo, 2008).

The development of more efficient and complete extraction methods could result in different raw materials with technological properties. In *D. Gigas*, protein solubilization in aqueous, salt, alkaline and acidic conditions and its further concentration by isoelectric precipitation has been extensively studied (Palafox, Cordova-Murueta, del Toro, & García-Carreno, 2009; Sánchez-Alonso, Careche, & Borderías, 2007); whereas in seaweeds, almost every study has focused in the optimization of the carrageenan and alginate extraction in red and brown algae respectively (Hilliou, Larotonda, Abreu, Ramos, Sereno, & Gonçalves, 2006; Vauchel, Leroux, Kaas, Arhaliass, Baron, & Legrand, 2009).

Moreover, in the past few years, raw materials of various origins have been studied as an alternative to synthetic materials for the development of edible and/or biodegradable film coatings (Petersen, Væggemose Nielsen, Bertelsen, Lawther, Olsen, Nilsson, et al., 1999). It has been studied, on the one hand, the filmogenic capacity of certain muscle proteins, such as those recovered from the waste of the fish processing industry (Artharn, Benjakul, & Prodpran, 2008) and of the cephalopod *Todadores pacificus* (Leerahawong, Arii, Tanaka, & Osako, 2011); and on the other hand, that of the traditionally extracted carrageenan and alginate (Han & Kim, 2008; Nakamura, Nishimura, Hatakeyama, & Hatakeyama, 1995). It has never been previously considered to maximize the extraction of *D. gigas* muscle proteins and all the seaweed compounds, aside from the phycocolloids, with technological potential (proteins, other polysaccharides…), for the development of edible film coatings.

In addition, it has been proven that both sulfated polysaccharides (carrageenan in red seaweeds, and fucoidan in brown species) and polyphenols and peptides contained in algae, among others, can show biological activity, such as, for example, antioxidant and antihypertensive activity (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011; Ngo, Wijesekara, Vo, Van Ta, & Kim, 2011). Since this activity is sometimes susceptible to sudden changes in temperature and pH, it would be interesting to develop

extraction methods suitable for its preservation that, at the same time, would allow being combined with the more extreme conditions of the extractions of polymeric materials. For example, the combination of aqueous extractions and/or enzymatic hydrolysis followed by an extraction of carrageenan at high temperatures and short times, would be a good alternative treatment for *M. stellatus* (Herrero, Cifuentes, & Ibáñez, 2006; Wang, Jónsdóttir, Kristinsson, Hreggvidsson, Jónsson, Thorkelsson et al., 2010).

Given that edible film coatings are less stable than synthetic ones, the possibility of adding active compounds in their formula would improve the quality and functional properties of the films (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). Nevertheless, the direct addition of hydrolysates to edible film coatings could be detrimental to their filmogenic properties (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009), and besides, it could sometimes confer a bitter taste (Sun-Waterhouse & Wadhwa, 2013); thus it is that new methods of active peptide addition, such as microencapsulation, have to be considered.

Microencapsulation is a technology which started being used in the food industry as a vehicle for certain compounds in order to protect them for different reasons and, in the case of active ingredients, to give them shelter from external elements, either during the food processing or in the final product (Gibbs, Kermasha, Alli, & Mulligan, 1999). There are many types of encapsulation procedures (coacervation, spray-drying, liposomes…), but recently the inkjet printing technology has been applied in the generation of a new method of microencapsulation (Houben, 2012), which produces homogenous microcapsules according to the core-shell model in a controlled and constant process, though it has not been applied to food product development until now. Inkjet printing microencapsulation might be a good method for hydrolysates encapsulation.

Taking everything into account, the production of different polymeric and active extracts of *D. gigas, M. stellatus, L. digitata* and *A. nodosum* and their further microencapsulation, are considered novel opportunities to develop edible film coatings, with or without activity.

15.2.2. Objectives

The aim of this thesis was to develop edible and active protein and polysaccharide films, from resources of marine origin, such as waste from the cephalopod processing industry and underutilized seaweeds.

To this end, the following partial objectives were considered:

6. Valorization of polymer materials recovered from different marine resources to produce muscle protein extracts from the waste from *Dosidicus gigas* industrial processing, and carrageenan and alginate unrefined extracts from red (*Mastocarpus stellatus*) and brown (*Laminaria digitata* and *Ascophyllum nodosum*) seaweeds.

7. Development of films based on biopolymers extracted from marine products, and their further physicochemical characterization to explore for different applications in the food industry.

8. Production and characterization of aqueous extracts and hydrolysates with active properties (antioxidant and antihypertensive) from the waste from *D. gigas* industrial processing and *M. stellatus*, to be utilized as functional ingredients.

9. Development of antioxidant films by the incorporation of active compounds into the biopolymer matrixes.

10. Microencapsulation of bioactive peptides extracted from *D. gigas* tunics by the *core-shell* method using the inkjet printing technology, characterization of the microcapsules and its addition to edible films.

15.2.3. Results

Extraction of raw materials and development of films from *Dosidicus gigas* muscle

D. gigas proteins were recovered by two methods consisting in: i) solubilization under aqueous, salt, alkaline and acidic conditions, and ii) concentration by isoelectric precipitation of the proteins solubilized under alkaline and acidic conditions. While an integral use of the raw material is achieved with the solubilization method, the concentration method is restricted only to those proteins susceptible to precipitation, which would guarantee the removal of most of the nitrogenated compounds responsible for this species' characteristic flavour. Alkaline and acidic conditions maximized the extraction yields; and when extracts were concentrated, more structurally preserved proteins, and thereby more functional, were recovered.

While proteins recovered in the alkaline solubilized extract were partially denatured, with unfolded myosin and signs of intermolecular aggregation; acidic solubilized proteins suffered hydrolysis, obtaining a lower molecular weight polymer material. Concentration by isoelectric precipitation led to extracts with more protein stability, especially significant in acidic solubilized proteins.

Both solubilized and concentrated extracts obtained from *D. gigas* muscle, were good raw materials for edible film development, absolutely easy to handle, transparent and with total ultraviolet light absorption. The water solubilized extract films showed a high water barrier, although these films, together with those obtained by salt solubilization, failed to be microbiologically stable and did not result in as good mechanical properties as those made of extracts obtained under alkaline or acidic pH.

Alkaline solubilized extracts resulted in films with more water resistance, tensile and puncture strength. These properties were considerably improved in alkaline concentrated films, probably due to an increased number of protein-protein interactions. Acidic solubilized extracts resulted in malleable films with more protein-plasticizer interactions. The acidic concentrated extraction lowered this water affinity, slightly improving the film water barrier properties.

After 4 months of storage under controlled temperature and humidity conditions, alkaline and acidic concentrated films suffered protein aggregation, which entailed an increase in solubility. While alkaline concentrated films underwent a loss of protein-protein interactions producing a total loss of mechanical and water barrier properties; acidic concentrated films experienced a matrix reorganization

that resulted in a slight improvement of the mechanical properties and of the protein retention in the polymer matrix when contacting water. In general, acidic concentrated films were more stable.

Extraction of raw materials and development of films from *Mastocarpus stellatus*

With the aim of exploiting to the maximum all its components, *M. stellatus* was subjected to two different types of extractions: i) Stepped aqueous extraction and ii) Hydrolysis with carrageenan precipitation.

The stepped aqueous extraction resulted in two polymer extracts, rich in κ/ι-carrageenan hybrid and proteins, with a good filmogenic capacity: M3 and M4. While M3 showed chemical characteristics resembling commercial carrageenan, M4 presented a high content of other seaweed compounds such as proteins. In this same extraction, two antioxidant extracts were also obtained: M1 and M2. M1 showed higher extraction yields and antioxidant activity, and therefore was selected as an ingredient for the development of antioxidant films.

Two antioxidant and antihypertensive hydrolysates were obtained in the hydrolysis: H (with a partially discarded phenolic contents) and Hp (with full phenolic contents). Whereas H showed the highest angiotensin-converting enzyme (ACE) inhibitory capacity, Hp presented the highest antioxidant capacity and extraction yield. Hp was selected for the development of antioxidant films, although it is obvious that any of them could constitute food ingredients by themselves.

Based on the two polymer rich extracts, two films with a different behaviour were developed. While M3 was suitable to develop transparent and flexible films, M4 produced a matrix with more water resistance and mechanical strength due to a higher proportion of protein-carrageenan bonds. The M3 and M4 mixture in the film formulation, resulted in intermediate film properties; and lowering the plasticizer ratio considerably increased the water resistance and mechanical strength of the M3+M4 film.

M1 and Hp presented a high sulfated compounds content, which might be due to two reasons: i) a small carrageenan fraction extracted collaterally; ii) the presence of phycobiliproteins rich in sulfated amino acids (Carra, Ó Heocha, & Carroll, 1964) with antioxidant activity (Hirata, Tanaka, Ooike, Tsunomura, & Sakaguchi, 2000; Lin & Stekoll, 2011). Even though M1 antioxidant activity was lower than Hp's, its inclusion in the films resulted in the highest antioxidant activity. This effect was probably due to a lower interaction between M1 compounds and the polymer matrix. Hp peptides interacted more efficiently with the carrageenan helices and plasticizer in the films, lowering its capacity to react with oxidant agents.

The inclusion of M1 in the films improved their mechanical strength and water resistance. Hp addition had a plasticizing effect on the films that improved their tensile elongation; but depending on the Hp ratio included the filmogenic properties were affected in a different way. For example, a 15% Hp addition, with respect to the dry film content, lowered their water vapour permeability; while a 30% addition improved the optical properties, the water resistance and the puncture strength of the films.

Extraction of raw materials and development of films from *Laminaria digitata* and *Ascophyllum nodosum*

Various extraction procedures were analysed in *L. digitata* and *A. nodosum* brown seaweeds, with the purpose of softening as much as possible the pretreatment acidic conditions and optimizing both the temperature and the type of alkaline treatment. Apart from an alginate content degraded to a greater or lesser extent, these extracts would also contain most of the rest of each seaweed compounds. A new alkaline extraction, using sodium hydroxide, was designed and compared in each seaweed species with the traditional sodium carbonate extraction procedure. From each extraction method, those extracts presenting the best yields and filmogenic capacity, were selected.

While carbohydrates were more efficiently extracted with NaOH, double ash content was obtained with Na_2CO_3 extraction. A higher proportion of non-degraded alginate with a high content in guluronic units was recovered with the NaOH treatment, which contributed to form polymer interactions in the film. However, a high percentage of degraded alginate and dispersed uronic acid units was recovered with Na_2CO_3 extraction, which contributed to their interaction with the plasticizer.

The type of alkaline treatment determined the water barrier differences among films, while the seaweed species affected the mechanical properties of the films. The Ascophyllum Na_2CO_3 extract resulted in transparent and flexible films, while the Laminaria NaOH extract produced the strongest and least water vapour permeable film.

