UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Nutrición y Bromatología II (Bromatología)

TESIS DOCTORAL

Evaluación nutricional y propiedades biológicas de algas marinas comestibles. Estudios *in vitro* e *in vivo*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Eva Gómez Ordóñez

Directores

Pilar Rupérez Antón
Antonio Jiménez Escrig

Madrid, 2013

EVALUACIÓN NUTRICIONAL Y PROPIEDADES BIOLÓGICAS DE ALGAS MARINAS COMESTIBLES. ESTUDIOS *IN VITRO* E *IN VIVO*









Tesis Doctoral

Eva Gómez Ordóñez

Consejo Superior de Investigaciones Científicas (CSIC)
Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN)
Departamento de Metabolismo y Nutrición

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y

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

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EN LA CUBIERTA, de derecha a izquierda y de arriba abajo;
Algas pardas: *Himantalia elongata* (Linnaeus) S.F.Gray, *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders, *Bifurcaria bifurcata* R.Ross; Algas rojas: *Mastocarpus stellatus* (Stackhouse) Guiry, *Gigartina pistillata* (S.G.Gmelin) Stackhouse, *Chondracanthus acicularis* (Roth) Fredericq, *Nemalion helminthoides* (Velley) Batters, *Osmundea pinnatifida* (Hudson) Stackhouse y *Dumontia contorta* (S.G.Gmelin) Ruprecht (Fuente: www.algaebase.org)

DRES. Mª PILAR RUPÉREZ ANTÓN, INVESTIGADOR CIENTÍFICO Y ANTONIO JIMÉNEZ ESCRIG, TÉCNICO SUPERIOR ESPECIALIZADO, DEL DEPARTAMENTO DE METABOLISMO Y NUTRICIÓN DEL INSTITUTO DE CIENCIA Y TECNOLOGÍA DE ALIMENTOS Y NUTRICIÓN DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC),

CERTIFICAN QUE:

Dª EVA GÓMEZ ORDÓÑEZ, ha realizado bajo nuestra dirección y en este Departamento el trabajo que lleva por título "**Evaluación nutricional y propiedades biológicas de algas marinas comestibles. Estudios *in vitro* e *in vivo***" que constituye su Memoria de Tesis Doctoral. Dicho trabajo reune las condiciones necesarias para su presentación y defensa.

Y para que conste a los efectos oportunos firman el presente certificado en Madrid a dos de marzo de dos mil doce.

SEDE CIUDAD UNIVERSITARIA:

C/ JOSÉ ANTONIO NOVÁIS, 10
CIUDAD UNIVERSITARIA
28040 MADRID, ESPAÑA
TELS.: 91 544 56 07 – 91 549 23 00
FAX: 91 549 36 27

SEDE JUAN DE LA CIERVA:

C/JUAN DE LA CIERVA, 3
28006 MADRID, ESPAÑA
TELS.: 91 562 29 00
FAX: 91 564 48 53

"Sólo comprendemos aquellas preguntas
que podemos responder"

Friedrich Nietzsche

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RESUMEN/SUMMARY

Resumen

El objetivo principal de esta tesis doctoral es la caracterización y evaluación *in vitro* e *in vivo* de la calidad nutricional de algas marinas españolas como fuente de compuestos biológicamente activos, principalmente polisacáridos sulfatados. Se ha analizado la composición química de las algas pardas y rojas de partida y especialmente la fibra alimentaria. Además mediante una extracción química secuencial se han obtenido fracciones solubles de los principales polisacáridos de la pared celular de algas. Adicionalmente, se ha desarrollado y validado metodología específica para determinar los principales aniones de algas por cromatografía iónica, para identificar los principales polisacáridos y grupos funcionales de algas por espectroscopía FTIR, y para la distribución de pesos moleculares por HPSEC. También se han determinado *in vitro* las principales propiedades funcionales (capacidad de hinchamiento, retención de agua y de aceite) y biológicas atribuibles a los polisacáridos sulfatados de la fibra de algas (capacidad antioxidante y anticoagulante). Asimismo, se han realizado ensayos *in vivo* con ratas Wistar alimentadas con un concentrado de algas pardas o rojas, determinando su influencia beneficiosa sobre el perfil lipídico, estado antioxidante general y efecto prebiótico potencial. Los resultados obtenidos pueden ser de gran interés para la obtención a partir de algas de nuevos ingredientes, que podrían ser usados por la industria alimentaria en la elaboración de alimentos funcionales.

Summary

The main aim of this PhD thesis is the *in vitro* and *in vivo* characterization and evaluation of the nutritional quality of Spanish seaweeds as a source of biologically active compounds, mainly sulfated polysaccharides. Chemical composition of brown and red algae and most particularly dietary fibre was analyzed. Moreover, soluble fractions of the main cell wall polysaccharides from seaweeds have been obtained by a sequential chemical extraction. Additionally, we have developed and validated methodology in order to determineg the major algal anions by ion chromatography, to identify the main functional groups of polysaccharides and algae by FTIR spectroscopy, and to assess their molecular weight distribution by HPSEC. Also, the main functional properties (swelling, water and oil retention capacity) and biological properties attributable to the sulfated polysaccharides of seaweed fibre (antioxidant and anticoagulant), have been determined *in vitro*. Moreover, *in vivo* studies have been carried out with Wistar rats fed a concentrate of red or brown algae, assaying its beneficial influence on lipid profile, overall antioxidant status and potential prebiotic effect. This results can be of great interest to obtain new ingredients from algae, which could be used by the food industry in the development of functional foods.

ABREVIATURAS

Lista de Abreviaturas (*)

ABTS	2,2′-azinobis-(3-etilbenzotiazolin-6-sulfónico)
AI	Índice aterogénico
APTT	Tiempo de protrombina parcial activada
CV	Coeficiente de variación
FRAP	Ferric Reducing Antioxidant Assay
FTIR	Espectroscopia infrarroja con transformada de Fourier
GLC	Cromatografía de gases
HDL	Lipoproteína de alta densidad
HPSEC	Cromatografía líquida de exclusión molecular
IC	Cromatografía iónica
IDF	Fracción insoluble de la fibra
IR	Infrarrojo
KL	Lignina Klason
LDL	Lipoproteína de baja densidad
LOD	Límite de detección
LOQ	Límite de cuantificación
MUFA	Ácidos grasos poliinsaturados
NS	Azúcares neutros
ORC	Capacidad de retención de aceite
PCL	Fotoquimioluminiscencia
PT	Tiempo de protrombina
SCFA	Ácidos grasos de cadena corta
SDF	Fracción soluble de la fibra
SW	Capacidad de hinchamiento
RP	Poder reductor
RSA	Capacidad de secuestro de radicales
RSD	Desviación estándar relativa
TC	Colesterol total
TGL	Triglicéridos
TPTZ	Tripiridiltriazina
TT	Tiempo de trombina
UA	Ácidos urónicos
WRC	Capacidad de retención de agua

(*) Se respeta la nomenclatura en inglés que está en las publicaciones

CAPÍTULO 1

INTRODUCCIÓN GENERAL

1. Las algas marinas como alimento: Situación actual y Legislación

Las algas han sido utilizadas como alimento desde tiempo inmemorial en los países orientales como Japón, China y Corea así como en algunos países americanos como México. Mientras estos países han mantenido la tradición de incluir las algas en su dieta, en España concretamente, a pesar de poseer amplias costas y gran abundancia de especies, no hay tradición en el consumo de algas. Aunque en los últimos años se está revalorizando este producto marino y consiguiendo introducir las algas poco a poco en la dieta.

En Europa las algas principalmente se han empleado y se continúan utilizando para la extracción de ficocoloides, sustancias exclusivas de estos vegetales que no tienen equivalente sintético. Los ficocoloides se emplean como agentes gelificantes, espesantes y estabilizantes, interviniendo en multitud de aplicaciones en la industria alimentaria, en artículos del hogar y productos biomédicos. Es interesante ver que la presencia de las algas marinas en productos de uso cotidiano como pasta dentífrica, cosméticos, champú, alimentos para animales, comidas para bebés, derivados lácteos, cremas, sopas de preparación instantánea y muchos otros productos, constituyen claros ejemplos de que las algas se utilizan desde hace muchos años a través de las industrias alimentaria y cosmética (McLachlan, 1985).

No obstante, conforme aumenta la población del planeta se pone de manifiesto la importancia de los limitados y preciados recursos naturales de la Tierra y la necesidad de conocer e introducir nuevos alimentos que cubran las necesidades de una población que va en aumento y que al mismo tiempo aporten algún beneficio para la salud. Esto hace muy interesante el uso de alimentos funcionales, que son los alimentos que contienen algún componente, sea nutriente o no, con efecto beneficioso para el organismo humano (American Dietetic Association, 2004). Esta búsqueda de nuevos sabores y de alimentos más saludables nos ha llevado a volver la mirada al consumo de algas.

La demanda de algas, ya sea para el consumo humano o para la elaboración de diferentes productos industriales, se ha intensificado en los últimos años, llegando a la producción mundial en 2010 de 15,8 millones de toneladas (FAO, 2010). Las tres algas marinas más ampliamente utilizadas como alimento humano son varias especies de *Porphyra* spp. (Nori), *Laminaria* spp. (Kombu) y *Undaria* (Wakame), conocidas mundialmente por sus nombres Japoneses entre paréntesis. *Porphyra* es un alga roja, mientras que *Laminaria* y *Undaria* son algas pardas. Estas tres algas se obtenían al principio de especies silvestres, pero en la actualidad sólo es posible cubrir la demanda utilizando métodos de cultivo en gran escala. En algunos países como Japón y China, el cultivo de las algas representa una industria que se encuentra en expansión. Sólo en Japón la demanda de algas para el consumo es muy elevada, con una ingesta media de 14,3 g/día por adulto (Fukuda et al., 2007), alcanzando los valores recomendados de consumo de fibra dietética de 20-25 g/día (Fukuda et al., 2007). En otras partes del mundo se está trabajando intensamente para lograr cultivarlas tanto con fines alimentarios como industriales. Por ejemplo, Chile es el productor de algas más importante fuera de Asia llegando a una producción de 21 700 toneladas en 2008 (FAO, 2010).

Las algas también llamadas "las verduras del mar" son un alimento de alto valor nutritivo, que proporcionan gran cantidad de vitaminas y minerales. No obstante, el consumo de algas requiere de ciertas normas de seguridad alimentaria. Con tal fin, existen legislaciones específicas para la puesta en el mercado de las algas para el consumo humano en algunos países miembros de la Unión Europea. Tal es el caso de Francia, que fue el primer país Europeo en la elaboración de una legislación específica para el consumo de algas (Mabeau & Fleurence, 1993). Un aspecto importante que no hay que olvidar es la capacidad de las algas, debido al hábitat en el que viven, de bioacumular no sólo elementos esenciales y minerales sino también elementos tóxicos como metales pesados y arsénico. En este sentido, en Francia y en otros países (Hold & Kraan, 2010) están regulados los valores máximos permitidos en las algas de

metales pesados y otros minerales considerados tóxicos, como el plomo, cadmio, mercurio, cobre, manganeso, zinc y arsénico (Hold & Kraan, 2010).

En España, sin embargo no hay legislaciones específicas para el consumo de algas y productos derivados, y por tanto, tampoco hay pautas que regulen los valores máximos permitidos de estos elementos tóxicos en las algas. Recientemente estudios en algas consumidas en España han revelado que algunas algas y productos derivados contienen cadmio y arsénico inorgánico en valores más altos que los permitidos por la legislación en otros países (Almela et al., 2002; Almela et al., 2006). Sobre todo en *Hizikia fusiforme* que excede los límites permitidos de arsénico inorgánico (Almela et al., 2006), de forma que el consumo diario de 1,7 g de producto seco podría alcanzar los Valores Provisionales de la Ingesta Diaria Recomendada por la OMS en una persona con un peso medio de 68 kg (Almela et al., 2002).

Hay otras legislaciones europeas que consideran las algas como alimento debido a su puesta en el mercado para el consumo humano antes del 15 de mayo 1997, y por tanto no están consideradas como 'Nuevo Alimento' o Novel Food (Reglamento 258/97 del Parlamento Europeo). Sin embargo, legislaciones específicas en ciertos países pueden limitar su introducción en los mercados.

En resumen, las algas constituyen un recurso abundante y a la vez infrautilizado de nuestras costas. A diferencia de otros países de nuestro entorno, donde representan una fuente de riqueza para muchas empresas, la valoración y explotación de este recurso en España está todavía lejos de su verdadero potencial. Es la intención de esta tesis profundizar en la evaluación nutricional, así como los beneficios para la salud, que derivan del consumo de este recurso natural presente en nuestras costas.

2. Evaluación nutricional de las algas marinas

La composición química de las algas marinas depende de la especie, lugar de cultivo, condiciones atmosféricas y periodo de recolección. Desde un punto de vista nutricional, las algas son muy interesantes por su alto contenido en fibra alimentaria (33-50% peso seco), por ser una fuente importante de proteínas (pardas 5-24%; rojas y verdes 10-47%) (Mohamed, Hashim & Rahman, 2012) y minerales (8-40%), y por su bajo contenido lipídico (1-2%) (Rupérez & Saura-Calixto, 2001).

2.1. Carbohidratos de alto peso molecular. Fibra alimentaria

La fibra alimentaria se conoce hoy, como un elemento importante para la nutrición sana. Sin embargo, no existe una definición totalmente aceptada a nivel europeo, ni a nivel internacional, ni tampoco un método analítico que mida todos los componentes alimentarios que ejercen los efectos fisiológicos de la fibra, pero sí existe un consenso acerca de que la definición debe incluir el papel fisiológico de la fibra alimentaria.

El concepto original de fibra -paredes celulares vegetales o polisacáridos excepto almidón y lignina- (Trowell et al. 1976) ha evolucionado científicamente de forma paralela a la metodología empleada para su determinación, y actualmente existe la tendencia a considerarlo de una forma más amplia, incluyendo otras sustancias que tampoco son atacadas por las enzimas digestivas y llegan al colon sin degradar.

Se puede decir, por tanto, que la fibra alimentaria está constituida por una serie de compuestos que comprenden una amplia mezcla de carbohidratos y polímeros presentes en las plantas, que incluyen tanto oligosacáridos como polisacáridos, como por ejemplo, celulosa, hemicelulosa, sustancias pécticas, gomas, almidón resistente e inulina, los cuales pueden estar asociados a la lignina y/o a otros componentes no carbohidratos (polifenoles, ceras, saponinas, cutinas y proteína resistente) (Elleuch et al., 2011). Otros autores

proponen un método alternativo al análisis de fibra alimentaria de la AOAC (Prosky et al., 1988), utilizando condiciones fisiológicas, denominado método de la *fracción indigerible* (Saura-Calixto et al., 2000) entendiendo como fracción indigerible 'la parte de los alimentos vegetales que no son digeridos ni absorbidos en el intestino delgado, alcanzando el colon, donde se convierte en el sustrato para la microbiota fermentativa' (Saura-Calixto et al., 2000; Rupérez & Toledano, 2003)

En resumen, se podría decir que la fibra alimentaria la constituyen sustancias de origen vegetal, concretamente de la pared celular, hidratos de carbono o derivados de los mismos que resisten la digestión y absorción en el intestino delgado humano y que experimentan una fermentación parcial o total en el intestino grueso.

La fibra alimentaria consta de dos fracciones (soluble e insoluble) y sus propiedades vienen determinadas principalmente por la proporción de estas dos fracciones. Así, la fibra soluble se caracteriza por su habilidad de formar geles viscosos, en contacto con el agua, en el tracto intestinal. La fibra insoluble no forma geles en contacto con el agua, sino que es capaz de retener agua en su matriz estructural, produciendo un aumento de la masa fecal que acelera el tránsito intestinal. Estas diferencias en el comportamiento de las fibras en el tránsito intestinal derivan en diferentes propiedades. La fibra insoluble es escasamente fermentada y tiene un marcado efecto laxante y regulador intestinal, mientras que la fibra soluble es fermentada en alta proporción y sus principales propiedades se relacionan con disminución de colesterol y glucosa en sangre y desarrollo de la microbiota intestinal (Cho & Drehar, 2001).

2.1.1. Fibra alimentaria en plantas terrestres

Como se ha visto, existe una gran heterogeneidad de compuestos que componen la fibra alimentaria en las plantas terrestres y con las nuevas definiciones, el número de sustancias que se incluyen en el concepto de fibra ha

aumentado haciendo más difícil su clasificación. La clasificación propuesta por Ha, Jarvis & Mann (2000), recoge de forma global los conocimientos actuales teniendo en cuenta los principales componentes de la fibra alimentaria.

Así, según Ha et al. la fibra se puede clasificar en: (1) polisacáridos no almidón, como la celulosa, β-glucanos, hemicelulosas, pectinas, gomas y mucílagos; (2) oligosacáridos resistentes, principalmente fructooligosacáridos (FOS), inulina y galactooligosacáridos; (3) ligninas; (4) sustancias asociadas a polisacáridos no almidón, como la suberina, la cutina y fenoles; y (5) almidones resistentes.

Otra clasificación también apropiada sería según el grado de hidrosolubilidad, ya que estas propiedades juegan un papel muy importante en los beneficios fisiológicos de la fibra. Así, entre los componentes de la fibra soluble encontramos polisacáridos del tipo de la hemicelulosa (tipo A), pectinas, gomas, mucílagos y otros polisacáridos junto con oligosacáridos como la inulina y algunos fructooligosacáridos y almidón resistente. Por otro lado componen la fibra insoluble polisacáridos como la celulosa, la lignina y la hemicelulosa (tipo B).

2.1.2. Fibra alimentaria en macroalgas

Atendiendo al color de sus pigmentos, las macroalgas se pueden clasificar en tres grandes grupos: Algas pardas (Phaeophyceae), algas rojas (Rhodophyceae) y algas verdes (Chlorophyceae). Tanto las algas pardas como rojas son las algas más comúnmente utilizadas para la alimentación humana. Las algas pertenecientes a cada grupo se distinguen por la composición específica de los polisacáridos estructurales de la pared celular y de reserva. La mayor parte de estos polisacáridos que forman parte de la composición de las algas, pueden ser considerados como fibra, ya que no son digeridos por el equipo enzimático humano, aunque en parte son degradables por las enzimas producidas por las bacterias colónicas (Jiménez-Escrig & Goñi, 1999). En la Tabla 1.1 se detallan los

diferentes polisacáridos específicos de la pared celular y de reserva clasificados como fibra soluble e insoluble en los tres grandes grupos de macroalgas.

Tabla 1.1. Clasificación de los principales polisacáridos que constituyen la fibra alimentaria en las macroalgas

	Fibra soluble	Fibra insoluble
Algas pardas (*Fucus*, *Laminaria*, *Undaria*, *Himanthalia*)	**Laminarano** *(1,3)-β-glucosa y (1,6)- β-glucosa, manitol* **Alginato** *β-(1,4)-ácido manurónico y a-(1,4)- ácido gulurónico* **Fucano /Fucoidano /Furano** *Fucosa sulfato, ácido manurónico, xilosa*	**Celulosa** *β-(1,4) glucosa*
Algas rojas (*Chondrus, Porphyra, Mastocarpus*)	**Agar** *D-galactosa y (3,6)-anhidro-D-galactosa sulfato* **Carragenano** *D-galactosa y (3,6)-anhidro-L-galactosa sulfato* **Xilanos y Mananos**	**Celulosa** *β-(1,4) glucosa*
Algas verdes (*Ulva*, *Enteromorpha*)	**Xilano** *Xilosa, ramificaciones arabinosa, glucosa, galactosa, acidos urónicos* **Ulvano** *Ramnosa, xilosa, ácidos urónicos, sulfato* **Galactanos sulfatados**	**Celulosa** *β-(1,4) glucosa*

Tomado de Jiménez-Escrig y Sánchez-Muniz, 2000; Holdt & Kraan, 2011; Jiao et al., 2011.

Así, la fibra alimentaria en las algas pardas se compone de cuatro familias de polisacáridos: laminaranos, alginatos, fucanos y celulosa. Los laminaranos constituyen polisacáridos de reserva, mientras que el resto son polisacáridos estructurales que forman parte de la pared celular.

La fibra alimentaria en las algas rojas está compuesta principalmente por galactanos sulfatados (carragenanos y agar) y en menor medida de xilanos, mananos y celulosa (Jiménez-Escrig & Sánchez-Muniz, 2000). El principal polisacárido de reserva en las algas rojas es el almidón floridiano. Las algas verdes contienen almidón, celulosa, xilanos, mananos y polisacáridos iónicos que contienen grupos sulfato y ácidos urónicos (Jiménez-Escrig & Sánchez-Muniz, 2000).

Los contenidos en fibra alimentaria de las macroalgas están comprendidos entre 33-62% del peso seco, siendo superiores a los que se encuentran en las frutas y hortalizas más comunes (Holdt & Kraan, 2011). Siendo además la fibra soluble la de mayor importancia (Dawczynski, Schubert, & Jahreis, 2007; Holdt & Kraan, 2011) en contraposición a las plantas terrestres de mayor riqueza en fibra insoluble (Jiménez-Escrig & Sánchez-Muniz, 2000). Dentro de las algas, el contenido en fibra soluble suele ser mayor en las rojas (15-22% en peso seco) como en *Chondrus* y *Porphyra* (Nori) (Rupérez & Saura-Calixto, 2001; Holdt & Kraan, 2011). En cambio las algas pardas como *Fucus* o *Laminaria* /*Saccharina*, tienen un mayor contenido en fibra insoluble (27-40% en peso seco) (Murata & Nakazoe, 2001; Holdt & Kraan, 2011). Sin embargo, estos contenidos son variable-dependientes y pueden aparecer fluctuaciones en función de la especie, la estación del año, zona geográfica, etc., lo cual dificulta a la hora de hacer estudios en conjunto de todas las macroalgas, ya que el contenido nutricional de cada alga es en la mayoría de los casos especie-dependiente.

2.2. Polisacáridos

Las macroalgas marinas contienen polisacáridos específicos, tanto estructurales como de reserva, que no están presentes en otros vegetales terrestres. Estos polisacáridos son polímeros de azúcares unidos por enlaces glicosídicos y que poseen numerosas aplicaciones comerciales como estabilizantes, emulsionantes, espesantes, gelificantes, etc. A continuación se

detallan los principales polisacáridos presentes en las algas pardas y rojas de mayor interés para la industria y para el consumo humano.

2.2.1. Laminaranos

Constituyen el principal polisacárido de reserva de las algas pardas, concretamente se encuentra en especies del orden Laminariales (*Laminaria* y *Undaria*) así como en especies del orden Fucales como *Fucus* y *Ascophyllum* (Holdt & Kraan, 2011). Su contenido varía con el hábitat y la época del año y puede alcanzar hasta un 36% en peso seco (Devillé et al., 2004). Como polisacárido de reserva, alcanza valores máximos en otoño, entre los meses de septiembre a noviembre, mientras que durante los meses de febrero y junio alcanza valores mínimos o casi nulos (Holdt & Kraan, 2011). Estas variaciones son importantes a la hora de escoger la fecha de recolección de estas algas para la extracción de este polisacárido con fines comerciales o industriales.

La estructura y composición del laminarano, también llamado laminarina, varía de acuerdo con la especie. En general el polímero está constituido por unidades de D-glucosa unidas por enlaces β-(1,3) y ramificado mediante enlaces β-(1,6) (Rioux, Turgeon & Beaulieu, 2007). Es un polímero de bajo peso molecular (3-6 kDa) dependiendo del grado de polimerización (entre 20-25 unidades de glucosa). Su solubilidad por tanto está condicionada por el grado de ramificación. Así, los laminaranos muy ramificados son solubles en agua fría, mientras que los menos ramificados son solubles en agua caliente (Rupérez, Ahrazem & Leal, 2002).

Una de las aplicaciones comerciales de este polisacárido es como fibra alimentaria, como sustrato de bacterias prebióticas en el colon (Devillé et al., 2007). Otras propiedades funcionales que se han descrito son: actividad anticoagulante, antitumoral (Miao et al., 1999) y capacidad de reducir el colesterol en sangre (Holdt & Kraan, 2011).

2.2.2. Alginatos

Los alginatos constituyen el principal polisacárido de la pared celular de las algas pardas, y no se encuentran en los vegetales terrestres. Su homólogo en las plantas terrestres serían las pectinas. Se extraen de especies como *Ascophyllum nodosum, Undaria pinnatifida, Fucus vesiculosus, Laminaria* spp, *Sargassum* spp, etc. pudiendo alcanzar entre 40-47 % del peso seco (Holdt & Kraan, 2011). Aunque estos valores sufren variaciones a lo largo del año y de la estación, alcanzando valores máximos en primavera, entre los meses de marzo a junio, y valores mínimos durante los meses de septiembre a octubre (Holdt & Kraan, 2011).

Los alginatos pueden disponerse en dos formas, en forma ácida o en forma de sal, y ambas pueden estar presentes en las algas. El nombre alginato, hace referencia a la sal del ácido algínico. Los alginatos (en forma de sal) son estables a pH 6-9, pero a pH ácido (3-4) se vuelven insolubles y precipitan en la forma de ácido libre.

El ácido algínico es un copolímero lineal compuesto por dos unidades de ácido β-D-manurónico (M) y ácido α-L-gulurónico (G) unidos mediante enlaces (1,4) (Figura 1.1). Estas dos unidades de ácidos urónicos están dispuestas en la molécula en tres tipos de secuencias o regiones, también llamadas bloques. Los bloques G contienen solo unidades derivadas del ácido L-gulurónico, los bloques M se basan enteramente en ácido D-manurónico y las regiones MG, consisten en unidades alternadas de ambos ácidos (Leal et al., 2008).

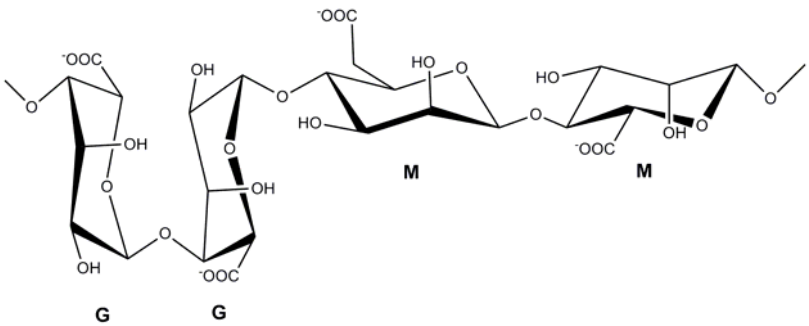


Figura 1.1. Estructura química del ácido algínico: ácido β-D-manurónico (M) y ácido α-L-gulurónico (G)

En la figura 1.1 también se muestran las configuraciones espaciales que adoptan los bloques M y G debido a los diferentes enlaces glicosídicos entre los carbonos C-1 y C-4 de las unidades monoméricas. Las regiones de bloques M corresponden a cadenas lineales, mientras que los bloques G presentan una estructura en forma de bucle. Con esta configuración, cuando dos cadenas de bloques G se alinean lado a lado resulta un hueco en forma de diamante, el cual tiene la dimensión ideal para acomodar en su interior un ión calcio, formándose una estructura dimérica. Éste modelo fue propuesto por Grant en 1973 ("egg-box model") para explicar las propiedades gelificantes de los alginatos al reaccionar con sales cálcicas.

Por este motivo, los alginatos se utilizan especialmente en la industria alimentaria por sus propiedades como espesantes y gelificantes. En este sentido, según las proporciones de regiones de bloques G y M, que varían según la especie, serán las características de los geles de alginatos, siendo el índice M/G un parámetro comúnmente utilizado para conocer la naturaleza de los geles de alginatos (Haug et al., 1967). En general, alginatos con un índice M/G bajo forman geles más fuertes y rígidos, mientras que alginatos con un alto índice M/G forman geles más elásticos y blandos (Draget et al., 2006). Este índice no sólo varía según las especies sino también con la edad y la estación del año (Haug et al., 1976).

Otras aplicaciones, además de como espesantes y gelificantes, pueden derivarse de estos polisacáridos especialmente como fibra alimentaria. Otras propiedades funcionales que se han descrito son: efecto antihipertensivo, antiinflamatorio, capacidad de reducir el colesterol en sangre, y para prevenir la absorción de sustancias tóxicas como metales pesados, que quedarían atrapados en la molécula no siendo absorbidos por el organismo (Holdt & Kraan, 2011).

2.2.3. Fucanos/Fucoidanos

Estos polímeros constituyen uno de los más fascinantes polisacáridos sulfatados de las algas. El fucano o fucoidano es un heteropolisacárido de L-fucosa sulfatada, que se encuentra en la pared celular o matriz extracelular de las algas pardas, pudiendo alcanzar hasta el 40% peso seco del alga (Holdt & Kraan, 2011). Debido a que se descubrió por primera vez en las algas del orden *Fucales* (Phaeophyceae) se le llamó fucano o fucoidano. Algunos autores diferencian estos dos términos, de forma que usan el término '*fucano*' para describir heteropolímeros sulfatados que contienen además de L-fucosa, D-xilosa, D-galatosa, D-manosa y ácido D-glucurónico (Chevolot et al., 1999; Bilan et al., 2010); mientras que utilizan el término '*fucoidano*' para referirse a homopolímeros sulfatados de algas constituidos por L-fucosa y grupos sulfato.

Sin embargo, a pesar de que hay muchos estudios acerca de la composición y estructura química de los fucoidanos o fucanos de algas en los últimos años (Li et al., 2008; Eluvakkal, Sivakumar & Arunkumar, 2010; Rioux, Turgeon & Beaulieu, 2010; Croci et al., 2011), todavía no se ha conseguido elucidar completamente su composición. Esto es debido en gran parte a su gran heterogeneidad y complejidad estructural que está condicionada no sólo por diferencias entre las especies, sino también entre partes de la planta e incluso por el método de extracción empleado (Eluvakkal et al., 2010). De tal forma que cada fucoidano extraído de una determinada especie con un método en concreto, será único en cuanto a estructura y composición, dando lugar por tanto a diferencias en cuanto a actividades biológicas. También se conoce que el número y la posición de los grupos sulfato parece ser un factor importante en la actividad biológica de los polisacáridos sulfatados (Li et al., 2008). Una técnica empleada para determinar de forma rápida y sencilla la posición de los grupos sulfato en los polisacáridos es la espectroscopía infrarroja (Li et al., 2008), pudiendo diferenciar bandas características de sulfatos en posición axial o equatorial dentro de la molécula.

Con todo ello, factores como el peso molecular, la cantidad y posición de grupos sulfato y la composición y conformación de los azúcares que lo componen, parecen tener una gran influencia en la actividad biológica del fucoidano (Rioux et al., 2010). Con respecto a las actividades biológicas, son muchas y muy variadas las que se le atribuyen a estos polisacáridos sulfatados de las algas, tales como actividad anticoagulante, antiviral, antiadhesiva, antiinflamatoria, antiproliferativa, antitumoral y antioxidante (Li et al., 2008; Eluvakkal et al., 2010; Croci et al., 2011; Holdt & Kraan, 2011; Wijesekara, Pangestuti & Kim, 2011) entre otras.

2.2.4. Carragenanos

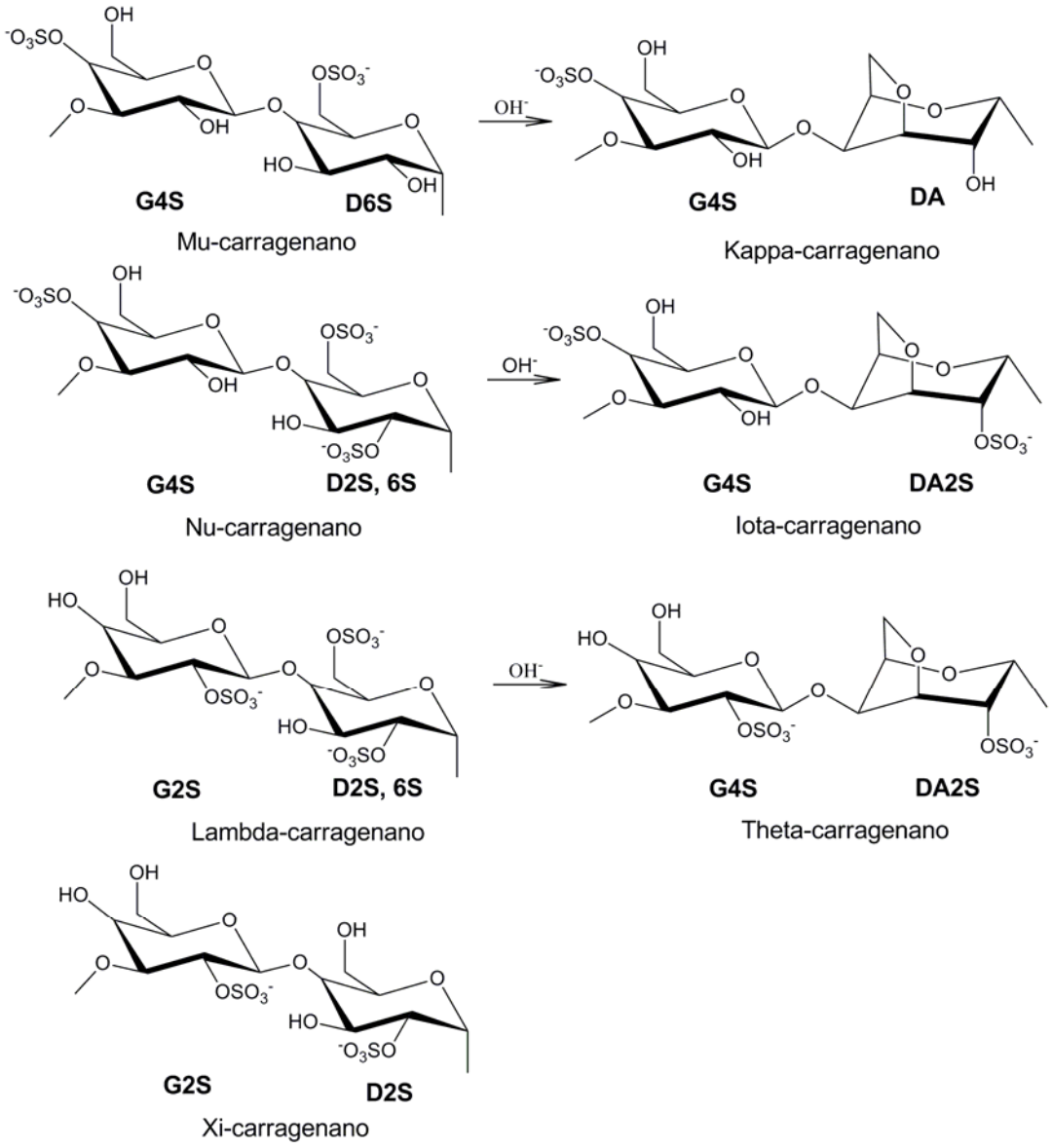
Junto con el agar constituyen la principal familia de polisacáridos sulfatados, también llamados galactanos, de elevado peso molecular que se pueden extraer de las algas rojas. La principal diferencia entre los carragenanos y el agar es la presencia de D-galactosa y anhidro-D-galactosa en los carragenanos y de D-galactosa, L-galactosa o anhidro-D-galactosa en el agar, además del grado de sulfatación que es mucho mayor en los carragenanos.

Por tanto, los carragenanos están formados por unidades de D-galactosa y de 3,6-anhidro-D-galactosa, sulfatadas y unidas mediante enlaces alternos α-(1,3) y β-(1,4) (Li et al., 2008; Tuvikene et al., 2009; Pereira & Van De Velde, 2011). Dependiendo del grado de sulfatación, de las posiciones de los grupos sulfato y de la presencia de 3,6-anhidrogalactosa se distinguen varios tipos de carragenanos, representados en la Figura 1.2. Actualmente existen alrededor de quince estructuras idealizadas de carragenanos que tradicionalmente se identifican con letras Griegas (Chopin et al., 1999), de ellas las más importantes desde el punto de vista industrial son tres tipos: kappa, lambda e iota.