The film antioxidant activity was conferred by the presence of pigments like fucoxanthin and sulfated polysaccharides such as fucoidans (Le Tutour, Benslimane, Gouleau, Gouygou, Saadan, & Quemeneur, 1998; Rocha De Souza, Marques, Guerra Dore, Ferreira Da Silva, Oliveira Rocha, & Leite, 2007).

The film obtained with the Ascophyllum NaOH extract had the highest antioxidant capacity among all the brown seaweed films developed, probably due to the presence of a higher proportion of sulfated compounds. This activity was highlighted for its resemblance to that previously reported in Mastocarpus films when adding antioxidant hydrolysate to the formula.

A bioactive peptides microencapsulation procedure by inkjet printing technology in a stearic acid and carnauba wax mixture, following the core-shell model, was studied. An encapsulation efficiency of 84.7 ± 3.4% and a ratio of 13.3:1 microencapsulation material:peptide (on dry basis) were obtained. The microcapsules were homogeneous and with a 110-140 μm diameter.

Depending on the environmental pH and temperature, the microcapsules showed different stability, being more stable at low temperatures and pH 7. In order to avoid microcapsule agglomeration and breakage during the development of films from Laminaria Na_2CO_3 extract, the development of a new and specific film inclusion procedure was required, obtaining films with better tensile strength and more malleable.

All films developed in the present work might be susceptible of use in the final food serving presentation, whether as part of the packaging material or as ingredients by themselves in food design.

Due to their high content in nitrogenated compounds, *D. gigas* solubilized extracts showed a characteristic fish flavour that would limit their applications. The rest of the polymer extracts might have more versatile uses, directly in various types of food, being possible to modify their formula to suit the culinary needs; and therefore extending the possibilities of mixing protein rich and polysaccharide rich extracts to obtain intermediate properties, or even the best of each.

Antioxidant films might be used in functional food development, as part of the final food presentation, improving the final product shelf-life and nutritional value. But they could also be applied technologically to prevent oxidation during the culinary process or for short shelf-life terms, such as in catering food products, or even as preservatives themselves for longer terms. Besides, the hydrophilic character of the developed films renders them good hydrophobic microcapsule carriers, offering the functional food market a new alternative for active compound inclusion in food products.

15.2.4. Conclusions

11. *Dosidicus gigas* muscle proteins, either as solubilized or concentrated extracts, present filmogenic properties, although each type shows different characteristics. The concentrated films highlights are their greater mechanical strength and water resistance. The acidic concentrated films are elastic and malleable, while the alkaline concentrated films are stronger and very water resistant.

12. The stability of the *Dosidicus gigas* acidic concentrated films is much higher than that obtained with alkaline concentrated films.

13. Extractions performed in several steps, from both red and brown seaweeds, result in a complex polymeric material with a filmogenic capacity similar to and, in some cases, improved in relation to that of the commercial carrageenan and alginate.

14. The aqueous active extracts and the hydrolysates obtained, with antioxidant and antihypertensive properties, can constitute in themselves food ingredients.

15. The integral Mastocarpus extraction allows the recovery of hybrid κ/ι-carrageenan and proteins, which results in stiffer and more water resistant films, due to the carrageenan-protein interactions; while films developed with more purified carrageenan extracts are more malleable and transparent. These differences in their properties allow more diverse applications.

16. The sodium hydroxide treatment of brown seaweeds represents a novel and alternative alginate extraction method, wherein the structure of the uronic acid units is more preserved. This favours inter-polymeric interactions, and consequently, a higher mechanical strength in the films, especially in *Laminaria digitata*. On the contrary, with the classic sodium carbonate extraction, smaller polymeric fractions are obtained, which produce a higher plasticizing effect, resulting in more malleable films, especially in *Ascophyllum nodosum*.

17. *Dosidicus gigas* protein films are more transparent, colourless, with a higher water barrier and more malleable; while seaweed polysaccharide films present the characteristic colour of the source species and show more tensile strength.

18. The development of films with active extracts (aqueous or hydrolysates) of *Mastocarpus stellatus* confers antioxidant properties, increases the light (UV/V) barrier properties, water resistance, and puncture strength, being these last physical properties mainly attributed to protein-carrageenan interactions.

19. The inkjet printing technology by the *core-shell* model, using stearic acid/carnauba wax as encapsulation materials, resulted in a very efficient process for the homogeneous microencapsulation of active peptides.

20. A procedure for the homogeneous inclusion of microcapsules in the films while preserving their integrity, either in the filmogenic solution or during the drying process, has been developed. The inclusion of microcapsules improves the tensile strength, the water vapour impermeability and the opacity of these films.

General conclusion

It is possible to develop films with highly varied properties and susceptible of many applications, at an industrial scale, using proteins recovered from *Dosidicus gigas* waste and red and brown seaweed extracts.

15.2.5. Fundamental contributions of the doctoral thesis

The present work proposes valorization methods for industrial waste and underutilized resources with protein and polysaccharide rich materials. Some of these procedures consider as an alternative the integral use of the resources, increasing their yields, their versatility, and sometimes even their functionality. Besides, mild and environmental friendly processes have been selected. Given all these reasons, it is believed that the extracts obtained in this research are highly competitive at an industrial scale.

Polymeric extracts serve as the building blocks of the filmogenic material, resulting in films with highly varied properties, thus allowing the diversification of the products design and their applications.

For the first time, it has been studied the development of films from *D. gigas* muscle concentrates, as a possible industrial application, opening new market possibilities for such an abundant waste as it is the muscle adhered to tunics and mantles, and finding a novel application for proteins concentrated after alkaline solubilization.

The extractions developed for *M. stellatus*, can be easily scaled up to an industrial level, opening the possibility of their application in other red seaweed species. In addition, the present work proposes a novel brown seaweed extraction procedure with sodium hydroxide, which is equally easy to scale up to an industrial level, and renders high yields and good technological properties.

The active aqueous extracts and the hydrolysates obtained, with antioxidant and antihypertensive properties, can constitute food ingredients by themselves. Specifically, the algae hydrolysates obtained in this study showed a much higher antihypertensive activity than that previously obtained with other algae extracts referred to in the literature.

For the first time, the food application of the *core-shell* encapsulation model has been studied and achieved by encapsulating bioactive peptides with the inkjet printing technology, and using as encapsulation material stearic acid and carnauba wax.

The inclusion of microcapsules in the films has required the development of a homogeneous inclusion method preventing the loss of capsule integrity both in the filmogenic solution and during the drying process. The films protect the capsules and offer the possibility of being used as main ingredient in the development of functional food products, being also possible the design of different release models for the encapsulated contents.

All these extracts and microcapsules may be used alone or in combination with each other for the development of different film coating materials, depending on the type of desirable final product and its future applications. Regarding these novel possibilities, several studies are currently ongoing, with the intention of turning these films into a real culinary choice in the near future.

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XVI. Appendix

16.1. Research paper

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Effect of different protein extracts from *Dosidicus gigas* muscle co-products on edible films development

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ABSTRACT

The co-products produced in the processing industry of *Dosidicus gigas* muscle, is a good source of polymeric material for film developing. The objective of this work was to compare different ways of protein recovery to find the best conditions of material for edible film developing. The proteins were recovered by water, salt, alkaline and acidic solubilisation. The highest protein solubilisation was obtained at alkaline conditions (≥70%) and DSC confirmed a partial denaturalization in saline and alkaline solution and total at acidic conditions, while SDS-PAGE confirmed a hydrolysis effect at pH 3. According to FTIR, the loss of secondary structure at pH 10 led to a stronger bonding film network and the hydrolysis at pH 3 resulted in more protein-plasticizer and protein–water interactions. Both alkaline and acidic conditions led to transparent and microbiologically stable films, the alkaline-film being more water resistant and with less protein release in water contact. Both alkaline- and acidic-films resulted in the more flexible and more resistant, especially at alkaline conditions. While salt-extract did not improve any mechanical property of the corresponding film compared with water-film, both films presented the lower solubility and the more water resistance but were not microbiologically stable and had poorer mechanical properties.

1. Introduction

Nowadays, in the processing industry of fishery products a lot of co-products are produced, mostly protein, that is a source of environmental pollution. In order to get value-added products and eliminate pollution from discarded material, numerous strategies to create new alternatives are studied (Cortes-Ruiz, Pacheco-Aguilar, Lugo-Sanchez, Carvallo-Ruiz, & Garcia-Sanchez, 2008; De la Fuente-Betancourt, Garcia-Carreno, Del Toro, & Cordova-Murueta, 2009; De la Fuente-Betancourt, Garcia-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sanchez, 2009; Felix-Armenta et al., 2009).

This is the case of jumbo squid (*Dosidicus gigas*) which is the largest and most abundant squid specie found in the pelagic zone of eastern Pacific, from Chile up to Oregon coasts (Nigmatullin, Nesis, & Arkhipkin, 2001). The percentage of the edible portion of cephalopods is exceptionally high, between 60 and 80% of their total weight.

In the last decades many products have been developed from *D. gigas* muscle, such as gel-based products (Cortes-Ruiz et al., 2008; De la Fuente-Betancourt, Garcia-Carreno, Del Toro, Cordova-Murueta, & Lugo-Sanchez, 2009) and gel-emulsion products (Felix-Armenta et al., 2009; Gomez-Guillen, Borderias, & Montero, 1997), *surimi* and mince (Campo-Deaño, Tovar, Pombo, Solas, & Borderías, 2009; Gomez-Guillen et al., 1997; Gomez-Guillen, Solas, Borderias, & Montero, 1996) and also other products have been made from skins and tunics (collagen, gelatin) (Denavi et al., 2009; Gimenez, Aleman, Montero, & Gomez-Guillen, 2009; Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011). The use of the mantle muscle as a polymeric material to film developing is a good asset, ensuring an added value and minimizing the discards during their process.

Edible and biodegradable films can be developed from different materials such as proteins, polysaccharides, lipids, and resins (Krochta, 2002). Among these materials, some proteins have been extensively studied because of their relative abundance, film-forming abilities, and nutritional qualities (Hamaguchi, Weng, & Tanaka, 2007).

At the moment muscle protein films have been developed from different marine species such as the bigeye (*Priacanthus tayenus*) (Chinabhark, Benjakul, & Prodpran, 2007), the Indo-Pacific blue marlin (*Makaira mazara*) (Hamaguchi et al., 2007), the round scad (*Decapterus punctatus*) (Artharn, Benjakul, & Prodpran, 2008) or the squid (*Todadores pacificus*) (Leerahawong, Arii, Tanaka, & Osako, 2011); finding differences depending on the specie and the

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protein myofibrillar/sarcoplasmic proportion (strong protein–protein interaction), such as a decreasing of films solubility when the sarcoplasmic content increased or a higher elongation at break when sarcoplasmic proportions under 20% (Artharn et al., 2008). Obviously other factors could be affected, for example the raw material conditions (proteolysis degree, ammonia compounds), protein extraction methods and so on. Other authors observed that film-forming by myofibrillar proteins produce a continuous matrix while the sarcoplasmic fraction tend to develop an added superimposed network (Sobral, dos Santos, & Garcia, 2005) or could be placed in the space left in the myofibrillar protein matrix due to their globular structure and small size. Denavi et al. (2009) found the same behaviour with soy protein in a gelatin matrix.

So far, muscle protein films from giant squid have never been developed before despite it would be an interesting alternative use. Several methods have been used to solubilized muscle proteins of *D. gigas*, it can be performed by a simple homogenization changing the water proportion used because its myofibrillar protein is highly water-soluble, or by solubilizing myofibrillar proteins at low ionic strength (Sanchez-Alonso, Careche, & Borderias, 2007), and at low (1–3) or high (9–11) pH values (Cortes-Ruiz et al., 2008; De la Fuente-Betancourt, Garcia-Carreno, Del Toro, & Cordova-Murueta, 2009; Palafox, Cordova-Murueta, del Toro, & Garcia-Carreno, 2009). However, no information regarding the film ability of *D. gigas* muscle co-products neither the adequate extraction methods to obtain film properties of interest have been described before.

The objective of this study was to evaluate the film forming ability of the *D. gigas* muscle proteins. For this purpose both different methods of protein extraction and properties of the resulting protein edible films were tested.

2. Materials and methods

2.1. Materials

Muscle proteins were recovered from the frozen mantle of giant squid (*D. gigas*), which was caught in the coast of Peru in January 2009. Following capture, the specimens were gutted and mantles separated from tentacles and frozen on board to −20 °C. The frozen mantles were shipped to the industrial plant PSK Océanos S.A. (Pozuelo de Alarcón, Madrid, Spain) and, after processing fishing byproducts, the pieces of discarded mantles were frozen to −20 °C and then sent to our Institute, where were kept frozen as raw material until analysis.

Analytical grade HCl, NaOH, NaBr, glycerol and sorbitol, and food-standard NaCl were from Panreac Química S.A. (Montplet and Estaben S.A., Montcada i Reixac, Barcelona, Spain). DOW 1510 silicon antifoaming agent was from DOW Corning Europe (Brussels, Belgium).

2.2. Methods

2.2.1. Proximate analyses

Moisture, fat and ash content of the raw material were determined according to official methods (A.O.A.C., 1995). Total nitrogen content was determined by Dumas' method (A.O.A.C., 2000) using a combustion oven apparatus (Model FP-2000, Leco Corporation, St Joseph, MI, USA). Nitrogen-to-protein conversion factor of 6.25 was employed to quantify total protein content. Analysis was performed at least in triplicate, and results expressed as percentages.

2.2.2. Microbiological assays

Raw material (10 g) or each film (1 g) were aseptically weighed and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 9 ml of buffered 0.1% peptone water (Oxoid, Basingtoke, UK), and four decimal dilutions were made. The total number of mesophilic microorganisms was determined with plate count agar (PCA, Merck) following the pour plate method, incubating at 30 °C for 72 h. The number of Enterobacteriaceae microorganisms was also determined in the films, using double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h. All microbiological counts were determined at least in triplicate and expressed as the log of the colony-forming units per gram of sample (log CFU/g).

2.3. Muscle protein extraction

Squid mantles were kneaded in a vacuum homogenizer (Stephan UM5, Stephan u Söhne GmbH & Co., Hameln, Germany) at temperatures lower than 10 °C. Distilled water was added in the 1:1 (v:w) proportion, and also DOW 1510 at 1 drop/100 mL to reduce the foam appearance. Extractions were carried out by using four different media: H_2O, 0.1 M NaCl, pH 10, and pH 3. Water extraction (water-E) was performed at pH 6.50 ± 0.05. For saline extraction (salt-E), 0.1 M NaCl was added in 1% proportion with a final pH of 6.58 ± 0.05. For alkaline and acidic extractions, NaOH and HCl dilutions were respectively added until reaching pH 10.0 ± 0.2 (alkaline-E) and pH 3.0 ± 0.2 (acidic-E). Two homogenization cycles (30 s at 1500 rpm and 90 s at 3000 rpm each one) were necessary to completely homogenize the muscle. The tunic rests were removed manually and the muscle extract was kept under 5 °C during less than 2 h until film preparation. A portable pH-meter series 3 Star Orion with an electrode pH ROSS (Thermo Fisher Scientific Inc., Landsmeer, Netherlands) was used for pH measurements.

2.4. Film preparation

Protein film-forming solutions (2% w/v) were prepared by the same method as in muscle extraction. A plasticizer mixture (glycerol and sorbitol at the same proportion) was added at 50% (w/w) of the total protein.

The pH of the film-forming solutions (FS) were 6.59 ± 0.05 for water-FS, 6.54 ± 0.05 for salt-FS, 9.63 ± 0.05 for alkaline-FS, and 3.39 ± 0.05 for acidic-FS. Finally, film-forming solutions were filtered to remove air bubbles, and 50 mL aliquots were then cast into methacrylate plates (120 × 120 mm) (Plexiglas® GS Röhm GmbH & Co. KG, Darmstadt, Germany) through a gauze for exhaustive bubbles removing. Plates were left for 21–23 h at 4.0 ± 0.5 °C and 85 ± 5% RH prior to further drying in a ventilated oven (FD 240 Binder, Tuttlingen, Germany) at 45.0 ± 0.8 °C and 12 ± 3% of relative humidity (RH) for 21–23 h. All films were conditioned at 58.0 ± 0.2% RH and 22 ± 1 °C for 4 days prior to analysis.

2.5. Protein solubility

Protein concentration in extracts and film-forming solutions was determined with the BCA kit (Meridian RD., Rockford, IL, 61101 USA) (Smith et al., 1985). Corresponding water-soluble protein fractions were expressed at least in triplicate as the percent protein solubilized with respect to total muscle protein, which was determined according to A.O.A.C. (Association of Official Analytical Chemists, 2000) and expressed as percentages in wet basis.

2.6. Electrophoretic analysis (SDS-PAGE under reducing conditions)

Extracts and film-forming solutions were mixed with a 2-fold concentrated loading buffer (2% SDS, 7% mercaptoethanol and 0.002% bromophenol blue) adjusting with distilled water to reach a final concentration of 2 mg/mL protein. Samples were heat-

denatured 5 min at 95 °C and analysed by PAGE-SDS under reducing conditions using 10% Mini-PROTEAN TGX™ gels in a Mini Protean II unit (Bio-Rad Laboratories SA, Alcobendas, Madrid, Spain) at 25 mA/gel. Protein bands were stained with Coomassie brilliant Blue R250. Precision Plus Protein Dual Xtra standards from 2 kD to 250 kD were used as markers (Bio-Rad Laboratories SA, Alcobendas, Madrid, Spain).

For electrophoretic profile of water-soluble film proteins, approximately 0.4 g of the films were placed in falcon tubes with 10 mL distilled water and shaken at 100 rpm in an orbital shaker at 22 °C for 24 h. The solution was then filtered through Whatman # 1 filter paper to discard the remaining un-dissolved material and the recovered water solution analysed like in the extracts and film-forming solutions.

Protein solubility was determined at least in triplicate by BCA and expressed in mg/mL.

2.7. Thermal properties

Calorimetric analysis of extracts and films were performed using a differential scanning calorimeter (DSC) model TA-Q1000 (TA Instruments, New Castle, DE, USA) previously calibrated by running high purity indium (melting point, 156.4 °C; melting enthalpy, 28.44 J/g). Samples of around 10–15 mg of extracts and protein films were weighed within ±0.002 mg by an electronic balance (Model ME235S Sartorius, Goettingen, Germany) and then tightly encapsulated in aluminium hermetic pans. An empty pan was used as reference. They were scanned under dry nitrogen purge (50 mL/min) between 5 and 90 °C at a heating rate of 10 °C/min. Peak temperatures (T_{peak}, °C) and denaturation enthalpies (ΔH) were measured at least in triplicate, the last data being normalized to dry matter content (J/g_{dm}) after desiccation of each particular capsule.

2.8. Film determinations

2.8.1. Total volatile basic nitrogen (TVB-N)

TVB-N determinations were carried out at least in triplicate by using the method of Ojagh, Nunez-Flores, Lopez-Caballero, Montero, and Gomez-Guillen (2011). Results were expressed in dry basis as mg TVB-N/100 g film.

2.8.2. Moisture content

It was determined at least in triplicate by drying samples of around 0.5 g at 105 °C for 24 h, according to A.O.A.C. (Association of Official Analytical Chemists, 1995). Water content was expressed as a percent of total weight.

2.8.3. Water activity

It was measured at least in triplicate placing circles cut at exactly the same shape of the equipment containers on each film stuck to the bottom, with a Lab MASTER-aw equipment (Novasina AG, Lachen, Switzerland) at constant temperature of 25 °C.

2.8.4. Thickness

It was measured using a micrometer (MDC-25M, Mitutoyo, Kanagawa, Japan), averaging the values of 4–6 random locations in 15 films for each treatment as described by Perez-Mateos, Montero, and Gomez-Guillen (2009).

2.8.5. ATR-FTIR spectroscopy

Film infrared spectra between 4000 and 650 cm^{-1} using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA) as was described by Ojagh et al. (2011). Data were recorded at least in triplicate and were processed using the Spectrum software calculating the second derivative.

2.8.6. Light absorption and transparency

The light barrier properties and transparency of the films were calculated at least in triplicate using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) at selected wavelengths from 200 to 700 nm following the method described by Perez-Mateos et al. (2009). Transparency was calculated by the equation:

$$\text{Trasparency} = -\log(T_{600}/X)$$

where T_{600} is the light transmission (T) at 600 nm, and X is the film thickness (mm).

2.8.7. Colour

The colour parameters L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were measured using a Konica Minolta CM-3500d colorimeter (Konica Minolta, Madrid, Spain). D65 illuminant (Daylight) and D10° standard observer were used. Measurements were taken at a number of 5 locations in different film portions and each reported value was the mean of at least 11 measurements.

2.8.8. Water vapour permeability

It was determined at least in triplicate following the method described by Sobral, Menegalli, Hubinger, and Roques (2001) at room temperature and in a desiccator with distiller water (100% RH). RH increment was calculated every hour during 7 h using the following equation: $w \cdot x \cdot t^{-1} \cdot A^{-1} \cdot \Delta P^{-1}$ where w is the gained mass (g), x is the film thickness (mm), t is the time (h), A is the film area exposed (cm^2) and ΔP is water vapour partial pressure difference between the atmosphere and silica gel (2642 Pa at 22 °C). Results were expressed in g mm h^{-1} cm^{-2} Pa^{-1}.