Las diferencias en composición y configuración de los diferentes tipos de carragenanos son responsables de las diferencias en cuanto a sus propiedades reológicas como agentes espesantes, gelificantes y estabilizantes que han

permitido su aplicación desde hace décadas en la industria alimentaria, farmacéutica y cosmética (Pereira & Van De Velde, 2011). Así, los carragenanos tipo kappa e iota contienen 3,6-anhidro-galactosa y se consideran gelificantes, mientras que el tipo lambda formado sólo por unidades de galactosa es un espesante (Prado-Fernández et al., 2003). La principal característica de los carragenanos que forman gel (tipo kappa e iota) es la presencia del puente de anhidro-galactosa en posición 4 del residuo de galactosa (Figura 1.2).

Figura 1.2. Estructura química de los principales tipos de carragenanos.



Esta conformación es crucial para la formación de la estructura helicoidal necesaria para formar el gel. Sin embargo, los carragenanos tipo lambda y

también los tipo mu- y nu-, considerados los precursores de los tipos kappa- e iota-, respectivamente (Figura 1.2), no tienen el puente formado en el residuo de anhidrogalactosa, y por tanto adoptan otra conformación distinta (Pereira & Van De Velde, 2011) que dificulta la formación del gel.

Debido a la gran variabilidad en la estructura y el número y posición de los grupos sulfato en los diferentes tipos de carragenanos, Knusten et al. (1994) desarrolló una nomenclatura binomial mucho más versátil y que se sigue utilizando hoy día. En la Figura 1.2 aparece dicha nomenclatura debajo de cada unidad binomial del carragenano, y en la Tabla 1.2 se detalla la estructura química que corresponde a cada código empleado por Knusten. En general se utilizan las letras G y D para referirse a cada una de las unidades de galactosa en una misma unidad binomial y la letra DA para las unidades de anhidrogalactosa añadiéndole el número de la posición del grupo sulfato (S) (Tabla 1.2).

Tabla 1.2. Nomenclatura binomial usada para clasificar los principales tipos de carragenanos de algas rojas

Código*	Estructura química	Tipo de carragenano
S	Ester sulfato ($O\text{-}SO_3^-$)	κ, λ, ι, μ, ν, θ, ξ
DA	3,6-anhidro-D-galactosa	κ
G4S	D-galactosa-4-sulfato	κ, ι, μ, ν
G2S	D-galactosa-2-sulfato	λ, θ, ξ
D6S	D-galactosa-6-sulfato	μ
D2S	D-galactosa-2-sulfato	ξ
D2S,6S	D-galactosa-2,6-disulfato	λ, ν
DA2S	3,6-anhidro-D-galactosa-2-sulfato	ι, θ

*Adaptado de Knusten et al. (1994)

Generalmente, las algas no producen carragenanos puros tal y como se muestran en la Figura 1.2, sino más bien una variedad de estructuras híbridas, que son mezcla de dos o más tipos de carragenanos. Así monómeros del tipo kappa- o iota- se pueden encontrar en una misma alga con sus precursores, los

monómeros mu- y nu- y también en diferentes proporciones entre sí. Por ejemplo, se han descrito diferencias en la composición de los carragenanos en las algas que pertenecen al orden Gigartinales entre la fase gametofítica, en la que predominan los carragenanos tipo kappa/iota/mu/nu (familia kappa) y la fase esporofítica en la que domina la familia lambda de carragenanos (lambda, theta, xi) (Chopin et al., 1999; Van De Velde et al., 2002). Por tanto, las cantidades relativas de cada uno de ellos va a depender no sólo de la especie, sino también de la época del año (fase gametofítica/esporofítica) así como del proceso de extracción empleado (Hilliou et al., 2006), ya que mediante modificación con álcali se puede inducir la formación del puente de anhidrogalactosa y producir carragenanos tipo kappa- o iota- a partir de sus precursores mu- y nu- (Figura 1.2) (Jiao et al., 2011; Pereira & Van De Velde, 2011).

Aparte de su capacidad como espesantes, gelificantes y estabilizantes, de gran interés para la industria, otras aplicaciones se han encontrado en los polisacáridos sulfatados de las algas rojas. Las actividades biológicas de mayor repercusión en estos compuestos son como anticoagulantes y antioxidantes (Jiao et al., 2011; Wijesekara et al., 2011; Holdt & Kraan, 2011). También se ha encontrado que poseen actividad antiinflamatoria (Holdt & Kraan, 2011) y antiproliferativa (Costa et al., 2010).

2.2.5. Agar

El agar consiste en una mezcla de dos tipos de polisacáridos, la agarosa y la agaropectina con propiedades estructurales y funcionales similares a los carragenanos. La agarosa es el componente principal, representando alrededor del 70% del total. Se trata de un polímero lineal de D-galactosa y 3,6-anhidro-L-galactosa con enlaces β-(1,3) y α-(1,4). Tanto la agarosa como la agaropectina tienen la misma estructura básica, pero se diferencian en la presencia de grupos sulfato y piruvato, relativamente abundantes en la agaropectina y muy escasos (idealmente ausentes) en la agarosa.

El agar se extrae principalmente de las algas rojas *Gelidium* spp y *Gracilaria* spp, pudiendo alcanzar en las especies de *Gracilaria* el 31% en peso seco (Holdt & Kraan, 2011). El agar extraído de estas algas, también llamado agar-agar, se ha utilizado en la cocina tradicional japonesa, por sus propiededes gelificantes, desde hace muchos siglos. Actualmente es muy utilizado en las industrias como gelificante, en productos cárnicos y de pescado, para mimetizar la gelatina, así como en otros productos gelatinosos. También se usa como estabilizante. Además, debido a que es un polisacárido no digerible, desde el punto de vista nutricional forma parte de la fibra alimentaria pues son muy raras las enzimas capaces de degradar el agar, incluso entre los microorganismos. Por eso el agar es también un valioso medio de cultivo en bacteriología, utilizándose en esta aplicación desde 1880. Otras aplicaciones descritas para el agar-agar son: capacidad de disminuir la concentración de glucosa en sangre y potencial como antioxidante y anticancerígeno (Holdt & Kraan, 2011).

2.3. Carbohidratos de bajo peso molecular.

Las algas marinas debido a que viven en un medio con una concentración muy alta de sales, necesitan acumular ciertos solutos que le permitan regular el balance osmótico entre sus células y el medio marino. En este proceso están involucrados muchos iones como el sodio, el cloro y el potasio, pero también están implicados ciertos carbohidratos de bajo peso molecular (Reed et al., 1995) que se acumulan en las células en respuesta a variaciones de salinidad. Entre ellos se encuentran por ejemplo, la sacarosa en las algas verdes, los alditoles como el manitol, en las pardas y rojas (Reed et al., 1995) y hexitoles como digeneásidos y fluorósidos en las rojas (Ascêncio et al., 2006).

Manitol. El manitol es el principal carbohidrato de bajo peso molecular presente en muchas especies de algas pardas, especialmente en *Laminaria* y *Ecklonia*. El manitol es un azúcar alcohol, un alditol, que se diferencia de la manosa por tener en el C-1 un grupo alcohol, en vez de un aldehído. El contenido en manitol suele ser < 10% del peso seco en especies de *Ascophyllyum*

nodosum y *Laminaria hyperborea*, aunque también está sujeto a muchas variaciones estacionales y en otoño se pueden alcanzar valores máximos de hasta el 25% del peso seco (Holdt & Kraan, 2011).

2.4. Minerales

Las algas obtienen del ambiente marino en el que viven una gran riqueza de elementos minerales, siendo conocidas por su elevado contenido mineral (8-40% del peso seco del alga). Esta gran abundancia tanto de minerales esenciales como de elementos traza incluyen macronutrientes como sodio, calcio, magnesio, potasio, cloruro, sulfato, fósforo, y micronutrientes como yodo, hierro, zinc, cobre, selenio, molibdeno, fluoruro, manganeso, boro, níquel y cobalto, entre otros. Sin embargo la composición mineral puede variar en función del grupo taxonómico al que pertenece la especie, en función a variaciones geográficas, estacionales y fisiológicas (Mabeau y Fleurence, 1993) e incluso con el tipo de procesado y método de mineralización aplicado (Rupérez, 2002).

El contenido mineral en las algas marinas es muy elevado en comparación con los vegetales terrestres (Murata & Nakazoe, 2001). En la mayoría de las plantas el contenido en cenizas varía entre 5-10% del peso seco, lo que coloca a las algas como una importante fuente de minerales, que no son tan abundantes en las plantas terrestres. Las algas por tanto se pueden utilizar como suplemento alimentario para contribuir a alcanzar las cantidades diarias recomendadas de algunos macrominerales y elementos traza (Rupérez, 2002).

Debido a la capacidad de las algas para absorber de un modo selectivo las sustancias inorgánicas del mar, estos minerales están en forma de iones asociados a los polisacáridos cargados de la pared celular. Sin embargo, el tipo de unión de ciertos minerales y elementos con los polisacáridos aniónicos (alginato, fucoidano, agar, carragenano) puede limitar la disponibilidad y

absorción de esos minerales (Mabeau & Fleurence, 1993; Jiménez-Escrig & Goñi, 1999).

Debido a esta capacidad que tienen de unirse a ciertos minerales y elementos, las algas también se consideran bioacumuladores de metales pesados y por este motivo se han utilizado también como bio-indicadores de contaminación medioambiental en ambientes marinos (Riget, Johannsen & Asmund, 1997). En ese sentido hay diversos estudios sobre contaminación por metales pesados en diferentes especies de importancia comercial y medioambiental (Hou & Yan, 1998; Sánchez-Rodríguez et al., 2001), y la contaminación por metales pesados es un aspecto a tener en cuenta en la seguridad para el uso de algas en la alimentación (Almela et al., 2006).

El sulfato es un componente típico de las algas marinas derivado principalmente de los polisacáridos fucoidanos en las algas pardas, carragenanos o agar en las algas rojas y ulvanos en las algas verdes (Jiao et al., 2011). Este anión, que se encuentra unido al polisacárido mediante enlace éster ($O\text{-}SO_3^-$) juega un papel muy importante desde el punto de vista de las propiedades biológicas de las algas. En ese sentido, en diversos estudios se ha encontrado una correlación positiva entre el contenido en sulfato y las propiedades biológicas como antioxidantes y anticoagulantes, entre otras (Costa et al., 2010; Jiao et al., 2011). Como consecuencia los polisacáridos sulfatados de las algas suelen ser modificados estructuralmente mediante procesos de 'sobresulfatación' para aumentar o mejorar las propiedades biológicas (Jiao et al., 2011).

2.5. Proteínas

El contenido en proteínas en las algas puede variar mucho entre los grandes grupos de algas (pardas, rojas y verdes). El contenido proteico en las algas pardas es generalmente bajo (5-24% del peso seco), mientras que las algas rojas y verdes tienen un contenido mayor en proteínas (10-47% del peso seco)

(Mohamed et al., 2012). Estos niveles son comparables con los niveles de proteína encontrados en vegetales ricos en proteína como la soja llegando incluso al 40% del peso seco (Holdt & Kraan, 2011). Por ese motivo las algas se consideran una interesante fuente potencial de proteínas (Rupérez & Saura-Calixto, 2001).

Tal y como ocurre con otros componentes nutricionales de las algas, el contenido de proteínas, péptidos y aminoácidos se ve influenciado por diversos factores, especialmente por la variación estacional (Holdt & Kraan, 2011). Por ejemplo se ha visto que el contenido proteico en algas pardas *Saccharina* y *Laminaria* muestra un máximo durante los meses de febrero a mayo (Holdt & Kraan, 2011). Una variación similar se ha encontrado en especies de algas rojas, con un máximo en verano y una considerable reducción durante el invierno (Mohamed et al., 2012).

2.6. Polifenoles

Los polifenoles constituyen un componente minoritario de las algas. Las algas verdes y rojas contienen bajas concentraciones de polifenoles (< 1% peso seco) (Mabeau & Fleurence, 1993; Rupérez & Saura-Calixto, 2001) en comparación con las algas pardas, pudiendo llegar hasta el 14% peso seco, en especies de *Ascophyllum* y *Fucus* (Holdt & Kraan, 2011). El origen de los polifenoles en las plantas terrestres es a partir de los ácidos gálico y elágico, sin embargo los polifenoles de algas derivan de unidades polimerizadas de floroglucinol (1,3,5-trihidroxibenceno). Los florotaninos son los polifenoles más ampliamente descritos de las algas pardas, especialmente en especies del género *Ecklonia*, que debido a su gran heterogeneidad estructural promueven un gran abanico de propiedades biológicas (Holdt & Kraan, 2011; Wijesinghe & Jeon, 2011). Pero de entre ellas la más destacable es como antioxidantes. Además de los florotaninos se han descrito otros polifenoles en las algas como: fucol y sus derivados, flavonoides y derivados como la catequina y epicatequina (Holdt & Kraan, 2011).

2.7. Lípidos

El contenido lipídico en las algas es también minoritario (< 5% peso seco) permitiendo a las algas ser un alimento potencial de bajo contenido calórico. Al igual que ocurre con otros componentes de las algas, el contenido en ácidos grasos varía de acuerdo con la estación y otros factores medioambientales (Holdt & Kraan, 2011).

De forma notable, las algas rojas contienen cantidades muy significativas de ácidos grasos poliinsaturados como el ácido eicosapentanoico (EPA 20:5) de la serie omega-3 y ácido araquidónico (20:4). Tanto las algas pardas como las rojas, son fuente de ácidos grasos omega-3 y -6 (Mabeau & Fleurence, 1993; Sánchez-Machado et al., 2004) con un efecto beneficioso para la salud (Murata & Nakazoe, 2001). Las algas marinas contienen especialmente ácidos grasos poliinsaturados (PUFAs) del tipo 18:4 omega-3, los cuales no se incluyen en otros organismos, y se cree que tienen un efecto beneficioso en el sistema inmune de los humanos (Holdt & Kraan, 2011).

Las algas también contienen en la fracción insaponificable carotenoides, como β-caroteno, luteína y violaxantina en las algas rojas y verdes y fucoxantina en las algas pardas, así como tocoferoles y esteroles (Holdt & Kraan, 2011).

3. Propiedades biológicas de las algas marinas

3.1. Propiedades de la fibra alimentaria

Las propiedades físicas que se derivan de la fibra alimentaria dependen de la naturaleza de sus componentes y estas características van a ser responsables de los posibles efectos fisiológicos que se presenten a lo largo del tracto gastrointestinal. Como se ha visto, la fibra alimentaria en las algas está constituida por polisacáridos específicos que difieren de los hallados en plantas terrestres pudiendo por tanto presentar efectos fisiológicos diferentes.

3.1.1. Propiedades físico-químicas

Muchas de estas propiedades tienen que ver con el grado de solubilidad e hidratación de las fibras en agua, siendo muy variable entre las distintas fibras.

3.1.1.1. Solubilidad y viscosidad

Las fibras se clasifican en soluble e insoluble, en función de su capacidad de formar o no una solución cuando se mezclan con agua. La solubilidad está relacionada con la estructura del polisacárido, de forma que la presencia de grupos de sustitución como grupos carboxilo (COO^-) o sulfato (SO_4^{2-}) pueden aumentar su solubilidad (Elleuch et al., 2011). En el caso de las algas, los polisacáridos insolubles de la pared celular están compuestos mayoritariamente por celulosa, mientras que las fibras solubles se componen principalmente de fucanos, laminaranos, alginatos (pardas), carragenanos y agar (rojas). Las fibras solubles se caracterizan por su capacidad para aumentar la viscosidad y reducir la respuesta glucémica y el colesterol en sangre (Elleuch et al., 2011). Las fibras insolubles se caracterizan por su porosidad y baja densidad que les permiten aumentar la masa fecal y disminuir el tránsito intestinal (Elleuch et al., 2011)

La fracción soluble que se encuentra en una proporción mayor en las algas respecto de frutas, hortalizas y cereales, tiene una gran capacidad de aumentar la viscosidad debido a su capacidad para formar geles y actuar como emulsionante y gelificante, y por ello pueden ser incorporados fácilmente tanto en alimentos como en bebidas.

3.1.1.2. Propiedades de hidratación y capacidad de retención de aceite

Las principales propiedades de hidratación de la fibra son la capacidad de retención de agua y de hinchamiento. La capacidad de retención de agua (WRC) se define como la cantidad de agua retenida por gramo de fibra bajo ciertas condiciones de temperatura (Robertson et al., 2000; Rupérez & Saura-Calixto, 2001; Elleuch et al., 2011). La capacidad de hinchamiento (SW) se

calcula como el aumento de volumen por gramo de fibra que experimenta la fibra al hidratarse a una temperatura y tiempo dados (Robertson et al., 2000; Rupérez & Saura-Calixto, 2001).

Estas propiedades de la fibra están íntimamente relacionadas con la estructura química de los polisacáridos que la componen, además de otros factores como porosidad, tamaño de partícula, fuerza iónica, pH, temperatura, tipo de iones en solución, etc. (Elleuch et al., 2011). De esta forma, se ha visto que las fibras de las algas tienen una mayor afinidad por el agua que otras fibras, por ejemplo las de cereales (Elleuch et al., 2011). Además se ha visto que estas propiedades de afinidad por el agua aumentan conforme aumenta la solubilidad de la fibra (Fleury & Lahaye, 1991), y que la presencia de iones puede afectar dicha solubilidad.

Estas propiedades confieren a la fibra la habilidad de absorber y retener agua, por lo que los polisacáridos que la componen pueden ser potencialmente beneficiosos en la salud gastrointestinal, contribuyendo al tránsito intestinal con una influencia positiva en la prevención del cáncer de colon (Goñi et al., 2001). Además las fibras con elevada WRC, como es el caso de las fibras de algas, se pueden usar como alimentos funcionales que permitan modificar la viscosidad y textura de algunos alimentos (Elleuch et al., 2011).

Otro parámetro que se suele medir en las fibras junto con WRC y SW es la capacidad de retención de aceite (ORC). En el caso de las algas se ha visto que ORC es baja (Rupérez y Saura-Calixto, 2001) debido en gran parte a la naturaleza hidrofílica de los polisacáridos cargados que forman parte de la fibra soluble (alginatos, fucanos, agar y carragenanos).

3.1.1.3. Capacidad de intercambio iónico

La mayoría de las fibras solubles de algas la constituyen polisacáridos solubles aniónicos y debido a su naturaleza iónica, la capacidad de unión a sales de las algas es bien conocida. Diversos estudios *in vitro* han demostrado que

algunas algas pardas son capaces de liberar potasio y capturar sodio del medio ambiente (Jiménez-Escrig & Sánchez-Muniz, 2000). De la misma forma, los alginatos tienen una especial afinidad por el calcio que ayuda a la conformación de "egg-box model" de las cadenas de ácido manurónico y glucurónico (Grant et al., 1973) y que explican las propiedades gelificantes de los alginatos. Con respecto a las algas rojas, los carragenanos tipo kappa forman geles rígidos en presencia de potasio, mientras que el tipo iota forma geles más elásticos en presencia de calcio (Pereira & Van De Velde, 2011).

Debido a estas propiedades, otro parámetro que suele medirse en las fibras de algas es la capacidad de intercambio catiónico (CEC), y se ha visto que suele ser mayor en las fibras de algas respecto de las fibras de otros vegetales (Rupérez & Saura-Calixto, 2001).

3.1.2. Fermentabilidad y efecto prebiótico

Las fibras de algas están compuestas por polisacáridos que son poco degradados o fermentados por la microbiota intestinal humana (Lahaye & Kaeffer, 1997), de forma que llegan al intestino grueso de forma inalterada donde las bacterias del colon, con sus numerosas enzimas de gran actividad metabólica pueden digerirla en mayor o menor medida dependiendo de su estructura. Es por tanto la fermentabilidad, la propiedad más importante, ya que de ella se derivan un gran número de efectos tanto locales como sistémicos.

Los principales productos de la fermentación de la fibra son ácidos grasos de cadena corta (SCFA), fundamentalmente acético, propiónico y butírico, y gases (dióxido de carbono, hidrógeno y metano). Estos productos dan lugar a una serie de cambios en el colon. Los ácidos grasos son los encargados de bajar el pH y la propia fibra, los gases y los SCFA son capaces de estimular el crecimiento de ciertos microorganismos respecto de otros, produciéndose un cambio en el metabolismo bacteriano en el colon (Holdt & Kraan, 2011). Esto es también lo que se denomina *efecto prebiótico*, considerando a los prebióticos

como: "componentes no digeribles de la dieta que resultan beneficiosos para el huésped porque producen el crecimiento selectivo y/o la actividad de una o un número limitado de bacterias del colon" (Gibson & Roberfroid, 1995; Gibson, 2004). Además se ha visto que los SCFA pueden tener un efecto beneficioso en el metabolismo del colesterol (Devillé et al., 2007).

3.2. Principales propiedades de los polisacáridos sulfatados

Los polisacáridos sulfatados además de ser componentes estructurales y de reserva de la pared celular de las algas y jugar un importante papel como fibra alimentaria, presentan muchas propiedades biológicas de gran interés para el consumo humano. Varios estudios han demostrado que tanto la composición – polisacáridos sulfatados y otros nutrientes – como las propiedades biológicas de las algas dependen de muchos factores como la variación estacional, la localización geográfica, la calidad nutricional del agua de mar y otros factores relacionados con los procesos de obtención y extracción de los polisacáridos (Rioux et al., 2007). Además la bioactividad de los polisacáridos sulfatados de las algas está relacionada con una compleja interacción entre diferentes factores como el grado de sulfatación, la distribución de los grupos sulfato en la molécula, el peso molecular, etc. (Jiao et al., 2011). Aunque son muchos los estudios sobre la estructura química de los polisacáridos sulfatados de algas (Lechat et al., 2000; Deniaud, Fleurence & Lahaye, 2003; Lahaye y Robic, 2007), todavía no se han podido esclarecer las relaciones entre estructura y actividad biológica (Jiao et al., 2011).

Son muchas las propiedades biológicas atribuibles a los polisacáridos sulfatados de las algas, entre ellas las más relevantes son: actividad antioxidante, anticoagulante, antiviral, anticancerígena e inmunomodulatoria que se recogen en estudios recientes de revisión bibliográfica (Jiao et al., 2011; Wijesekara et al., 2011). Además se han descrito otras propiedades menos conocidas para los polisacáridos de algas como pueden ser: actividad antimicrobiana, antiproliferativa, anti-inflamatoria (Wijesekara et al., 2011),

protección hepática (Charles & Huang, 2009), efecto en glucosa (Hoebler et al., 2000; Vaugelade et al., 2000), en el metabolismo lipídico (Hoebler et al., 2000; Amano et al., 2005; Bocanegra, Benedí & Sánchez-Muniz, 2006; Huang, 2010) y efecto prebiótico (Devillé et al., 2007).

3.2.1. Estudios *in vitro*

A continuación se detallan las dos propiedades mejor conocidas y estudiadas en las algas como son la capacidad antioxidante y anticoagulante.

3.2.1.1. Capacidad antioxidante

Las algas marinas en respuesta a las condiciones altamente oxidativas en que viven, han desarrollado fuertes sistemas antioxidantes de defensa. Como organismos fotosintéticos, las algas están expuestas a una combinación de luz y altas concentraciones de oxígeno, que permiten la formación de radicales libres y otros agentes oxidantes fuertes (Rozema et al., 2002), pero la ausencia de daños oxidativos en las membranas tilacoidales de sus cloroplastos sugiere que sus células han desarrollado potentes mecanismos de protección (Matsukawa et al., 1997).

Muchos autores han comprobado que los extractos de algas marinas presentan en su composición química cantidades apreciables de compuestos antioxidantes, entre los que se encuentran compuestos lipofílicos como ácidos grasos insaturados, clorofila y carotenoides; compuestos hidrofílicos como polifenoles y vitamina C; y polisacáridos (Batista et al., 2009; Jiménez-Escrig et al., 2001).

En los últimos años, se ha demostrado que los polisacáridos de las algas desempeñan un papel importante como agentes secuestrantes de radicales libres y antioxidantes, para la prevención del daño oxidativo en organismos vivos. Se ha visto que polisacáridos presentes en algas pardas como fucoidanos, laminaranos y ácido algínico presentan actividad antioxidante *in vitro* (Rupérez

et al., 2002; Rocha De Souza et al., 2007; Wang et al., 2010), pudiendo ser considerados como potentes antioxidantes potenciales.

La actividad antioxidante de los polisacáridos sulfatados depende de varios factores como el grado de sulfatación, el peso molecular, el tipo de azúcar y enlace glicosídico (Wijesekara et al., 2011). Se ha visto, por ejemplo que polisacáridos de bajo peso molecular muestran mayor actividad antioxidante que los de mayor peso molecular (Sun et al., 2009). Esto se puede explicar porque los polisacáridos de menor peso molecular se pueden incorporar más fácilmente a las células y donar protones de forma más eficaz que los polisacáridos de mayor peso molecular (Wijesekara et al., 2011). Además se ha encontrado una correlación positiva entre el contenido en sulfato y la actividad secuestrante de radicales libres en fracciones de fucoidano del alga parda *Laminaria japonica* (Wang et al., 2008). De forma similar se ha demostrado la relación entre la capacidad antioxidante y los polisacáridos sulfatados de algas rojas recogidas en la India (Chandini et al., 2008; Rocha De Souza et al., 2007) y en algas tropicales (Costa et al., 2010).

3.2.1.2. Capacidad anticoagulante

El mecanismo de coagulación de la sangre está compuesto fundamentalmente por dos vías de actuación: intrínseca y extrínseca, en donde intervienen numerosos factores de coagulación y enzimas. El proceso de coagulación se lleva a cabo a través de ciertos factores de coagulación que permiten detener el flujo de sangre allí donde se ha producido un daño tisular del vaso sanguíneo. En la medida que los anticoagulantes, bien endógenos o exógenos, interfieren con estos factores de coagulación inactivándolos o limitándolos, la coagulación de la sangre se prolonga o se para (Wijesekara et al., 2011).

El uso de anticoagulantes tiene interés desde el punto de vista terapéutico, y la heparina (un polisacárido glucosaminoglucano sulfatado) es el

anticoagulante comercial más ampliamente usado desde hace más de cincuenta años en la prevención y tratamiento de enfermedades cardiovasculares (Wijesekara et al., 2011). No obstante su uso produce una serie de efectos colaterales o secundarios (Pereira et al., 2002; Jiao et al., 2011) y en los últimos tiempos se están buscando compuestos naturales alternativos con capacidad anticoagulante.

La capacidad anticoagulante de los polisacáridos sulfatados de las algas ha sido una de las propiedades más estudiadas, con el fin de encontrar un sustituto de origen natural para la heparina. Al igual que como ocurre con la capacidad antioxidante y con otras propiedades biológicas, la capacidad anticoagulante de los polisacáridos de las algas va a depender de muchos factores como el peso molecular, la composición de azúcares, grado y distribución de grupos sulfato en la molécula, etc. (Jiao et al., 2011), siendo difícil establecer una relación clara entre estructura y propiedad biológica. No obstante, se han podido aislar y caracterizar varios polisacáridos sulfatados de las algas con capacidad anticoagulante (Wijesekara et al., 2011). Principalmente se han identificado dos tipos de polisacáridos sulfatados: los galactanos sulfatados o carragenanos de las algas rojas (Wijesekara et al., 2011) y los fucoidanos sulfatados de las algas pardas (Colliec, Boisson-Vidal & Jozefonvicz, 1994; Chevolot et al., 1999).

Dicha capacidad anticoagulante se mide usando los ensayos de prolongación del tiempo de protrombina parcial activada (APTT), tiempo de protrombina (PT) y tiempo de trombina (TT), aunque los más utilizados son el APTT y PT. La prolongación del APTT sugiere la inhibición de los factores intrínsecos de la coagulación, mientras que la prolongación de PT está relacionada con la vía extrínseca de la coagulación. En diversos estudios se ha visto que tanto los polisacáridos sulfatados de algas pardas (fucoidanos) como los de algas rojas (carragenanos), tienen un mayor efecto sobre la prolongación de la vía intrínseca que sobre la prolongación de la vía extrínseca (Melo et al., 2004; Wang et al., 2010; Mohamed et al., 2012), siendo su acción principalmente vía antitrombina.

La relación entre estructura y capacidad anticoagulante de algunos polisacáridos sulfatados de algas ha sido estudiada por varios grupos (Colliec et al., 1994; Melo et al., 2004; Silva et al., 2010) y se ha visto que la presencia de grupos sulfato parece ser un factor determinante en dicha capacidad. Los resultados obtenidos hasta ahora demuestran que los polisacáridos de las algas pueden ser una alternativa a la heparina debido a su uso potencial como sustancias naturales anticoagulantes en la industria farmacéutica (Wijesekara et al., 2011; Jiao et al., 2011)

3.2.2. Estudios *in vivo*

Numerosos estudios *in vivo* han demostrado los efectos beneficiosos que proporcionan algunos polisacáridos sulfatados de las algas. Algunos han visto que la presencia de polisacáridos sulfatados en diferentes algas presentan un efecto destoxificador de gran importancia en la protección hepática en un modelo animal con ratas Wistar (Costa et al., 2010). Otros estudios han evidenciado su papel en la protección hepática frente a la toxicidad mediada por galactosamina de un fucoidano presente en el alga parda *Laminaria* en un modelo animal con ratas (Kawano, Egashira & Sanada, 2007). Además se ha ensayado la capacidad antioxidante de los polisacáridos sulfatados de las algas en un modelo animal con ratas estresadas (Veena et al., 2007).

También se ha observado su efecto en el metabolismo de la glucosa en un modelo animal con cerdos a partir de la ingesta de algas (Hoebler et al., 2000; Vaugelade et al., 2000; Amano et al., 2005). Y otros estudios han evidenciado el efecto de la ingesta de las algas comestibles Kombu (*Laminaria*) y Nori en el metabolismo lipídico en un modelo con ratas hipercolesterolémicas (Hoebler et al., 2000; Amano et al., 2005; Bocanegra et al., 2006), así como el efecto prebiótico en el colon (Devillé et al., 2007).

Por lo tanto, aunque es mucho lo que queda por estudiar todavía con respecto a las propiedades biológicas de las algas marinas comestibles, los resultados obtenidos hasta ahora demuestran que sus polisacáridos sulfatados pueden considerarse potencialmente beneficiosos para la salud.

CAPÍTULO 2

OBJETIVOS

1. Objetivo general

El objetivo principal de este trabajo de investigación es el estudio de la composición nutricional, así como de las propiedades biológicas de algas marinas comestibles españolas, a fin de revalorizar las algas como alimento funcional al dar a conocer los beneficios para la salud que se derivan del consumo de este recurso natural infrautilizado presente en nuestras costas.

2. Objetivos específicos

El objetivo general se podrá alcanzar gracias a la realización los siguientes objetivos específicos:

2.1. Caracterización y evaluación nutricional de algas

- *Composición centesimal (proteína, cenizas, grasa, humedad)*
- *Fibra alimentaria total y sus fracciones (soluble e insoluble)*
- *Polifenoles y carbohidratos totales en extractos acuosos y orgánicos*
- *Determinación de aniones por cromatografía iónica (IC)*
- *Identificación de los principales polisacáridos por espectroscopía infrarroja (FTIR)*
- *Fraccionamiento secuencial de los principales polisacáridos de la pared celular del alga parda Saccharina latissima y del alga roja Mastocarpus stellatus*
- *Distribución de pesos moleculares en las fracciones solubles del alga parda Saccharina latissima y del alga roja Mastocarpus stellatus por cromatografía de exclusión molecular (HPSEC)*

2.2. Evaluación de las propiedades biológicas *in vitro*

- *Principales propiedades físico-químicas de la fibra alimentaria*
- *Actividad antioxidante mutifuncional en extractos acuosos y orgánicos*
- *Capacidad antioxidante y anticoagulante medida en las fracciones solubles del alga parda Saccharina latissima y del alga roja Mastocarpus stellatus*

2.3. Evaluación de las propiedades biológicas *in vivo*

- *Ensayos experimentales en ratas Wistar sanas para evaluar los efectos fisiológicos producidos por la ingesta de dietas suplementadas con un alga parda (Saccharina latissima) o con un alga roja (Mastocarpus stellatus)*
- *Incidencia de las dietas suplementadas con un alga parda o roja sobre el metabolismo lipídico, así como efecto prebiótico potencial y estado antioxidante en el suero y ciego de las ratas*

CAPÍTULO 3

VISIÓN GENERAL DEL TRABAJO EXPERIMENTAL

Esta tesis basada en la evaluación nutricional y propiedades biológicas de algas marinas comestibles se ha desarrollado íntegramente (excepto la estabulación y cuidado de los animales de experimentación) en el Departamento de Metabolismo y Nutrición, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de investigaciones Científicas (CSIC), Madrid, España.

La parte de trabajo experimental se puede dividir principalmente en tres fases: estudios *in vitro*, estudios *in vivo* y desarrollo de la metodología usada en la primera fase de caracterización de algas. Aunque los detalles de las técnicas, métodos y materiales utilizados en el trabajo experimental se detallan más exhaustivamente en cada publicación, este capítulo trata de dar una visión general de todo el trabajo experimental realizado (Figura 3.1.), así como de las algas estudiadas y las publicaciones obtenidas como resultado de ese trabajo.

1. Algas estudiadas

Un total de nueve algas, tres algas pardas y seis algas rojas, proporcionadas por la empresa española Porto-Muiños (La Coruña, España) han sido estudiadas. En la Tabla 3.1 se detallan los nombres (Especie, Orden y Familia) y el tipo de estudio realizado en cada una de ellas para la elaboración de esta tesis doctoral.

Las algas se recolectan principalmente en primavera y verano, se limpian bien de arena y epífitos con agua corriente y se secan en corriente de aire (< 50 ^{o}C) dejándolas listas para trocear y moler a un diámetro de partícula inferior a 1.0 mm. Las muestras así molidas se almacenan en bolsas de plástico selladas permitiendo su conservación y almacenamiento hasta 3 años de caducidad a temperatura ambiente.

Figura 3.1. Esquema general del trabajo experimental

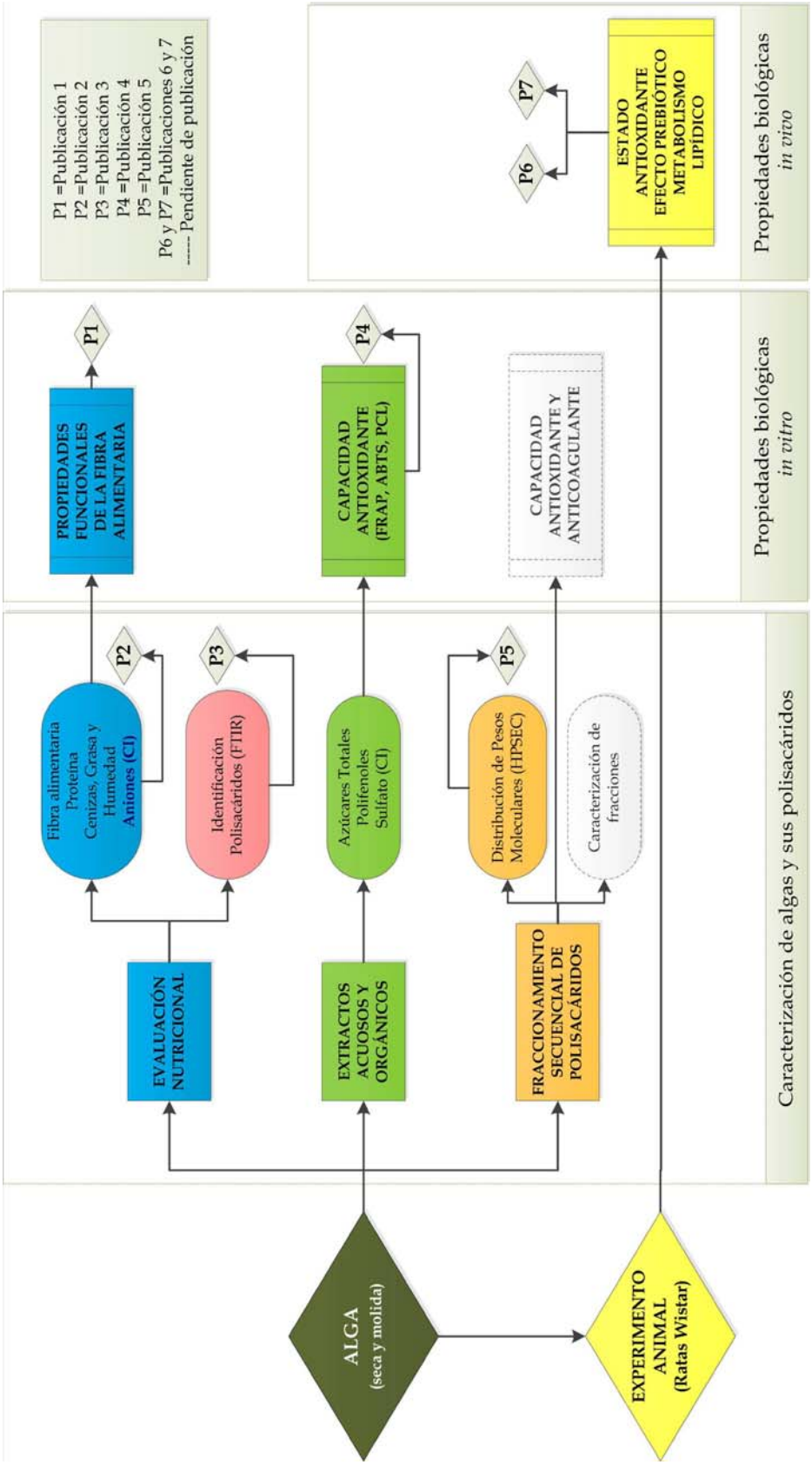


Tabla 3.1. Clasificación de las algas utilizadas y tipo de estudios realizados

Especie	Orden	Familia	Tipo de Estudio		
			Estudio *in vitro*	Metodología	Estudio *in vivo*
Filum Heterokonthophyta, clase Phaeophyceae					
Himanthalia elongata (L.) Gray	*Fucales*	*Himanthaliaceae*	• Caracterización • Capacidad antioxidante	•Aniones (IC) •FTIR	—
Saccharina latissima (L.) C.E.Lane, C.Mayes, Druehl & G.W.Saunders	*Laminariales*	*Laminariaceae*	•Caracterización •Capacidad antioxidante •Fraccionamiento	•Aniones (IC) •FTIR •HPSEC	SI
Bifurcaria bifurcata Ross	*Fucales*	*Sargassaceae*	•Caracterización •Capacidad antioxidante	•Aniones (IC) •FTIR	—
Filum Rhodophyta, clase Florideophyceae					
Mastocarpus stellatus (Stackhouse) Guiry	*Gigartinales*	*Gigartinaceae*	•Caracterización •Capacidad antioxidante •Fraccionamiento	•Aniones (IC) •FTIR •HPSEC	SI
Gigartina pistillata (Gmelin) Stackhouse	*Gigartinales*	*Gigartinaceae*	•Caracterización •Capacidad antioxidante	•Aniones (IC) FTIR	—
Chondracanthus acicularis (Roth) Fredericq	*Gigartinales*	*Gigartinaceae*	•Capacidad antioxidante	•FTIR	—
Nemalion helminthoides (Velley in Withering) Batters	*Nemalionales*	*Nemalionaceae*	•Capacidad antioxidante	•FTIR	—
Osmundea pinnatifida (Hudson) Stackhouse	*Ceramiales*	*Rhodomelaceae*	•Capacidad antioxidante	—	—
Dumontia contorta (Gmelin) Ruprecht	*Cryptonemiales*	*Dumontiaceae*	•Capacidad antioxidante	•FTIR	—

Todas las algas fueron recolectadas de su hábitat natural, excepto *Saccharina latissima*, que es un alga cultivada por la empresa Porto-Muiños en condiciones naturales (cultivo *outdoor*) (Figura 3.2), permitiendo el desarrollo natural del alga en el mar.

Figura 3.2. Parque de cultivo *outdoor* de *Saccharina latissima* en Porto-Muiños

2. Estudios *in vitro*

Los estudios in vitro se llevaron a cabo en dos fases, una primera fase de caracterización de las algas y evaluación nutricional, y una segunda fase para determinar las propiedades biológicas *in vitro* en diferentes extractos de algas.

2.1. Caracterización de las algas

2.1.1. COMPOSICIÓN CENTESIMAL

Cinco de las nueve algas fueron caracterizadas (Tabla 3.1) con los siguientes parámetros de composición: fibra alimentaria (soluble e insoluble), azúcares totales, proteína, polifenoles, cenizas, grasa y humedad.

- *Fibra alimentaria*: Se utilizó el método enzimático-gravimétrico de la AOAC (Prosky et al., 1988), omitiendo el tratamiento con α-amilasa y amiloglucosidasa en las algas pardas, ya que se sabe que prácticamente no contienen almidón.
    - Determinación de ácidos urónicos por el método colorimétrico de Scott (1979) con ácido galacturónico como patrón.

- Determinación de azúcares neutros por cromatografía de gases (GLC) previa hidrólisis ácida y derivatización de las muestras, utilizando el método de Englyst & Cummings (1988).

- *Azúcares totales*: Se utilizó el método de la antrona (Loewus, 1952) con pequeñas modificaciones (Rupérez et al., 2002)
- *Proteína*: Se utilizó el equipo instrumental Leco FP-2000 para determinar el nitrógeno total, y la proteína se calculó como nitrógeno x 6,25.
- *Polifenoles*: Se determinó en extractos acuosos y orgánicos obtenidos de las nueve algas objeto de estudio, mediante el método espectofotométrico de Folin-Coicalteu (Montreau, 1972) usando floroglucinol como solución patrón.
- *Cenizas*: Para obtener cenizas las muestras se incineraron en una mufla a 550 ºC durante 16 h.
- *Grasa*: Se extrajo con éter de petróleo en un equipo Soxtec (Tecator) y su contenido se determinó por gravimetría.
- *Humedad*: El contenido en humedad se determinó secando las muestras a 105 ºC en estufa hasta peso constante.

2.1.2. FRACCIONAMIENTO DE POLISACÁRIDOS DE LA PARED CELULAR

Una vez caracterizadas en cuanto a composición las algas objeto de estudio, se seleccionaron dos algas, una parda y otra roja, para realizar un fraccionamiento secuencial de los polisacáridos de acuerdo con su solubilidad. Se seleccionó el alga parda *Saccharina latissima* por su interés como especie cultivada y el alga roja *Mastocarpus stellatus* por su riqueza en proteína.

El procedimiento de fraccionamiento secuencial se basa en las diferencias en solubilidad de los polisacáridos de algas. Se aplicaron 4 tratamientos de extracción con: (1) agua a 22 ± 1 ºC; (2) agua a 60 ºC; (3) HCl 0,1 N, 16 h.; (4) KOH 2M, 16 h (Figura 3.2), obteniendo un total de cinco fracciones: dos solubles en agua (F1 y F2), una soluble en ácido (F3), una soluble en álcali (F4) y por

último una fracción insoluble (F5) (Figura 3.3). Una descripción más detallada del protocolo del fraccionamiento secuencial se encuentra en la Publicación 5. Los extractos acuosos así obtenidos (F1-F4) se caracterizaron con los siguientes parámetros de composición: Azúcares neutros (por GLC), ácidos urónicos (método de Scott), proteína (métodos Leco y Bradford), aniones (por IC), con los mismos métodos empleados en la composición centesimal de las algas. También se emplearon las técnicas de espectroscopía FTIR para la identificación de los polisacáridos y HPSEC para la distribución de pesos moleculares de los polisacáridos (ver sección 3, *Desarrollo de la metodología*). Los resultados del fraccionamiento en las dos algas, se encuentran en fase de preparación para su publicación. En el capítulo 5 de este tesis sólo se discutirán los resultados más relevantes relacionados con las propiedades biológicas de los polisacáridos.

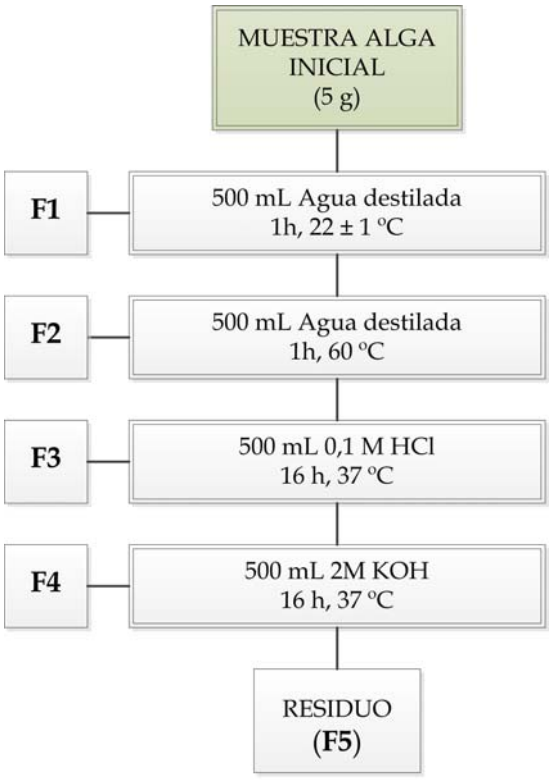


Figura 3.3. Esquema del fraccionamiento secuencial de polisacáridos de algas

2.2. Evaluación de las propiedades biológicas *in vitro*

2.2.1. PROPIEDADES FÍSICO-QUÍMICAS DE LA FIBRA

Se evaluaron tres propiedades físico-químicas de la fibra alimentaria en las algas: Capacidad de hinchamiento, capacidad de retención de agua y capacidad de retención de aceite. Las capacidades de hinchamiento y de retención de agua se determinaron siguiendo el protocolo experimental de un estudio Europeo (Robertson et al., 2000). De manera similar a la capacidad de retención de agua, se evaluó la capacidad de retención de aceite.

2.2.2. CAPACIDAD ANTIOXIDANTE

Se emplearon tres métodos para evaluar la capacidad antioxidante multifuncional en dos tipos de extractos: acuosos y orgánicos-acuosos, en las nueve algas objeto de estudio.

- ***Poder reductor***: por el método de FRAP (*Ferric Reducing Antioxidant Power*). Con este método se determina la capacidad de reducción frente al semisistema Fe(III)/Fe(II). A pH bajo y en presencia de un reductor (antioxidante) el complejo incoloro de tripiridiltriazina (TPTZ) con Fe(III) se reduce a la forma Fe(II), desarrollando un intenso color con una absorción máxima a 595 nm.
- ***Actividad de secuestro de radicales***: por los métodos, ABTS y PCL.

El método ABTS se basa en la decoloración del radical **ABTS$^{\bullet+}$** (2,2'-azinobis-(3-etilbenzotiazolin-6-sulfónico), pre-formado por oxidación de ABTS con persulfato potásico, cuando es reducido por la presencia de antioxidantes. La decoloración es detectada a 658 nm a 30 ºC.

El método de Fotoquimioluminiscencia (PCL), combina la (1) generación fotoquímica de radicales con la (2) detección sensible por quimioluminiscencia. En ambos procesos el luminol juega un papel fundamental. (1) La generación fotoquímica de radicales tiene lugar cuando el fotosintetizador (luminol) es fotoinducido (luz UV-A, 365 nm), mediado por el oxígeno. El luminol oxidado

de esta manera genera radicales superóxido. (2) El luminol también actúa como un detector de radicales de oxígeno, a través de la quimioluminiscencia. En esta reacción intervienen el luminol, los radicales superóxido y algunos intermediarios excitados, emitiendo luminiscencia. Por lo tanto, este método se basa en la inhibición temporal de la quimioluminiscencia que emana de la oxidación del luminol, por la presencia de un antioxidante que tiene lugar en el equipo automático Photochem®.

2.2.3. PROPIEDADES BIOLÓGICAS EN FRACCIONES SOLUBLES DE ALGAS

En las fracciones solubles de algas ricas en polisacáridos (F1-F4, Figura 3.3) se determinó tanto la capacidad antioxidante multifuncional (por los mismos métodos descritos anteriormente), como la actividad anticoagulante.

Actividad anticoagulante: Con este método se determinó el retraso en el tiempo de formación del coágulo de las fracciones solubles del fraccionamiento de polisacáridos utilizando plasma humano control normal (Spinreact, Girona, España) y heparina sódica (Sigma-Aldrich) como control positivo. Para ello se emplearon dos métodos ampliamente utilizados:

- *Tiempo de tromboplastina parcial activado (APTT)* se utiliza para análisis de los defectos en la vía intrínseca de la coagulación. El ensayo evalúa la función de fibrinógeno, la protrombina y la de los factores V, VIII, IX, X, XI y XII. Un defecto en cualquiera de estos factores o la presencia de un anticoagulante como la heparina da lugar a una prolongada APTT.
- *Tiempo de protrombina (PT)* es un ensayo diseñado para detectar defectos en el fibrinógeno, la protrombina, y los factores V, VII, y X, y por lo tanto las medidas de las actividades de la vía extrínseca de la coagulación. Cuando cualquiera de estos factores es deficiente o por la presencia de un anticoagulante entonces el PT se prolonga.

3. Desarrollo de la metodología

Durante el transcurso de esta tesis doctoral, ha sido necesario el desarrollo y puesta a punto de tres métodos que permitieron conocer y caracterizar mejor a las algas en estudio. Los métodos analíticos e instrumentales fueron:

- Determinación de aniones inorgánicos por cromatografía iónica (IC) (**Publicación 2**)

Es un método que de una manera simple y rápida nos permitió analizar simultáneamente los aniones mayoritarios, cloruro y sulfato, así como otros minoritarios (fluoruro, nitrito, bromuro, nitrato y fosfato) en las cenizas obtenidas a partir de las muestras de algas. Este método también se aplicó al análisis de sulfato y otros aniones en extractos acuosos y fracciones solubles de algas.

- Aplicación de la espectroscopia infrarroja (FTIR) para la identificación de los principales polisacáridos presentes en las algas (**Publicación 3**)

La espectroscopía infrarroja con transformada de Fourier es una técnica muy útil, basada en el análisis de bandas de absorción específicas en el rango de frecuencias 4000-650 cm^{-1} (número de onda) del espectro infrarrojo y que nos permitió llegar a la identificación estructural de los principales polisacáridos y a la estimación de los grupos sulfato presentes en las algas estudiadas.

- Determinación de la distribución del peso molecular de los polisacáridos de algas mediante cromatografía líquida de exclusión molecular (HPSEC) (**Publicación 5**)

El desarrollo de este método permitió conocer de una manera relativamente rápida y sencilla la distribución de los pesos moleculares de los polisacáridos de algas estudiadas, utilizando patrones de pullulano y dextrano para la estimación de los pesos moleculares, y patrones comerciales de polisacáridos de algas (alginato sódico, fucoidano, laminarano y carragenanos) para realizar la cuantificación de los principales polisacáridos en nuestras muestras.

4. Estudios *in vivo*

Diseño experimental: Un total de doce ratas hembras Wistar fueron alimentadas bien con una dieta base (grupo control) o una dieta base suplementada con un 10% del alga (*Saccharina latissima* o *Mastocarpus stellatus*) (grupo tratado) durante un periodo de cuatro semanas. Después de lo cual se determinaron en el ciego, el pH y contenido de ácidos grasos de cadena corta (SCFA) y en el suero, los parámetros bioquímicos. El estado antioxidante se determinó tanto en el ciego como en el suero. Asimismo también se llevó a cabo un recuento de bacterias (lácticas, enterobacterias y coliformes) presentes en las heces (Figura 3.4).

Metodología e Instrumentación:

- Determinación de SCFA en contenidos cecales por cromatografía de gases (GLC). Se utiliza una mezcla de patrones en ácido fórmico 12%: ácido acético, propiónico, butírico, iso-butírico, valérico, iso-valérico y metil-valérico, siendo este último el patrón interno. Las muestras cecales se preparan de igual forma en fórmico 12%.

Figura 3.4. Esquema del modelo animal en ratas Wistar empleado con las algas *Saccharina* y *Mastocarpus*

- Determinación pH en los contenidos cecales: pHmetro *Crison micro pH2001* equipado con un microelectrodo.
- Recuento de bacterias: Las heces se recogen en condiciones asépticas sobre tubos de vidrio con agua de peptona, se homogenizan y se realizan diluciones seriadas. A continuación se realiza la siembra en diferentes medios selectivos en función del tipo de bacteria:

Coliformes totales y E. coli β-glucuronidasa positivas: medio chromID Coli estéril (COLI ID-F, Biomérieux). Incubación en estufa a 37 ºC durante 24 h en placas Petri de forma invertida. Al ser cromogénico el medio permite diferenciar las colonias de *E. coli* β-glucuronidasa positivas (color rosa a violeta) de otros coliformes (color azul/azul-gris). El número de coliformes totales corresponde a la suma de las colonias rosa a violeta y las colonias azul a azul/gris (Figura 3.5-A).

Enterobacterias: medio VRBG estéril (Agar Rojo Bilis Violeta Glucosa, Cultimed). Incubación en estufa 30 ºC, 72 h. Recuento de unidades formadoras de colonias (UFC) características de color rosa o rojo con o sin halo de precipitación (Figura 3.5-B)

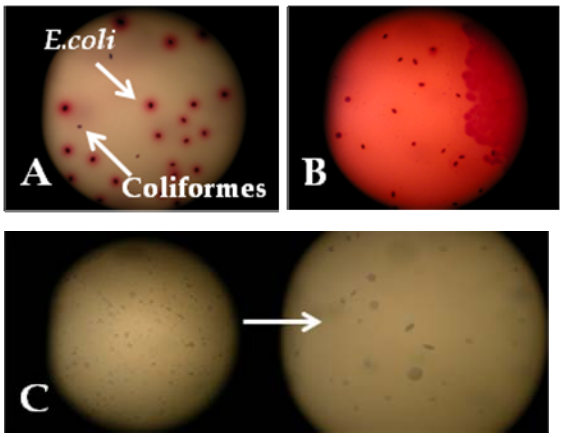


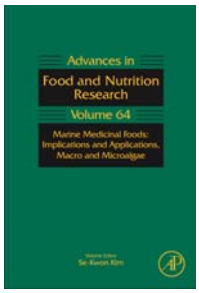
Figura 3.5. Medios selectivos utilizados para el recuento de bacterias. (A), Coliformes y *E. coli β-glucoronidasa-positivos*; (B), Enterobacterias; (C), Bacterias lácticas

Bacterias lácticas: medio MRS agar estéril (De Man Rogosa & Sharpe, 1960; Merck). Incubación en estufa a 30 ºC durante 72 h de forma invertida en placas Petri. Recuento de UFC (Figura 3.5-C).

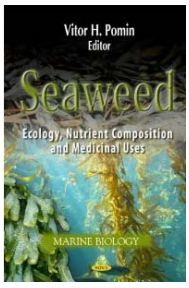
- Parámetros bioquímicos en el suero: Proteína total, albúmina y glucosa se midieron en el suero de las ratas usando un Autoanalizador (RA-500, Bayer, España). Además se utilizaron kits comerciales (Spinreact, Girona, España) para la determinación de la concentración de ácido úrico, triglicéridos, colesterol total (TC), LDL- y HDL-colesterol con un equipo espectrofotométrico Beckman de acuerdo con las indicaciones del fabricante (Spinreact). También se determinó el índice aterogénico (AI) usando la fórmula: AI = TC/HDL-colesterol.
- Estado antioxidante por el método FRAP en suero y ciego
- Capacidad anticoagulante en plasma

Las siete publicaciones incluidas en esta tesis doctoral contienen los resultados de los estudios *in vitro* (**Publicaciones 1** y **4**) e *in vivo* (**Publicaciones 6** y **7**) y del desarrollo de la metodología (**Publicaciones 2, 3** y **5**). Las publicaciones derivadas del fraccionamiento secuencial de la pared celular de las algas se encuentran en fase de preparación. En la Tabla 3.2 se muestra un resumen gráfico de las publicaciones.

Asimismo, como consecuencia de la actividad investigadora durante el periodo de realización de esta tesis doctoral, se publicaron dos capítulos de libro (por invitación del editor) sobre trabajos recopilatorios o bibliográficos de algas:



Jiménez-Escrig, A., Gómez-Ordóñez, E. & Rupérez P. (2011) *Seaweed as a Source of Novel Nutraceuticals: Sulfated Polysaccharides and Peptides*. In: Book Series: "Advances in Food and Nutrition Research" Volume 64, Chapter 26, Marine Medicinal Foods, Kim S.K. (ed.), Elsevier Inc., ISBN: 978-0-12-387669-0



Rupérez P., Gómez-Ordóñez E. & Jiménez-Escrig A. (2011) *Nutritional quality and biological properties of edible seaweeds*. In: "Seaweed: Ecology, Nutrient Composition and Medicinal Uses" Nova Science Publishers, Inc. ISBN: 978-1-61470-878-0

Tabla 3.2. Resumen gráfico de las publicaciones incluidas en esta tesis doctoral

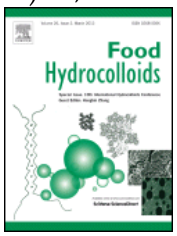
	Publicación 1	**Publicación 2**	**Publicación 3**	**Publicación 4**
Título	*Dietary fibre and physicochemical properties of several edible seaweeds from the northwestern Spanish coast*	*A simple ion chromatography method for inorganic anion analysis in edible seaweeds*	*FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds*	*Brown and red seaweeds as potential sources of antioxidant nutraceuticals*
Revista	Food Res Int (2010) 43, 2289-2294 	Talanta (2010) 82, 1313-1317 	Food Hydrocolloids (2011) 25, 1514-1520 	J Appl Phycol (2011) DOI 10.1007/s10811-011-9742-8 
Tipo de Estudio	Estudio *in vitro*. Caracterización y propiedades físico-químicas de la fibra	Metodología. Determinación aniones por CI	Metodología. Técnica de espectroscopia IR para la identificación de polisacáridos algas	Estudio *in vitro*. Capacidad antioxidante
Conclusión Principal	Las algas estudiadas constituyen una buena fuente de fibra alimentaria, minerales y proteína para el consumo humano	El cloruro y el sulfato son los principales aniones de las algas. Se validan los parámetros de linealidad, sensibilidad, precisión y exactitud del método	El principal polisacárido identificado por FTIR de las algas pardas es el alginato, mientras que en las rojas es el carragenano	La capacidad antioxidante está correlacionada con los polifenoles y los polisacáridos sulfatados en las algas pardas y con los polisacáridos sulfatados en las algas rojas

Tabla 3.2. Resumen gráfico de las publicaciones incluidas en esta tesis doctoral (continuación)

	Publicación 5	**Publicación 6**	**Publicación 7**
Título	*Molecular weight distribution of polysaccharides from edible seaweeds by high-performance size-exclusion chromatography (HPSEC)*	*Antioxidant and prebiotic effects of dietary fiber co-travellers from Sugar Kombu in healthy Wistar rats*	*Effect of the red seaweed Mastocarpus stellatus intake on lipid metabolism and antioxidant status in healthy Wistar rats*
Revista	Talanta (*In press*, 2012) DOI:10.1016/j.talanta.2012.01.067	J Agr Food Chem (*Enviado*, 2012)	Food Chem (*Enviado*, 2012)
Tipo de Estudio	Metodología. Determinación del peso molecular en fracciones de polisacáridos de algas	Estudio *in vivo*. Experimento animal con una dieta suplementada con el alga parda *Saccharina latissima*	Estudio *in vivo*. Experimento animal con una dieta suplementada con el alga roja *Mastocarpus stellatus*
Conclusión Principal	Las fracciones de polisacáridos de las algas estudiadas muestran gran heterogeneidad de pesos moleculares. Se establecen los parámetros de validación del método	La ingesta del alga parda *Saccharina* produce una mejora del estado antioxidante y un efecto prebiótico en el ciego de ratas sanas	La ingesta del alga roja *Mastocarpus* produce una mejora del perfil lipídico en el suero y estado antioxidante en el ciego de ratas sanas

CAPÍTULO 4

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Dietary fibre and physicochemical properties of several edible seaweeds from the northwestern Spanish coast

Eva Gómez-Ordóñez, Antonio Jiménez-Escrig, Pilar Rupérez *

Metabolism and Nutrition Department, Instituto del Frío (IF-ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, E28040-Madrid, Spain

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ABSTRACT

Proximate composition (moisture, ash, protein and oil content), total dietary fibre content and physicochemical properties of three brown and two red edible Spanish seaweeds, namely: *Himanthalia elongata* (sea spaghetti), *Bifurcaria bifurcata*, *Laminaria saccharina* (sweet kombu), *Mastocarpus stellatus* and *Gigartina pistillata* were studied. Ashes (24.9–36.4%) were high in all samples. Protein content ranged from 10.9 to 25.7%, being much higher for *Laminaria* (25.7%) followed by the red seaweeds (15.5–21.3%). Minor components were lipids (0.3–0.9%) in all samples except for *Bifurcaria* (5.6%). Total dietary fibre content ranged from 29.3 to 37.4% of which 39.1–74.7% was soluble. For brown algae, the soluble fibre contained uronic acids from alginates and neutral sugars from sulphated fucoidan and laminarin. For red seaweeds, the main neutral sugars corresponded to sulphated galactans (carrageenan or agar). Insoluble fibres (7.4–22.7%) were essentially made of cellulose with an important contribution of Klason lignin especially in brown seaweeds (9.5–10.8%). Regarding the main physicochemical properties, swelling and water retention capacity were high in all samples, while oil retention was low, related to the hydrophilic nature of fibre polysaccharides. In conclusion, these seaweeds can be estimated as a good source of food fibre, protein and minerals for human consumption.

1. Introduction

While marine algae have traditionally formed part of the Oriental diet, especially in Japan, China and Korea (Nisizawa, Noda, Kikuchi & Watanabe, 1987; Murata, & Nakazoe, 2001; FAO, 2002); their major use in Western countries has traditionally concentrated on the extraction of compounds used by pharmaceutical, cosmetics, and food industries as source of phycocolloids, thickening and gelling agents (production of agar, alginate, carrageenan, etc) (Mabeau & Fleurence, 1993; Jiménez-Escrig & Goñi, 1999; Jiménez-Escrig & Sánchez-Muniz, 2000). However, in recent decades there has been an increase in direct consumption of marine algae as food in Western countries and most recently as components of functional foods (Shahidi, 2009) because of their especial nutritional properties. Although varying from one type to another, edible marine algae, sometimes referred to as seaweeds, are known for their richness in polysaccharides, proteins (Fleurence, 1999), minerals and vitamins (Mabeau & Fleurence, 1993; Rupérez & Saura-Calixto, 2001; Rupérez, Ahrazem, & Leal, 2002) and their low lipid content (1–3% algal dry weight) (Jiménez-Escrig, & Goñi, 1999; Dawczynski, Schubert, & Jahreis, 2007) with high concentrations of certain long-chain polyunsaturated fatty acids (Bocanegra, Bastida, Benedí, Ródenas, & Sánchez-Muniz, 2009).

The consumption of seaweeds is subject to specific regulation (Mabeau & Fleurence, 1993) and the details of their chemical composition and variations among species are required to obtain authorisation for its use in human nutrition. France has been the first European country to establish a specific regulation concerning the use of seaweeds for human consumption (Mabeau & Fleurence, 1993). Currently, in Spain seaweeds are considered as novel foods and for the purposes of controlling the maximum limit of contaminants they have been included in the canned vegetables group (RD, 2420/78); but there are no specific regulations for seaweed and derived products and their consumption is limited.

Seaweeds contain large amounts of polysaccharides, most of which are not digested by humans, whose gastrointestinal tract does not produce the required degradation enzymes; therefore, they can be regarded as dietary fibres. From a nutritional point of view, seaweeds are interesting because of their high content in dietary fibre (33–75%) (Jiménez-Escrig & Goñi, 1999; Jiménez-Escrig & Sánchez-Muniz, 2000), particularly rich in the soluble fractions (50–85% of total dietary fibre content); which in red seaweeds (Rhodophyta) are mostly composed of sulfated galactans, such as agar and carrageenans. In brown seaweeds (Phaeophyta), soluble dietary fibre polysaccharides

Abbreviations: EDTA, ethylene-diamine-tetra-acetic acid; SDF, soluble dietary fibre; IDF, insoluble dietary fibre; TDF, total dietary fibre; NS, neutral sugars; UA, uronic acids; KL, Klason lignin; GLC, gas–liquid chromatography; FID, flame ionization detector; SC, swelling capacity; WRC, water retention capacity; ORC, oil retention capacity; ANOVA, analysis of variance.

* Corresponding author. Tel.: +34 915445607; fax: +34 915493627.
E-mail address: pruperez@if.csic.es (P. Rupérez).

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are alginates, fucans, and laminarans; the insoluble fibres are essentially made of cellulose (Jiménez-Escrig & Sánchez-Muniz, 2000). This dietary fibre content can be calculated as non-starch polysaccharides plus lignin (Rupérez & Saura-Calixto, 2001) according to the AOAC dietary fibre method (Prosky, Asp, Schweizer, DeVries, & Furda, 1988).

Dietary fibre from different algal sources is known for the capacity to lower serum cholesterol levels (Jiménez-Escrig & Sánchez-Muniz, 2000; Ginzberg, Cohen, Sod-Moriah, Shany, Rosenshtrauch, & Arad, 2000) and the potential to be used as natural antioxidants by the food industry (Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001; Rupérez, Ahrazem, & Leal, 2002). These properties of seaweed polysaccharides seem to be due to their ability to disperse in water, retain cholesterol and related physiologically active compounds and inhibit lipid absorption in the gastrointestinal track, and also due the antioxidant bioactivity of sulphated polysaccharides and polyphenols (Rupérez et al., 2002; Díaz-Rubio, Pérez-Jiménez, & Saura-Calixto, 2009).

These properties confer on the seaweed the potential to be used in food technology for the acquisition of low-calorie food (Kadam & Prabhasankar, in press; Murata & Nakazoe, 2001; Sakata, 1995) and might be important in body weight control, as well as in prevention of gastrointestinal (Lahaye & Kaeffer, 1997) and cardio-vascular (Bocanegra et al., 2009) diseases.

Several edible Spanish seaweeds which are commonly used in the oriental diet are known world-wide by their Japanese names as Kombu (*Laminaria* spp.), Wakame (*Undaria pinnatifida*) and Nori (*Porphyra* spp.). They are a good source of food ingredients and new dietary fibre-rich products (Rupérez & Saura-Calixto, 2001). Indeed, many other brown and red types of seaweed are present in the Spanish coasts and the details of their chemical composition and physicochemical properties are required in order to fulfill the increasing demand for Spanish seaweed products. The aim of the present study was to evaluate the nutritional composition, namely dietary fibre, of several brown and red Spanish seaweeds, which are not so commonly consumed, and their physicochemical properties (swelling, water and oil retention capacity) that are mainly responsible for their physiological effects.

2. Materials and methods

2.1. Raw material

Brown seaweeds (Phaeophyta) *Himanthalia elongata* (L.) S.F. Gray (sea spaghetti; Fucales, Himanthaliaceae), *Bifurcaria bifurcata* R. Ross (Fucales, Cystoseiraceae) and *Laminaria saccharina* (L.) J.V. Lamouroux (sweet Kombu; Laminariales, Laminariaceae); and red seaweeds (Rhodophyta) *Mastocarpus stellatus* (Stackhouse) Guiry (Gigartinales, Gigartinaceae) and *Gigartina pistillata* (S.G. Gmelin) Stackhouse (Gigartinales, Gigartinaceae) were obtained from a local supplier (Porto-Muiños, Cambre, Coruña, Spain). All the seaweeds were collected during spring–summer 2008 and 2009, except for *L. saccharina* which was cultured under sea natural conditions. In the supplier industry, seaweeds were cleaned from epyphytes and sand, washed with tap running water, air-dried at 50 °C and milled to less than 1.0 mm particle size. The milled seaweed samples were stored in sealed plastic bags at 2 °C several months until analysis. Residual moisture content was determined by drying at 105 °C in an oven to constant weight.

2.2. Chemical analysis

2.2.1. Protein, oil and ashes

Protein content was determined with a Leco FP-2000 Instrument. In brief, powdered samples were weighed (50–100 mg) into ceramic boats and loaded into the FP-2000, where they were combusted in the pure oxygen environment of the furnace. After passing through a thermoelectric cooler to drop out water, an aliquot from the combustion gasses was taken. Gasses were scrubbed, and all nitrogen-containing materials were reduced to N_2, and detected by a thermal-conductivity cell. An air blank was carried out and the instrument calibrated with EDTA. Protein was calculated as nitrogen × 6.25.

Oil was extracted from 1 g (dry weight) seaweed sample with 50 mL petroleum ether in a Soxtec system (Tecator). For ashes, seaweed samples were incinerated in a furnace at 550 °C for 16 h and weighed.

2.2.2. Dietary fibre

Soluble and insoluble dietary fibre fractions were determined according to the AOAC enzymatic-gravimetric method (Prosky et al., 1988), omitting α-amylase and amyloglucosidase treatment for brown seaweeds, because they contain negligible amounts of starch (Goñi, Valdivieso, & Gudiel-Urbano, 2002). After the enzymatic treatment the samples were dialysed against water for 48 h. Soluble dietary fibre (SDF) dialysates were then hydrolysed with 1 M sulphuric acid (100 °C, 1.5 h). In order to quantify a precipitate which appeared after the hydrolysis, the hydrolysates were centrifuged and the insoluble residue obtained was washed with distilled water to almost neutral pH and then dried at 60 °C in an oven to constant weight.

Insoluble dietary fibre (IDF) residues obtained after enzymatic treatment and centrifugation were hydrolysed with 12 M sulphuric acid (30 °C, 1 h) and 1 M sulphuric acid (100 °C, 1.5 h). The residual material was dried (105 °C, overnight) and quantified as Klason lignin (KL).

Neutral sugars (NS) from the hydrolysates of fibre fractions (SDF and IDF) were identified and quantified by gas–liquid chromatography (GLC) as alditol acetates with inositol as internal standard. A Shimadzu gas chromatograph model GC-14A equipped with a flame ionization detector (FID), an automatic injector (AOC-14) and a Hewlett-Packard HP-Chem Station with an HP-Deskjet 600 printer were used. The column was a Supelco SP-2330 capillary fused silica, 30 m × 0.32 mm i.d., 0.2 μm film thickness. The oven, injector and detector temperatures were: 240 °C (isothermal), 270 °C, and 270 °C, respectively. The split ratio was 1:10 and the carrier gas (nitrogen) head column pressure was 0.75 kg/cm^2.

Uronic acids (UA) in the hydrolysates were quantified colorimetrically by the Scott method, with galacturonic acid as standard and 3,5-dimethylphenol as the reagent (Scott, 1979), and corrected for incomplete recovery of uronic acids (26.9 g/100 g) from alginate hydrolysis (Rupérez et al., 2002).

Soluble dietary fibre was calculated as NS, plus UA, plus the residue (resistant to the hydrolysis), and insoluble dietary fibre was calculated as NS, plus UA, plus KL.

2.3. Physicochemical properties

Swelling and water retention capacity in seaweed samples were assessed following the experimental protocol used in a European collaborative study (Robertson, De Monredon, Dysseler, Guillon, Amadò & Thibault, 2000). In addition oil retention capacity was measured.

2.3.1. Swelling capacity (SC)

The dry powdered sample (500 mg) was weighed in a 10 mL measuring cylinder (0.1 mL graduations) and 10 mL distilled water, containing 0.02% sodium azide as bacteriostatic was added. Then, it was stirred gently to eliminate trapped air bubbles and left on a level surface at room temperature overnight (18 h) to allow sample to settle. The volume (mL) occupied by the sample was measured and SC was expressed as mL/g of dry sample.

2.3.2. Water retention capacity (WRC)

Thirty millilitres of distilled water, containing 0.02% sodium azide was added to 500 mg of dry powdered samples in a 50 mL centrifuge tube. The sample was stirred and left at room temperature for 18 h.

After centrifugation at 3,000×*g* for 20 min, the supernatant was discarded, the residue was weighed and WRC calculated as g water/g of dry sample.

2.3.3. Oil retention capacity (ORC)

The same protocol as above was followed, but using commercial virgin olive oil (0.4°) instead of water. ORC was expressed as g olive oil retained/g of dry sample.