2.8.9. Water solubility

Film circumferences of 40 mm in diameter were placed in plastic containers with 50 mL distilled water and placed at 22 °C for 24 h. The solution was then filtered through Whatman # 1 filter paper to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h. Film solubility FS (%) was calculated using the expression $[(W_o - W_f)/W_o] \times 100$, where W_o was the initial weight of the film expressed as dry matter and W_f was the weight of the undissolved desiccated film residue. All tests were carried out at least in triplicate.

2.8.10. Water resistance

Films were fixed onto the opening of calibrated cells (area 15.90 cm^2) and the cells placed in desiccators and exposed over distilled water. Distilled water (5 mL) were poured over the film surface. The film deformation due to the water effect, the time when the water started to leak and the time when the film broke were annotated. All tests were carried out at least in triplicate.

Table 1
Water-soluble protein (SP, %) in water (water-E), salt (salt-E), alkali (alkaline-E) and acid (acidic-E) extracts, and corresponding film-forming solutions water-FS, salt-FS, alkaline-FS and acidic-FS.

SP (%)		
Sample	Extract (E)	Film-forming solution (FS)
Water-	60.39 ± 2.00 a/x	56.70 ± 2.12 a/x
Salt-	65.41 ± 0.79 b/x	54.60 ± 0.62 a/y
Alkaline-	70.11 ± 0.50 c/x	100.82 ± 1.20 b/y
Acidic-	66.07 ± 2.57 b/x	83.03 ± 4.07 c/y

Results are the mean ± standard deviation. Two-ways ANOVA: Different letters (a,b,c) in the same column indicate significant differences among the different treatments ($P < 0.05$). Different letters (x,y) in the same row indicate significant differences among extracts and film-forming solutions ($P < 0.05$).

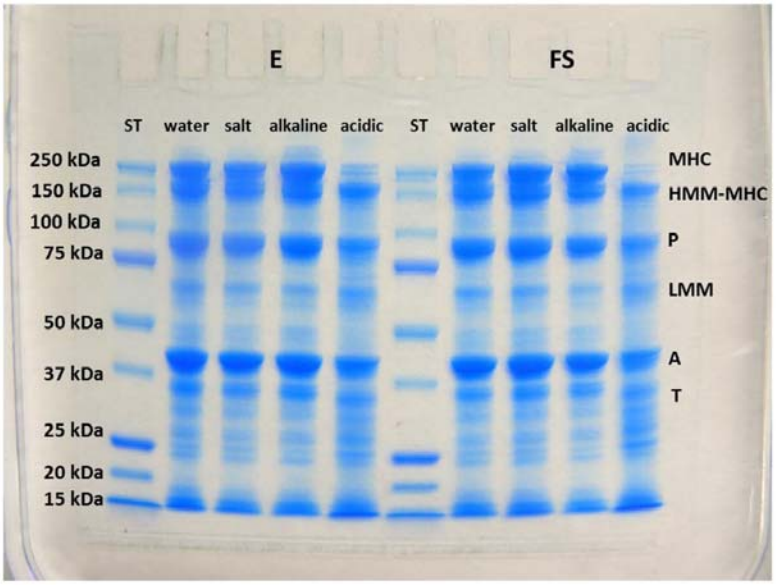

Fig. 1. SDS-PAGE patterns under reducing conditions of extracts (E) and film-forming solutions (FS). Left: Water-E, salt-E, alkaline-E and acidic-E extracts. Right: Water-FS, salt-FS, alkaline-FS and acidic-FS film-forming solutions. ST: Standard; MHC: Myosin heavy chain; HMM-MHC: Heavy meromyosin-myosin heavy chain; LMM: Light meromyosin; P: Paramyosin; A: Actin; T: Tropomyosin.

2.8.11. Mechanical properties

Tensile and puncture tests were run using a texture analyzer TA.XT plus TA-XT2 (Texture Technologies Corp., Scarsdale, NY, USA) (58% RH and room temperature) controlled by the Texture Exponent Software (Texture Technologies and by Stable Micro Systems, Ltd., Scarsdale, NY, USA), using a 5 kg load cell. Tensile test: At least three probes were cut rectangular (100 mm × 20 mm), leaving initial grips separation (l_0) of 60 mm and using cross-head speed of 100 mm/min. The tensile strength (*TS*, MPa) (break force/initial cross-sectional area) and elongation at break [$(l_{break} - l_0)/l_0$] × 100, (*EAB*, %), were determined from the stress *vs* strain curves at the breaking point, and the elastic modulus or Young's module (*Y*, MPa) calculated as the slope of the linear initial portion (elastic response zone) of the curve $(l_{break} - l_0)/l_0$. Puncture test: Films of 100 × 100 mm were fixed in a 35 mm diameter cell and punctured to breaking point with a round-ended stainless steel plunger (5 mm) at a cross-head speed of 100 mm/min, for breaking force (*F*, N), and breaking deformation (*D*, %) data according to Sobral et al. (2001), which were carried out at least in triplicate at room temperature and keeping the samples at 58% RH until the text performance.

2.8.12. Microstructure

Low temperature scanning electron microscopy (LowT-SEM) (Oxford CT1500 Cryosample Preparation Unit, Oxford Instruments, Oxford, England) was used to examine representative film cross sections. Samples were mounted with an optical coherence tomography (OCT compound Gurr®) and mechanically fixed onto the specimen holder and cryo-fractured after mounted as described by Gomez-Guillen, Borderias & Montero (2007).

2.9. Statistical analysis

Statistical tests were performed using the SPSS computer programme (SPSS Statistical Software Inc., Chicago, Illinois, USA.) for one-way analysis of variance. The variance homogeneity was made using the Levene test or, the Brown-Forsythe when variance conditions were not fulfilled. Paired comparisons were made using the Bonferroni test or the Tamhane test (depending on variance homogeneity), with the significance of the difference set at $P \leq 0.05$.

3. Results and discussion

The muscle used as raw material showed 83.46 ± 1.21% moisture, 0.80 ± 0.01% fat, 0.87 ± 0.05% ash, and 14.87 ± 0.31% protein, of which 37.10 ± 1.35% was water-soluble protein. Total viable bacteria count was 4.4 log CFU/g. This load was lower than the allowed limits for fresh and frozen fishery products (m = 5.7 log CFU/g) (International Commission on Microbiological Specifications for Foods, 1986), which indicated a relatively good quality for co-products from the processing of squid.

3.1. Protein extracts (E) and film-forming solutions (FS)

3.1.1. Protein solubility

Table 1 shows protein solubility data for each extraction. The highest solubility was obtained at pH 10 (alkaline-E) while water extraction (water-E) caused the lowest solubility. Extractions at pH 3 (acidic-E) and 0.1 M NaCl (salt-E) produced similar ($P > 0.05$) protein solubility, being slightly lower than at alkaline conditions. Palafox et al. (2009) reported similar protein solubility in giant squid muscle at basic (11) and acidic (3) pHs. However, Sanchez-

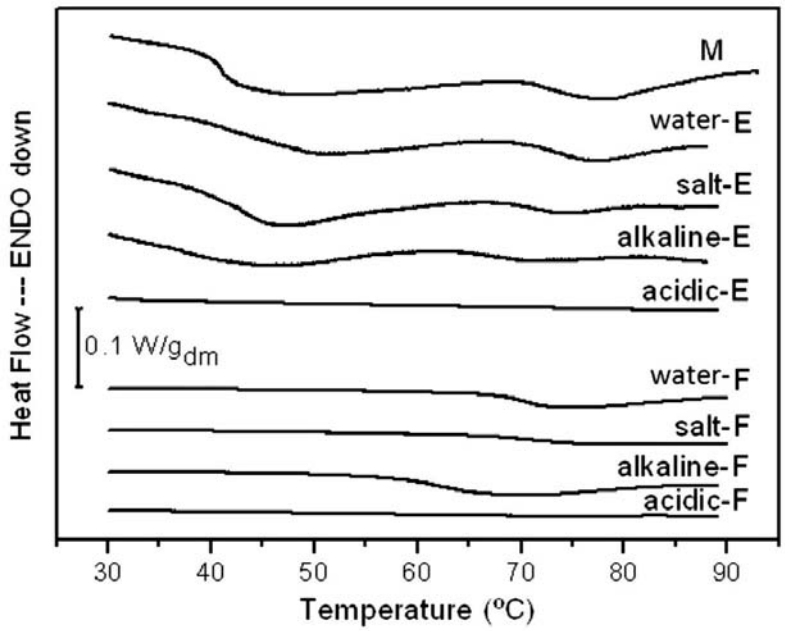

Fig. 2. DSC of extracts (E) and films (F). Top: *Dosidicus gigas* muscle (M), water-E, salt-E, alkaline-E and acidic-E extracts. Bottom: Water-F, salt-F, alkaline-F and acidic-F films.

Alonso et al. (2007) reported considerably lower protein solubility (~55%) in giant squid muscle extracted with 0.1 M NaCl. De la Fuente-Betancourt, Garcia-Carreno, Del Toro, and Cordova-Murueta (2009) have also observed a high functionally, evaluated as foaming and emulsifying properties, of giant squid muscle protein at alkaline pH (10–11), reaching the highest stability; however water extraction was not as good.

Regarding film-forming solutions, protein solubility at 0.1 M NaCl (salt-FS) was lower than before but similar to water extraction (water-FS), which may be due to the dilution effect from 0.1 M to 0.04 M whereas pH remained similar in the extracts ($P \leq 0.05$). This dilution also caused a total solubilisation (100%) at pH 10 (alkaline-FS), while pH 3 (acidic-FS) obtained a limited but rather high protein solubility (~83%). Hamaguchi et al. (2007) also observed higher solubility (percentages not given) at pH values of 1–4 and 10–12 in films forming solutions of 2% of blue marlin (*M. mazara*) muscle protein and 1% glycerol than pH values between 4 and 10.

Solubility differences between protein extracts and film-forming solutions were detected ($P \leq 0.05$) in all cases except in water extraction.

3.1.2. Electrophoretic patterns

Molecular weight protein distribution of extracts and film-forming solutions are shown in Fig. 1.