2.4. Statistical analysis

All determinations were performed at least in triplicate, the data were expressed as means ± standard deviations, and reported on a dry matter basis.

One-way analysis of variance (ANOVA) was carried out to assess for any significant differences between the means. Differences between means at the 5% ($P<0.05$) level were considered significant.

3. Results and discussion

3.1. Proximate composition (moisture, ashes, protein and oil content)

Moisture contents were very similar in all samples (see Table 1) with the lowest value for *Laminaria* (6.64% dry weight) and the highest for *Gigartina* (9.86% dry weight).

3.1.1. Ash

Ashes contents were high and ranged from 24.99% (*Mastocarpus*) to 36.41% (*Himanthalia*) (Table 1). Similar values have been reported by other authors for red and brown seaweeds (Mabeau & Fleurence, 1993; Rupérez & Saura-Calixto, 2001; Cofrades, López-López, Solas, Bravo, & Jiménez-Colmenero, 2008; Sánchez-Machado, López-Cervantes, López-Hernández, & Paseiro-Losada, 2004). This high ash content is a general feature of seaweeds, and these values are generally much higher than those of terrestrial vegetables other than spinach (Rupérez, 2002). Besides, they vary between species, between geographical locations and between seasons (Ito & Hori, 1989; Kaehler, & Kennish, 1996). Inorganic anion profile of these five seaweeds has been determined by ion chromatography and reported recently by our group (Gómez-Ordóñez, Alonso & Rupérez, 2010). Brown seaweeds are characterized by higher chloride content up to 33.7–36.9% ash dry weight, while red seaweeds are characterized by higher sulphate content (45–57% ash dry weight). Besides chloride and sulphate, small amounts of fluoride, nitrate and phosphate and trace amounts of nitrite and bromide are found in all the seaweeds (Gómez-Ordóñez et al., 2010). Related to their mineral content, these seaweeds my serve as food supplement to help meet the recommended daily intakes of some minerals and trace elements, as well as in other seaweeds studied previously (Rupérez, 2002).

3.1.2. Protein

For brown seaweeds, the contents ranged from 10.9 to 25.7% (Table 1), while being much higher for *Laminaria* (25.7%) followed by the red ones (15.5–21.3%), within the values reported for other authors (Fleurence, 1999; Sánchez-Machado, Lopez-Cervantes et al., 2004). Sánchez-Machado, López-Cervantes, et al. (2004) have reported lower protein content (5.46 % dry weight) in *H. elongata* dried seaweeds, and also lower values than their respective canned seaweeds (10.95 % dry weight), than those reported here. According to Fleurence, most brown seaweeds industrially exploited (*Laminaria digitata*, *Ascophyllum nodosum*, *Fucus vesiculosus* and *H. elongata*) have a protein content lower than 15%, (dry weight), except for *U. pinnatifida* (Wakame) and in the present study *L. saccharina*. The favourable conditions of the cultured *L. saccharina* could be responsible for this higher protein content; red seaweeds such as *Porphyra* spp. (Nori) are relatively high in proteins (Rupérez & Saura-Calixto, 2001; Sánchez-Machado, López-Cervantes, et al., 2004). These levels may vary according to the species, geographic area, season or environmental conditions (Ito & Hori, 1989). Indeed, protein content seems to be subject to large variations during the year, with the maximum concentration during winter and the beginning of spring, and the minimum concentration during summer and early autumn period (Galland-Irmouli et al., 1999; Denis et al., 2010). Nevertheless, seaweeds, especially the red seaweeds appear to be an interesting potential source of food proteins.

3.1.3. Oil

Lipid content was very low in all seaweed samples (0.3–0.9%), except for *Bifurcaria* (5.6%, Table 1), but this fell within the ranges reported previously (Jiménez-Escrig & Goñi, 1999; Rupérez & Saura-Calixto, 2001). Studies from lipid extracts of *B. bifurcata* have shown that this species contains a rich array of acyclic diterpens (geranylgeraniol and derived diterpenes) (Valls, Piovetti, Banaigs, Archavlis, & Pellegrini, 1995; Culioli, Di Guardia, Valls, & Piovetti, 2000) and their content can vary between 4.5 and 6.6 mg/g algal dry weight from different coastal locations (Ireland, Spain, and France) (Daoudi et al., 2001). Also, high levels of fucosterol have been reported in *Bifurcaria* and in some brown seaweed (Sánchez-Machado, López-Hernández, Paseiro-Losada, & López-Cervantes, 2004).

3.2. Dietary fibre

Soluble, insoluble and total dietary fibre contents of brown and red seaweeds are shown in Table 2. The total dietary fibre content (TDF, 29.3 to 37.4% dry weight) was slightly lower than previously reported (33–50% algal dry weight; Rupérez & Saura-Calixto, 2001). Besides higher TDF values (50.3% dry weight) have been reported for *H. elongata* (Cofrades et al., 2008) than those reported here, yet these values were still higher than levels reported in most higher plants and terrestrial foodstuffs (Mabeau & Fleurence, 1993; MacArtain, Gill, Brooks, Campbell, & Rowland, 2007).

Also, seaweeds showed higher levels of SDF than IDF as is already known (Jiménez-Escrig & Sánchez-Muniz, 2000; Rupérez & Saura-Calixto, 2001), the opposite of which usually happens in land plants (Anderson & Bridges, 1988). The ratio of SDF to TDF ranged from 39.1 to 74.7%, being higher for red seaweeds, due to their higher SDF content. Slightly lower values of this ratio (43.1–64.9 %) were reported previously for red algae *Chondrus crispus*, and Nori (Rupérez & Saura-Calixto, 2001). Besides, there were statistical

Table 1
Moisture, ashes, protein and oil content (% dry weight) in edible Spanish seaweeds.

Seaweed	Moisture	Ashes	Protein	Oil
Himanthalia	9.08 ± 0.02^{ac}	36.41 ± 0.15^{a}	14.08 ± 0.21^{a}	0.94 ± 0.07^{a}
Bifurcaria	8.73 ± 0.04^{a}	34.31 ± 0.21^{b}	10.92 ± 0.10^{b}	5.67 ± 0.32^{b}
Laminaria	6.64 ± 0.06^{b}	34.78 ± 0.08^{c}	25.70 ± 0.11^{c}	0.79 ± 0.07^{a}
Mastocarpus	8.86 ± 0.04^{a}	24.99 ± 0.12^{d}	21.30 ± 0.18^{d}	0.39 ± 0.02^{c}
Gigartina	9.86 ± 0.07^{c}	34.56 ± 0.47^{bc}	15.59 ± 0.28^{e}	0.57 ± 0.06^{a}

Data are mean values of triplicate determinations ± standard deviation.
Means with different letters in each column differ significantly ($P<0.05$).

Table 2
Soluble, insoluble and total dietary fibre (% dry weight) in edible Spanish seaweeds.

Seaweed	SDF	IDF	TDF
Himanthalia	23.63 ± 0.48^{a}	13.51 ± 0.45^{a}	37.14 ± 0.86^{a}
Bifurcaria	14.64 ± 0.68^{b}	22.79 ± 0.97^{b}	37.42 ± 0.78^{a}
Laminaria	17.12 ± 0.84^{c}	13.11 ± 0.56^{a}	30.23 ± 0.85^{b}
Mastocarpus	22.85 ± 0.19^{a}	8.85 ± 0.67^{c}	31.70 ± 0.23^{c}
Gigartina	21.90 ± 0.22^{a}	7.41 ± 0.12^{c}	29.31 ± 0.34^{b}

Data are mean values of triplicate determinations ± standard deviation.
Means with different letters in each column differ significantly ($P<0.05$).
SDF = soluble dietary fibre; IDF = insoluble dietary fibre; TDF = total dietary fibre.

Table 3
Soluble and insoluble dietary fibre fractions (% dry weight) in edible Spanish seaweeds.

Seaweed	SDF			IDF		
	NS	UA	Residue	NS	UA	KL
Himanthalia	5.14 ± 0.75^{a}	12.65 ± 0.95^{a}	5.84 ± 0.22^{a}	3.31 ± 0.64^{a}	0.71 ± 0.03^{a}	9.54 ± 0.40^{a}
Bifurcaria	2.35 ± 0.25^{b}	7.67 ± 0.69^{b}	4.62 ± 0.45^{b}	7.04 ± 0.27^{b}	4.43 ± 0.87^{b}	10.83 ± 0.84^{b}
Laminaria	2.15 ± 0.15^{b}	9.64 ± 0.74^{c}	4.91 ± 0.32^{ab}	5.89 ± 0.75^{bc}	2.14 ± 0.33^{c}	3.89 ± 0.12^{c}
Mastocarpus	19.00 ± 0.35^{c}	2.35 ± 0.28^{d}	0.09 ± 0.03^{c}	5.18 ± 0.24^{ac}	0.62 ± 0.24^{a}	3.45 ± 0.90^{c}
Gigartina	19.48 ± 0.34^{c}	1.27 ± 0.25^{d}	0.25 ± 0.12^{c}	4.73 ± 0.29^{a}	0.21 ± 0.09^{a}	3.24 ± 1.13^{c}

Data are mean values of triplicate determinations ± standard deviation.
Means with different letters in each column differ significantly ($P<0.05$).
SDF = soluble dietary fibre; IDF = insoluble dietary fibre; NS = neutral sugars; UA = uronic acids; KL = Klason lignin.

significant differences in SDF values between brown and red seaweeds, except for *Himanthalia* with the highest value (23.63%, Table 2) followed by the red ones (21.9–23.1%). This difference in the amount of SDF is related to differences in polysaccharide solubility and composition between brown and red seaweeds. In this way, sulphated polysaccharides in red seaweeds, with a higher proportion in the soluble fraction of dietary fibre, turn up to be more soluble than the polysaccharides of brown algae due to their ability to form viscous gels in the intestinal track (Jiménez-Escrig & Sánchez-Muniz, 2000). For brown seaweeds, the AOAC method for dietary fibre analysis presented some difficulties and after dialysis of the soluble fibre two fractions could be distinguished: a soluble fraction and a sulphuric acid-insoluble residue, this sulphuric acid-insoluble residue ranged 24–31% of the total soluble fibre in the different seaweed tested (Table 3). The higher fraction corresponded to uronic acids (7.6–12.6% dry weight) that came from alginates and the acid-insoluble residue was probably composed of alginic acid. In comparison, only 1.2–2.3% of uronic acids were quantified in the soluble fibre of red seaweeds (Table 3).

Also, soluble fibre in brown seaweeds is composed of neutral glucans, like laminarin and sulphated fucoidans (Lahaye & Kaeffer, 1997). Neutral sugar contents determined by GLC are shown in Table 4. The main sugars detected in brown seaweed were fucose, glucose and galactose, respectively, which possibly corresponded to sulphated fucoidan and laminarin. For red seaweeds the main neutral sugar identified by GLC in SDF was galactose in both algae (Table 4) due to sulphated galactans like agar and carrageenans, as described in this type of algae (Jiménez-Escrig & Goñi, 1999). These values in neutral sugars of SDF were much higher in red seaweeds (19–19.4%; Table 3) than in brown seaweeds (2.15–5.14%; Table 3), and corresponded with values reported previously (Rupérez & Saura-Calixto, 2001).

Insoluble fibre (7.4–22.7% dry weight; Table 2) was composed of neutral sugars (3.3–7.04%), residual uronic acids (0.21–4.43%) from alginates in brown seaweeds and varying amounts of Klason lignin (Tables 2 and 3). The main neutral sugars determined by GLC were glucose and fucose for brown seaweeds and galactose and glucose for the red ones (Table 5). Insoluble fibres are essentially made of cellulose, plus residual polysaccharides. KL was high, especially in *Bifurcaria* (10.83%) and *Himanthalia* (9.54%), most probably due to protein and other compounds resistant to protease treatment. There were statistically significant differences between IDF in brown and red seaweeds, with the highest value for *Bifurcaria* (22.79%; Table 2) and the lowest values for the red ones (7.41 and 8.85%). Various amounts of polyphenols (Jiménez-Escrig et al., 2001) and other compounds usually are associated to Klason lignin (Pinelo, Arnous, & Meyer, 2006) in brown algae that might be responsible for their higher IDF content. Thus, the highest value for IDF of *B. bifurcata* (IDF = 22.7%, Table 2) may be related to the higher KL content (KL = 10.8%, Table 3).

3.3. Physicochemical properties

The physiological effects of dietary fibre are related to their physicochemical properties. The water associated with fibre is an important consideration when studying the effects of fibre in the diet. Such water will influence the metabolic activity of fibre along the gut. The swelling, water and oil retention capacity of the brown and red seaweed samples are shown in Table 6. SC and WRC of the seaweed samples ranged from 7.2 to 11.4 mL/g dry weight and 4.9 to 10.2 g/g dry weight, respectively, with no statistically significant differences between the two groups (brown and red seaweed). Both *Bifurcaria* (brown alga) and *Mastocarpus* (red alga) with the higher IDF values in each group (Table 2), also showed the lowest (ANOVA one-way, $P<0.05$) SC and WRC values, indicating the possible relationship between insoluble fibre and water retention capacity. The SC and WRC of the seaweed samples were not only similar to those of *Fucus*, Kombu, Wakame, *Chondrus* and Nori (5.7–10.5 mL/g and 5.1–10.9 g/g dry weight, respectively) reported previously (Rupérez & Saura-Calixto, 2001), but also comparable to those of some soybean by-products (dietary fibre concentrates) as okara (10.54 mL/g and 8.87 g/g dry weight, respectively) also with an important contribution in

Table 4
Neutral sugars determined by GLC (% dry weight) in soluble dietary fibre from edible Spanish seaweeds.

Neutral sugars	Brown seaweeds			Red seaweeds	
	Himanthalia	*Bifurcaria*	*Laminaria*	*Mastocarpus*	*Gigartina*
Rhamnose	0	0	0.05 ± 0.01	0	0
Fucose	2.37 ± 0.45^{a}	1.16 ± 0.29^{b}	1.18 ± 0.07^{b}	0.06 ± 0.01^{c}	0.11 ± 0.02^{c}
Arabinose	0	0	0.06 ± 0.01	0	0
3,6-Anhydro-Gal*	0	0	0	0.05 ± 0.02	0
Xylose	0.17 ± 0.04^{ac}	0.14 ± 0.10^{ab}	0.10 ± 0.02^{b}	0.43 ± 0.06^{d}	0.19 ± 0.02^{c}
Mannose	0.21 ± 0.04^{a}	0.25 ± 0.09^{a}	0.22 ± 0.05^{a}	0.82 ± 0.02^{b}	0.68 ± 0.15^{c}
6-*O*-Me-Gal*	0	0	0	0.26 ± 0.03	0
Galactose	0.56 ± 0.08^{a}	0.50 ± 0.10^{a}	0.49 ± 0.03^{a}	16.79 ± 2.23^{b}	17.75 ± 1.53^{c}
Glucose	1.84 ± 0.23^{a}	0.30 ± 0.07^{b}	0.08 ± 0.01^{c}	0.58 ± 0.07^{d}	0.75 ± 0.04^{e}
Total sugar	5.14 ± 0.75^{a}	2.35 ± 0.25^{b}	2.15 ± 0.15^{c}	19.00 ± 0.35^{d}	19.48 ± 0.34^{d}

Data are mean values of triplicate determinations ± standard deviation.
Means with different letters in each row differ significantly ($P<0.05$).
* Tentatively identified as 3,6-anhydrogalactose and 6-*O*-methylgalactose by their retention times.

Table 5
Neutral sugars determined by GLC (% dry weight) in insoluble dietary fibre from edible Spanish seaweeds.

Neutral sugars	Brown seaweeds			Red seaweeds	
	Himanthalia	*Bifurcaria*	*Laminaria*	*Mastocarpus*	*Gigartina*
Rhamnose	0	0	0.02 ± 0.01	0	0
Fucose	0.29 ± 0.08^{ab}	3.53 ± 0.69^{c}	0.37 ± 0.05^{a}	0	0.03 ± 0.01^{b}
Arabinose	0	0	0	0	0
Xylose	0.05 ± 0.03^{a}	0.12 ± 0.02^{b}	0.07 ± 0.01^{c}	0.21 ± 0.03^{d}	0.04 ± 0.01^{a}
Mannose	0.14 ± 0.06^{ab}	0.18 ± 0.04^{a}	0.07 ± 0.01^{c}	0.08 ± 0.01^{bc}	0.58 ± 0.17^{d}
6-*O*-Me-Gal*	0	0	0	0.09 ± 0.02	0
Galactose	0.12 ± 0.06^{a}	0.26 ± 0.07^{a}	0.13 ± 0.02^{a}	3.72 ± 0.89^{b}	3.46 ± 1.09^{b}
Glucose	2.70 ± 0.47^{a}	3.11 ± 0.53^{b}	6.19 ± 0.51^{c}	1.08 ± 0.25^{d}	0.59 ± 0.12^{e}
Total sugar	3.31 ± 0.64^{a}	7.04 ± 0.27^{b}	6.83 ± 0.09^{b}	5.18 ± 0.24^{c}	4.73 ± 0.29^{c}

Data are mean values of triplicate determinations ± standard deviation.
Means with different letters in each row differ significantly ($P<0.05$).
* Tentatively identified as 6-*O*-methylgalactose by its retention time.

total indigestible fraction (41.6% dry weight, Espinosa-Martos & Rupérez, 2009). Moreover, SC was comparable to other vegetable food samples such as pea hulls, apple pulp or citrus pulp (10 mL/g dry weight), and higher than those for pea (5.26 mL/g) and chickpea (4.28 mL/g) ((Tosh & Yada, 2010)). Reported WRC values of the IDF of chickpeas, peas and lentils (10.1–13.4 g/g) (Tosh et al., 2010) were slightly higher than those obtained in the present work for brown and red seaweeds. However, values of WRC are difficult to compare with each other, because they depend on the experimental conditions (temperature, pH, time, centrifugation) as well as on sample preparation and particle size, being the temperature the factor with major influence (Wong & Cheung, 2000; Carvalho et al., 2009).

Fleury and Lahaye (1991) reported that the physicochemical properties of powdered seaweed could be assumed to reflect those of the fibre present. These properties could be related to the hydrophilic nature of the charged polysaccharides of SDF: alginates and fucans (see uronic acid content, Table 3) in brown seaweeds, and agar and carrageenan (see neutral sugar content, Tables 3 and 4) in red seaweeds; although protein is also closely associated with cell wall polysaccharides (Fleury & Lahaye, 1991; Carvalho et al., 2009). In this study, the total contents of protein and TDF in the seaweed samples were up to 48.3–55.9% dry weight, so the physicochemical properties of the brown and red seaweeds might be mainly determined by these two chemical components. These properties confer on the fibre the ability to absorb and hold water, and these polysaccharides can be potentially beneficial in gut health, contributing to water binding, faecal bulking and decreasing transit time, which is a positive factor in preventing colon cancer (Goñi, Gudiel-Urbano, Bravo, & Saura-Calixto, 2001).

Oil retention capacity was low and similar in all seaweed samples (1.22–1.67 g/g dry weight, Table 6). There were statistically significant differences between brown and red seaweeds, with slightly lower values for the red ones, indicating that the nature of their constituents was much more hydrophilic than lipophilic. In view of these results, this hydrophilic nature might work more clearly in the sulphated galactans in red seaweeds than in the polysaccharides (alginate, laminarans and fucans) in the brown ones. Reported ORC values of soybean (0.23 g/g) and okara (0.20 g/g) (Espinosa-Martos & Rupérez, 2009) are lower than those found in the brown and red seaweeds tested in the present work.

Table 6
Physicochemical properties of edible Spanish seaweeds.

Seaweed	Swelling capacity (mL/g dry weight)	Water retention capacity (g/g dry weight)	Oil retention capacity (g/g dry weight)
Himanthalia	10.97 ± 0.62^{a}	7.26 ± 0.13^{a}	1.61 ± 0.07^{a}
Bifurcaria	7.75 ± 0.73^{b}	4.89 ± 0.12^{b}	1.65 ± 0.11^{a}
Laminaria	10.20 ± 0.37^{a}	8.93 ± 0.52^{c}	1.67 ± 0.11^{a}
Mastocarpus	7.20 ± 0.42^{b}	5.42 ± 0.06^{b}	1.22 ± 0.04^{b}
Gigartina	11.43 ± 0.63^{a}	10.22 ± 0.67^{d}	1.32 ± 0.03^{b}

Mean values of triplicate determinations ± standard deviation.
Means with different letters in each column differ significantly ($P<0.05$).

4. Conclusion

All the brown and red seaweeds selected from the Spanish northwestern Atlantic coast were a good source of dietary fibre, minerals and protein. The study of their physicochemical properties, together with their chemical composition, reveals their suitability to be a good source of food fibre for human consumption. Yet further studies are necessary (e.g. polysaccharides, fatty acids and amino acids composition) to improve our knowledge about the nutritional value of these marine algae, traditionally consumed in Japan.

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A simple ion chromatography method for inorganic anion analysis in edible seaweeds

Eva Gómez-Ordóñez, Esther Alonso[1], Pilar Rupérez*

Departamento de Metabolismo y Nutrición, Instituto del Frío (IF-ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, E28040-Madrid, Spain

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ABSTRACT

A new, simple, fast and sensitive ion chromatography (IC) method, for the simultaneous analysis of fluoride, chloride, nitrite, bromide, nitrate, phosphate and sulphate in edible seaweeds was developed and reported for the first time. The validation of the analytical method was studied in terms of linearity, sensitivity, precision and accuracy. All standard calibration curves showed very good correlation between anion peak area and concentration ($r > 0.999$). Limits of detection and quantitation ranged between 0.002–0.05 mg/L and 0.01–0.1 mg/L, respectively and indicated the high sensitivity of the method. Relative standard deviation values of repeatability and inter-day precision for standard anions with the same sample were less than 2%. Anion recoveries ranged from 97 to 113% for chloride and from 87 to 105% for sulphate, respectively and showed the fairly good accuracy of the method. The method was applied to the analysis of inorganic anions in brown and red edible seaweeds. Brown seaweeds were characterized by higher chloride content up to 33.7–36.9%, while red seaweeds were characterized by higher sulphate content (45–57%). Sulphate content in seaweeds is related to the presence of sulphated polysaccharides of biological importance. The method developed was well applicable to mineral anion analysis in edible seaweeds and shows suitability and reliability of use in other food samples of nutritional importance.

1. Introduction

Seaweeds draw from the environment were they live, the sea, an incomparable wealth of mineral elements [1], some of which are essential for the proper functioning of the body. This rich array of essential nutrient minerals and trace elements, include mineral macronutrients such as sodium, calcium, magnesium, potassium, chlorine, sulphur and phosphorus; and micronutrients such as iodine, iron, zinc, copper, selenium, molybdenum, fluoride, manganese, boron, nickel and cobalt. Mineral fraction can account for up to 40% dry weight of some seaweeds [2], however, in some cases mineral content of seaweeds is recorded even higher than that of land plants and animal products [3]. Minerals in marine algae are attributed to ions associated with charged polysaccharides [4,5]. Specifically, edible brown and red seaweeds can be used as a food supplement to help meet the recommended daily intakes of some macrominerals and trace elements [6].

Sulphate and chloride are the main anions found in seaweeds. Sulphate may account for a 50% of the ashes [2], and it is an important constituent of charged polysaccharides in marine algae, related to high salt concentration in the environment and with specific functions in ionic regulation. Such sulphated mucilages are not found in land plants [7]. Sulphate is mainly derived from charged polysaccharides, such as fucan, alginate or laminaran in brown seaweeds or from agar and carrageenan (galactans) in red ones [6].

Physicochemical properties of dietary fibre in edible seaweeds have been related to the hydrophilic nature of the charged polysaccharides [2]. Sulphated polysaccharides from edible marine algae are not toxic for humans and, especially fucans and alginic acid derivatives, are known to exhibit different biological properties, such as: anticoagulant, anti-inflammatory, antiviral, or antitumoral activities [8–12]. As an attempt to find a substitute for heparin, the anticoagulant activity of sulphated polysaccharides from seaweeds has been the most researched property. Anticoagulant capacity has been shown to be related to sulphate content in fucans [13]. Also, sulphated polysaccharides from brown and red seaweeds have been reported to exhibit antioxidant capacity *in vitro* and potentially could be used as natural antioxidants by the food industry [14].

Many different methods have been reported in the literature for the analysis of mineral cations and anions in water and in plant materials. Flame atomic absorption spectrophotometry has been traditionally used for cation determination either in water or in

Abbreviations: IC, Ion chromatography; RSD, Relative standard deviation; *r*, correlation coefficient; LOD, limit of detection; LOQ, limit of quantitation.

* Corresponding author. Tel.: +34 915445607; fax: +34 915493627.
E-mail address: pruperez@if.csic.es (P. Rupérez).
[1] Current address: Ministerio de Ciencia e Innovación, Dirección General de Transferencia de Tecnología y Desarrollo Empresarial, Ramírez de Arellano 29, E28043 Madrid, Spain.

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vegetable ashes [6,15–18]. Regarding anions, many methods have been developed which are in most cases specific for the separate analysis of every single anion. The Association of Official Analytical Chemists (AOAC, 2005) [19] and the American Public Health Association (APHA, 1998) [20] defined individual methods, which have been traditionally used, for the determination of chloride, fluoride, nitrate, sulphate and phosphorus in water. Since then, there have been many efforts to improve the utilization of these methods. In this way, potentiometry with ion-selective electrodes (ISE) seems to be the most popular and convenient method for fluoride determination [21]; Volhard method is a classical way to determine chloride in food [22]; while the automated ascorbic acid colorimetric method is more extensively used for phosphate determination in water and soil [23]. However, some of these methods suffer from many interferences with other anions.

Also, sulphate ions are measured using several direct and indirect analytical principles, including volumetric, gravimetric, colorimetric and turbidimetric methods. Currently, volumetric [24], colorimetric [25] and gravimetric methods for sulphate in water [19a] are not widely used because they are time consuming and require a skilled person to determine the end point of the analysis. Turbidimetric method is a well known quantification technique for sulphate analysis based on the precipitation of sulphate ions as insoluble barium sulphates [19b], which was improved for the determination of sulphate in plants and soils [26] and also used in seaweeds [6].

A comparative study of sulphate quantification methods, i.e. turbidimetric, ion chromatography (IC) and inductively coupled plasma atomic emission spectrometry (ICP-AES) indicates clearly that the widely used turbidimetric method is limited by its quantification range and suffers from interference of nitrate ions, while IC method shows a higher precision and lack of interferences in quantifying sulphate [27].

From its inception in 1975 [28], IC is currently a well-established technique in analytical chemistry for the separation and determination of inorganic anions. Because the most common mode of detection has been conductivity, this technique was usually applied to the determination of common anions (fluoride, chloride, nitrate and sulphate). Recent developments such as the use of higher capacity columns, larger loop injection, and more complex detection schemes have increased its utilization in a variety of areas. IC analysis of anionic and cationic species in plant materials has already been reported [29–32], using different sample preparation techniques and detection methodologies. IC is a direct way to determine sulphate concentration; moreover, this chromatographic method also permits in a single run the simultaneous determination of most anions like: fluoride, chloride, nitrite, bromide, nitrate, phosphate and sulphate.

In view of the nutritional relevance to determine accurately mineral anion content, especially sulphate in edible seaweed and other food resources, and the usefulness of ion chromatography for the aqueous determination of ions, the aim of the present study was to develop a new, simple, fast and sensitive ion chromatography method for the simultaneous analysis of main anions in seaweeds, where no complex handling of the sample is required except for the preparation of ashes and filtration.

2. Material and methods

2.1. Reagents and standards

Deionized water from Millipore Milli-Q (18.2 MΩ cm, equipped with a Millipack 0.22 μm filter) was used for the preparation of solutions. Eluent solution was prepared by dissolving appropriate amounts of sodium carbonate (Na_2CO_3, Merck) and sodium hydrogen carbonate ($NaHCO_3$, Merck) powders in Milli-Q water and degassing in an ultrasonic bath for 10 min. Accurate weighing of chemicals was performed on a Boeco BBC 22 model (Boeco, Germany) analytical balance.

Standard solutions of fluoride, chloride, nitrate, phosphate and sulphate were prepared by appropriate dilution of their anion standard stock Fluka (Sigma–Aldrich, Buchs, Switzerland) solution of analytical grade (1000 mg/L, TraceCERT®) for IC to obtain the desired concentrations of each analyte. Composition of commercial standard anions is described as follows: Fluoride Standard, 1000 mg/L F^- in water, prepared with high purity NaF and water *Trace*SELECT®Ultra. Chloride Standard, 1000 mg/L Cl^- in water, prepared with high purity NaCl and water *Trace*SELECT®Ultra. Nitrate Standard, 1000 mg/L NO_3^- in water, prepared with high purity $NaNO_3$ and water *Trace*SELECT®Ultra. Phosphate Standard, 1000 mg/L PO_4^{3-} in water, prepared with high purity Na_2HPO_4 and water *Trace*SELECT®Ultra. Sulphate Standard, 1000 mg/L SO_4^{2-} in water, prepared with high purity Na_2SO_4 and water *Trace*SELECT®Ultra. Moreover, standard solutions containing 25, 50, 75 and 100 mg/L of chloride and sulphate anions were prepared for the recovery assay.

2.2. Instrument and ion chromatographic conditions

A Metrohm Advanced compact ion chromatographic instrument (IC-861 model, Metrohm AG, Switzerland) controlled using Metrodata IC Net 2.3 software and attached to an Advance Sample Processor (IC-838) with an injection Valve Unit (IC-812) with a 20 μL sample loop was used in all analyses. The instrument was also equipped with a Pump (IC-818), an Eluent Degasser (IC-837) and a Liquid Handling Unit (IC-833) with a 0.45 μm filter that required a minimal volume of 10 mL for the samples. Detection was performed with a Conductivity Detector (IC-819) Advanced from Metrohm.

Separation was performed in a Metrosep A Supp 5–250 column (250 × 4 mm, 5 μm particle size). The carrier material was an anion-exchange polymer of polyvinyl alcohol with quaternary ammonium groups. All measurements were carried out at 32 °C (column temperature) under the following elution conditions: 3.2 mM sodium carbonate/1 mM sodium hydrogen carbonate at 0.70 mL/min as mobile phase, prepared as previously described. In order to adjust the baseline to 15 μS/cm, 50 mM sulphuric acid solution and ultrapure water (Milli-Q) were used for automatic chemical suppression.

Under the working conditions all anions were separated completely and total analysis time was 28 min. Anions in samples were identified by the coincidence of their retention times with those of commercial standard anions. Peak areas were utilized for quantitative analysis.

2.3. Validation of IC method

2.3.1. Linearity and sensitivity

The external standard calibration method used was based upon commercial Fluka standard solutions (1000 mg/L) and appropriate dilutions in ultrapure water. Calibration curves of different anions were prepared in seven levels evenly distributed from: 1 to 120 mg/L for chloride and sulphate, 0.75 to 90 mg/L for nitrate and phosphate, 0.2 to 24 mg/L for bromide, and 0.02 to 2.4 mg/L for fluoride and nitrite.

In order to check the sensitivity of the method under the working conditions used, the limits of detection (LOD) and quantitation (LOQ) were studied. LODs were calculated as the concentration corresponding to three times the peak height of the baseline noise (signal-to-noise ratio equals 3); whereas LOQs were set as 10 times the noise height (signal-to-noise ratio equals 10).

Table 1
Summarized calibration data of different anions in standard solution by IC.

Analyte	Range (mg/L)	Regression equation	Correlation coefficient (r)	LOD (mg/L)	LOQ (mg/L)
Fluoride	0.02–2.4	$y = 1.12899x$	0.99934	0.002	0.01
Chloride	1–120	$y = -4.33317e^{-5}x^2 + 1.21667x$	0.99965	0.002	0.01
Nitrite	0.02–2.4	$y = 32.8006x$	0.99971	0.052	0.17
Bromide	0.2–24	$y = 3.66138x$	0.99995	0.006	0.02
Nitrate	0.75–90	$y = 2.49096x$	0.99903	0.004	0.01
Phosphate	0.75–90	$y = 4.97956x$	0.99904	0.008	0.03
Sulphate	1–120	$y = -22.1121e^{-5}x^2 + 2.0793x$	0.99908	0.003	0.01

IC = Ion chromatography; y = concentration (mg/L); x = area (μS/cm $\times$ sec).
LOD = limit of detection; LOQ = limit of quantitation. LOD and LOQ were determined at a signal to noise ratio of about 3 and 10, respectively.

2.3.2. Precision

The precision of the IC method was assessed in terms of repeatability and intermediate (inter-day) precision by analyzing three replicate determinations on the same day and two determinations on two different days with the same sample of anion standards. Only most common anions in seaweeds were taken under consideration (fluoride, chloride, nitrate, phosphate and sulphate). Random concentrations of each analyte were prepared. The relative standard deviation (RSD) percentage of individual measurements was determined and expressed for intra-day precision and for inter-day precision. Also, RSD (%) corresponding to retention time, peak height and peak area were determined. To consider a good precision of the method, RDS values must be less than 5%.

2.3.3. Accuracy

To study the reliability and suitability of the IC method, recovery experiments were carried out. Recovery percentage was obtained by measuring anion content in one brown (*Laminaria*) and in one red alga (*Mastocarpus*), before and after the addition of four different concentrations (25, 50, 75 and 100 mg/L) of chloride and sulphate standard solutions. Each test was repeated three times. For this assay we only focused on these two anions because of their quantitative importance in each type of algae. Experimental values found were compared with the corresponding theoretical concentration.

2.4. Sample material

The brown seaweeds (Phaeophyta) *Himanthalia elongata* (L.) S.F. Gray (sea spaghetti), *Laminaria saccharina* (L.) J.V. Lamouroux (sweet Kombu) and *Bifurcaria bifurcata* R. Ross; and the red seaweeds (Rhodophyta) *Mastocarpus stellatus* (Stackhouse) Guiry and *Gigartina pistillata* (S.G. Gmelin) Stackhouse were obtained from a local supplier (Porto-Muiños, Cambre, Coruña, Spain). Marine algae were cleaned from epyphytes and sand, washed with tap running water, dried and milled to less than 1.0 mm particle size before analysis. Residual moisture content was determined by oven drying to a constant weight at 105 °C.

2.5. Preparation of seaweed sample for anion analysis by IC

Generally, ash content is the percentage of inorganic matter in a sample, and so, it can be used to analyze anion or cation content. To determine ash content, seaweed samples (0.5 g) were incinerated in a digitally controlled Hobersal HD-230 muffle furnace (Barcelona, Spain) at 550 °C for 16 h and weighed. Ash content was calculated and expressed as a percentage of the seaweed sample dry weight. Just before injection, ashes were milled with pestle and mortar and an aliquot (25 mg) dissolved in 50 mL Milli-Q water. To separate particulate matter, filtration was performed through Whatman No. 44 ashless cellulose filter (3 μm particle size, 110 mm diameter). Then all samples were appropriately diluted in ultrapure water and adjusted to a final volume of 10 mL to give an approximate concentration of 100 mg/L, poured to auto-sampler polyethylene tubes and analyzed by ion chromatography.

2.6. Statistical analysis

All determinations were performed at least in triplicate. Data were expressed as mean values ± standard deviations and reported on a dry matter basis. Relative standard deviation (RSD) percentage was calculated as standard deviation/mean value.

3. Results and discussion

3.1. Validation of IC method

3.1.1. Linearity and sensitivity

The linearity of the IC method was evaluated for the following anions: fluoride, chloride, nitrite, bromide, nitrate, phosphate and sulphate (Table 1). The calibration curves showed a linear relationship (linear through zero) between the peak area and concentration over a wide range of concentrations for fluoride, nitrite, bromide, nitrate and phosphate. In order to adjust more properly to the whole and wider working range (1–120 mg/mL), chloride and sulphate presented a quadratic relationship (quadratic through zero) in their calibration curve.

Calibration data of different anions as summarized in Table 1, showed a good correlationship between peak area (x) and concentration (y, mg/L), with a correlation coefficient $r > 0.999$ for all analytes under the IC working conditions used.

The LODs results showed that the high sensitivity of IC method was obtained for all anions with values between 0.002 and 0.05 mg/L (Table 1). Also the LOQs values (0.01–0.1 mg/L), corresponding to the lowest level on the calibration curve that could be determined with appropriate precision and accuracy under the experimental conditions, confirmed the high sensitivity of the method.