Similar behaviour was observed in all extracts, mainly in those in water and salt. A different degradation pattern was appreciated depending on the pH; in alkaline pH a higher band density at high molecular weight (including myosin and paramyosin) was observed. In acid-aided processes a high proportion of myosin heavy chains (MHC) (205 kDa) disappeared, and paramyosin (P) (108 kDa) and actin (A) (45 kDa) band intensities decreased; meanwhile higher band intensities between 50 and 75 kDa of light meromyosin (LMM) and below 45 kDa were observed possibly due to protein degradation by endogenous enzymes and the low pH. The fact that the solubility capacity behaved in different ways along the pH scale (De la Fuente-Betancourt, Garcia-Carreno, Del Toro, & Cordova-Murueta, 2009) suggests that the degradation level might affect the functional properties. Others authors observed a reduction of MHC and a consequently increase of LMM and HMM (heavy meromyosin) which may indicate a stronger metalloprotease activity at pH 3 (Cortes-Ruiz et al., 2008). This would tend to support

Table 2
Light transmission (T, %) at several wavelengths (nm) and transparency ($-\log(T_{600}/X)$) Of water-F, salt-F, alkaline-F and acidic-F films.

Film	T at selected wavelengths							Transparency
	200	280	350	400	500	600	700	
Water-F	0.01 ± 0.001 a	0.01 ± 0.003 a	13.51 ± 0.86 a	23.49 ± 1.87 a	35.83 ± 3.77 a	39.63 ± 4.15 a	41.59 ± 3.94 a	3.48 ± 0.42 a
Salt-F	0.01 ± 0.001 a	0.02 ± 0.006 a	19.98 ± 1.28 b	30.52 ± 1.91 b	40.51 ± 2.51 ac	43.78 ± 2.62 a	45.16 ± 2.62 a	3.40 ± 0.44 a
Alkaline-F	0.01 ± 0.001 a	0.01 ± 0.001 a	15.26 ± 1.67 a	41.64 ± 2.44 c	77.63 ± 3.32 b	87.42 ± 3.34 b	89.60 ± 3.47 b	1.14 ± 0.26 b
Acidic-F	0.01 ± 0.001 a	0.01 ± 0.001 a	20.04 ± 3.76 b	33.81 ± 3.25 b	45.95 ± 2.93 c	50.91 ± 3.42 c	53.82 ± 3.94 c	0.73 ± 0.05 b

Results are the mean ± standard deviation. One-way ANOVA: Different letters indicate significant differences among the different films ($P < 0.05$).

Table 3
L^*, a^* and b^* of water-F, salt-F, alkaline-F and acidic-F films.

Film	L^*	a^*	b^*
Water-F	34.44 ± 0.24 a	−1.00 ± 0.05 a	0.06 ± 0.01 a
Salt-F	35.98 ± 0.64 b	−1.15 ± 0.13 ab	0.10 ± 0.1 a
Alkaline-F	34.23 ± 0.12 a	−1.24 ± 0.03 b	1.26 ± 0.07 b
Acidic-F	35.93 ± 0.87 b	−0.69 ± 0.03 c	−0.56 ± 0.09 c

Results are the mean ± standard deviation. One-way ANOVA: Different letters in the same column indicate significant differences among the different films ($P < 0.05$).

the idea that pH induces conformational changes in protein structures making them more liable to enzyme hydrolysis (Cortes-Ruiz et al., 2008). Additionally, more intense bands were detected below the 50 kDa region, which is the expected location for derivatives from such hydrolysis. Degraded myofibrillar fragments due to acid treatment were also found in acid-aided protein recovery from enzyme-rich pacific whiting (Choi & Park, 2002), where numerous new and low-molecular-weight bands appeared and even actin band was degraded into two bands. On the other hand, Ramirez-Suarez et al. (2008) found a disappearance of the 50–58 and 85 kDa bands, which they attributed to a cross-linking caused by endogenous transglutaminase (TGase) enzyme action (although the pH was not optimum), forming dimmers and trimers with an approximate molecular weight of 153 kDa.

However, it was not clear whether the reduction of myosin heavy chain resulted from the degradation of myosin by acidic proteases or acid hydrolysis. Extracts (E) and corresponding film forming solutions (FS) presented highly similar electrophoretic behaviours (Fig. 1).

3.1.3. DSC

DSC thermograms of extracts (E) had the typical profiles shown in Fig. 2: water-E, salt-E and alkaline-E depicted two main (rounded shaped) endothermic transitions at the ranges of 45 – 50 °C (mainly myosin) and 70–80 °C (mostly actin), with an overlapping zone in between (paramyosin, collagen (nearly inexistent) and sarcoplasmic proteins), that resembled the general pattern of actomyosin systems. Acidic-E had however a flat trace with no sign of transitions. Respective T_{peak} (°C) values were: 51.08 ± 0.77 and 77.52 ± 0.33 in water-E; 46.63 ± 0.46 and 74.64 ± 0.39 in salt-E; 45.54 ± 1.92 and 69.86 ± 1.79 °C in alkaline-E. Transition temperatures were significantly different except for the low transition data in salt-E and alkaline-E which were not significantly different. Corresponding ΔH (J/g_{dm}) values were 5.05 ± 0.48, 4.70 ± 0.68, and 4.21 ± 0.71 respectively for water-E, salt-E, and alkaline-E extracts. Denaturation enthalpies were not significantly different among the extracts.

DSC traces of water-E, salt-E and alkaline-E closely resembled typical profiles of frozen muscle proteins from dissected mantles of giant squid (*D. gigas*) of likely the same source and processing than current material (Fernández-Martín, 1998) (Fig. 2, line M). Corresponding thermal denaturation data were: 47.28 ± 0.52 °C and 77.60 ± 0.43 °C for the main transitions T_{peak} endotherms, with around 60/40 enthalpy ratio and total ΔH of 8.16 ± 0.12 J/g_{dm}. These data recorded usual grinding/freezing and frozen-storage effects on myofibrillar proteins and differed to previous reports by others on different cephalopod species and experimental conditions (Hastings, Rodger, Park, Matthews, & Anderson, 1985; Paredi, Tomas, Crupkin, & Anon, 1996; Ramírez Olivas, Rouzaud Sánchez, Haard, Pacheco Aguilar, & Ezquerra Brauer, 2004). From the calorimetric point of view, water-E extracts seemed to consist of considerably structure-preserved proteins (~62%) relative to those of frozen mantle muscle of giant squid (Fernández-Martín, 1998). It is well known that squid proteins differ from fish and mammals proteins of being more water-soluble, and more prone to thermal denaturation. Despite the general fact that proteins are more soluble in salt (depending on ionic strength) at normal pH, salt-E yield (~58%) was not significantly different than that of water-E concerning enthalpy data, but transition temperatures underwent a significant down-shifting, in agreement to generally described salt effects on myofibrillar proteins. Regarding extracts at high pH (alkaline-E), the yield (~52%) was not significantly lower (~10%) than the above case and consisted of mainly preserved myosin and paramyosin, and considerably degraded high-thermostable proteins (sarcoplasmic proteins and actin) which underwent, as expected, a great reduction in the transition temperatures. These findings seemed to be accordant to the high functionality ascribed to this protein fraction by De la Fuente-Betancourt, Garcia-Carreno, Del Toro, and Cordova-Murueta (2009). It is worth nothing that the intermediate endothermic DSC zone appeared reduced with respect to water-E in salt-E and, even more, in alkaline-E (Fig. 2), which could likely be due to a significant suppression of sarcoplasmic proteins solubility in the presence of high salt concentration (Kim, Yongsawatdigul, Park, & Thawornchinsombut, 2005), and their partial unfolding during alkaline extraction since some sarcoplasmic proteins are alkali stable according to Tadpitchayangkoo, Park, and Yongsawatdigul (2010). It is also well known that low pH treatments may produce a high protein recovery but with great structural degradations, as in acidic-E (see the review from Totosaus, Montejano, Salazar, & Guerrero, 2002) depending on the pH level. Additionally, sarcoplasmic proteins are also significantly suppressed in solubility at an acidic pH (Kim et al., 2005). Effects of processing by pH-shifting (3 and 11) in giant squid have been recently reported by Palafox et al. (2009). References on thermal data of giant squid subjected to acid treatment are very scarce however since the only precedent was found in Fernández-Martín, Deaño-Campos, Tovar, and Borderías (2011): two cases were reported on frozen giant squid mantle processed at ~pH 5 under different conditions, with the result of different protein yields and denaturation effects; considerably higher acidic conditions may likely cause entire protein denaturation, as in current acidic-E.

These thermal results on extracts confirmed the respective solubility data but could not necessarily match with the corresponding electrophoretic patterns due to the different protein nature (native and denatured respectively).

3.2. Film (F) properties

3.2.1. Light barrier properties

Light transmission in UV and visible ranges at selected wavelength of 200–700 nm as well as transparency are shown in Table 2. Generally, films exhibited the lower transmission in the UV range (200–280 nm), irrespective of pH or NaCl presence, which could decrease lipid oxidation in food system. These results are consistent with earlier works (Artharn et al., 2008; Benjakul, Artharn, & Prodpran, 2008; Hamaguchi et al., 2007; Shiku, Hamaguchi, Benjakul, Visessanguan, & Tanaka, 2004) reporting that fish muscle protein films had very good UV barrier properties, owing to their high content of aromatic amino acids that absorb UV light. This is interesting because most of synthetic polymer films do not prevent the passage of UV light above 280 nm (Shiku et al., 2004; Shiku, Hamaguchi, & Tanaka, 2003).

Although every film showed high transparency, they became more transparent at pH 3 (acidic-F) and pH 10 (alkaline-F) (0.73 and 1.14 respectively) than in water (water-F) and salt (salt-F), as shown in Table 2. The lack of pigments in the muscle might favour the transparency of the films. Artharn et al. (2008) observed that higher proportion of solubilized myofibrillar protein gave place to higher transparency, which is in agreement with the present study. Shiku

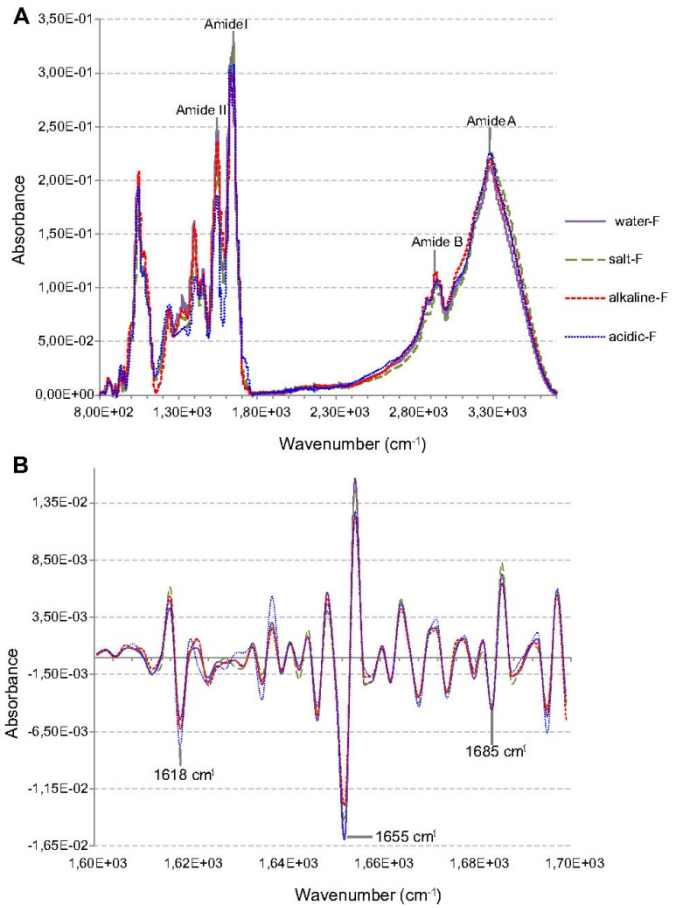

Fig. 3. A. ATR-FTIR spectra of water-F, salt-F, alkaline-F and acidic-F films. B. Second derivative of amide I band (1700–1600 cm^{-1}) from FTIR spectra of water-F, salt-F, alkaline-F and acidic-F films.

et al. (2003) claimed that blue marlin muscle protein films prepared at acid (2–3) or alkaline (11–12) pH led to more stable protein networks, with superior transparency close to synthetic films.