3.1.2. Precision and accuracy

RSD (%) of repeatability was below 2.1% and RSD (%) of inter-day was below 1.4%, which indicated that the IC method has both good repeatability and inter-day precision (Table 2). Besides, assays of repeatability for retention times, peak heights and peak areas were carried out and results are shown in Table 3. Likewise, RSD (%) values were less than 2% and illustrated the good precision of the analytical method.

To determine the accuracy, ash solutions of *Laminaria* and *Mastocarpus* spiked with four different concentrations of chloride and sulphate standards were used. Recovery values amounted to 97.5–113% for chloride and 87.6–105% for sulphate (Table 4). Closeness of the results to 100% confirmed the fairly good accuracy of the IC method.

Table 2
Precision of the IC method.

	Repeatability ($n=3$)			Inter-day precision ($n=2$)		
Analyte	Prepared (mg/L)	Found (mg/L)	RSD (%)	Prepared (mg/L)	Found (mg/L)	RSD (%)
Fluoride	1	1.0 ± 0.0	0.2	1	1.0 ± 0.0	0.6
Chloride	50	49.4 ± 0.3	0.6	50	49.4 ± 0.3	0.5
Nitrate	10	8.4 ± 0.0	0.1	10	8.4 ± 0.1	0.9
Phosphate	6	5.9 ± 0.1	2.1	6	5.9 ± 0.1	1.4
Sulphate	50	49.0 ± 0.1	0.1	50	49.2 ± 0.4	0.8

Data are mean values ± standard deviation.
IC = Ion chromatography; RSD = Relative standard deviation.

Table 3
Repeatability ($n=3$) of retention time, peak height and peak area.

Analyte	Prepared (mg/L)	Retention time (min)	RSD (%)	Peak height	RSD (%)	Peak area	RSD (%)
Fluoride	1	6.13	0.4	1.87	1.64	18.23	0.8
Chloride	50	9.51	0.2	68.76	1.19	832.74	0.9
Nitrate	10	16.59	0.3	2.82	1.25	67.44	0.5
Phosphate	6	21.33	0.3	0.84	0.68	23.53	1.4
Sulphate	50	25.42	1.1	16.22	1.73	496.43	0.6

Peak height = μS/cm; Peak area = μS/cm × sec.
RSD = Relative standard deviation.

Table 4
Recovery ($n=3$) of the IC method.

Seaweed	Chloride in sample (mg/L)	Added chloride (mg/L)	Found (mg/L)	Recovery (%)	Sulphate in sample (mg/L)	Added sulphate (mg/L)	Found (mg/L)	Recovery (%)
Laminaria	36.6 ± 0.2	25	63.2 ± 0.5	108.2 ± 0.4	5.3 ± 0.1	25	29.6 ± 0.2	100.7 ± 0.7
		50	92.4 ± 0.4	108.6 ± 0.2		50	57.5 ± 0.3	105.3 ± 0.5
		75	119.6 ± 1.0	108.6 ± 0.7		75	85.6 ± 0.7	103.3 ± 0.6
		100	145.8 ± 0.7	107.1 ± 0.6		100	113.2 ± 1.0	105.8 ± 0.7
Mastocarpus	3.4 ± 0.1	25	28.8 ± 0.1	113.7 ± 0.2	55.5 ± 0.7	25	83.7 ± 0.7	105.0 ± 1.6
		50	56.0 ± 0.5	107.9 ± 1.0		50	105.6 ± 1.0	100.8 ± 1.6
		75	84.3 ± 1.7	109.4 ± 2.0		75	135.8 ± 4.4	102.0 ± 3.3
		100	100.5 ± 0.5	97.5 ± 0.5		100	137.8 ± 0.6	87.6 ± 0.1

Data are mean values ± standard deviation; IC = Ion chromatography.

3.2. Application of IC method to seaweed analysis

Differences in composition between brown and red seaweeds related to their polysaccharide composition are well known. Red seaweeds are mostly composed of sulphated galactans, such as agar and carrageenans, while brown seaweeds are composed of alginates, fucans, and laminarans [8,2,33]. Sulphate seems to be a typical component of marine algal polysaccharides, mostly derived from fucans in brown algae or from galactans in red ones. Therefore, differences in seaweed polysaccharides are translated into differences in anion composition. Fig. 1 shows a typical ion chromatogram for different standard anions and seaweed samples showing these differences. All anions in the samples were separated completely.

Ash and anion content in seaweeds are shown in Table 5. Ash content ranged from 24.9 to 36.4% algal dry weight, with the lowest value for the red alga *Mastocarpus* and the highest for the brown alga *Himanthalia*. Ash content in most land vegetables, with an average value of 5–10 g/100 g dry weight [34], is usually much lower than in marine algae. The ash values obtained fit well within the wide ranges, from 8 to 40% of algal dry weight, reported for seaweeds [3–5]. Also these values are in agreement with previous results [2,6].

Total anions content (% ash dry weight, Table 5) was lower for brown (40.6–48.9%) than for red seaweeds (55.4–61.7%). Besides, differences in relative anion abundance between brown and red seaweeds were found (Table 5, Fig. 1). Thus, brown seaweeds were characterized by a higher chloride content (33.7–36.9%), while red ones by a higher sulphate content (45–57%). These sulphate values agree with those reported previously by the AOAC gravimetric method [2] and by the gelatin-barium chloride spectrophotometric method [6,14] applied to seaweed samples, showing the characteristic difference in composition between brown and red seaweeds. In this way, this pattern agrees with that found in Hawaiian seaweeds [35] with a higher sulphate content in red (2–8% dry weight) than in brown algae (1–2% dry weight). Also, other red seaweeds, such as *Grateloupia lithophila* in the Gulf of Mannar (India),

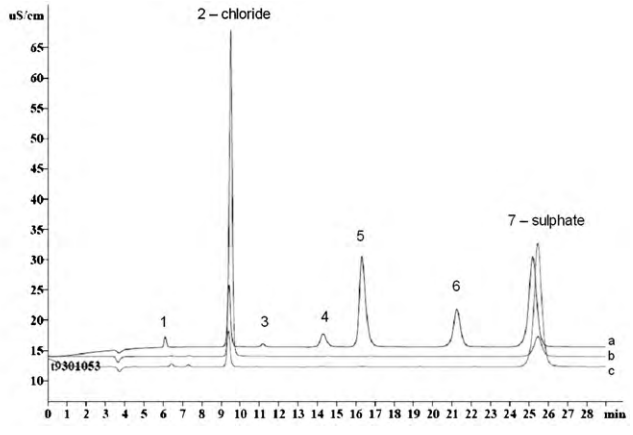

Fig. 1. A typical ion chromatogram for different samples: (a) standard solution of anions, (b) *Laminaria saccharina* (brown seaweed), (c) *Mastocarpus stellatus* (red seaweed). Identified peak anions: (1) fluoride; (2) chloride; (3) nitrite; (4) bromide; (5) nitrate; (6) phosphate; (7) sulphate.

Table 5
Ash and anion content in edible seaweeds by IC.

Anion[a]	Brown seaweeds			Red seaweeds	
	Himanthalia	*Bifurcaria*	*Laminaria*	*Mastocarpus*	*Gigartina*
Ash[b]	36.4 ± 0.2	34.3 ± 0.2	34.8 ± 0.1	25.0 ± 0.1	34.6 ± 0.5
Fluoride	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
Chloride	37.0 ± 1.1	33.7 ± 0.2	34.8 ± 0.5	4.1 ± 0.1	9.9 ± 0.7
Nitrite	nd	0.1 ± 0.0	nd	nd	nd
Bromide	nd	0.1 ± 0.0	nd	0.1 ± 0.0	0.1 ± 0.0
Nitrate	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Phosphate	0.1 ± 0.0	nd	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Sulphate	11.6 ± 1.2	13.5 ± 0.7	5.5 ± 0.5	57.1 ± 0.8	45.1 ± 1.0
Total anions	48.9 ± 0.3	47.8 ± 0.2	40.6 ± 0.2	61.7 ± 0.2	55.4 ± 0.3

Data are mean value of triplicate determinations ± standard deviation. nd: not detected.
[a] Anion content expressed as % ash dry weight.
[b] Ash content expressed as % algal dry weight.

show higher sulphate values (162.8 mg/g algal dry weight), than the brown ones (0.88–61 mg/g algal dry weight) [36].

Chloride content in seaweeds may be related to the water salinity of the sea where they live [37], and can vary from one sea to another and with depth. Red seaweeds use to live in deeper waters with lower salinity levels that could explain the reason why they seem to have much lower chloride contents than brown seaweeds (Table 5). Moreover, mineral content has been shown to vary according to seaweed species, oceanic residence time, geographical place of harvest, seasonal, annual, environmental and physiological factors [1,5,38].

Besides chloride and sulphate, other minor anions were estimated (Table 5). Small amounts of fluoride, nitrate and phosphate were found in all the seaweeds. The brown seaweed *Bifurcaria* also presented trace amounts of nitrite and bromide, but no phosphate. Moreover, red seaweeds also exhibited trace amounts of bromide.

4. Conclusion

A simple, fast and sensitive ion chromatography method for the individual anion determination in edible seaweeds was developed. The brown and red edible seaweeds studied presented a rich array of inorganic anions. Chloride and sulphate were the main anions in brown and red seaweeds, respectively. The presence of sulphate is of especial relevance since it seems to play an important role in the biological properties of sulphated polysaccharides. This ion chromatography method showed good precision, accuracy, sensitivity and a lack of interference when applied to the anion analysis of seaweeds. This method could also be applied, with a similar preparation of the ashes, to the anion determination in other food samples of nutritional importance.

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FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds

Eva Gómez-Ordóñez, Pilar Rupérez*

Metabolism and Nutrition Department, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Nováis 10, Ciudad Universitaria, E28040 Madrid, Spain

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ABSTRACT

Phycocolloids present in three brown (*Himanthalia elongata*, *Bifurcaria bifurcata*, *Saccharina latissima*) and five red edible seaweeds (*Mastocarpus stellatus*, *Gigartina pistillata*, *Chondracanthus acicularis*, *Nemalion helminthoides* and *Dumontia contorta*) were studied by FTIR-ATR spectroscopy. Infrared spectra of polysaccharide standards (alginate, agar, iota-, kappa- and lambda-carrageenan) were obtained for comparison. The main polysaccharide found in brown seaweeds was alginate, a linear copolymer of mannuronic (M) and guluronic acid (G). Alginate M/G ratio was tentatively estimated from specific absorption bands (808/787 cm^{-1} and 1030/1080 cm^{-1}) in infrared spectra, suggesting higher values of mannuronic than guluronic acid blocks (M/G > 1) for brown seaweeds. According to their infrared spectra, all the red seaweeds studied were mainly carrageenan producers. Thus, *M. stellatus* showed absorption bands at 929.0, 844.7 and 803.2 cm^{-1} of a typical hybrid kappa/iota/mu/nu-carrageenan, meanwhile *G. pistillata* and *C. acicularis*, showed the characteristic broad band (830–820 cm^{-1}) of lambda-type carrageenan. Moreover, when the second-derivative was obtained to improve resolution of overlapped bands in the original FTIR spectra, this band was divided into several sharper signals, indicating the presence of lambda-/theta-/xi-carrageenans. Accordingly with their FTIR spectra, *N. helminthoides* contained sulphated polysaccharides, such as carrageenan or mannans, while *D. contorta* produced lambda- with lesser amounts of kappa-carrageenan. Therefore, FTIR-ATR spectroscopy is proposed as a useful tool for the food, pharmaceutical and cosmetics industry to check the phycocolloid quality of a raw seaweed material by a quick and non-destructive method.

1. Introduction

Phycocolloids (e.g. alginate, carrageenan, agar) are polysaccharides associated with the cell wall and intercellular spaces of some seaweed species, which have been used extensively as gels and thickeners in food and industrial preparations.

The major structural polysaccharide of brown seaweeds (Phaeophyta) is alginate. Alginate is the salt of alginic acid, a linear copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) (1 → 4)-linked residues, arranged either in heteropolymeric (MG) and/or homopolymeric (M or G) blocks (Larsen, Salem, Sallam, Mishrikey, & Beltagy, 2003; Leal, Matsuhiro, Rossi, & Caruso, 2008).

Carrageenan and agar are the principal sulphated polysaccharides produced by red seaweeds (Rhodophyta); the main difference between the highly sulphated carrageenans from the less sulphated agars is the presence of D-galactose and anhydro-D-galactose in carrageenans and of D-galactose, L-galactose or anhydro-L-galactose in agars.

The structure of the various types of carrageenans is defined by the number and position of sulphate groups, the presence of 3,6-anhydro-D-galactose and conformation of the pyranosidic ring. There are about fifteen idealised carrageenan structures traditionally identified by Greek letters (Chopin, Kerin, & Mazerolle, 1999). However, a more versatile binomial version, incorporating the substitution pattern of each sugar unit was developed by Knutsen, Myslabodski, Larsen, and Usov (1994). FTIR band assignment and letter code nomenclature of the different carrageenans are summarised in Table 1.

Thus, commercial carrageenans are normally divided into three main types: kappa (κ)-, iota (ι)- and lambda (λ)-carrageenan. Their differences on chemical composition and configuration are responsible for their interesting rheological properties as gelling, stabilising and thickening agents used in the food, pharmaceutical and cosmetics industry. Kappa- and iota-carrageenan contain the

* Corresponding author. Tel.: +34915445607; fax: +34915493627.
E-mail address: pruperez@ictan.csic.es (P. Rupérez).

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Table 1
FTIR band assignment and letter code nomenclature of the different carrageenans.[a]

Wave number (cm^{-1})	Assignment	Letter code[b]	Found in carrageenans
1210–1260	Sulphate ester (O–SO_3^-)	**S**	κ, ι, λ, μ, ν, θ, ξ
928–933, 1070 (shoulder)	3,6-anhydro-D-galactose	**DA**	κ, β
840–850	D-galactose-4-sulphate	**G4S**	κ, ι, μ, ν
830	D-galactose-2-sulphate	**G2S**	λ, θ, ξ
820, 825 (shoulder)	D-galactose-2,6-disulphate	**D2S,6S**	λ, ν
810–820, 867 (shoulder)	D-galactose-6-sulphate	**D6S**	μ
800–805, 905 (shoulder)	3,6-anhydro-D-galactose-2-sulphate	**DA2S**	ι, θ

[a] Adapted from Knutsen et al. (1994).
[b] Disaccharide repeating unit is composed of alternating: 3-linked β-D-galactopyranose (**G**-units) and 4-linked α-D-galactopyranose (**D**-units) or 4-linked 3,6-anhydro-α-D-galactopyranose (**DA**-units).

3,6-anhydro-galactose unit and are gel forming, whereas lambda-carrageenan with only galactose residues is a thickener (Prado-Fernández, Rodríguez-Vázquez, Tojo, & Andrade, 2003; Tojo & Prado, 2003).

Generally, seaweeds do not produce pure carrageenans, but more likely a range of hybrid structures. Thus, differences in carrageenan composition have been described between the gametophytic (kappa family) and tetrasporophytic (lambda family) growth phase in Gigartinales (Chopin et al., 1999; Van De Velde, Knutsen, Usov, Rollema, & Cerezo, 2002).

One of the most useful techniques for identifying polysaccharide structures is infrared (IR) spectroscopy, which is based on the analysis of absorption peaks at certain wave numbers (expressed in cm^{-1}). In the last years, the combination of Fourier transform algorithm with attenuated total reflectance (ATR) techniques has improved the conventional IR spectroscopy with various and important advantages. Thus, FTIR-ATR (Fourier transformed IR from attenuated total reflectance) spectroscopy is direct and non-destructive, requires only small amounts of dried material (just a few milligrams) and is a quick method (a few minutes vs. several days), thus avoiding lengthy extractions and further sample preparation as a film or KBr pellet (Pereira, 2006; Pereira & Mesquita, 2004). Therefore, carrageenans and other phycocolloids present in an alga can be identified rapidly by FTIR-spectroscopy directly on only a few milligrams of dried, ground algal material, avoiding time consuming sample preparation procedures (Chopin & Whalen, 1993).

Moreover, software for FTIR spectrometers allows processing of spectral data, especially conversion to second-derivative spectra, which provides more information by better resolving into sharper signals the corresponding bands and shoulders in parent spectra (Chopin et al., 1999; Matsuhiro & Rivas, 1993). Also, the second-derivative mode of the FTIR spectra can be applied to distinguish agar-producing from carrageenan-producing seaweeds (Matsuhiro, 1996; Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003). Besides, infrared spectroscopy has been used in a quantitative method for estimating total sulphate content of carrageenans and agars from characteristic infrared absorption bands (Rochas, Lahaye, & Yaphe, 1986).

In the structural analysis of carbohydrates, five frequency regions (Mathlouthi & Koenig, 1987) can be distinguished in the normal spectra (4000–650 cm^{-1}): (1) region of OH and CH stretching vibrations at 3600–2800 cm^{-1}; (2) region of local symmetry at 1500–1200 cm^{-1}; (3) region of CO stretching vibration at 1200–950 cm^{-1}; (4) fingerprint or anomeric region at 950–700 cm^{-1}; and (5) skeletal region below 700 cm^{-1}.

IR absorption bands at 2960, 2920, 2845, 1640, 1370, 1250, 930, 900, 845, 805 and 705 cm^{-1} are used to obtain information on the structure of agars and carrageenans. Besides, FTIR-spectroscopy in the second-derivative mode may be applied to distinguish agar-from carrageenan-type of seaweed galactans. Moreover, not only the major types of carrageenans, but also smaller fractions, such as μ- and ν-carrageenan (the biological precursors of κ- and ι-carrageenan, respectively) can be detected by FTIR (Chopin et al., 1999; Pereira, Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009).

There is currently an increasing demand for seaweed polysaccharides by the food industry. Also a quick and reliable non-destructive method to assess the quality of raw algal material is needed. Information on polysaccharide composition and structure, as well as protein or sulphate content, can be gained from algal infrared spectra. Thus, this technique would allow to preliminary identify the main polysaccharides in an unknown seaweed sample. Our aim was to use FTIR-ATR spectroscopy as a tool for the direct identification of the main natural phycocolloids, namely alginate, agar and carrageenan, in ground and dried samples from several brown and red seaweeds.

2. Material and methods

2.1. Seaweed sample

Brown seaweeds (Heterokonthophyta, Phaeophyceae) *Himanthalia elongata* (L.) S.F. Gray (sea spaghetti; Fucales), *Bifurcaria bifurcata* R. Ross (Fucales) and *Saccharina latissima* (*Linnaeus*) *C.E. Lane*, *C. Mayes*, *Druehl* & *G.W. Saunders* (sweet kombu, formerly *Laminaria saccharina* (L.) J.V. Lamouroux) (Laminariales) and red seaweeds (Rhodophyta) *Mastocarpus stellatus* (Stackhouse) Guiry (Gigartinales), *Gigartina pistillata* (S.G. Gmelin) Stackhouse (Gigartinales), *Chondracanthus acicularis* (Roth) Fredericq (Gigartinales), *Nemalion helminthoides* (Velley in Withering) Batters (Nemalionales) and *Dumontia contorta* (Gmelin) Ruprecht (Cryptonemiales) were obtained from a local supplier (Porto-Muiños, Cambre, A Coruña, Spain). Fresh brown and red seaweeds were collected in June 2007 and 2009, respectively. In the supplier industry, these marine algae were cleaned from epiphytes and sand, washed with tap running water, air-dried at 50 °C and milled to less than 1.0 mm particle size. The dried and milled seaweed samples were stored in sealed plastic bags at 2 °C until analysis.

2.2. Commercial standards of polysaccharides

Commercial standards of polysaccharides, alginate (alginic acid sodium salt, D-7924), agar (A-7002), kappa- (type I, C-1013) and iota-carrageenan (type II, C-1138) were obtained from Sigma–Aldrich Chemicals (Alcobendas, Madrid, Spain). According to previously published FTIR spectral reports (Pereira et al., 2003; Tojo & Prado, 2003), current commercially available standard of lambda-carrageenan was not found to be of the highest purity (spectrum not shown), and therefore the FTIR spectrum of lambda-carrageenan used for comparison was obtained from the literature (Prado-Fernández et al., 2003).

Alginic acid used as standard, was prepared in our laboratory from the commercial polysaccharide alginate (alginic acid sodium salt). In summary, sodium alginate (50 mg) was dissolved in 17 mL 0.08 M phosphate buffer solution at room temperature. Then, 1 mL of 18 M H_2SO_4 was added, so that final acid concentration was 1 M. The solution was left at room temperature for 30 min to allow sample to precipitate. Then it was centrifuged (1000×*g*) for 15 min, the insoluble residue obtained was washed with distilled water to almost neutral pH and then dried at 60 °C overnight. The residue

thus prepared was finely ground with pestle and mortar and used as a standard of alginic acid for FTIR-spectroscopy.

2.3. FTIR-ATR spectra acquisition

The FTIR spectra of dried and ground algal material and polysaccharide standards were recorded using the Perkin–Elmer® Spectrum™ 400 FT-IR/NIR spectrometer (Perkin Elmer Inc., Tres Cantos, Madrid) in mid-IR mode, equipped with a Universal ATR (attenuated total reflectance) sampling device containing diamond/ZnSe crystal. Besides, for powdered samples an extra accessory plate with a conic awl was used which required only a few milligrams without any previous sample preparation. The pressure applied to squeeze the powdered sample towards the diamond was approximately 148 ± 1 N.

Spectra were acquired and then processed with the Spectrum software version 6.3.2. The spectra were scanned at room temperature in transmission mode over the wave number range of 4000–650 cm^{-1}, with a scan speed of 0.20 cm/s, and 30 accumulations at a resolution of 4 cm^{-1}. Triplicates of each sample were averaged to obtain an average spectrum. A background spectrum of air was scanned under the same instrumental conditions before each series of measurements.

2.4. M/G ratio of alginate

Alginates show several characteristic bands in the IR spectra assigned to mannuronic (M) and guluronic acid (G), respectively at 808 and 787 (Mackie, 1971) and at 1030 and 1080 cm^{-1} (Sakugawa, Ikeda, Takemura, & Ono, 2004). These authors suggest that it would be possible to gain a fairly good estimation of the M/G ratio of alginate from the quotient of these bands intensity. Likewise, other authors (Lijour, Gentric, Deslandes, & Guezennec, 1994; Rochas et al., 1986) have also applied IR spectroscopy to estimate sulphate content of carrageenans and agars from characteristic IR absorption bands (Lijour et al., 1994; Rochas et al., 1986). Therefore, previous work on sulphate estimation was adapted to tentatively estimate the M/G ratio in alginate from brown seaweeds. Base line method for determining transmittance at the above mentioned wave numbers in the average IR spectra was applied (Rochas et al., 1986). M/G ratio was calculated from the quotient of absorbance at the corresponding wave numbers according to the following equation:

$$A = \log T_b/T_p$$

Symbols refer to transmittance (T) and absorbance (A) of base line (b) and peak (p).

2.5. FTIR second-derivative spectra

Second-derivatives of FTIR spectra are generally used as an aid for wave number determination of weak absorption bands or to improve resolution of overlapped bands in the original spectra (Matsuhiro & Rivas, 1993). Therefore, derivation including Savitzky–Golay algorithm with twenty five smoothing points, was performed using the Spectrum software version 6.3 incorporated into the hardware of the instrument.

3. Results and discussion

Common to all polysaccharide standards and seaweed samples two bands appeared in the 4000–2000 cm^{-1} region of the FTIR spectra (data not shown): a broad band centred at 3260 cm^{-1} assigned to hydrogen bonded O–H stretching vibrations and a weak signal at 2926 cm^{-1} due to C–H stretching vibrations. In addition, the medium to strong IR absorption bands at 1200–970 cm^{-1} are mainly due to C–C and C–O stretching in pyranoid ring and to C–O–C stretching of glycosidic bonds. An intense absorption in this spectral region is common for all polysaccharides (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998; Synytsya et al., 2010).

3.1. Infrared spectra of brown seaweeds

The FTIR-ATR spectra of polysaccharide standards (alginic acid, sodium alginate) and brown seaweeds in the range 2000–650 cm^{-1} are presented in Fig. 1. Alginates are stable in solution between pH 6 and 9 but they form insoluble precipitates at acid pH; thus, soluble alginate in salt form is converted into insoluble alginic acid in free acid form. This conversion of soluble alginate into insoluble alginic acid also happens naturally in seaweeds; therefore it is important to use both standards for FTIR-spectroscopy of polysaccharides.

Sodium alginate standard presented seven characteristic bands consecutively numbered in Fig. 1b. The most remarkable difference observed between sodium alginate and alginic acid standard (Fig. 1a) was in the relative position of the band 1, which corresponds to a carboxylic ester band (Leal et al., 2008). This carbonyl group can shift into two forms: as carboxylic acid ester form (C=O, 1730 cm^{-1}) in alginic acid standard or as carboxylate anion form (COO^-, 1600 cm^{-1}) in alginate standard.

According to literature reports (Leal et al., 2008; Mathlouthi & Koenig, 1987), the band 2 at 1406.6 cm^{-1} (Fig. 1b–e) is assigned, to C–OH deformation vibration with contribution of O–C–O symmetric stretching vibration of carboxylate group. The weak bands 3 (1083.0 cm^{-1}) and more intense 4 (1024.1 cm^{-1}) may be assigned to C–O and C–C stretching vibrations of pyranose ring

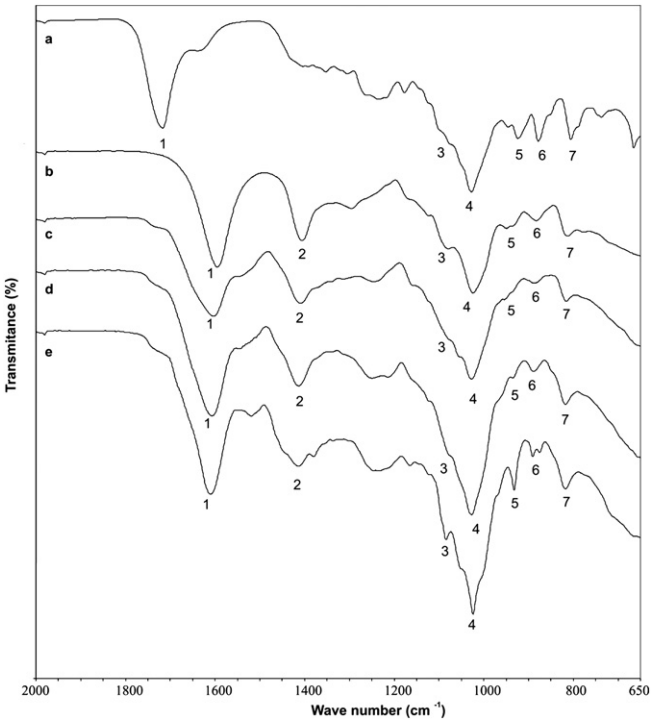

Fig. 1. Infrared spectra of polysaccharide standards: (a) alginic acid; (b) sodium alginate and brown seaweed samples: (c) *Saccharina latissima*; (d) *Himanthalia elongata* and (e) *Bifurcaria bifurcata*. Band numbers (1–7) in FTIR spectra indicate most characteristic bands.

(Fig. 1a–e). The anomeric region of fingerprint (950–750 cm^{-1}) showed three characteristic absorption bands in all polysaccharide standards and brown seaweeds (Fig. 1, bands 5–7) of alginate polysaccharides. The band 5 at 947.9 cm^{-1} is assigned to the C–O stretching vibration of uronic acid residues, the one at 878.1 (Fig. 1, band 6) is assigned to the C1-H deformation vibration of β-mannuronic acid residues. Finally, the band 7 at 817.1 cm^{-1} is characteristic of mannuronic acid residues (Chandía, Matsuhiro, Mejías, & Moenne, 2004).

Thus, alginate was the main polysaccharide found in brown seaweed samples, despite of the little shoulder which appeared around 1730 cm^{-1} in *S. latissima*, *H. elongata* and *B. bifurcata* (Fig. 1c–e) showing the presence of alginic acid, the free acid form of alginate. These algae also exhibited a broad band around 1220–1260 cm^{-1}, assigned to the presence of sulphate ester groups (S=O) which is a characteristic component in fucoidan and sulphated polysaccharides other than alginate in brown seaweeds (Chandía & Matsuhiro, 2008; Rupérez, Ahrazem, & Leal, 2002; Synytsya et al., 2010).

3.1.1. M/G ratio of alginate

IR spectroscopy has proven useful for the quantitative estimation of mannuronic to guluronic acid (M/G) ratio in alginates (Mackie, 1971; Sakugawa et al., 2004).

In the present study the ratio of band intensities in FTIR spectra at approximately 808/787 cm^{-1} and at 1030/1080 cm^{-1} were used to tentatively estimate the M/G ratio of brown seaweeds. According to this, M/G ratio of sodium alginate standard was 1.2 and 1.7, respectively; similar values were obtained in seaweed samples: 1.5–1.8 for *H. elongata,* 2.4–2.1 for *B. bifurcata* and 1.4–1.6 for *S. latissima,* respectively. Thus, brown seaweed samples with M/G > 1, which corresponded to higher values of mannuronic (M) than guluronic acid blocks (G), appeared similar to alginate standard.

M/G ratio can vary within brown seaweed alginates from 0.5 to 2.5, due to the heterogeneity between chain length and distribution of G and M blocks (Miller, 1996). Besides, mannuronic to guluronic acid ratio is an index of the nature of gels produced (Haug, Larsen, & Smidsrød, 1967) and these different M/G ratios mean that alginates obtained from different seaweeds would have different physico-chemical properties. In general alginic acid, with a low M/G ratio (<1) and a large proportion of guluronic acid blocks, forms a strong and rigid gel (Draget, Skjåk-Bræk, & Stokke, 2006). On the contrary, alginate with a low number of guluronic acid blocks and a high M/G ratio (>1) produces a soft and elastic gel. The ratio of M- to G-units is not only known to vary among species but also to some extent with the age or part of the plant and season of collection (Haug, Larsen, & Smidsrød, 1974; Jothisaraswathi, Babu, & Rengasamy, 2006; Miller, 1996; Minghou, Yujun, Zuhong, & Yucai, 1984). This heterogeneity of alginates can be useful in many food and non food industrial applications.

3.1.2. FTIR second-derivative spectra of brown seaweeds

Signals assigned in the FTIR second-derivative spectra of brown seaweeds and polysaccharide standards (sodium alginate, alginic acid) are shown in Table 2. The second-derivative spectra showed twelve signals in the region 2000–650 cm^{-1}, while the parent ones only showed seven bands (Fig. 1). According to the literature (Femenia, García-Conesa, Simal, & Rosselló, 1998; Kačuráková & Wilson, 2001), signals at 1650 and 1550 cm^{-1} are assigned to the amide I and amide II bands, proposed for identification of proteins. These absorption bands with a very low intensity in the original spectra, gave well defined signals in the second-derivative spectra. The signal at 1650 cm^{-1} may appear overlapped in the original spectra with the strong band 1 (Fig. 1c–e) of carbonyl group. Protein content in these brown algae, which has been recently reported (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010), fairly agrees with these results. In addition, a number of signals was found at 1200–970 cm^{-1} in all samples (Table 2), due to stretching vibration of glycosidic bonds, common to all polysaccharides.

3.2. Infrared spectra of red seaweeds

FTIR-ATR spectra of polysaccharide standards (agar, carrageenans) and red seaweeds in the range 2000–650 cm^{-1} are presented in Fig. 2A and B, respectively. Agar, iota-, kappa- and lambda-carrageenan presented ten characteristic bands (consecutively numbered in Fig. 2). Agars differ from carrageenans as they have the L-configuration for the 4-linked galactose residue; nevertheless, they have some structural similarities with carrageenans. The characteristic broad band of sulphate esters in general (Chopin et al., 1999) between 1210 to 1260 cm^{-1} (Fig. 2A, band 2) was much stronger in carrageenan standards than in agar. Especially in the anomeric region (950–700 cm^{-1}) agar and carrageenan standards showed several similar bands. Thus, the strong band 5 (Fig. 2A) at 930.3 cm^{-1} assigned to the presence of 3,6-anhydro-galactose residue (**DA**, Table 1) was common to agar and carrageenans; the band 6 (Fig. 2A) at 891.8 cm^{-1} corresponded to anomeric CH of β-galactopyranosyl residues and bands 9–11 (Fig. 2A) at 770.0, 740.7 and 693.8 cm^{-1} are assigned to the skeleton bending of pyranose ring (Matsuhiro, 1996) both in agar and carrageenans. Also, the bands 3 (1150.0 cm^{-1}) and 4 (1030–1010 cm^{-1}) in Fig. 2 may be assigned to C–O and C–C stretching vibrations of pyranose ring common to all polysaccharides.

Carrageenan standards showed a number of bands in the anomeric region characteristic of the type of carrageenan (iota-, kappa- and lambda-carrageenan) and the degree of sulphation. According to Knutsen et al. (1994) (Table 1) the letter code nomenclature for the three main types are: **G4S-DA2S**; **G4S-DA** and **G2S-D2S,6S** for iota-, kappa- and lambda-carrageenan, respectively. Thus, iota- and kappa-carrageenan standards showed two characteristic bands at 845 cm^{-1} and 805 cm^{-1} (Fig. 2A, b–c, bands 7, 8) that corresponded to galactose-4-sulphate (**G4S**) and 3,6-anhydro-galactose-2-sulphate (**DA2S**), respectively. The commercial kappa-carrageenan standard used contained predominantly kappa- but lesser amounts of iota-carrageenan, accordingly with our results. Lambda-carrageenan standard showed a characteristic broad band at 845–830 cm^{-1} (Fig. 2A, d, band 7) and the band at 930 cm^{-1}

Table 2
Signals assigned in the FTIR second-derivative spectra of brown seaweeds and alginate standards.

Wave number (cm^{-1})												
Band number[a]	1		2		3	4			5	6	7	
H. elongata	1653.7	1600.1	1543.2	1414.4	1082.9	1028.2	998	960.9	931.4	890.4	819.7	781.0
B. bifurcata	1660.50	1614.9	1545.4	1411.0	1087.5	1023.7	998.7	964.4	931.9	890.4	820.8	777.6
S. latissima	1650.2	1600.1	1543.2	1411.0	1082.9	1028.2	994	950.7	932.5	885.8	819.7	781.0
Alginate sodium salt	–	1595.0	–	1406.7	1085.1	1026.0	995.1	950.7	930.2	881.2	819.7	778.7
Alginic acid	–	1715.2	–	–	1079.5	1028.2	–	948.5	924.5	879.0	807.2	787.8

[a] In original spectra, according to Fig. 1.

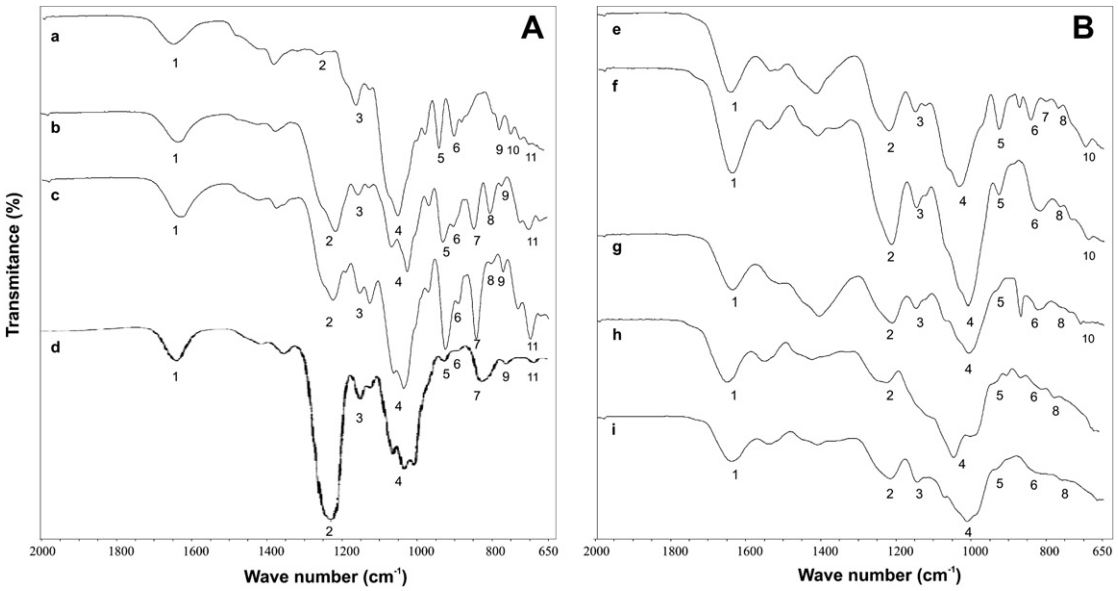

Fig. 2. Infrared spectra of (A) polysaccharide standards: (a) agar; (b) iota-carrageenan; (c) kappa-carrageenan; (d) lambda-carrageenan and red seaweed samples (B): (e) *Mastocarpus stellatus*; (f) *Gigartina pistillata*; (g) *Chondracanthus acicularis*; (h) *Nemalion helminthoides* and (i) *Dumontia contorta*. Band numbers (1–11) in FTIR spectra indicate most characteristic bands.

(Fig. 2A, d, band 5) much weaker than those of kappa- and iota-carrageenan standards.

Additionally, agar standard showed in the region 800–700 cm^{-1} two characteristic diagnostic bands of agar-type producers, according to the literature (Matsuhiro, 1996) at 790.9 cm^{-1} (Fig. 2A, a, before band 9) and 715.0 cm^{-1} (Fig. 2A, a, after band 10).

FTIR spectra of red seaweeds showed in the anomeric region, characteristic bands of the carrageenan standards used. The bands 7 and 8 in *M. stellatus* (Fig. 2B, e) were similar to those in kappa-carrageenan standard. The broad band 7 at 830–820 cm^{-1}, characteristic of lambda-carrageenan standard was found with different relative intensity in *G. pistillata*, *C. acicularis*, *N. helminthoides* and *D. contorta* (Fig. 2B, f–i). The band at 930 cm^{-1} in the spectra, corresponding to 3,6-anhydro-galactose, was more intense in *M. stellatus* than in *G. pistillata* (Fig. 2B, e–f, band 5). In a previous report on these seaweeds 3,6-anhydro-galactose was tentatively identified according to its retention time by GLC (Gómez-Ordóñez et al., 2010). Therefore, small differences in sugar content between *M. stellatus* and *G. pistillata* were reflected in FTIR-ATR spectroscopic analysis.

Also the presence of the broad band 2 associated to the sulphate ester groups of sulphated polysaccharides was characteristic in all seaweeds. The chemical composition previously studied in the red seaweeds *M. stellatus* and *G. pistillata* suggest the presence of galactans with galactose as major component (Gómez-Ordóñez et al., 2010) and sulphate (Gómez-Ordóñez, Alonso, & Rupérez, 2010). FTIR-ATR spectra obtained in the present study would confirm the presence of galactose and sulphate in both seaweeds.

None of them showed in the FTIR spectra the diagnostic bands of agar-type producers. Thus, in view of these preliminary results, all the red seaweeds tested were mainly carrageenan-type producers. Nevertheless, in order to better characterise the phycocolloids produced by the red seaweeds tested it was necessary to evaluate the second-derivative spectra.

3.2.1. FTIR second-derivative spectra of red seaweeds

Signals assigned in the FTIR second-derivative spectra of red seaweeds and commercial standards of agar, iota- and kappa-carrageenan are shown in Table 3. The second-derivative spectra showed eighteen signals only in the region 1300–650 cm^{-1}, while the parent ones only showed eleven bands (Fig. 2). Agar standard showed two diagnostic bands in the second-derivative mode in the region 800–700 cm^{-1} at 790.9 and 715.3 cm^{-1} (Table 3) characteristic of agar-type polysaccharides (Matsuhiro, 1996). These bands are usually used to distinguish between agar- and carrageenan-type galactans in red seaweeds within a few minutes (Matsuhiro, 1996).

Table 3
Signals assigned in the FTIR second-derivative spectra of red seaweeds and commercial agar and carrageenan standards.

Wave number (cm^{-1})																		
Band number[a]	2		3			5	6		7			8		9	10		11	
M. stellatus	1263.2	1216.7	1154.2	–	970.2	929.0	895.5	874.5	844.7	–	–	803.2	–	771.5	738.6	–	697.9	660.7
G. pistillata	1261.7	1212.3	1154.2	–	970.2	931.1	891.2	–	843.3	–	817.1	–	–	770.6	740.1	–	695.0	662.0
C. acicularis	1263.2	1210.9	1150.9	–	–	931.9	–	873.7	–	830.2	817.1	–	–	769.1	740.1	715.3	693.5	–
N. helminthoides	1260.3	1210.9	–	–	970.9	926.1	–	860.7	–	826.6	–	805.4	–	770.6	740.5	720.9	699.4	668.8
D. contorta	1260.3	1210.9	1151.3	984.2	–	931.9	899.9	–	850.6	834.2	814.2	–	796	766.2	741.5	710	695	668.8
Agar	1249.4	1218.3	1151.1	987.1	967.1	930.4	891.2	–	–	–	–	–	790.9	769.9	740	715.3	692.1	–
ι-carrageenan	1260.7	1210.7	1154.8	–	965.9	928.4	899.4	–	845.8	–	–	803.1	–	770.8	726.6	–	699.8	669.8
κ-carrageenan	1260.2	1220.6	1154.2	–	970.2	929.7	891.3	–	845.5	–	–	803.4	–	771.3	739.1	–	698.2	668.8

[a] In original spectra, according to Fig. 2.

M. stellatus, *G. pistillata* and *C. acicularis* belong to the Gigartinaceae family. Our spectroscopic analysis showed that the carrageenophytes studied seem to present a similar variation to that existing in other species of Cystocloniaceae, Gigartinaceae, Petrocelidaceae, Phyllophoraceae and Solieriaceae (Chopin et al., 1999; Pereira & Mesquita, 2004). The gametophyte and non-fructified stages produce carrageenans of the kappa family (hybrid kappa/iota/mu/nu-carrageenan), whereas the tetrasporophyte stages produce carrageenans of the lambda family (hybrid xi/theta- or xi/lambda-carrageenan).

Accordingly, in the case of *M. stellatus*, the presence of the bands at 929.0, 844.7 and 803.2 cm^{-1} (Table 3) revealed that this alga produces a hybrid kappa/iota-carrageenan with galactose-4-sulphate (**G4S**) and 3,6-anhydro-galactose-2-sulphate (**DA2S**). Also, in the second-derivative spectrum it is more evident the presence of the band at around 874.5 cm^{-1} (Table 3), corresponding to the existence of carrageenan precursors (μ-carrageenan: **G4S-D6S**, Table 1). Thus, *M. stellatus* produces a hybrid kappa/iota/mu/nu-carrageenan in the gametophytic growth phase.

On the other hand, *G. pistillata* and *C. acicularis*, showed the characteristic lambda-type carrageenan with a broad band in the normal spectra at 830–820 cm^{-1} (Fig. 2B, f–g). In the second-derivative mode, this broad band was divided into two sharper signals at 843.3 (**G4S**) and 817.1 cm^{-1} (**DA2S**) and at 830.2 (**G2S**) and 817.1 cm^{-1} (**DA2S**) (Table 3) in *G. pistillata* and *C. acicularis*, respectively. This may correspond with the tetrasporophytic growth phase (Correa-Diaz, Aguilar-Rosas, & Aguilar-Rosas, 1990; Pereira & Mesquita, 2004). Besides, the presence in both seaweeds of the signal with different relative intensity at around 930 cm^{-1} (**DA**), and the strong signal at 873.7 cm^{-1} in *C. acicularis* (Table 3, Fig. 2B, g), suggested the existence not only of lambda-/theta-/xi-carrageenans but also of hybrid kappa/iota/mu-carrageenans. Pereira et al. (2009) also conclude that these carrageenophytes in the North West of the Iberian Peninsula are a potential source of industrial co-polymers of kappa/iota- and xi/theta-carrageenan.

The FTIR spectrum of *N. helminthoides* (Fig. 2B, h) showed some carragenophyte bands. The weak band 7 in the original spectra gave a well defined signal at 826.6 cm^{-1} (**G2S**) and a signal at 805.4 cm^{-1} (**DA2S**) (Table 3), these bands along with the signal at 926.1 cm^{-1} (**DA**) (Table 3, Fig. 2B, h, band 5) suggested the production of theta-carrageenan type (Table 1). *N. helminthoides* polysaccharides have not been previously characterised by infrared spectroscopy. Nevertheless, a recent study about sulphated polysaccharides extracted from *N. helminthoides* has found sulphated mannans and xylomannans with the following structure: (1 → 3)-linked α-D-mannans, sulphated at positions 4 and 6 with (1 → 3) and (1 → 4) D-xylose linked residues (Pérez-Recalde, Noseda, Pujol, Carlucci, & Matulewicz, 2009). This may also correspond with our spectroscopy results. Sulphate groups at an axial position on C-4 of galactose usually can be detected in FTIR spectrum due to the band at 845–830 cm^{-1} (Chopin & Whalen, 1993). Accordingly with its FTIR spectrum it could be deduced that *N. helminthoides* produces sulphated polysaccharides, possibly carrageenan-type, with sulphated sugars on axial position (Fig. 2B, h, band 7), although the presence of sulphated mannans cannot be ruled out.

The FTIR spectrum of *D. contorta* showed some carragenophyte bands (Fig. 2B, i, bands 5, 7 and 9). The broad and weak band 7 in the parent spectrum gave well defined signal at 850.6 (**G4S**), 834.2 (**G2S**) and 814.2 cm^{-1} (**D2S,6S**) in the second-derivative (Table 3). These signals along with the weak signal at 931.9 cm^{-1} (**DA**) (Fig. 2B, i, band 5) suggested the production of mainly lambda-carrageenan type and lesser amounts of kappa-carrageenan. *D. contorta* was reported by other authors to produce lambda- and hybrid lambda-/theta-carrageenan (Chopin et al., 1999). Besides, two additional signals were found in the second-derivative spectrum in the range 800–700 cm^{-1} characteristic of agar-type producers at 796 and 710 cm^{-1} (Table 3). Some structural studies have demonstrated the co-occurrence of agaroid and carrageenan structures in several cryptonemialean algae (Takano, Iwane-Sakata, Hayashi, Hara, & Hirase, 1998). Agars differ from carrageenans as they have the L-configuration in the 4-linked galactose residue. These polysaccharides (agaroids) seem to contain both D- and L-derivatives (Takano et al., 1998) that may correspond with the bands appearing in our spectroscopic analysis.

4. Conclusions

Vibrational FTIR-ATR spectroscopy is a useful tool for a preliminary identification of the main phycocolloids (namely alginate, agar and carrageenan) produced by edible brown and red seaweeds. This technique allowed to distinguish between agar and carrageenan producers. Besides FTIR spectra provided additional information in comparative studies of carrageenan-types in red seaweeds. Moreover, certain bands of weak intensity or overlapped in the original spectra appeared much clearer in FTIR second-derivative spectra, thus increasing the information obtained and allowing the correct interpretation of the vibrational spectra. Yet further studies are necessary, along with other complementary techniques such as FT-Raman and NMR spectroscopy, in order to characterise completely these phycocolloids in seaweeds. FTIR-ATR spectroscopy has proven its suitability to be used in food and non food industries for a rapid characterisation of polysaccharides in seaweeds.

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List of abbreviations

ATR = attenuated total reflectance; FTIR = Fourier transformed infrared spectroscopy; G = guluronic acid; M = mannuronic acid; IR = infrared spectroscopy; NIR = near infrared; NMR = nuclear magnetic resonance spectroscopy; GLC = gas liquid chromatography.

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Brown and red seaweeds as potential sources of antioxidant nutraceuticals

Antonio Jiménez-Escrig · Eva Gómez-Ordóñez · Pilar Rupérez

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Abstract Multifunctional antioxidant potential of several brown and red edible seaweeds was evaluated in organic and aqueous soluble extracts. The great reduction power and radical scavenging activity of *Bifurcaria bifurcata*—a Sargassaceae brown algal species—in both organic and aqueous extracts were emphasized. In addition, two Gigartinaceae red algal species, *Gigartina pistillata* and *Mastocarpus stellatus* showed relatively high reduction power in the aqueous extracts. When all of the variables of the aqueous extracts were combined in a principal component analysis, a clear differentiation pattern among the tested seaweeds was observed. In the Phaeophyceae, the correlation found among reduction power, radical scavenging activity and total phenolic content is in favour of the involvement of phenolic compounds in the antioxidant mechanisms, whereas in the case of the Florideophyceae, the role of sulphate-containing polysaccharides in reduction power is presumably shown. Nevertheless, the evidence of some taxonomy-based clustering (class and order levels) in this study may prove that polyphenol and sulphate content, besides multifunctional antioxidant profile, are related to specific groups of seaweeds. This evidence could help the search of suitable sources of phytochemicals from seaweeds for further nutraceutical applications.

Keywords Edible seaweed · Chemometrics · Polyphenols · Sulphated polysaccharides

A. Jiménez-Escrig (✉) · E. Gómez-Ordóñez · P. Rupérez
Metabolism and Nutrition Department, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN),
Consejo Superior de Investigaciones Científicas (CSIC),
José Antonio Novais 10, Ciudad Universitaria,
ES-28040 Madrid, Spain
e-mail: a.jimenez.escrig@csic.es

Introduction

Since ancient times, brown and red seaweeds have been part of the diet in Asian countries, especially China, Japan and Korea (Nisizawa et al. 1987). In European and other Western countries, seaweeds are utilized in pharmaceutical, cosmetic and food industries as a source of hydrocolloids such as agar, carrageenan and alginate (Juanes and Borja 1991; Marinho-Soriano and Bourret 2005). Furthermore, seaweeds are traditionally used as feed for animal nutrition (Ventura et al. 1994) or as amendments to increase crop production (Khan et al. 2009). Nowadays, around 18 million tonnes (wet weight) of seaweeds and other aquatic plants are produced/harvested annually with an estimated value of US $ 5,000 million (FAO 2011); however, seaweed species are often regarded as under-exploited bioresources (Khan et al. 2009; Cardozo et al. 2007). Over the past few decades, seaweeds also have been considered as promising organisms for providing both novel biologically active substances and essential compounds for human nutrition, with high potentially economical impact in food and pharmaceutical industry and public health (e.g., Cardozo et al. 2007; MacArtain et al. 2007; Smit 2004). However, much research, such as on their role in nutrition and disease prevention, remains to be done before science-based dietary recommendations can be given for edible seaweeds (Smit 2004). In this regard, systematic research on certain nutritional and health-promoting attributes of specific seaweeds commonly consumed in several European (Denis et al. 2010) and other countries (Gressler et al. 2010) has been recently reported as a prelude to future rational economic exploitation.

Seaweeds have to survive in a highly competitive environment, and therefore, they need to develop defence strategies that result in a tremendous diversity of antioxi-

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dant compounds from different metabolic pathways such as carotenoids, phenols, minerals, sulphur compounds, vitamins, etc. (Cardozo et al. 2007). In addition, seaweeds are often located in the intertidal zones and consequently must be able, through protective antioxidant defence systems, to cope with a constantly changing environment, and fluctuations in light and oxygen (Blanchette 1997). In this context, the use of seaweeds as a source of antioxidants may be revitalized in the growing public consciousness in Western countries, regarding the health impact of consuming vegetables and fruits (MacArtain et al. 2007). Besides, consumers prefer natural to synthetic antioxidants, although safety limits of natural antioxidants are largely unknown (Pokorný 2007). However, there are few systematic studies reporting on the potential antioxidant activity of seaweeds.

In order to search for the health benefits or the efficacy of antioxidants in protecting food, the use of several *in vitro* models to screen for highly potential antioxidant activities of natural compounds could be a useful approach (Jeffery and Keck 2008). This search could be a necessary first step to select a specific antioxidant dietary source to assess more conclusive studies in cell, animal or human models (Mortensen et al. 2008). Therefore, the aim of this study was to screen for the potential antioxidant activity of several brown and red edible seaweeds commonly collected from the Northwestern Atlantic coast of Spain, by measuring the reduction power—ferric reducing/antioxidant power (FRAP)—and radical scavenging activity—2,2′-azinobis(3-ethylbenzothiazolin-6-sulphonate) (ABTS) and photochemiluminescent methods—in organic and aqueous soluble extracts. Polyphenol content, total carbohydrate, sulphate content and sulphation degree of polysaccharide were also determined. Furthermore, principal component analysis (PCA) was performed to simplify the data set and also to investigate if the parameters studied allowed grouping of the seaweeds according to their taxonomy.

Materials and methods

Nine seaweed species (Table 1) were provided by Porto-Muiños, a local food processing company (Cambre, A Coruña, Spain). Samples were collected from April 2008 to July 2009, at the intertidal zone at the Gulf of Artrabo, a marine bight formed by the Bay of A Coruña and the estuary of Ferrol and Ares in the Atlantic coastal region (latitude 43°22′ N, longitude 08°23′ W) of Galicia, Spain (Bode and Varela 1998). One of the brown seaweeds [*Saccharina latissima*] was cultivated under natural conditions. The seaweeds were rinsed with tap water to remove sand, epiphytes and encrusting material and then air-dried at 50°C. The dried samples were milled and passed through a 1-mm mesh sieve. At the laboratory, the milled seaweed samples were stored in plastic bags and kept at 2°C for further analysis.

Table 1 Taxonomy of tested seaweeds

Phylum Heterokontophyta, class Phaeophyceae	
Bifurcaria bifurcata R.Ross	Fucales, Sargassaceae
Himantalia elongata (Linnaeus) S.F.Gray	Fucales, Himanthaliaceae
Saccharina latissima (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders [formerly *Laminaria saccharina* (Linnaeus) J.V.Lamouroux)]	Laminariales, Laminariaceae
Phylum Rhodophyta, class Florideophyceae	
Chondracanthus acicularis (Roth) Fredericq	Gigartinales, Gigartinaceae
Dumontia contorta (S.G.Gmelin) Ruprecht	Gigartinales, Dumontiaceae
Gigartina pistillata (S.G.Gmelin) Stackhouse	Gigartinales, Gigartinaceae
Mastocarpus stellatus (Stackhouse) Guiry	Gigartinales, Phyllophoraceae
Nemalion helminthoides (Velley) Batters	Nemaliales, Liagoraceae
Osmundea pinnatifida (Hudson) Stackhouse	Ceramiales, Rhodomelaceae

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Preparation of algal extracts

Organic extracts Half a gram of dried algal powder was placed in a test tube; 20 mL methanol/water (50:50 *v*/*v*) plus HCl were added to obtain a final pH of 2.0. The solution was thoroughly shaken at room temperature (1 h) and centrifuged (2,500×*g*, 10 min) and the supernatant was recovered. To the residue 20 mL acetone/water (70:30 *v*/*v*) was added, and shaking and centrifugation steps were repeated. Both organic extracts were combined (Jiménez-Escrig 2007) (Fig. 1).

Aqueous extracts Half a gram of dried algal powder was placed in a test tube; 50 mL water plus HCl (0.1 M final concentration) was added. The solution was thoroughly shaken at room temperature (1 h) and centrifuged (2,500×*g*, 10 min), and the supernatant was recovered (Fig. 1).

The solid content from the organic and aqueous extracts was determined by gravimetric measurement after drying an aliquot (2 mL) at 60°C overnight. From this data, the extraction yield was calculated as gram of dry extract per 100 g of dry algal powder. Total polyphenol content was estimated in the organic and aqueous extracts. Besides, total carbohydrate and anion sulphate content were measured in the aqueous extracts as described below. Both aqueous and organic extracts were used for the determination of antioxidant activities.

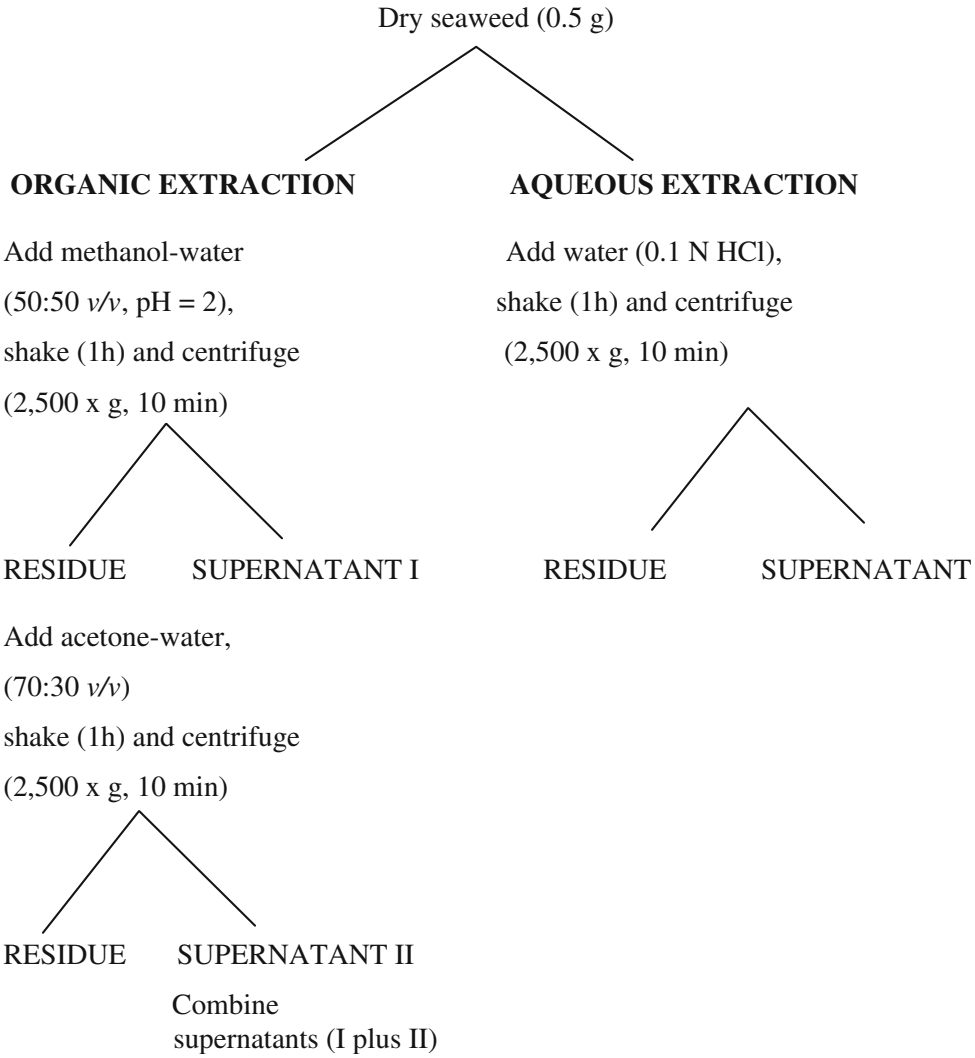

Fig. 1 Extraction procedure for the determination of bioactive compounds and antioxidant activities in selected seaweeds

Total polyphenol Total polyphenol content of the organic and aqueous extracts was determined spectrophotometrically according to the Folin–Ciocalteu procedure (Montreau 1972). A standard curve with phloroglucinol solutions (20–100 mg L^{-1}) was used for calibration (Jiménez-Escrig et al. 2001). Results were expressed as gram of phloroglucinol equivalents (PGE) per 100 g of seaweed dry extract.

Total carbohydrate Total carbohydrate content of the aqueous extracts was quantified colourimetrically according to the anthrone method (Loewus 1952) with minor modifications (Rupérez et al. 2002). A standard curve with glucose solutions (25–150 mg L^{-1}) was used for calibration. Results were expressed as gram of glucose equivalents (GE) per 100 g of seaweed dry extract.

Anion sulphate (SO_4^{2-}) Anion sulphate content in aqueous extracts from selected seaweeds was determined by an ion chromatography method previously developed for inorganic anions in seaweeds (Gómez-Ordóñez et al. 2010). A Metrohm Advanced compact ion chromatographic instrument (IC-861 model, Metrohm AG, Switzerland) controlled using Metrodata IC Net 2.3 software and attached to an Advance Sample Processor (IC-838) with an injection valve unit (IC-812) with a 20-μL sample loop was used in all analyses. The instrument was also equipped with a pump (IC-818), an eluent degasser (IC-837) and a liquid handling unit (IC-833) with a 0.45-μm filter that required a minimal volume of 10 mL for the samples. Detection was with a conductivity detector (IC-819) Advanced from Metrohm. Separation was performed in a Metrosep A Supp 5-250 column (250×4 mm, 5 μm particle size). The carrier material was an anion-exchange polymer of polyvinyl alcohol with quaternary ammonium groups. All measurements were carried out at 32°C (column temperature) under the following elution conditions: 3.2 mM sodium carbonate/1 mM sodium hydrogen carbonate at 0.70 mL min^{-1} as mobile phase. In order to

adjust the baseline to 15 $\mu S\,cm^{-1}$, 50 mM sulphuric acid solution and ultrapure water (Milli-Q) were used for automatic chemical suppression. Results were expressed as gram of sulphate equivalents (SE) per 100 g of seaweed dry extract. The degree of substitution or degree of sulphation (DS), which indicated the average number of sulphonic groups attached to each glucose residue, was calculated from the sulphur content on the basis of Schöniger's formula (Tao et al. 2006), as follows: $DS = (1.62 \times S\%)/(32 - 1.02 \times S\%)$, where S% is the sulphate percentage in the samples, 32 the sulphur atomic weight, and 1.62×100 and 1.02×100 represent the molecular weight of the sugar residue and the attached sulphonic residue, respectively.

Multifunctional antioxidant activity

Reduction power The ability of seaweed—aqueous and organic extracts—to act as reducing compounds was assessed by means of the FRAP assay (Benzie and Strain 1996). This method is based on the principle that the reduction of ferric ion (FeIII) to ferrous ion (FeII) at low pH can form a coloured ferrous–2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) complex that absorbs maximally at 595 nm. The FRAP reagent contained 2.5 mL of a 10 mmol L^{-1} TPTZ solution in 40 mmol L^{-1} hydrochloric acid, plus 2.5 mL of 20 mmol L^{-1} ferric chloride hexaaquo, plus 25 mL of 0.3 mol L^{-1} acetate buffer pH 3.6. Readings at the absorption maximum were taken every 15 s using a Beckman DU-640 spectrophotometer thermostatized at 37°C. The readings at 4 and 30 min were selected for calculation of reduction power (RP) values (Jiménez-Escrig et al. 2001). A standard curve with 6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid (Trolox) in methanol solution (2.5–200 μM) was used for calibration. Results were expressed as μmol of Trolox equivalents (TE) per g of algal dry extract.

Radical scavenging activity The ability of seaweed extracts to act as radical scavengers was tested by two assays: the ABTS decolourization assay and the automated photochemiluminescent (PCL) assay:

(a) *ABTS assay.* The analysis was performed using the ABTS decolourization protocol (Re et al. 1999) with some modifications (Sánchez-Alonso et al. 2008). ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS (66 mg) with a 2.45 mM solution of potassium persulphate (10 mL). The mixture was left in the dark at room temperature for 12–16 h before use. The $ABTS^{\cdot+}$ solution was diluted with water to an absorbance of 0.70±0.02 at 658 nm on a Beckman DU-340 spectrophotometer. Radical scavenging activity (RSA) of the seaweed extracts was measured by mixing 0.1 mL of the sample extracts with 3.9 mL of diluted $ABTS^{\cdot+}$ solution, and the absorbance reading was taken at 1 min intervals for a total of 10 min. A standard RSA curve was prepared by reacting Trolox (10–600 μM) with $ABTS^{\cdot+}$ solution. Results were expressed as μmol of TE per g of algal dry extract.

(b) *PCL assay.* The principle of PCL is based on optical excitation (UV-A light, 365 nm) of a suitable photosensitizer (luminol), an excited state aminophthalate anion is produced, followed by chemiluminescence and generation of the superoxide radical $O_2^{\cdot-}$. All radical scavenging substances that are able to react with luminol or oxygen radicals affect the light output. The photosensitized chemiluminescence was measured with the device Photochem® (Popov and Lewin 2005). This system can be used for both water-soluble and lipid-soluble antioxidative substances. In the case of water-soluble antioxidants, a standard PCL curve was prepared with vitamin C (0.5–3.0 nM). Results are expressed as μmol of vitamin C equivalents (VCE) per g of algal dry extract. In the case of lipid-soluble antioxidants, a standard PCL curve was prepared with Trolox solution (0.5–3.0 nM). Results were expressed as μmol of TE per g of algal dry extract.

Statistical analysis

Statistical analysis was carried out with the Statistical Package for the Social Sciences (SPSS 17.0). Results are expressed as mean values±standard deviation. Comparison of means of at least three independent extractions and measurements was performed by ANOVA. In order to test the null hypothesis, the significance of differences was defined at the 5% level ($P<0.05$). In addition, the multivariate analysis PCA was applied to summarize the information in a reduced number of principal components, selecting those values with eigenvalues larger than 1.0. Then, the factors were rotated using Varimax method to obtain the expected weight for each extraction factor.

Results and discussion

Extraction yield

There was a high variability in the extraction yields among different seaweed species (Table 2). Extractants also have an impact on the yield. The highest extraction yield was recorded for the water extract of *Nemalion helminthoides*,

Table 2 Yield of extraction (% *w/w* of seaweeds on a dry weight basis) with organic and aqueous solvents in selected brown and red seaweeds

Seaweeds	Organic extract	Aqueous extract
B. bifurcata	64.71 ± 0.52^{ah}	44.61 ± 1.29^{abi}
H. elongata	53.40 ± 0.79^{bh}	57.46 ± 035^{ch}
S. latissima	45.26 ± 0.48^{bh}	42.44 ± 0.22^{ah}
C. acicularis	24.25 ± 0.30^{dh}	29.26 ± 1.16^{di}
D. contorta	50.83 ± 0.72^{eh}	42.74 ± 0.54^{ai}
G. pistillata	33.39 ± 0.65^{fh}	28.59 ± 0.63^{di}
M. stellatus	34.76 ± 0.33^{fh}	44.43 ± 0.08^{abi}
N. helminthoides	42.02 ± 0.55^{gh}	68.02 ± 0.66^{ei}
O. pinnatifida	52.09 ± 0.75^{bei}	33.70 ± 0.27^{fi}

Results are expressed as mean±standard deviation (n=3). Column wise values of same letters (a–g) of this type indicate no significant difference (P<0.05) among seaweed species. Row wise values of same letters (h–i) of this type indicate no significant difference (P< 0.05) between extracts

whereas the lowest was for the organic extract of *Chondracanthus acicularis*. When comparison was made with literature values, a similar variability in yield of extraction is given in the aqueous or acetone extracts of several Icelandic seaweed species (44.7% to 10.5%) (Wang et al. 2009). In contrast, lower yield (2.85% to 5.01%) is given in methanol extracts from selected Indian red seaweeds (Ganesan et al. 2008). In our work, the extraction yield percentage could not be attributed to the brown or red type of seaweed. In the case of aqueous extracts, extraction yields were significantly correlated to the anion sulphate content in both red and brown seaweeds (P<0.058, r= 0.448; P<0.079, r=0.453, respectively). This may be explained by the relatively high solubility in water of the polysaccharides to which sulphate groups are linked. Consistently, it is described that sulphation improves the water solubility of polysaccharides in the mushroom *Ganoderma lucidum* (Liu et al. 2010).

Total phenolic content

The concentration of polyphenols in seaweeds depends on many variables such as habitat, season of harvesting and environmental conditions (light, temperature and salinity). In addition, the distribution of phenolics varies with the species (Rodríguez-Bernaldo de Quirós et al. 2010). Significant differences were found in total polyphenol content among different seaweed species (Fig. 2). The brown seaweeds showed higher total polyphenol contents than red seaweeds. Among the species studied, two Fucales, *Bifurcaria bifurcata* and *Himantalia elongata*, displayed the highest polyphenol content. This feature was in agreement with previous studies in some Phaeophyceae from Brittany coasts (Zubia et al. 2009), reporting the Fucales as those among brown seaweeds with the highest polyphenol content. Also, a higher polyphenol content in brown than in red seaweeds has been reported (Jiménez-Escrig et al. 2001). In our work, except for *S. latissima* cultured in natural conditions, brown seaweeds showed on average 4.6-fold higher total polyphenol content than red seaweeds.

The organic solvent was more efficient than aqueous extraction for polyphenolic compounds in all species tested. This was in agreement with previous studies reporting the aqueous mixtures of methanol, ethanol or acetone as the more effective extractants of polyphenol compounds (Wang et al. 2009; Koivikko et al. 2005). In this sense, acetone is shown to break down hydrogen bonds formed between phenolic and protein carboxylic groups during extraction, leading to an increase in yield of extraction (Kallithraka et al. 1995).

Total carbohydrate and anion sulphate content

Polysaccharides are generally present in the cell walls of terrestrial plants and seaweeds, showing structural, matrix or storage functions. Seaweed phycocolloids are used as thickening, stabilizing or gelling agents in food and cosmetics industry (Jiménez-Escrig and Sánchez-Muniz 2000). Marine algae are the most important source of non-animal sulphated polysaccharides. Those sulphated polysaccharides demonstrate a wide spectrum of biomedical properties, i.e. as phytochemical analogues of heparin,

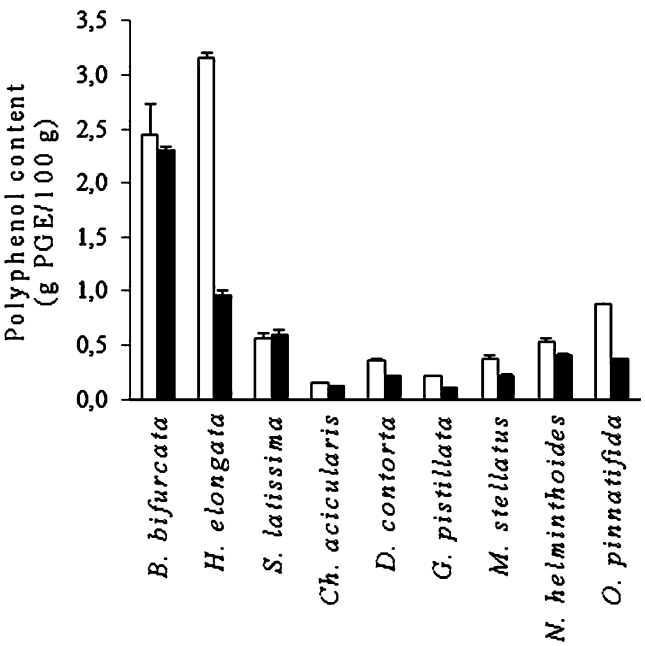

Fig. 2 Total phenolic content (g PGE 100 g^{-1}) in organic (*white*) and aqueous (*black*) extracts from selected seaweeds

which acts as an anticoagulant drug in mammalians (Preetha and Devaraj 2010) or as potential antioxidants (Rupérez et al. 2002). Total carbohydrate and sulphate content of aqueous extracts from the nine seaweeds tested is given in Figs. 3 and 4a. In addition, in Fig. 4b, the degree of substitution by sulphonic groups or degree of sulphation (DS) in each sugar residue, calculated according to Schöniger's formula (Tao et al. 2006), is shown. Interestingly, a strong correlation was found between carbohydrate, sulphate content and DS in brown seaweeds ($P<0.0001$, $r=0.829743$). In contrast, no correlation was found in red seaweeds. Therefore, it could be deduced that the distribution of sulphated polysaccharides among the six red seaweeds tested is heterogeneous.