L^* (lightness), a^* (reddish/greenish) and b^* (yellowish/bluish) values are shown in Table 3, where it is revealed that all films had low lightnesses (~35). In general, films had a lack of reddish tendency ($+a^*$). Acidic-F specimens had the higher a^* value while alkaline-F had the lowest a^* value ($P \leq 0.05$). Alkaline-F specimens had the most yellowish tendency ($+b^*$) and acidic-F type the least ($P \leq 0.05$). No significant differences ($P > 0.05$) were found between water-F and salt-F films. A possible reason which could explain the yellowish tendency at pH 10 might be a higher solubilised myofibrillar protein proportion in the corresponding film-forming solution (Artharn et al., 2008).

Data obtained in this study seemed to indicate that giant squid protein films are highly transparent, UV barrier and have the adequate colour for their use as see-through packaging or coating materials.

3.2.2. ATR-FTIR

Fig. 3A shows ATR-FTIR spectroscopic patterns (4000–800 cm^{-1}) of water-F, salt-F, alkaline-F and acidic-F films. Factors as pH and NaCl led to important changes in the spectra. The Amide A band (~3300 cm^{-1}) and amide B (~3079 cm^{-1}), attributed fundamentally to N–H stretching of protein vibrations, with contribution from O–H stretching of intermolecular hydrogen bonding, are related to free water. Salt-F, acidic-F and alkaline-F showed a slight shift to the lower wave numbers of amide A (~3280–3273 cm^{-1}), specially the last one. This was possibly caused by the higher formation of hydrogen bonding interaction between polymer molecules in the film, causing higher hydration at alkaline conditions. In this study, films contained glycerol as one of

Table 4
Thickness, moisture content, film solubility, protein release and water vapour permeability (WVP) of water-F, salt-F, alkaline-F and acidic-F films.

Film	Moisture content (%)	Thickness (μm)	Film solubility (%)	Protein release (%)	WVP (× 10^{-7} g m^{-1} s^{-1} Pa^{-1})
Water-F	21.35 ± 1.19 a	109.0 ± 9.0 a	40.75 ± 0.51 a	4.41 ± 0.91 a	1.35 ± 0.05 a
Salt-F	22.63 ± 0.97 a	126.8 ± 7.4 b	47.67 ± 0.38 b	2.37 ± 0.26 b	1.77 ± 0.03 b
Alkaline-F	27.42 ± 0.56 b	108.8 ± 8.7 a	45.48 ± 0.78 c	3.11 ± 0.01 c	1.55 ± 0.02 c
Acidic-F	20.99 ± 1.36 a	125.6 ± 6.5 b	42.95 ± 0.56 d	12.98 ± 0.28 d	1.71 ± 0.04 b

Results are the mean ± standard deviation. One-way ANOVA: Different letters in the same column indicate significant differences among the different films ($P < 0.05$).

the plasticizers; as a consequence, some water might be bound to the film protein network as Hoque, Benjakul, and Prodpran (2010) stated.

The amide I band, located in the region ~1650 cm^{-1}, arises predominantly from C=O stretching vibrations, being weakly coupled with in-plane N–H bending and C–N stretching vibrations. Spectral changes in the amide I region have been associated with myofibrillar protein conformational changes and widely used for the spectroscopic analysis of the secondary structure of proteins (Bertram, Kohler, Bocker, Ofstad, & Andersen, 2006; Bocker, Ofstad, Bertram, Egelandsdal, & Kohler, 2006; Ojagh et al., 2011; Palaniappan & Vijayasundaram, 2008). To enhance the spectral resolution, a second derivative spectrum (Fig. 3B.) was used to investigate the amide I region (1700–1600 cm^{-1}). Alkaline-F showed the highest wave number at ~1655 cm^{-1}, which indicates a higher denaturation due to the loss of α-helical structure (secondary structure). In this case no difference was found among water-F, salt-F, and acidic-F (~1651 cm^{-1}). Alkaline-F also showed intra-molecular aggregation of β-sheet structures (~1683 cm^{-1} at pH 10 and 1682 cm^{-1} the rest), but less pronounced than in the α-helix band, which may indicate a more organized structure. However, salt-F had the tendency to exhibit higher intermolecular β-sheet aggregation (~1618 cm^{-1}), which might have relation to nonhydrogenated C=O groups (Bocker, Kohler, Aursand, & Ofstad, 2008).

The amide II bands (~1545 cm^{-1}) (Fig. 3A) represent N–H bending vibrations coupled to C–N stretching vibrations. Generally, the lower wave number showed the existence of hydrogen bonds, with stronger hydrogen bonded peptide groups and collagen absorbing. Amide II band is less susceptible to secondary structure changes, but more affected by hydration. Regarding the extraction procedure, changes in these bands might be affected by the collagen rests. It is known that acidic pH condition helps collagen solution and protein hydrolysis, as there has been shown in the SDS-PAGE, producing short protein fragments more suitable for hydrogen bonding. While amide II band remained at ~1548 cm^{-1} in alkaline-F, it decreased to ~1539 cm^{-1} in acidic-F which may be due to the reduction of the number of non-bonded peptide groups caused by more extensive hydrogen bonding between the protein and glycerol (Chunli et al., 2006), causing a higher hydration.

The peak situated around ~1000–1100 cm^{-1} might be related to possible interactions arising between plasticizer (OH groups of glycerol and sorbitol) and film structure (Bergo & Sobral, 2007). Acidic-F presented the lowest wave number (~1040 cm^{-1}), which were related to the possible extra interactions between short protein fragments and the plasticizers. On the other hand, alkaline-F presented the highest wave number (~1043 cm^{-1}) could be associate to less interaction with plasticizers.

Fourier-transform infrared study indicated that pH had some differences in functional groups and inter- and intra-molecular interaction, resulting in more protein-plasticizer and protein-water interactions at pH 3 and more protein–protein interactions at pH 10, which is due to the higher secondary structure lost reported at alkaline conditions, and the protein hydrolysis found at pH 3.

3.2.3. Microbiological index

Total viable bacteria count was 6.52 log CFU/g in conditioned water-F and 6.57 log CFU/g in conditioned salt-F, which were higher than the limits in similarly conditioned edible gelatin films (3.7 log CFU/g). This is due to environmental conditions: time, temperature during processing and, particularly, the nature of extracts that are very suitable for the microbial growth. On the contrary, counts in conditioned alkaline-F and acidic-F (2.5 log CFU/g and 2.86 log CFU/g respectively) were lower than the recommended limits in the fishery products (5 log CFU/g). Regarding Enterobacteriaceae counts, it was 4.13 log CFU/g in water-F, while only 0.4 log CFU/g grew in salt-F, and the growth was totally inhibited at pH 10 (alkaline-F) and pH 3 (acidic-F).

The nitrogen of total volatile bases (TVB-N) quantified in marine products had also been utilized as indicator of bacterial spoilage for some fish species, where amounts over 30 mg/100 g of muscle are considered the maximum allowed. In this study, the values observed were from 1135 to 274.5 mg N/100 g both raw material and film. Marquez-Rios, Moran-Palacio, Lugo-Sanchez, Ocano-Higuera, and Pacheco-Aguilar (2007) related the high level found (243.7–278.8 mg N/100 g *D. gigas* muscle) to the intrinsic high level of NH_4Cl, which acts as a physiological tool to regulate squid buoyancy. Therefore, the high TVB-N value detected in this specie cannot be utilized as a quality/spoilage index for squid muscle material since it is not an exclusive consequence of bacterial activity.

Concerning their use as food packaging, only alkaline-F and acidic-F preparations would be considered harmless adequate to avoid microbial growth; however addition of an antimicrobial edible agent should be needed in the case of water-F and salt-F preparations.

3.2.4. Physical properties

The water activity evolution of the different films along the conditioning process at 58% RH and 20 °C for 3 days followed the typical profiles asymptotically. Acidic-F was the only film practically getting the RH equilibration (0.56 ± 0.01) and it seemed clear that the rest needed considerably longer conditioning times (salt-F 0.53 ± 0.03 and alkaline-F 0.54 ± 0.01), water-F in particular (0.50 ± 0.03). Moisture contents were however not significantly different among the films (Table 4, first column) except for alkaline-F which showed higher value, which confirms the elevated hydrogen bonding stated in the Amide An FTIR results. Since the plasticizer amount and the plasticizer/protein ratio were the same in all the films, it seemed that any different behaviour exhibited by the films may be likely due to different conformational states of corresponding proteins derived from the different extraction method used and the amount of hydrogen and protein bondings developed during the drying step. The highest film moisture (Table 4) was found at pH 10 ($P \leq 0.05$) which might have been influenced by the higher formation of hydrogen bonding interaction between polymer molecules as it has been seen by FTIR (Amide A).

Film thicknesses examined in this study ranged between 108.8 and 126.8 μm (Table 4). Salt-F and acidic-F were thicker ($P \leq 0.05$)

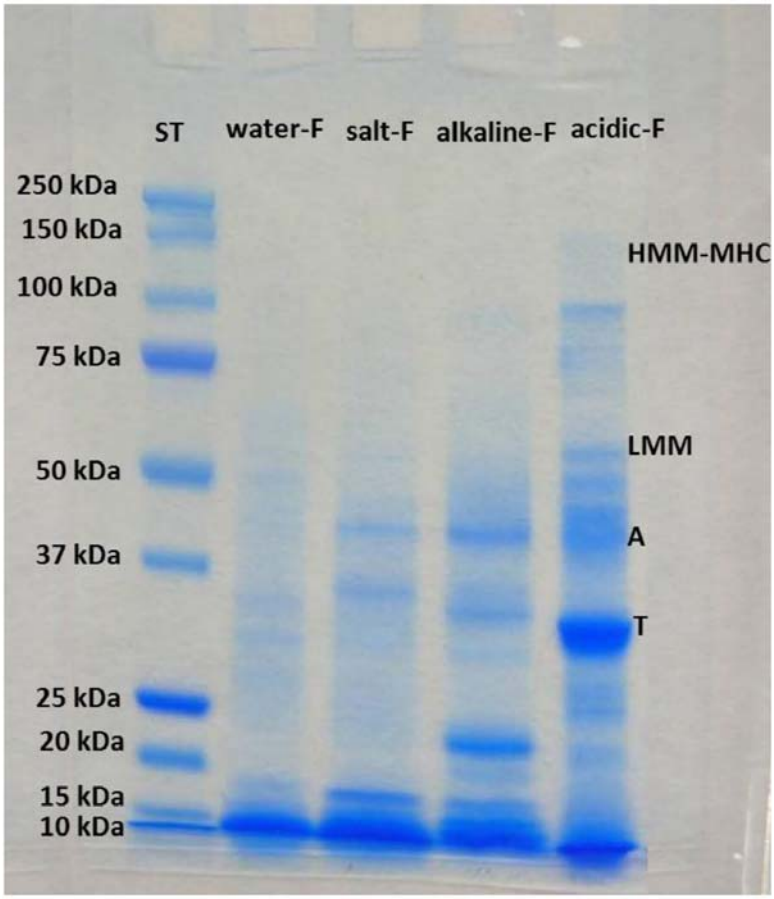

Fig. 4. SDS-PAGE patterns under reducing conditions of proteins released in water from water-F, salt-F, alkaline-F and acidic-F films. ST: Standard; MHC: Myosin heavy chain; HMM-MHC: Heavy meromyosin-myosin heavy chain; LMM: Light meromyosin; P: Paramyosin; A: Actin; T: Tropomyosin.

and no significant difference was found between water-F and alkaline-F. This difference might be caused by the protein–protein, protein–water and protein–plasticizer interactions and the different protein size resulting in different compaction. Alkaline pH led to a better film aspect and compact thickness (Bourtoom, 2009), which might justify the higher density reached at pH 10; and probably films made at pH 3 were thicker due to the presence of more interactions with plasticizers.

Protein films have been associated with high water vapour permeability (WVP), which is caused by the high number of hydrophilic groups (Hamaguchi et al., 2007). Despite the thickness was higher in salt- and acidic-F, the salt crystallization (Leerahawong et al., 2011) and the hydrolysis caused by pH 3 increased their water vapour permeability ($P \leq 0.05$) (Table 4). A higher solubilized sarcoplasmic (globular and lighter) and the loss of secondary structure protein fraction at pH 10 might improve the network matrix by filling the little holes left in the myofibrillar aggregation structure.

Depending on the raw material, protein film water vapour permeability was different (Paschoalick, Garcia, Sobral, & Habitante, 2003), other author did not find significant difference between alkaline and acidic treatments; such as in 2% Indo-Pacific blue marlin (*M. mazara*) muscle protein and 1% glycerol film (Hamaguchi et al., 2007; Iwata, Ishizaki, Handa, & Tanaka, 2000; Shiku et al., 2003). Alkaline and acidic treatment did not lead to low water vapour permeability, contrary to Bourtoom (2009) findings. Adding different concentration of organic salts (0–10%) in films with 4% squid (*T. pacificus*) muscle protein and 2% glycerol, Leerahawong et al. (2011) did not observe a permeability reduction.

Film solubility increased with pH treatment and more drastically with NaCl ($P \leq 0.05$). Despite the significant differences, film solubility resulted in the same range and the differences were not

Table 5
Water resistance parameters: Elongation time (h), elongation (cm), water filtration time (h) and breakage time (h), for water-F, salt-F, alkaline-F and acidic-F films.

	Water-F						Salt-F						Alkaline-F						Acidic-F					
Elongation time (h)	0.13	0.50	4	24	51	120	192	288	0.05	0.10	1	24	0.08	0.13	25	0.02	0.03	0.05	0.06	0.07	0.08	0.1
Elongation (cm)	0.3 ± 0.01	0.7 ± 0.1	1 ± 0.1	11.3 ± 0.5	1 ± 0.01	0.5 ± 0.01	0.3 ± 0.01	0.1 ± 0.01	0.3 ± 0.01	0.5 ± 0.02	1 ± 0.1	0.7 ± 0.02	0.5 ± 0.02	1 ± 0.2	1.3 ± 0.1	0.5 ± 0.01	1 ± 0.1	1.33 ± 0.1	1.4 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	2 ± 0.2
Water filtration time (h)	5 ± 0.5								2 ± 0.3				0.17 ± 0.03			No filtration						
Breakage time (h)	Unbreakable								50 ± 2				48 ± 1			0.09 ± 0.01						

Results are the mean ± standard deviation.

higher than 7%. Current films were dried at 45 °C for 21–23 h which might boost hydrophobic and covalent bonds. Covalent bonds are mainly formed at higher temperatures but longer exposure times may also provide suitable conditions at lower temperatures (Gomez-Guillen, Montero, Solas, & Borderías, 1998). Moreover, sarcoplasmic proteins presence entails remained enzymes, one of those muscle endogenous transglutaminase (TGase) which catalyses the formation of a covalent bond between a free amine group and the gamma-carboxyamide group of protein- or peptide-bound glutamine at low and moderate temperatures (25–40 °C) (Montero, Lopez-Caballero, Perez-Mateos, Solas, & Gomez-Guillen, 2005), depending on the breed (Montero et al., 2005) which might also cause a reduction in water vapour permeability. From the total release material, only 2–4% was protein in water-, salt-, and alkaline-F, and 12% in acidic-F (Table 4), which indicated that the rest should mainly be plasticizer. The acidic treatment led to a higher protein release due to the increase of the network free volume (Cuq, Gontard, Cuq, & Guilbert, 1997). Nevertheless the results showed more interactions with plasticizers only in acidic-F. Shiku et al. (2003) observed in alkaline and acidic films with 1% Indo-Pacific blue marlin (*M. mazara*) myofibrillar proteins and 0.5% glycerol dried at 25 °C for 24 h, that pH affected secondary, hydrophobic and hydrogen bonds, which might also lead to a weaker network compared to water-F.

Fig. 4 illustrates the electrophoretic patterns of the proteins released in water, where water-F presented the lowest band intensities. This might mean a higher loss of sarcoplasmic proteins and plasticizers proportion instead of myofibrillar proteins, which seems to be strongly aggregated and was not released from the matrix. Both water- and salt-F showed bands below 50 kDa, which could be due to a sarcoplasmic protein release. While salt-F showed Tropomyosin (30–35 kDa) and actin (45 kDa) soft bands, they were not visible in water-F, which indicated changes and/or interactions in proteins as a consequence of salting (Llorca et al., 2007) making water-F more insoluble. 17 kDa band corresponds to light chains of myosin (Llorca et al., 2007), and was hardly visible in water-F but quite intense in salt- and alkaline-F, which indicated myosin degradation to little fragments. Alkaline-F also showed the light chain of myosin, which might came from LMM which, in turn, showed lower band intensity in respective alkaline-FS, leading to a different cross-linking pattern and forming weaker bonds facilitating their release in water contact. The acidic-FS protein hydrolysis previously observed both in the extract and DSC results, led to the weakest network in acidic-F, releasing more proteinic material, including a large amount of tropomyosin and even actin and paramyosin. Acidic-F retained more plasticizers and water, which may explain the unique swelling effect caused when they came in contact with water, partially losing its network integrity but not getting fully dissolved. Kristinsson and Hultin (2003) also found that, as a result of the HMM dissociation, the relative viscosity and hydrodynamic volume were higher at acidic than at alkaline pH. According to the protein solubility determined by BCA, the maximum release of water-soluble proteins was observed in acidic-F, indicating a higher release of proteins than plasticizers; while alkaline-F and salt-F showed the lower values, possibly due to a higher proportion of plasticizers lost, being slightly higher in alkaline-F due to the denaturalization effect of alkaline conditions.

Table 5 describes film water resistance. Water-F samples did not break and resisted more time before water soaked through. Water-F, salt-F and alkaline-F presented short and similar elongation while acidic-F rapidly stretched until breakage. Experimental results could not be compared to others because the inexistence of previous data. This can be explained by the fact that protein interactions were modified at alkaline and acidic treatment due to the proteinic chain extension and degradation (Bourtoom, 2009;

Table 6
Tensile strength (*TS*), elongation at break (*EAB*), Young's module (*Y*), puncture force (*F*) and puncture deformation (*D*) of water-F, salt-F, alkaline-F and acidic-F films.

Film	*TS* (MPa)	*EAB* (%)	*Y* (MPa)	*F* (N)	*D* (%)
Water-F	1.36 ± 0.32 a	1.82 ± 0.28 a	83.8 ± 16.9 a	6.59 ± 0.44 a	8.88 ± 0.59 a
Salt-F	0.97 ± 0.33 ac	1.90 ± 0.89 a	51.0 ± 16.7 b	4.80 ± 0.66 a	8.52 ± 2.50 a
Alkaline- F	3.10 ± 0.53 b	12.09 ± 3.25 b	55.0 ± 11.9 ab	15.78 ± 0.46 b	24.06 ± 2.36 b
Acidic-F	0.85 ± 0.60 c	13.28 ± 1.68 b	34.0 ± 4.9 b	6.78 ± 1.02 a	12.69 ± 2.96 a

Results are the mean ± standard deviation. One-way ANOVA: Different letters in the same column indicate significant differences among the different films ($P < 0.05$).

Cortes-Ruiz et al., 2008; De la Fuente-Betancourt, Garcia-Carreno, Del Toro, & Cordova-Murueta, 2009); hardly finding differences between water-F and salt-F, due to the effect of the dilution in salt-FS. Hydrophobic interactions became reduced at alkaline pH and was maximum without any treatment (water-F), finding a water resistance reduction in alkaline-F. The high loss of resistance in acidic-F might be caused by the hydrolysis of proteins and the higher interaction protein-plasticizer, affecting the distribution of protein conformations changes and resulting in a weaker film. In every film which did not get broken water progressively filtrated through the film in time. The filtration time was later in those films more resistant to breakage.