Multifunctional antioxidant activity

The antioxidant activity of plants includes several multifunctional mechanisms. Thus, to evaluate this potential antioxidant activity, it is necessary to use several antioxidant assays that include different antioxidant mechanisms (Sánchez-Moreno 2002). A useful way of viewing the interactions among various antioxidants is to take into account oxidation–reduction potentials, measuring the reduction power (RP). Another mechanism commonly used is the radical scavenging activity (RSA). Therefore, in order to evaluate the total antioxidant activity of seaweed extracts, three different functional systems were chosen: FRAP based on the standard redox potential of Fe(III)/Fe(II) (0.77 V), ABTS and PCL assays based on the transfer of one hydrogen to the synthetic radical $ABTS^{\cdot+}$ or the biological superoxide radical $O_2^{\cdot-}$, respectively, by the tested antioxidant. These systems measure the RP of reductants with an ionization potential above 0.77 V and the RSA of antioxidants containing an RH

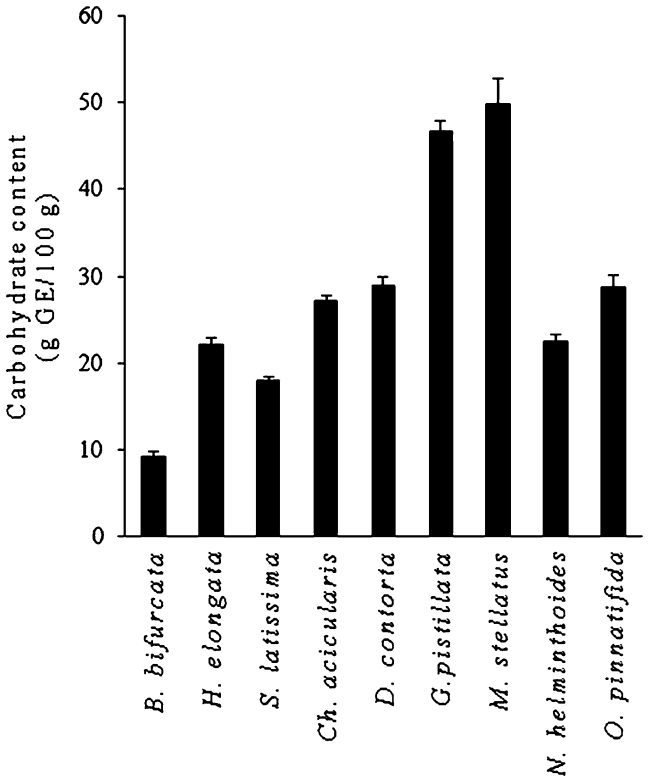

Fig. 3 Total carbohydrate (g GE 100 g^{-1}) of polysaccharides in aqueous extracts from selected seaweeds

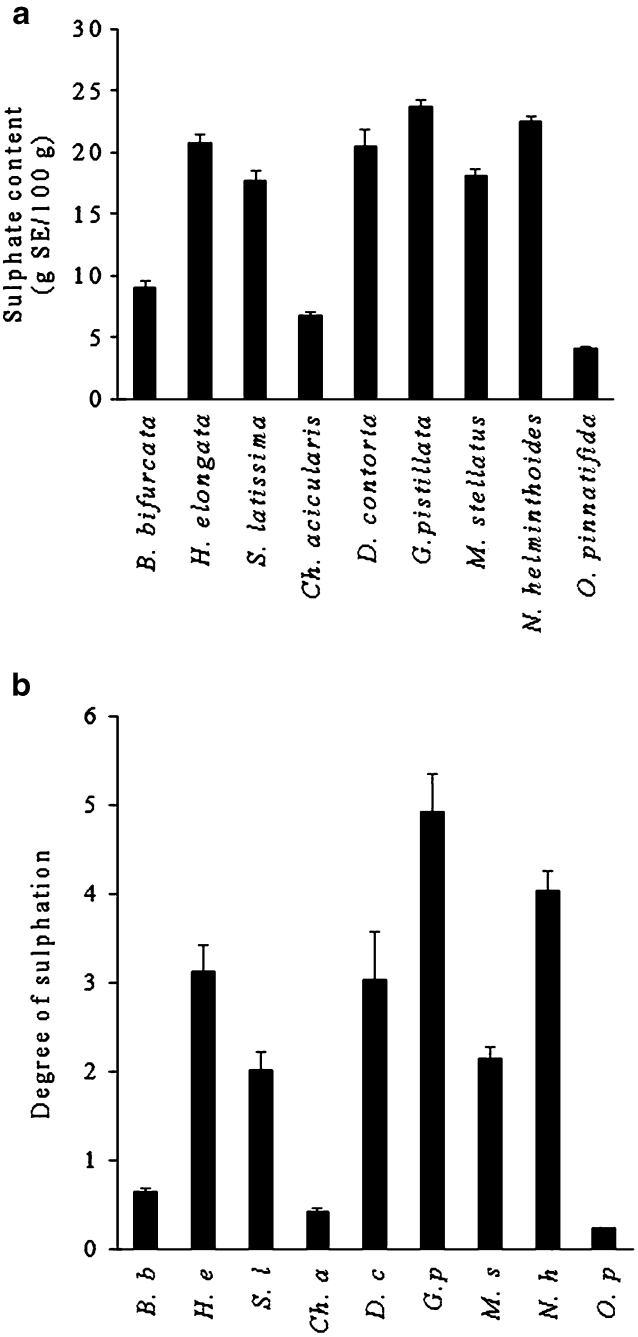

Fig. 4 **a** Total sulphate content (g SE 100 g^{-1}) and **b** DS of polysaccharides in aqueous extracts from selected seaweeds

group with an adequate enthalpy difference ($\Delta H_1 < 0$) in the scavenging reaction towards the radical (Trouillas et al. 2008).

Reduction power To study the kinetics of the organic (Table 3) and aqueous extracts (Table 4) in the FRAP assay, RP values at different times (4 and 30 min) were recorded and the ratio between 30 and 4 min (F30/F4) values was calculated. A significant difference in F30/F4 ratio between both types of seaweeds was found either in organic ($P<0.0034$), or in aqueous ($P<0.0001$) extracts. On average, F30/F4 ratio in organic extracts was 1.86±0.24 and 1.35±0.32 for brown and red seaweeds, respectively, whereas in aqueous extracts, it was 1.92±0.37 and 1.26±0.12 for brown and red seaweeds, respectively, indicating that brown seaweeds showed slower kinetics than red seaweeds for the RP assay in the organic extracts. This feature was independent of the polar nature of the extracts. Previous studies report the same characteristic of brown seaweeds in organic extracts (Jiménez-Escrig et al. 2001). The peculiar kinetics of brown seaweeds could be due to its content in polyphenols, which was relatively higher than that in red seaweeds (Fig. 2).

The RP (expressed as μm TE g^{-1}) of the organic (Table 3) and the aqueous extracts (Table 4) showed activity for all the seaweeds tested. The brown seaweed *B. bifurcata* showed the highest RP either in organic (464 μm TE g^{-1}) or in aqueous (195 μm TE g^{-1}) extracts. The second highest RP value among tested seaweed in aqueous extracts was shown by the red seaweed *Gigartina pistillata* (85.85 μm TE g^{-1}), whereas in organic extracts it was shown by the brown *H. elongata* (44.89 μm TE g^{-1}). In the case of *N. helminthoides*, the great viscosity of the aqueous extracts interfered with the measurement of the activity, and no value could be obtained. The organic extracts showed significantly higher or similar RP than the aqueous extracts for brown seaweeds, whereas the opposite tendency was shown for red seaweeds, in which the aqueous extracts of all the tested seaweeds, except for *N. helminthoides*, showed higher RP.

Reduction power versus polyphenol content *B. bifurcata* with the relatively highest polyphenol content in both tested extracts (Fig. 2) showed the highest RP value (Tables 3 and 4). In contrast, in the case of organic extracts, the brown seaweed with the highest polyphenol content (*H. elongata*) did not show the highest RP value. This could indicate qualitative differences in the polyphenol composition of *B. bifurcata* versus *H. elongata.* It is worth mentioning that literature data are controversial about relationships between polyphenol contents and antioxidant activity in seaweeds. Whereas, a positive correlation in certain seaweeds is observed (Jiménez-Escrig et al. 2001), this correlation is not systematic and clear because of the putative implication of phenol structures in the antioxidant activity and especially of polyphenol polymerization degree; an inverse correlation between polymerization degree and bioactivity in phlorotannins from seaweeds is observed (Connan et al. 2007). Apart from polyphenols, the presence of polar acyclic diterpenoids has been described in the case of *B. bifurcata* (Ortalo-Magné et al. 2005), which potential role as antioxidants in the organic extracts should not be ruled out.

Reduction power versus total carbohydrate and anion sulphate content High correlations were found among RP values and total carbohydrate ($P<0.0448$, $r=0.887091$) or sulphate ($P<0.0036$, $r=0.6820551$) content in red seaweeds. Indeed, a significant correlation between RP value and degree of substitution of sulphate in polysaccharides was found ($P<0.0768$, $r=0.837383$) in red seaweeds. The red seaweed with the highest sulphate content and DS (*G. pistillata*) showed the second highest RP among all seaweeds tested. Consistently, the possibility to enhance the RP of polysaccharides through sulphation is described. Introduction of sulphate group might enhance the electron cloud density of active hydroxyl groups and enhance the

Table 3 Multifunctional antioxidant activity of organic extracts from brown and red seaweeds

Seaweed	RP		RSA	
	4 min	30 min	ABTS	PCL
B. bifurcata	269±4^{a}	464±5^{a}	334±0.8^{a}	20.62±0.32
H. elongata	20.39±0.59^{b}	43.55±0.71^{b}	62.88±3.90^{b}	1.36±0.09^{a}
S. latissima	14.07±0.94^{c}	24.38±1.42^{c}	40.57±2.28^{c}	0.75±0.04^{b}
C. acicularis	9.42±0.82^{d}	9.61±0.79^{d}	nd	nd
D. contorta	12.34±0.53^{ec}	20.62±0.64^{e}	nd	nd
G. pistillata	21.15±1.09^{b}	29.07±1.93^{f}	nd	nd
M. stellatus	5.76±0.41^{f}	9.62±0.48^{d}	nd	nd
N. helminthoides	4.41±0.93^{f}	6.73±0.81^{g}	nd	nd
O. pinnatifida	12.56±1.02^{ec}	20.62±0.74^{e}	nd	nd

Results are expressed as mean± standard deviation ($n=3$). Column wise values of same letters (a–g) indicate no significant difference ($P<0.05$). RP, ABTS and PCL=μmol Trolox equivalent g^{-1} algal dry extract

nd not detected

Table 4 Multifunctional antioxidant activity of aqueous extracts from brown and red seaweeds

Seaweed	RP		RSA	
	4 min	30 min	ABTS	PCL
B. bifurcata	117±5.9[a]	195±13.6[a]	89.76±2.30[a]	1.80±0.13[a]
H. elongata	26.50±2.21[b]	44.89±1.20[b]	10.95±0.82[b]	1.88±1.10[a]
S. latissima	11.70±0.85[c]	22.24±1.32[c]	38.77±2.27[c]	0.32±0.02[b]
C. acicularis	7.67±0.35[c]	10.45±0.22[d]	nd	nd
D. contorta	20.43±1.78[d]	25.28±1.88[d]	nd	nd
G. pistillata	77.76±5.02[e]	85.85±5.20[e]	nd	nd
M. stellatus	44.92±2.01[f]	54.62±2.14[f]	nd	nd
N. helminthoides	nd	nd	nd	nd
O. pinnatifida	12.97±0.53[g]	22.80±1.69[c]	nd	nd

Results are expressed as mean± standard deviation (n=3). Column wise values of same letters (a–g) indicate no significant difference (P<0.05). RP and ABTS=μmol Trolox equivalent g^{-1} algal dry extract. PCL =μmol VCE g^{-1} algal dry extract

nd not detected

molecular electron-withdrawing activity, which can eliminate free radicals and terminate radical-mediated oxidative chain reactions (Liu et al. 2010). Also, it has been shown that sulphation of polysaccharides increases its RP activity in the mushroom *G. lucidum* (Liu et al. 2010). Thus, DS could modulate the relatively high RP found in the aqueous extracts of *G. pistillata*.

Radical scavenging activity

Regarding RSA, only brown seaweeds have shown activity towards the ABTS or the PCL systems in both solvent extracts (Tables 3 and 4). In general, these algae showed higher RSA in organic than in aqueous extracts. Among the nine seaweeds tested, *B. bifurcata* showed the highest RSA in the ABTS model, either in the organic (334 μm TE g^{-1}), or aqueous (89.76 μm TE g^{-1}) extracts, as in the case of RP assay. Also, the organic extracts of this seaweed showed the highest RSA in the PCL assay (20.62 μm TE g^{-1}). Consistently, *B. bifurcata* shows relatively high RSA (DPPH stable free radical) and RP among ten seaweeds tested (Zubia et al. 2009). *B. bifurcata* belongs to the Sargassaceae which have been extensively studied because of their natural diversity in bioactive compounds. In our case, no RSA was found in the aqueous extracts containing sulphated polysaccharides from red seaweeds. Algal polysaccharides, mainly from brown seaweeds, have been reported to play an important role as free radical scavengers for the prevention of oxidative damage in living organisms. This activity depends on several structural parameters, such as the type of sugar and glycosidic branching, the molecular weight, the degree and position of sulphation (Kumar et al. 2008).

The presence in the extracts of other bioactive compounds apart from polyphenols or sulphated polysaccharides, such as peptides or pigments, which might be involved in the antioxidant activity of seaweeds, could also be possible.

Principal component analysis

Principal component analysis (PCA) identifies patterns in data, expressing them in such a way as to highlight similarities and differences. Thus, PCA was carried out on the aqueous extracts to investigate the relation between the bioactive compounds and the antioxidant activities (RP) in the species tested. PCA was carried out by selecting those PCs with eigenvalues larger than 1.0; the higher eigenvalue, the higher percentage of the total variance explained. The first two components explained 84.96% of the total variance in the data set (eigenvalues=2.708 and 2.390, respectively). The first component (PC 1) accounted for 45.13% of the variance and correlated positively with all the variances except for total phenolics. The second component (PC 2) accounted for 39.83% of the total variance and correlated positively with the total phenolics and the reduction power, and negatively with the rest of the variances. Using the rotated component matrix (Table 5), we could infer that the variables related to

Table 5 Rotated component matrix for the analysis of the relation between bioactive compounds and reduction power in seven species of seaweeds

Variable	Component	
	PC 1	PC 2
Total phenolics	−0.162	0.900
Total carbohydrate	0.343	−0.833
Anion sulphate content	0.920	−0.101
Degree of sulphation	0.936	−0.147
Reduction power at 4 min	0.636	0.639
Reduction power at 30 min	0.538	0.770

Factors were extracted with the principal component analysis method using the Varimax rotation

PC principal component

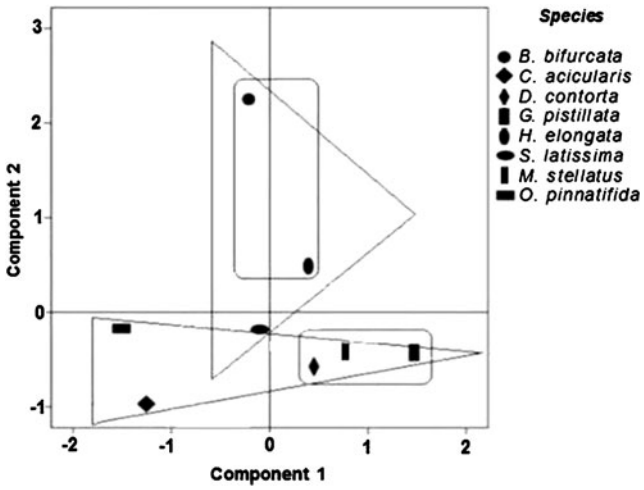

Fig. 5 PCA scatter plot using six variables in the study of the bioactivity of aqueous extracts in eight species of seaweeds. Principal component 1 accounted for 45.13% of the variance and is related to the variables related to the presence of sulphate linked to the sugar residue and to the activity of reduction power. Principal component 2 accounted for 39.83% of the variance and it is related to the polyphenol content and to the activity of reduction power. *Triangle* class: *up* Phaeophyceae, *down* Florideophyceae. *Rectangle* order: *up* Fucales, *down* Gigartinales

the presence of sulphate in the sugar residue and to the reduction power of the aqueous extracts had the highest weightings in the PC 1. Accordingly, by referring to the PCA scatter plot (Fig. 5), the red seaweeds *Dumontia contorta*, *M. stellatus* and *G. pistillata*, and the brown *H. elongata* with the highest DS, sulphate content and RP (mainly RP-4 min value) were located in the positive part of the PC 1, with *G. pistillata* located further to the right along PC 1. Consequently, this red seaweed showed the highest values among all tested seaweeds in the three variables described previously defining the PC 1. On the other hand, *C. acicularis* and *Osmundea pinnatifida*, with rather relatively low DS and sulphate content, as well as weak RP, were located on the negative/opposite site of the PC 1. In the PC 2, the highest weightings were for polyphenol content and RP (mainly PR-30 min value), showing firstly that polyphenol content was unrelated to the presence of sulphates and secondly that RP attributed to polyphenols was more related to the PR-30 value than to the PR-4 value. Accordingly, two brown seaweeds (*B. bifurcata* and *H. elongata*) were placed in the positive part of the PC 2, indicating their relatively high polyphenol content and RP value. *H. elongata* was the only seaweed among those tested placed in the positive part of both PC 1 and PC 2; this peculiar position indicated the contribution of this brown seaweed to all the variances which defined both components. Regarding *B. bifurcata*, it was placed on the top position of the PC scatter plot, specifically at the positive part of PC 2 and at the negative part of PC 1. This position is justified because this seaweed exhibited the highest polyphenol content and the highest RP (PC 2), along with a relatively low DS and sulphate content (PC 1) among all of the seaweeds tested. Plotting the scores according to their class and order drew some distinctions between the different seaweeds tested. The Phaeophyceae appeared close to the vertical axis (PC 1=0) indicating the low influence of the variables defining PC 1 (mainly sulphate content) whereas the Florideophyceae appeared on the negative side of PC 2, indicating the weak influence of the variables defining PC 2 (polyphenol and RP-30). More specifically, the brown Fucales appeared on the positive side of PC 2 indicating the strong influence of polyphenol content and RP-30 in this order, whereas red *Gigartinales* appeared mostly at the far right of the positive side of PC 1 indicating the strong influence of the sulphate content and RP-4 in this order. Other factors such as seasonal changes, exact localization, and cultural practices are likely to play important roles in defining the sulphate and phenolic content and the resulting antioxidant properties of the seaweed. Nevertheless, the evidence of some taxonomy-based clustering in this study may prove that polyphenol and sulphate content, besides multifunctional antioxidant profile, are related to specific taxa of seaweeds. The tightness of the clustering could be improved by adding more seaweed samples to these data.

In summary, this novel approach on the search of nutraceuticals, taking into account different aspects such as multifunctional antioxidant activity, polyphenol and sulphated polysaccharide content, in brown and red seaweeds, can allow a more complete picture of the potential bioactive compounds involved than the usual approaches based only on polyphenols and antioxidant properties. In general, the correlation found between marked radical scavenging activities, reduction power and total polyphenol content is in favour of the involvement of phenolic compounds in the antioxidant mechanisms in Phaeophyceae, whereas in the case of Florideophyceae, the involvement of sulphate-containing polysaccharides in the reduction power is presumably evidenced.

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Molecular weight distribution of polysaccharides from edible seaweeds by high-performance size-exclusion chromatography (HPSEC)

Eva Gómez-Ordóñez, Antonio Jiménez-Escrig, Pilar Rupérez*

Metabolism and Nutrition Department, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, E28040, Madrid, Spain

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ABSTRACT

Biological properties of polysaccharides from seaweeds are related to their composition and structure. Many factors such as the kind of sugar, type of linkage or sulfate content of algal biopolymers exert an influence in the relationship between structure and function. Besides, the molecular weight (MW) also plays an important role. Thus, a simple, reliable and fast HPSEC method with refractive index detection was developed and optimized for the MW estimation of soluble algal polysaccharides. Chromatogram shape and repeatability of retention time was considerably improved when sodium nitrate was used instead of ultrapure water as mobile phase. Pullulan and dextran standards of different MW were used for method calibration and validation. Also, main polysaccharide standards from brown (alginate, fucoidan, laminaran) and red seaweeds (kappa- and iota-carrageenan) were used for quantification and method precision and accuracy. Relative standard deviation (RSD) of repeatability for retention time, peak areas and inter-day precision was below 0.7%, 2.5% and 2.6%, respectively, which indicated good repeatability and precision. Recoveries (96.3–109.8%) also showed its fairly good accuracy. Regarding linearity, main polysaccharide standards from brown or red seaweeds showed a highly satisfactory correlation coefficient ($r > 0.999$). Moreover, a good sensitivity was shown, with corresponding limits of detection and quantitation in mg/mL of 0.05–0.21 and 0.16–0.31, respectively. The method was applied to the MW estimation of standard algal polysaccharides, as well as to the soluble polysaccharide fractions from the brown seaweed *Saccharina latissima* and the red *Mastocarpus stellatus*, respectively. Although distribution of molecular weight was broad, the good repeatability for retention time provided a good precision in MW estimation of polysaccharides. Water- and alkali-soluble fractions from *S. latissima* ranged from very high (>2400 kDa) to low MW compounds (<6 kDa); this high heterogeneity could be attributable to the complex polysaccharide composition of brown algae. Regarding *M. stellatus*, sulfated galactans followed a descending order of MW (>1400 kDa to <10 kDa), related to the different solubility of carrageenans in red seaweeds. In summary, the method developed allows for the molecular weight analysis of seaweed polysaccharides with very good precision, accuracy, linearity and sensitivity within a short time.

1. Introduction

Seaweeds are a rich and easily renewable source of structurally and functionally unique polysaccharides, which are increasingly used as natural thickeners, formulation stabilizers, or gelling agents in applications ranging from food industry to pharmaceutics [1]. For brown seaweeds, soluble polysaccharides are alginates, fucoidans and laminarans. For red seaweeds, soluble polysaccharides are sulfated galactans, agar and carrageenans [2,3].

Alginate is the salt of alginic acid, a gelling polyuronide, composed of mannuronic (M) and guluronic (G) acid [4]. Laminarans and fucoidans are considered as the main water-soluble polysaccharides of brown algae. Laminaran is the principal storage polysaccharide of brown seaweeds and is composed of β-glucan [5,6]. Fucoidans, a unique class of sulfated fucans isolated from many brown seaweeds, have not been found in other algae or plants [7]. Their composition varies with the species and the precise structural characteristics have not yet been elucidated, but essentially they always contain fucose and sulfate [8,9], with small proportions of galactose, xylose, mannose and uronic acids [6,10]. Carrageenan is a generic name given to a family of high-molecular-weight

Abbreviations: GPC, gel permeation chromatography; GFC, gel filtration chromatography; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; LOD, limit of detection; LOQ, limit of quantitation; MW, molecular weight; *r*, correlation coefficient; RI, refractive index; RSD, relative standard deviation; SEC, size-exclusion chromatography.

* Corresponding author. Tel.: +34 915445607; fax: +34 915493627.
E-mail addresses: pruperez@ictan.csic.es, pruperez@if.csic.es (P. Rupérez).

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sulfated polysaccharides isolated from red seaweeds, made up of repeating α-(1,3)-galactose and β-(1,4,3,6)-anhydro-D-galactose alternating units [6,11,12]. Carrageenans are classified in three industrially relevant types (κ-, λ- and ι-carrageenans), which differ in the amount and position of their ester sulfate substitutes and (3,6)-anhydrogalactose content.

These water soluble biopolymers possess important pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anticomplementary, anti-inflammatory, antiviral, antipeptic and antiadhesive activities [13–17]. The relationship between structure and biological activities of algal polysaccharides is not yet clearly established. Usually, water solubility, average molecular weight, chain conformation, and introduction of suitable ionic groups with appropriate degree of substitution can change the bioactivities of polysaccharides [18]. For example, anticoagulant activity of sulfated fucans increases with molecular weight [7,19]. As molecular weight plays an important role on the relationship between structure and function of polysaccharides, it is important to develop a simple but effective method for the molecular weight determination of soluble algal polysaccharides.

Several ways of measuring molecular weight of polysaccharides have been reported in the literature, such as size-exclusion chromatography (SEC), light scattering, sedimentation analysis in analytical ultracentrifugation and intrinsic viscosity [20]. Light scattering and analytical ultracentrifugation methods are reliable and provide an absolute value of molecular mass. The intrinsic viscosity method is easy to operate however it relies on the measurements of viscosity at low sample concentration with consequent difficulties in accurate measurements of low viscosity samples [20]. SEC is the most widely used method for the molecular characterization of polymers in general. The method appeared in the late 1950s and was named gel permeation chromatography (GPC) [21] or gel filtration chromatography (GFC) [22]. It provides information on relative or absolute molecular mass distribution depending on the standards used for calibration. Pullulans and dextrans are the most widely used standards for this purpose. Further breakthroughs in SEC have been the development of online detectors, especially light-scattering detectors [23] and viscometers. Light-scattering detectors yield absolute molecular weights (i.e., no need for a calibration curve) through low-angle laser light scattering, multi-angle laser light scattering, or triple detection [24]. Viscometers enable the determination of molecular weight using a universal calibration curve [24]. And more recently mass spectrometers detectors [24] with the great advantage of providing absolute value of molecular mass without the need for standards or other auxiliary measurements.

The HPSEC system used in this research employs refractive index (RI) detection. This is the most traditional and universal detector for SEC, and has a great advantage for the analysis of polymers since the signal is directly proportional to the polymer concentration (mainly in the case of homopolymers), thus providing an estimation of the molecular weight distribution of the polymer [25]. Moreover, the use of a polymer-based TSK-GEL PW column, more suitable for analyzing water-soluble polymers, gave us the advantage of using aqueous solvents, with fast and easy sample preparation. The column was calibrated with the pullulan and dextran molecular weight standards and commercially available polysaccharide standards of seaweeds, such as, sodium alginate, laminaran, fucoidan and carrageenan were used for the validation of the method. The method was then applied for the molecular weight estimation of soluble polysaccharide fractions from the brown seaweed *Saccharina latissima* and the red *Mastocarpus stellatus*, respectively.

In order to characterize better the relationship between structure and biological activities of algal polysaccharides, our aim was to develop and optimize a simple, reliable and fast HPSEC method with refractive index detection for determining the MW distribution of soluble polysaccharides from brown and red seaweeds.

2. Material and methods

2.1. Reagents and standards

Ultrapure water from Millipore Milli-Q (18.2 MΩ cm, equipped with a Millipack: 0.22 μm filter) was used for the preparation of solutions and mobile phase.

Standards of different molecular mass were used to perform the calibration curves. A Shodex pullulan standard P-82 kit (range of molecular weights in kDa: P-800 = 788, P-400 = 404, P-200 = 212, P-100 = 112, P-50 = 47.3, P-20 = 22.8, P-10 = 11.8, P-5 = 5.9; Showa-Denko, Japan) was obtained from Waters Chromatography, S.A. (Madrid, Spain). Dextran standards: blue dextran-2000, T-500, T-70 and T-10 with molecular mass ranging from 2000 to 10 kDa were obtained from Pharmacia Biotech Europe GmbH (Barcelona, Spain).

Commercial standards of seaweed polysaccharides, fucoidan (from *Fucus vesiculosus* F-5631), sodium alginate (D-7924), laminaran (from *Laminaria digitata* L-9634), carrageenan (type I, C-1013) and iota-carrageenan (type II, C-1138) were obtained from Sigma–Aldrich Chemicals (Alcobendas, Madrid, Spain). Each of these standards was separately injected at 0.5, 1.0 and 2.0 mg/mL (in ultrapure water solution) for the HPSEC analysis.

2.2. Instrument and chromatographic conditions

The HPLC system was equipped with the following instruments: Kontron autosampler 360, Agilent quaternary pump system 1200 Series with online degasser, Agilent differential refractometer 1100 Series (RI detector), Jones chromatography thermostatic oven, Agilent HPLC control unit 1100 Series (console table) and Kontron Data System 450-MT2.

The separation was performed on a TSK-Gel G 5000 PW stainless steel column (300 × 7.5 mm i.d.) with a TSK-Gel PWH guard column (75 × 7.5 mm i.d.) from TosoHaas (Tecknokroma, Barcelona, Spain). The column was eluted isocratically either with ultrapure water or 0.1 M sodium nitrate (vacuum-filtered through 0.45 μm and degassed), at 40 °C with a flow rate of 0.8 mL/min. Standards and polysaccharide fractions were filtered through 0.45 μm (cellulose acetate filters, 25 mm diameter, Análisis Vínicos, Tomelloso, Toledo, Spain) for aqueous samples and injected (50 μL) into the HPLC. Under the conditions used chromatographic runs took less than 15 min.

2.3. Validation of proposed method

2.3.1. Standard curves of polysaccharide for molecular weight estimation

Series of pullulan standards (788, 404, 212, 112, 47.3, 22.8, 11.8 and 5.9 kDa) and dextran standards (2000, 500, 70 and 10 kDa) were used to calibrate the system. Each of these standards was separately injected at 0.5, 1.0 and 2.0 mg/mL (in ultrapure water solution) in triplicate ($n = 9$). A standard calibration curve for the logarithm of the molecular weight versus the HPSEC retention time was obtained for each series of standards.

2.3.2. Standard curves of commercial polysaccharides for quantification

The external standard calibration method was based upon standard solutions of commercial polysaccharides from brown and red seaweeds, namely: alginate, fucoidan, laminaran, kappa-carrageenan and iota-carrageenan. Calibration curves (peak area versus concentration expressed in mg/mL) were prepared at 0.5, 1.0

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and 2.0 mg/mL. These solutions were separately injected in triplicate ($n = 9$). The regression curves of each polysaccharide separated according to its molecular weight were obtained. The correlation coefficients, slopes, intercepts and standard deviations of the curves were used to determine the linearity and limits of detection (LOD) and quantitation (LOQ).

2.3.3. Precision

The precision of the method was assessed in terms of repeatability and intermediate (inter-day) precision. Repeatability was expressed as relative standard deviation (RSD) percent of individual measurements of three replicate determinations on the same day and three determinations on three different days. Standard solutions of commercial polysaccharides at 2 mg/mL were used. The RSD (%) values of the results corresponding to retention time and peak area were determined.

2.3.4. Accuracy

To study the reliability and suitability of the HPSEC method, recovery experiments were carried out. The accuracy was determined as percent ratio of the commercial polysaccharide concentration calculated from the calibration line versus nominal polysaccharide concentration at three concentration levels (0.5, 1.0, 2.0 mg/mL) on three replicate measurements. Accuracy was acceptable when the determined concentration (% recovery) reached from 85% to 115% of nominal concentration.

2.4. Algal material

The brown seaweed sweet Kombu [*Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, formerly *Laminaria saccharina* (Linnaeus) J.V. Lamouroux)] and the red seaweed *Mastocarpus stellatus* (Stackhouse) Guiry were obtained from a local supplier (Porto-Muiños, Cambre, Coruña, Spain). Marine algae were cleaned from epyphytes and sand, washed with tap running water, dried and milled before analysis.

2.5. Sequential extraction of polysaccharide fractions from seaweeds and preparation for HPSEC analysis

The procedure was based on the different solubility of the polysaccharides from seaweeds, as previously reported [26]. Briefly, a dry algal sample (5 g) was extracted with distilled water (500 mL) with constant stirring at 22 °C for 1 h [fraction 1, (F1)]; then the insoluble residue was again extracted with water (500 mL) at 60 °C for 1 h [fraction 2, (F2)], to remove the bulk of water-soluble polysaccharides. The water-insoluble residue was then sequentially extracted with 0.1 M HCl (500 mL) [acid-soluble fraction 3, (F3)] and with 2 M KOH (500 mL) [alkali-soluble fraction 4, (F4)], each treatment at 37 °C for 16 h. After each extraction step a soluble fraction was obtained by centrifugation (12,000× *g*, 30 min). The final insoluble residue obtained was washed with 2 M HCl until neutral pH, exhaustively dialyzed (dialysis tubing size 9/28.6 mm, molecular weight cut-off 12–14 kDa, Medicell International Ltd., London) against running tap water (7 L/h) at 25 °C for 48 h and then freeze-dried [fraction 5, (F5)]. Thus, by this procedure four polysaccharide soluble fractions (F1–F4) and a final polysaccharide insoluble fraction (F5) were obtained from *S. latissima* and *M. stellatus* (yields: 2352.9 mg and 2947.5 mg, respectively).

The pH of the acid- (F3) and alkali-soluble (F4) fractions was adjusted to 5.5, either with 2 M KOH or with concentrated HCl, as appropriate. Each soluble fraction (F1 to F4) was evaporated to half-volume in a R-114 Buchi vacuum rotatory evaporator with a B-480 Buchi water bath and temperature not exceeding 50 °C. The concentrates were exhaustively dialyzed, against water either for 48 h or until conductivity (μS/cm) of water in the dialysis tank was equal to that of tap running water as measured with a portable conductimeter (Myron L Company, Model EP Meter). The dialyzed solutions were kept at −20 °C.

A portion of soluble fractions (F1 to F4) from *S. latissima* and *M. stellatus* were filtered through 0.45 μm filters just before injection. Average molecular weight of polysaccharide peaks in commercial standards and fractions were calculated by comparison of their retention time with the calibration curves of pullulans and dextrans. Peak areas were utilized for quantitative analysis.

3. Results and discussion

3.1. HPSEC method development

A major topic for method development in aqueous size-exclusion chromatography is mobile phase selection. Specifically, a usual aspect to be solved is the interaction between solvent and column packing. Ionic interactions between the sample and column packing are reduced with addition of salt, 0.1 M $NaNO_3$ is often added as an electrolyte to suppress ion exclusion, and is preferred to NaCl since the latter is corrosive. An addition of salt provides an ionic strength that adds degree of reproducibility to the system [27], especially when analysing unknown samples that can possibly contain ionic species, as alginate and carrageenan polysaccharides of seaweeds. The effect of ionic strength of mobile phase on the elution of alginate has been investigated [20]. Better reproducibility and peak shape is observed when a low pH and high ionic strength buffer is used as mobile phase [20]. In order to achieve the best separation and resolution of peaks related to the ionic interactions between the sample and column packing of ionic species, two different mobile phases were investigated: (1), ultrapure water and (2) 0.1 M $NaNO_3$. Same retention time and peak shape were observed for pullulan and dextran standards either with ultrapure water or 0.1 M $NaNO_3$ as mobile phase.

Main charged polysaccharides from brown and red seaweeds, such as alginates, fucoidans and carrageenans, were analysed in the current study. In this case, the ionic repulsion between the anionic molecules and the sorbent of the stationary phase caused early elution when using ultrapure water. This is due to inter- and intra-molecular ionic repulsions that tend to expand their size [20]. Also chromatograms of abnormal shapes were observed and the repeatability of retention time of the chromatograms was poor with ultrapure water as mobile phase. Fig. 1 shows the chromatograms obtained by using either ultrapure water (Fig. 1A) or 0.1 M $NaNO_3$ (Fig. 1B) as mobile phase for a carrageenan sample. In view of these preliminary results, hereafter 0.1 M $NaNO_3$ was selected as mobile phase and used for the validation of the method. Thereby the HPSEC method was optimized and the separation and resolution of the chromatograms was improved.