Alkaline-F shown significantly highest ($P \leq 0.05$) tensile strength (TS) (Table 6), followed by water-F and salt with an intermediate TS value, although salt- values were not always significantly different from acidic-F. Nevertheless, tensile values were lower than the results obtained in other studies on different muscle protein and plasticizer percentages from various raw materials, such as 1% glycerol and 2% Purple-spotted bigeye (*P. tayenus*) myofibrillar protein films at acidic and alkaline pH (*TS* ~3.5 MPa) (Chinabhark et al., 2007), 0.5% glycerol and 1% Indo-Pacific blue marlin (*M. mazara*) myofibrillar protein films (8–16.7 MPa) (Shiku et al., 2003) or ~1% glycerol and 2% Nile Tilapia (*Oreochromis niloticus*) myofibrillar protein films at acidic pH (2–10 MPa) (Sobral et al., 2005). Both species had a very superior muscle texture compared to *D. gigas*', which is extremely tender, meaning that intrinsic characteristics might influence their mechanical resistance.

The elongation at break values (Table 6) showed notable differences between those with pH shifting and the others without it, being much higher in alkaline-F and acidic-F. These results seem to refute the common belief that tensile strength and elongation at break are inversely related in edible protein films (Kester & Fennema, 1986; Krochta & De MulderJohnston, 1997). Experimental results could not be compared to others because the inexistence of previous data, but showed lower elongation than previous studies in fish muscle protein films, such as 1% glycerol and 2% Indo-Pacific blue marlin (*M. mazara*) muscle protein films (74.6 ± 7.4%) (Hamaguchi et al., 2007), and 0.3–1.3% glycerol and 2% Nile Tilapia (*O. niloticus*) muscle protein films (30–90%) (Sobral et al., 2005); but neither of those who compared acidic and alkaline pH found differences between their elongation at break.

As far as Young's module is concerned (Table 6), the highest elasticity corresponded to water-F ($P \leq 0.05$) while acidic-F obtained the lowest one ($P \leq 0.05$). Despite the alkaline-F and acidic-F flexibility, their stretchiness was not high, and water-F had less plasticity than alkaline- and acidic-F but more elasticity. These results might suggest that water-F might have stronger protein interactions and weaker interactions with the plasticizer and water than acidic-F, as it has been seen in FTIR results. These results were much higher than those in 1% glycerol and 2% Nile Tilapia (*O. niloticus*) muscle proteins at acidic pH (5–10 MPa) (Sobral et al., 2005).

Regarding puncture deformation (*D*) no significant differences were observed between water-F, salt-F, acidic-F films (Table 6), even higher than the tensile elongation at break: while in acidic-F the percentage was similar to the *EAB*. Respect to the puncture force (*F*) (Table 6), a similar behaviour to tensile test was detected, with the only significant difference in alkaline-F which showed the highest resistance to the puncture ($P \leq 0.05$). In general, deformation capacity was greater (2–9%) than the observed by other authors in 1% glycerol and 2% Nile Tilapia (*O. niloticus*) muscle protein films elaborated at acidic pH (Sobral, Garcia, Habitante, & Monterrey-Quintero, 2004; Sobral et al., 2005), or 0.3–1.3% glycerol and 1% Nile Tilapia (*O. niloticus*) myofibrillar protein films at pH 2.7 (Paschoalick et al., 2003; Sobral, 2000). Puncture force values at acidic conditions were however similar.

Artharn et al. (2008) found that higher sarcoplasmic protein solubilized proportion in film-forming solution reduced tensile strength while higher myofibrillar protein solubilized proportion increased strength due to its fibrillar structure and cross-linking capacity. Sarcoplasmic protein with low molecular weight would make possible their dispersion and insertion between myofibrillar proteins during drying, weakening myofibrillar protein–protein interactions and favouring network migrations (Orliac, Rouilly, Silvestre, & Rigal, 2002; Shiku et al., 2003; Sobral et al., 2005); which might be the reason for the highest elasticity in water-F. The low tensile strength obtained at pH 3 (acidic-F) is related to less protein–protein interactions and more protein-plasticizer and protein-water interactions due to the hydrolysis caused by acidic conditions. Leerahawong et al. (2011) found that films with 2% glycerol and 4% squid (*T. pacificus*) myofibrillar protein showed salt crystallization from 0.5% NaCl addition, which affected film physical properties. This may justify the mechanical behaviour obtained with 0.4% NaCl films, not being remarkable in any test.

These findings were consistent with the low-temperature scanning electron microscopy (LowT-SEM) images (Fig. 5) of the current series of edible films. Water-F and salt-F films presented a similar cross section and occasionally showed some bacterial growth as mentioned before. However, salt-F showed a more laminar structure while water-F seemed denser compact and disorganized. This water-F structure might favour the water resistance, film low solubility and low water vapour permeability. Salt-F surface had phosphate crystals, by its tendency to catch cations in detriment to NaCl. *Dosidicus* mantle is usually treated with phosphates during the process, and it is present in all muscles, although the crystals only are formed in presence of NaCl. This irregular salt-F surface aspect was congruent with the findings of Leerahawong et al. (2011) who hypothesized that it should be due to the salt crystallization produced from 0.5% NaCl concentration in 4% muscle protein squid (*T. pacificus*) and 2% glycerol films.

Despite the similar cross section appearance at 500x, alkaline-F tended to be more compact, evidencing tubular structures and little globules where the cut was irregular, indicating a strong and resistant structure. Higher protein network density might cause the reduction of thickness showed in water-F and alkaline-F films. Interestingly, acidic-F showed more resistance to the cut due to its high plastic behaviour causing a rough cross section probably affected by that difficulty. Despite their irregular cross section, acidic-F was homogeneous and less compact than water-F and

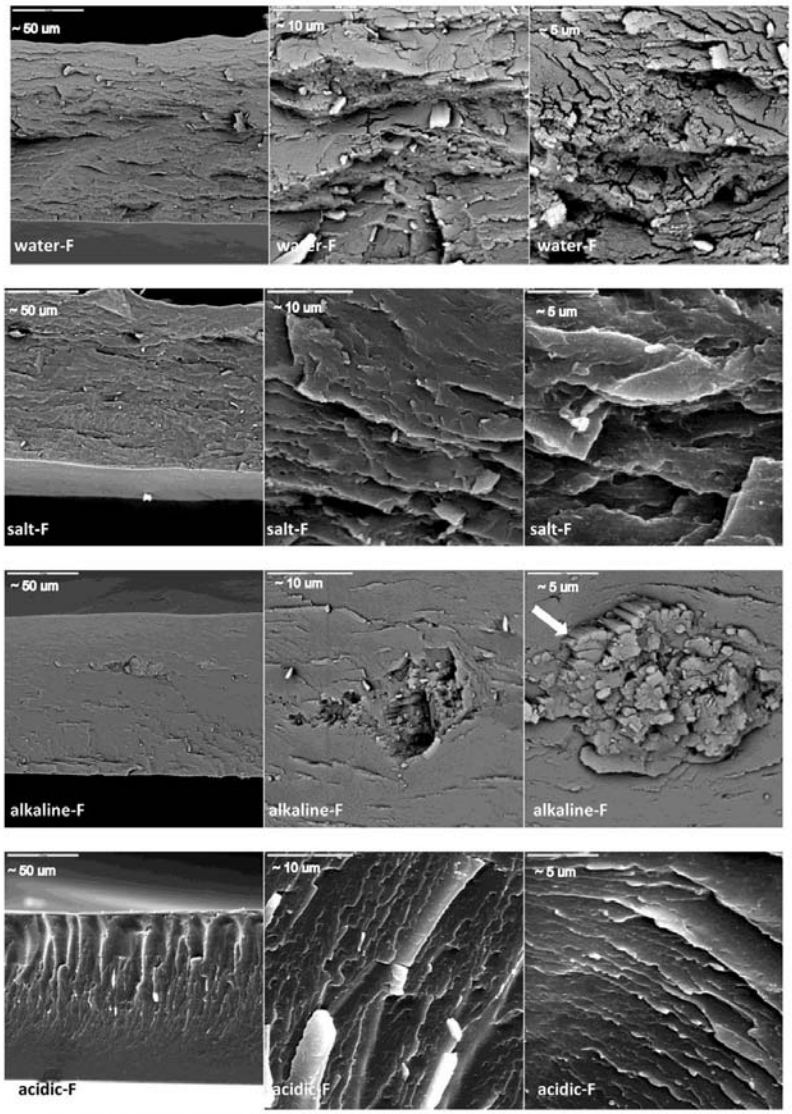

Fig. 5. Low temperature-scanning electron microscopy cross section images of water-F, salt-F, alkaline-F and acidic-F films.

alkaline-F films. This plastic behaviour and appearance might be due to the high proportion of protein-water and protein-plasticizer interactions.

3.2.5. DSC

Typical DSC traces of the films (F) are also shown in Fig. 2: a clear endothermic transition was evident with maximum temperatures at around 70 – 80 °C in the cases of water-F, salt-F and alkaline-F, while acidic-F obviously presented no transitions. It was consistent with the thermal behaviour of corresponding mother extracts (E) but with some additional effects: First, the presence of plasticizers may induce some crystalline inhibiting effects; secondly, higher denaturation was expected in low- thermostable proteins such as myosin, since the long drying time period at 45 °C.

Thermal denaturation data were: 74.57 ± 0.18 °C and 1.19 ± 0.06 J/g_{dm} in water-F; 76.47 ± 0.42 °C and 0.40 ± 0.03 J/g_{dm} in salt-F; 69.32 ± 0.16 °C and 2.88 ± 0.06 J/g_{dm} in alkaline-F. Transition temperatures T_{peak} and corresponding transition enthalpies ΔH were, on each side, significantly different among the films. Interesting to note is that salt-F was the most affected by processing in that film drying caused NaCl precipitation (LowT-SEM), suggesting that a reduced salt effect in decreasing transition temperatures may likely be derived as well as an increased plasticization (free-water) and subsequent vitrification effects in the system. These results match with the mechanical film behaviour previously discussed: higher interaction with plasticizer in acidic conditions led to plastic behaviour (a better elongation but less resistant); whereas in alkaline-F less denaturized proteins resulted in better mechanical behaviour.

Similarly to extracts E, thermal results on films F generally conformed to respective solubility data but did not necessarily match corresponding electrophoretic patterns.

4. Conclusion

The present study emphasizes on alkaline- and acidic-films. Water-F presented high water-resistance, lower solubility and water vapour permeability, which might be interesting for some particular applications, but both water-F and salt-films were not microbiologically stables and did not showed as good mechanical properties as alkaline and acidic-F. Alkaline pH led to a higher unfolded myosin inducing changes in the structure and also showed intramolecular aggregation, hence there were more functional groups available. This effect led to the highest water-soluble protein fraction in the film-forming solution, therefore an easier molecular orientation and hence a more mechanically resistant film.

On the other hand, pH 3 induced protein hydrolysis also enhancing its solubility in film-forming solution but leading to a weaker network due to more plasticizer–protein interactions, additionally increasing its hygroscopicity. Besides acidic-film had not as good behaviour in contact with water as alkaline film, both of them were transparent, a good UV barrier and had good mechanical properties.

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