3.2. HPSEC method validation

3.2.1. Linearity and sensitivity

The linearity of the method was calibrated using pullulan and dextran standards of different molecular weight. Table 1 shows retention time means and relative response factors of the standards and the calibration curve equations obtained. The calibration curves of pullulans and dextrans were plotted as the molecular weights on a log scale versus the retention time. The absolute response factor of the pullulan P-100 (112 kDa) and the dextran T-500 (500 kDa) were chosen to obtain the relative response factor of pullulans and dextrans, respectively (Table 1). Repeatability of retention time, expressed as mean values ± standard deviation, provided a good precision in the molecular weight estimation. Pullulan and dextran calibration curves showed good linearity

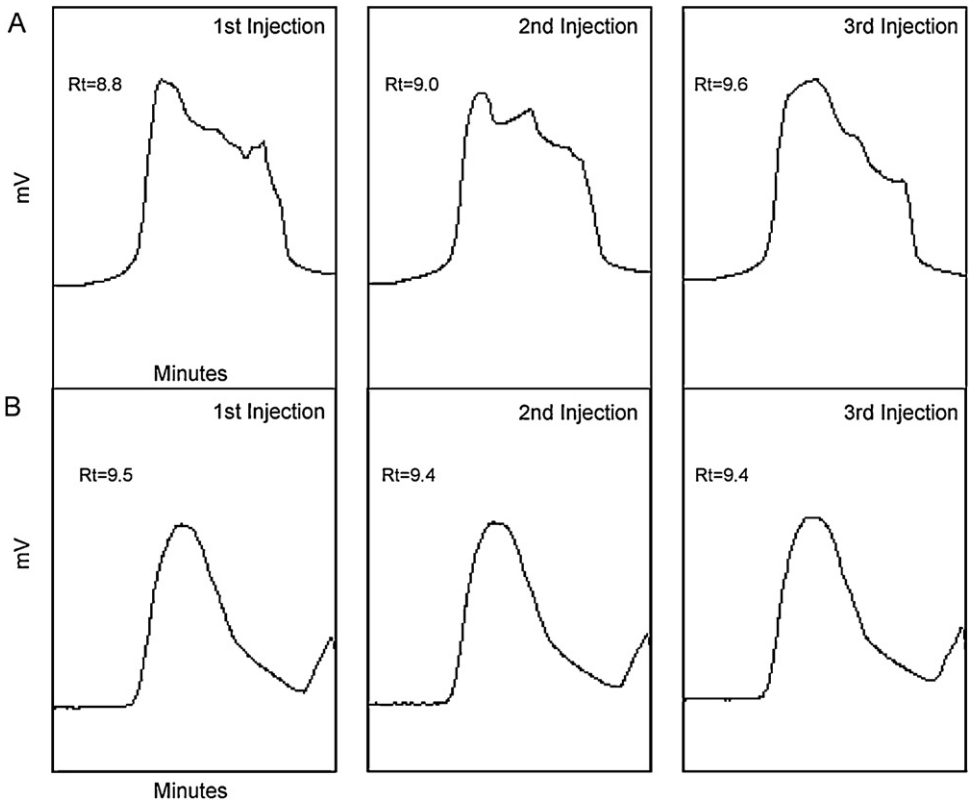

Fig. 1. Comparison of different chromatogram runs obtained from consecutive injections of Kappa-carrageenan standard with ultrapure water (A) or 0.1 M $NaNO_3$ (B) as mobile phase and refractive index (RI) detection.

(coefficient of correlation, $r = 0.9966$ and $r = 0.9933$, respectively) in the range of 5.9–2000 kDa. Although both calibration curves provided a good precision in the MW estimation just the closest one to the retention time of each peak was used for the optimum estimation of the MW.

The linearity of the calibration curve for quantification was evaluated by analyzing various concentrations of commercial brown and red seaweed polysaccharides. The regression curves of each commercial brown or red seaweed polysaccharide showed a satisfactory correlation between concentration (x, in mg/mL) and peak area (y) with $r > 0.999$ within the range in which the curves were established (0.5–2 mg/mL). The results are summarized in Table 2. Also, LOD and LOQ values achieved for the HPSEC method developed are presented in Table 2. The method showed to be sensitive enough for the determination of soluble polysaccharides from seaweed with LOD and LOQ values between 0.05–0.21 mg/mL and 0.16–0.31 mg/mL, respectively.

3.2.2. Precision and accuracy

Main polysaccharide standards from brown (alginate, fucoidan, laminaran) and red seaweeds (kappa- and iota-carrageenan) were used to calculate the precision and accuracy of HPSEC method.

The RSD (%) of repeatability of retention time was below 0.7% and inter-day RSD (%) was below 2.6%, which indicated that the method has both good repeatability and inter-day precision (Table 3). Also assays of repeatability for peak areas were carried

Table 1
Retention time, relative response factors and calibration curve equations of the polysaccharide standards by HPSEC method.

Standard	Grade	MW[a] (kDa)	Retention time (min)[e]	Relative response factor	Calibration curve
Pullulan	P-800	788	9.6 ± 0.0	1.10	$y = -0.46x + 7.333$
	P-400	404	10.2 ± 0.1	1.04	$r = -0.9966$
	P-200	212	10.9 ± 0.0	1.01	
	P-100	112[b]	11.6 ± 0.0	1.00	
	P-50	47	12.4 ± 0.0	1.10	
	P-20	22	13.1 ± 0.0	1.08	
	P-10	11.8	13.6 ± 0.0	1.03	
	P-5	5.9	14.0 ± 0.0	1.01	
Dextran	Blue dextran	2000	8.1 ± 0.2	0.95	$y = -0.392x + 6.512$
	T-500	500[c,d]	11.1 ± 0.0	1.00	$r = -0.9933$
	T-70	70	12.3 ± 0.0	1.04	
	T-10	10	13.8 ± 0.0	1.08	

x = retention time (min); y = log MW; r = correlation coefficient.
[a] MW according to the manufacturer specifications.
[b] Reference standard for response factors of pullulans.
[c] Reference standard for response factors of dextrans.
[d] This standard was not included in the calibration curve.
[e] Data are mean values ± standard deviation with $n = 9$.

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Table 2
Linearity, sensitivity, and detection and quantitation limits of polysaccharide standards from seaweeds by HPSEC method.

Seaweed polysaccharide	MW (kDa)	Linearity (r)	Sensitivity[a]		LOD (mg/mL)	LOQ (mg/mL)
			Slope (m)	Intercept (b)		
Alginate	213–277	0.9997	13.85	−0.37	0.07	0.16
Fucoidan	105–117	0.9986	9.87	−0.17	0.10	0.28
Laminaran	7.3–7.6	0.9996	16.27	−2.74	0.21	0.31
Kappa-carrageenan	917–1124	0.9994	9.13	0.44	0.05	0.17
Iota-carrageenan	944–1626	0.9984	9.72	1.15	0.08	0.28

LOD = limit of detection; LOQ = limit of quantitation.
[a] Linear equations: $y = mx + b$; y = area (mV min); x = concentration (mg/mL); range = 0.5–2 mg/mL.

out and RSD (%) values were less than 2.5% (Table 3) showing also a good precision for the analytical method.

Values of recovery percentage for the method ranged between 96.3% and 109.8% (Table 3) showing the fairly good accuracy of the determination.

3.3. Molecular weight distribution of alginate, fucoidan, laminaran and carrageenan

The good precision for retention time of commercial soluble polysaccharides provided a good precision in the molecular weight estimation. Each polysaccharide was injected in triplicate at three concentrations ($n = 9$) and estimation of MW was obtained from their retention time using the calibration curves of pullulan or dextran. Then the MW range was calculated using the formula: MW range = MW mean ± CV (coefficient of variation). The molecular weight distribution in all polysaccharides exhibited a broad distribution. Thus, sodium alginate presented a molecular weight range of 213–277 kDa (Tables 2 and 3). This MW confirms previous results obtained by other authors. High-molecular mass alginic acids extracts from *Macrocystis pyrifera* and *Lessonia nigrescens* have a molecular weight interval of 146–264 kDa and 177–290 kDa, respectively [28].

Commercial fucoidan polysaccharide presented a molecular weight interval of 105–117 kDa (Tables 2 and 3). Some researchers have measured fucoidan's molecular weight at approximately 100 kDa, others have observed a molecular weight range from 638 to 1529 kDa [5], while our group has measured in *Fucus vesiculosus* a major fraction of 1600 kDa [26]. Results by Rupérez et al. [26] were obtained on the same column and chromatographic conditions as currently with ultrapure water as mobile phase, probably causing an early elution of fucoidan sample, due to inter- and intramolecular ionic repulsions, as previously discussed (in Section 3.1).

Laminaran showed a range between 7.3 and 7.6 kDa (Tables 2 and 3). This was a little bit higher than the molecular weight previously reported for laminarans, usually within 3–6 kDa [29]. In the case of carrageenans a higher molecular weight (917–1626 kDa) was obtained (Tables 2 and 3). Commercially available food-grade carrageenans can have a MW distribution ranging from 400 to 600 kDa with a minimum of 100 kDa [30]. Also, some carrageenans can possess a molecular weight of 990 kDa [30].

3.4. Analysis of soluble polysaccharide fractions from seaweeds

3.4.1. Saccharina latissima

The procedure of extraction was based on the different solubility of polysaccharides from brown seaweeds [26]. Solubility of alginates can be influenced by factors such as pH, concentration, ions in solution, the presence of divalent ions and ionic force [18] although solubility of laminarans is influenced by the degree of branching. Thus, highly branched laminaran is soluble in cold water, and can be extracted at 22 °C (F1) whereas lower levels of branching induce solubility only in warm water (60 °C, F2) [26]. Fucoidans were extracted with diluted hydrochloric acid (F3), whereas alginates were extracted with potassium hydroxide (F4).

Retention time and molecular weight values obtained in F1, F2 and F4 are presented in Table 4. The four peaks in F1 and F2 and the triple peak in F4 indicated that soluble fractions contained either four or three components respectively, with different molecular weight eluting between 7.5 and 15 min, approximately. The molecular weight of F3 could not be determined due to the low yields of extraction (0.5% algal dry weight), which concentration was below fucoidan's quantitation limit (data not shown). Peak No. 1 was observed in all fractions with an average molecular weight of 2111–2428 kDa, which corresponded to the lowest area percentage: 5–6 in F1, F2 and 2.5 in F4 (Table 4).

The water soluble fractions (F1 and F2) had high heterogeneity on molecular weight. Peak No. 2 in F1 and F2 presented a molecular weight distribution of 310–433 kDa and peak No. 3 showed a 20–27 kDa molecular weight. Finally, peak No. 4 with a molecular weight distribution between 5 and 5.8 kDa showed the highest concentration (mg/mL). Peaks No. 3 and 4 amounted to more than 50% of total area in the water-soluble fractions. Peak No. 4 could be tentatively identified as laminaran, a low-molecular weight polysaccharide with a MW within 3–6 kDa [29]. Related to peak No. 3, some authors have obtained from various algae, water-soluble polyuronans with a low-molecular weight (30–40 kDa), 5–10 time lower than the typical high-molecular weight alginic acids [28]. According to our previous work, the main polysaccharide found in *S. latissima* is alginate (uronic acids) [3,31], so it is also possible to find polyuronans with a lower molecular weight in the water-soluble fractions (peak No. 3, Table 4). Shevchenko et al. also found a poly(mannuronic acid) with a molecular weight range of 20–50 kDa

Table 3
Repeatability of retention time and peak area, MW estimation and recovery of polysaccharide standards from seaweeds by HPSEC method.

Seaweed polysaccharide	Repeatability ($n = 3$)		Inter-day precision ($n = 9$)		Estimation MW (kDa)	Repeatability ($n = 3$)		Recovery (%)
	Retention time (min)	RSD (%)	Retention time (min)	RSD (%)		Peak area	RSD (%)	
Alginate	10.8 ± 0.0	0.1	10.8 ± 0.1	1.1	213–277	27.4 ± 0.1	0.5	101.1 ± 0.4
Fucoidan	11.4 ± 0.0	0.2	11.4 ± 0.1	0.6	105–117	19.5 ± 0.2	0.9	96.3 ± 2.3
Laminaran	14.0 ± 0.0	0.0	14.0 ± 0.0	0.1	7.3–7.6	29.9 ± 0.1	0.2	101.3 ± 0.2
Kappa-carrageenan	9.4 ± 0.1	0.7	9.4 ± 0.1	1.0	917–1124	18.6 ± 0.1	0.3	109.8 ± 0.3
Iota-carrageenan	9.3 ± 0.0	0.3	9.2 ± 0.2	2.6	944–1626	20.5 ± 0.5	2.5	102.1 ± 1.1

Data are mean values ± standard deviation. Peak area = mV min; RSD = relative standard deviation.

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Table 4
Yield (%), repeatability of retention time, MW estimation and amount of soluble polysaccharide fractions from the brown seaweed *S. latissima* by HPSEC method.

Fraction	Yield (%)	Peak No.	Retention time (min)	MW (kDa)	Area account (%)	Amount (mg/mL)
F1	9.6	1	8.1 ± 0.0	2111–2190	5.6 ± 0.4	0.09 ± 0.0
		2	10.4 ± 0.0	338–351	27.9 ± 2.1	0.49 ± 0.1
		3	12.9 ± 0.1	23–27	35.3 ± 2.4	0.28 ± 0.1
		4	14.3 ± 0.0	5.7–5.8	31.5 ± 0.7	0.62 ± 0.0
F2	5.0	1	8.0 ± 0.0	2269–2414	5.9 ± 0.6	0.05 ± 0.0
		2	10.4 ± 0.2	310–433	38.8 ± 4.6	0.23 ± 0.0
		3	13.1 ± 0.1	20–22	29.7 ± 1.6	0.22 ± 0.0
		4	14.4 ± 0.0	5.0–5.1	28.0 ± 8.9	0.30 ± 0.1
F4	25.0	1	8.0 ± 0.0	2228–2428	2.5 ± 0.6	0.04 ± 0.0
		2	12.4 ± 0.1	43–49	62.9 ± 1.1	0.40 ± 0.1
		3	14.0 ± 0.0	7.4–7.9	35.7 ± 1.4	0.11 ± 0.0

Mean value of three determinations ± SD. Yield (%) by gravimetry (g/100 g dry weight); F1, soluble in water at 22 °C; F2, soluble in water at 60 °C; F4, soluble in 2 M KOH at 37 °C.

[29]. As well, peak No. 2 could be related to high-molecular weight alginates (>300 kDa) or to water-soluble fucoidans [26].

The main peak in the alkali-soluble fraction (F4), presented a molecular weight interval of 43–49 kDa (Table 4). This may also correspond to alginates which are the main alkali-soluble polysaccharides in brown seaweeds [26,28]. Also a peak with a molecular weight around 8 kDa was found in this fraction (Table 4).

This alga and its soluble fractions exhibited high heterogeneity on molecular weight, which could be related to the heterogeneous polysaccharide composition (laminaran, fucoidan and alginate) of brown algae, as well as to their extraction process. It is known that molecular weight of laminarans, fucoidans and alginates in brown algae also depend on season of collection [32]. Further research work is necessary to completely characterize polysaccharides in these soluble fractions, in order to fully comprehend the relationships between structure and function. Nevertheless, the proposed method was very useful for determining the molecular weight distribution of soluble polysaccharides in *S. latissima* and could be extended to other brown seaweeds.

3.4.2. *Mastocarpus stellatus*

The same procedure of extraction used in *S. latissima* was applied to this red alga. *M. stellatus* belongs to the Gigartinaceae family, which members are mainly carrageenophytes. According to our previous work, the main polysaccharide in *M. stellatus* is a hybrid kappa-/iota-carrageenan [31], a sulfated galactan [3]. Retention time and molecular weight values estimated in *M. stellatus* fractions are presented in Table 5. Each fraction exhibited a single peak, with a broad molecular weight distribution (Table 5). It is interesting to note that the molecular weight distribution of the soluble polysaccharides in *M. stellatus* goes from the highest to the lowest values through the process of sequential extraction. This behaviour is explained because the polymerization degree of algal galactans varies significantly and is highly dependent on the extraction conditions [33]. Thus, through the process of sequential extraction, the degree of polymerization became lower, giving as a result lower molecular weight distributions. Mild-acid hydrolysis is a commonly used method to obtain low-molecular weight fractions of carrageenans [34]. This was consistent with F3, the acid-soluble fraction that showed a lower average molecular weight than the water-soluble fractions (F1, F2) (Table 5).

Table 5
Yield (%), repeatability of retention time, MW estimation and amount of soluble polysaccharide fractions from the red seaweed *M. stellatus* by HPSEC method.

Fraction	Yield (%)	Retention time (min)	MW (kDa)	Amount (mg/mL)
F1	6.7	8.6 ± 0.1	1248–1425	1.1 ± 0.0
F2	14.4	9.2 ± 0.2	707–983	3.8 ± 0.1
F3	25.5	13.2 ± 0.0	17–134	4.7 ± 0.1
F4	12.1	14.1 ± 0.0	8–10	2.3 ± 0.0

Mean value of three determinations ± SD. Yield (%) by gravimetry (g/100 g dry weight); F1, soluble in water at 22 °C; F2, soluble in water at 60 °C; F3, soluble in 0.1 M HCl at 37 °C; F4, soluble in 2 M KOH at 37 °C.

Commercial carrageenans have a molecular weight distribution ranging from 400 to 600 and 900 kDa. In the case of seaweed samples, the molecular weight distribution also varies from sample to sample, depending upon the sample history, e.g., age of the harvested seaweed, season of harvesting, way of extracting and length of heat treatment [30,33]. This heterogeneity may cause that different molecular weight was obtained by different processes of sequential extraction. As well as for the brown seaweed *S. latissima*, further research work on *M. stellatus* polysaccharides is necessary to correlate their molecular weight distribution to chemical or functional properties. The method could also be extended to the molecular weight estimation of polysaccharides in other red seaweeds.

4. Conclusions

A simple size-exclusion chromatography method for the molecular weight distribution of polysaccharides in seaweeds was proposed. The validation parameters, precision, accuracy, linearity and sensitivity, showed that the HPSEC method was adequate for the analysis of soluble polysaccharides in seaweeds according to their molecular weight. The method was optimized and repeatability of chromatograms was further improved by using 0.1 M $NaNO_3$ as mobile phase. The proposed method was very useful for determining the molecular weight distribution of polysaccharides from seaweeds. Soluble fractions from brown *S. latissima* and red seaweed *M. stellatus* presented high heterogeneity on molecular weight. Also the extraction conditions for carrageenans in *M. stellatus* showed differences in MW of soluble fractions. These preliminary results can provide additional information necessary to correlate the molecular weight of seaweed polysaccharides with their chemical or functional properties. Results of detailed chemical composition and biological activities will be reported elsewhere and should help in acquiring a better understanding of the interplay between chemical structure and functional properties of biopolymers from edible brown and red seaweeds.

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Antioxidant Capacity and Prebiotic Effect of Dietary Fiber Co-Travelers from Sugar Kombu in Healthy Rats

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Complete List of Authors:	Jimenez-Escrig, Antonio; Institute of Food Science and Technology and Nutrition. CSIC, Metabolism and Nutrition Dpt. Ruperez, P; Institute of Food Science and Technology and Nutrition, CSIC, Metabolism and Nutrition Dpt. Gomez-Ordoñez, Eva; Institute of Food Science and Technology and Nutrition, CSIC, Metabolism and Nutrition Dpt. Tenorio, Maria Dolores; Facultad de Farmacia, Universidad Complutense de Madrid, Universidad Complutense de Madrid, Nutrition and Bromatology II

TITLE: Antioxidant Capacity and Prebiotic Effect of Dietary Fiber Co-Travelers from Sugar Kombu in Healthy Rats

AUTHORS: Antonio Jiménez-Escrig[*§], Eva Gómez-Ordóñez[§], María Dolores Tenorio[#], Pilar Rupérez[§]

ADDRESSES:

[§]Metabolism and Nutrition Department, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, ES-28040 Madrid, Spain

[#]Nutrition and Bromatology II Department, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal, Ciudad Universitaria, ES-28040 Madrid, Spain

RUNNING TITLE: Health-promoting activities of edible seaweeds

*Corresponding author: a.jimenez.escrig@csic.es

ABSTRACT: Edible brown seaweed Sugar Kombu (*Saccharina latissima*) is a good source of dietary fiber (DF) and associated compounds. Besides it presents antioxidant capacity *in vitro* due to their sulfated polysaccharides and polyphenols. Effect of a DF-rich Sugar Kombu diet on biochemical parameters, antioxidant capacity and prebiotic effect in healthy rats was evaluated. Thus, rats were fed either a basal diet or a supplemented one with 10% Sugar Kombu during four weeks. Several health-promoting effects were found such as a decrease in triglycerides and uric acid, and an increase in antioxidant status both in serum and cecum. Regarding prebiotic effect, higher cecum weight and total short chain fatty acids content were evidenced in the seaweed-fed group, without significant differences on total bacterial count of feces. Sugar Kombu and sulfated polysaccharides from its DF could be used as functional ingredients for further nutraceutical applications.

KEYWORDS: *Edible brown seaweed, Biological activity, Reduction power, Prebiotic, Uric acid*

INTRODUCTION

Epidemiological studies have shown that consumption of fruits and vegetables is associated with reduced risk of chronic diseases. Association between dietary vegetable intake and chronic diseases is mainly attributed, along with the dietary fiber (DF) constituent, to a wide range of plant secondary compounds called phytochemicals. In consequence, an increased consumption of products from vegetable origin, which contain high levels of DF and associated phytochemical constituents, has been recommended (1-3).

DF is not a single compound but a combination of chemical substances with varied composition and structure. The definition of DF has evolved since 1972 before the publication of Trowell´s paper, but the debate over developing a comprehensive definition for DF still continues today. A general feature of all given definitions is the fact that whole plant cell walls are considered as the major source of DF (4, 5). Various DF-rich food from vegetable origin could be very good sources of phytochemicals which include polyphenols, carotenoids, plant sterols, lignans, terpenoids, or sulfated polysaccharides. These so-called co-passengers or co-travelers of DF reach the intestinal tract as non-digestible food, and are an essential part of the healthy DF complex, and thus contribute to the nutritionally benefits of DF-rich food (6-8).

Prebiotics are dietary carbohydrates which escape digestion in the small intestine, but undergo bacterial fermentation in the large intestine, and beneficially affect the intestinal microbiota (9-11). Fermentable carbohydrates could favor mineral absorption in the distal part of the digestive tract in several ways: hypertrophy of the cecal wall and greater surface area, increase in soluble mineral, and accelerated blood flow (12). In addition, the products

of fermentation in the colon, mainly short chain fatty acids (SCFA), have a role in the improvement of mineral absorption (11).

Since ancient times, brown and red seaweeds have been part of the diet in Asian countries, especially China, Japan and Korea (13). In Western and European countries seaweeds are utilized as a source of hydrocolloids (14, 15). Nowadays, around 16 million tonnes (wet weight basis) of seaweeds and other aquatic plants are produced/captured annually with an estimated value of US $ 7500 million (16); however, seaweed species are often regarded as under-exploited bio-resources (17, 18). Besides, it is worth to mention that due to their diversity of compounds, seaweeds have been considered over the past few decades as promising live organisms for providing both, novel biologically active substances and essential compounds for human nutrition, with high potentially economic impact in food and pharmaceutical industry and public health (17, 19-20). In addition, it is stated that much research, such as their role in nutrition and disease prevention, remains to be done before science-based dietary recommendations can be given for edible seaweeds (20).

A screening study for the potential antioxidant activity of several brown and red edible seaweeds commonly collected from the Northwestern Atlantic coast of Spain has been recently reported by our research group, being this activity attributed to non-digestible sulfated polysaccharides and polyphenol components (21). Besides, several health effects of edible seaweeds in animal models have been assessed. It has been recently shown in a hypercholesterolaemic rat model that red seaweed Nori would be the algal choice in dietary treatment of hypercholesterolaemia (22). In a similar rat model, apparent absorption of several minerals appeared significantly affected in Nori-fed rats, with a significant decrease in the ratio of Zn and Cu intakes and apparent absorption (23). In healthy rats, cholesterol-

adjusted total antioxidant capacity in plasma after the intake of brown seaweed Kombu or Nori-fed groups is significantly lower than those of control (24). Thus controversial evidences in the evaluation of health effects of seaweeds in animal models are described.

The evaluation of the protective effect from edible brown algae intake in the serum and cecum in healthy animal model could clarify the health-promoting attributes of seaweeds. These two compartments provide both a systemic representation and a partial representation of the gut environment, respectively. Therefore, the aim of this study was to evaluate the effect of diet supplementation with the edible brown seaweed *Saccharina latissima* on biochemical parameters, antioxidant status, prebiotic effect, and mineral balance in healthy rats.

MATERIALS AND METHODS

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E was purchased from Aldrich Co (St Louis, MO). All reagents used were of analytical grade.

Algal Material. The brown seaweed *Saccharina lattisima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders, [formerly *Laminaria saccharina* (Linnaeus) J.V.Lamouroux)] (25), commonly known by its Japanese name Sugar Kombu, was cultivated outdoor under natural conditions. The seaweed was provided by Porto-Muiños, a local food processing industry (Cambre, A Coruña, Spain). In the industry, the seaweed samples were rinsed with tap water to remove sand, epiphytes and encrusting material and then air-dried at 50 ^{o}C. The dried samples were milled and passed through a 1 mm mesh

sieve. At the laboratory the milled seaweed samples were stored in plastic bags and kept at 2 ºC for further assays. The proximate composition of *S. latissima* is given in Table 1.

Animals, Maintenance and Experimental Design. Female Wistar rats (n = 12; 193-202 g), ten and a half-week-old, were obtained from the feeding animal center of Facultad de Biología, Universidad Complutense de Madrid (UCM), Madrid, Spain. The use of animals was conducted in compliance with the Laboratory European guidelines for the care and use of laboratory animals, and protocols were approved both by the Experimental Animals Committee at UCM and by the Bioethical Committee from CSIC (Spanish National Research Council). The rats were housed individually in metabolic cages in a room at 22 ± 1 ºC, 60-65% humidity, with a controlled 12-h light-dark cycle. Rats were fed a basal diet (Panlab S.L., ref. A04, Barcelona, Spain), and after a one-week adaptation period (week 0) rats were divided into two groups. The control group (n = 5) and the *Saccharina* group (n= 7). The treated group was fed the control diet plus 10% of dried *Saccharina latissima*. The experimental period was four weeks. Diets and drinking water were provided *ad libitum*. The composition of the control and treated diets is shown in Table 1.

General Procedure. Body weight, food intake, fecal weight, and urine volume were recorded at the end of each experimental week, including the adaptation week. The feeding efficiency (FE), a parameter with reflects body weight gain *versus* food intake, was also calculated. Feces and urine samples were kept at -20 ºC until analysis. The rats were anesthetized with carbonic anhydride and killed by blood extraction via carotid puncture. Blood samples were collected into tubes and serum was separated by low speed centrifugation (1500 x *g*, 4 ºC, 10 min). Several internal organs (kidneys, spleen and liver) were rapidly removed and weighed. The gastrointestinal tract was removed and its

longitudinal measure recorded, also the whole cecum (including its content) was weighed and recorded. Serum samples and cecum were stored at -80 ºC until analysis. Also total bacterial count on feces was performed, namely: enterobacteria, lactic bacteria, fecal coliform and *E. coli* ß-glucuronidase-positive.

Biochemical Parameters in Serum. Total protein, albumin, glucose, uric acid (UrA), triglycerides (TGL), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were measured in the rat serum using either an Autoanalyzer (RA-500, Bayer, Spain) or a Beckman DU-640 spectrophotometer.

Antioxidant Status in Serum and Cecum. *Preparation of Extracts from Cecal Content* An aliquot of fresh cecal samples (0.300 g) was placed in a centrifuge tube; 40 mL of methanol/water (50:50; v/v) were added, and the mixture was thoroughly shaken at room temperature for 1h. The tube was centrifuged (2500 x *g,* 10 min) and supernatant recovered. Then, 40 mL of acetone/water (70:30; v/v) was added to the residue, and shaking and centrifugation steps were repeated. Both organic extracts were mixed and made up to 100 mL with water. Aqueous-methanol-acetone extracts of cecal content were produced in triplicate and used to measure the antioxidant capacity (26).

Reduction Power (RP). Antioxidant capacity, in terms of reduction power (RP) towards Fe(III), of serum and organic cecal extracts in *Saccharina*-fed rats was evaluated (27) (26). Briefly, 900 µL of FRAP reagent, freshly prepared and warmed at 37 ºC, was mixed with 90 µL water and either 30 µL (cecum)/10 µL (serum) of test sample or standard or appropriate reagent blank. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L

HCl, plus 2.5 mL of 20 mmol/L $FeCl_3$ $6H_2O$, plus 25 mL 0.3 mol/L acetate buffer pH 3.6. Readings at the absorption maximum (595 nm) were taken every 15 s using a Beckman DU-640 spectrophotometer thermostatized at 37 ºC. The readings at 4 and 30 min were selected for calculation of RP values. Methanolic solutions of known Trolox concentrations were used for calibration expressing the results as Trolox equivalents (TE).

pH, SCFA, Uronic acid and Lactate in Cecum. An aliquot of the cecal content was freeze-dried, weighed and results expressed as dry weight.

pH and SCFA Determination. A portion of the cecal content was treated as described below to measure pH and short chain fatty acids (SCFA), and the untreated sample left was stored at -80 ºC until analysis of the antioxidant status in the cecum was performed. After sampling, the cecal content was immediately diluted 1:3 (w/v) in milli Q water, then pH was measured using a microelectrode (Crison, micro pH 2001). To measure SCFA, the diluted samples were centrifuged (9000 x *g*, 15 min, 4 ºC) and the supernatants utilized for gas-liquid chromatography (GLC). A 0.4 mL sample with 0.5 mL internal standard, in 12% formic acid (4-methyl valeric acid, 2 μmol/mL) and made up to 1 mL with water, was centrifuged as above, and 1 μL of supernatant was injected into a gas chromatograph (5890 Hewlett Packard) equipped with a flame ionization detector, and a fused silica column (Carbowax 20 M, 10 m x 0.53 mm x 1.33 μm film thickness). The carrier gas was nitrogen with a flow rate of 15 mL/min. The injector and detector temperature was 250 ºC and the column temperature was isothermal at 120 ºC (28).

Uronic Acid Determination. Water-diluted cecal samples were centrifuged as above (9000 x *g*, 15 min, 4 ºC) and the insoluble residue was freeze-dried. Uronic acids (UA) in freeze-dried residues (10 mg) were quantified colorimetrically (29), with galacturonic acid

as standard and 3,5-dimethylphenol as the reagent, and results corrected for incomplete recovery of uronic acids (26.9 g/100 g) from standard alginate hydrolysis (30).

Lactate Determination. Water-diluted cecal samples were centrifuged as above (9000 x *g*, 15 min, 4 ºC) and supernatants used for lactate determination by ion chromatography (IC). Supernatants were filtered through 0.45 μm filters just before injection. A Metrohm Advanced compact ion chromatographic instrument (IC-861 model, Metrohm AG, Switzerland) controlled using Metrodata IC Net 2.3 software and attached to an Advance Sample Processor (IC-838) with an Injection Valve Unit (IC-812) with a 20 µL sample loop was used in lactate analyses. The instrument was also equipped with a Pump (IC-818), an Eluent Degasser (IC-837) and a Liquid Handling Unit (IC-833) with a 0.45 µm on line filter. Detection was performed with an Advanced Conductivity Detector (IC-819) from Metrohm. Separation was performed in a Metrosep Organic Acids column (250 x 7.8 mm, 5 µm particle size). The carrier material was a polystyrene-divinylbenzene copolymer with sulfonic acid groups. All measurements were carried out at 20 ºC (column temperature) under the following elution conditions: 0.5 mmol/L H_2SO_4 and acetone (85:15; v/v) at 0.50 mL min^{-1} as mobile phase. Results were expressed as lactate in g /100 g cecal dry weight.

Total Bacteria Count on Feces. Fecal samples (0.5 g) were collected in aseptic conditions into glass tubes with 4.5 mL sterile buffered peptone water (Cultimed). Samples were vortexed and serial dilutions from 10^{-1} to 10^{-7} were performed. Three bacterial culture media were used: 1.-, for enterobacteria a VRBG (Violet Red Bile Glucose) agar medium was prepared according to manufacturer's specifications (Cultimed) and set at 47 ± 2 ºC; 2.-, for fecal coliform and *E. coli* β-glucuronidase positive a chromogenic chromID Coli (COLI ID-F, Biomérieux) medium was prepared and set at 47 ± 2 ºC; 3.- for lactic bacteria

a MRS agar medium (31) was prepared according to manufacturer's specifications (Merck) and set at 47 ± 2 ºC.

Enterobacteria, lactic bacteria, fecal coliform and *E. coli* were harvested by adding 1 mL of each serial dilution and 15 mL of fresh culture medium in a Petri dish until solidification, and then 10 mL of fresh culture medium was again added to allow bacterial growth under anaerobic conditions.

The incubation conditions were as follows: 30 ºC during 72 h for enterobacteria and lactic bacteria, and 37 ºC during 24 h for fecal coliform and *E. coli* β-glucoronidase positive. Afterwards colony count was performed in Petri dishes with a growth range of 0-300 colony forming units (CFU) and results were expressed as *log* CFU/g fecal dry weight.

Mineral Composition in Diets, Feces and Urine. To precisely evaluate the digestibility of minerals, control and *Saccharina* diets, feces and urine samples were analyzed for mineral composition. Daily collected feces and urine samples of each animal were pooled (7-day samples) and kept frozen at -80 ºC until analysis. Fecal samples were freeze-dried, milled and then ashed at a temperature increased linearly to 550 ºC for 1 h and then at 550 ºC for 24 h in a microwave oven (Milestone MLS-1200 Pyro). The ashed samples, dissolved in 2 mL of HCl (50%):HNO3 (50%), (1:1; v/v) were diluted with water to 25 mL. Diets were ashed by the same method as that of feces. Urine was appropriately diluted with a 0.2% solution of HNO_3 in water (v/v) and subjected directly to atomization. Calcium and magnesium concentrations in control and *Saccharina* diets, fecal and urine samples were measured using an atomic absorption spectrophotometer (Perkin Elmer Aanalyst 200). Calcium and magnesium samples were previously diluted with a lanthanum oxide solution to 0.1% (w/v).

Mineral Balance. Apparent mineral absorption and balance were calculated as follows: Apparent mineral absorption (AMA) (%) = [(mineral intake – fecal excretion)/mineral intake]*100, whereas apparent mineral balance (AMB) (mg/day) = [(mineral intake – fecal mineral excretion) – urinary mineral excretion] and efficiency of mineral retention (EMR) (%) = 100*(AMB (mg/day)/mineral intake).

Statistical Analysis. Results are expressed as mean values ± standard deviation. Comparison of means of three measurements, using a significance level of $P < 0.05$, was performed by one-way analysis of variance (ANOVA). SPSS, version 14.0 was used.

RESULTS AND DISCUSSION

Weight Gain and Feed Efficiency. All animals treated with the *Saccharina latissima* diet were in good health throughout the experiment, and no side effects such as diarrhea were apparent. In addition, treated rats appeared to behave normal throughout the study. Both diets -the control and the treated diet- showed similar energy values (Table 1). During the four weeks of feeding, each rat consumed a similar amount of diet (treated group: 511 ± 49 g, and control group: 471 ± 20 g). By the end of the experiment, animals fed the algal-supplemented diet showed similar body weight gain and feeding efficiency to those fed the control diet (Table 2).

Internal Organs and Cecum Weights and Gastrointestinal Tract Longitudinal Measure. Several organs (kidneys, liver, and spleen) and the cecum were weighed in both groups of animals. Also the longitudinal measure of the gastrointestinal tract was recorded. As a result, no difference either in the organs weight or in the gastrointestinal longitudinal measure between both groups was found, except for kidney, in which a small but significantly higher weight was observed in the treated group (Table 2). Higher cecal weight values were found in treated rats in comparison to control. This fact is likely to be related to the physicochemical properties of the indigestible polysaccharides of dietary fiber (DF) (32), which are the main component of dry seaweeds. Supporting this, we have found a relatively high water retention capacity in *Saccharina latissima* among several edible seaweeds, correlating directly to the soluble DF component of seaweeds (30). However, owing to the relatively high protein content in *S. latissima* (Table 1), the role of protein component in the physicochemical behavior of the seaweed during the gut transit in the rat cannot be ruled out. In this sense we have reported high values of indigestible protein in edible brown seaweeds, specifically around 57% of total protein content in *Laminaria* spp (33), supporting a potential role for the protein component of seaweeds in the rat gut.

Serum Biochemical Parameters. No difference was observed either in total cholesterol, LDL-C, HDL-C, glucose, albumin, or total protein serum levels in seaweed-fed group as compared to control (data not shown). A significant decrease in serum TGL level (50.67%, $P < 0.05$) was observed in the treated group (Table 3). Also, uric acid (UrA) has been depleted (67.83%, $P < 0.05$) at the end of the experiment in rats fed the brown seaweed. Consistently, it is described a reduction in serum TGL by enriched-soluble DF from apple source in healthy rats (34). In addition, serum UrA has been depleted in

enriched-DF diets in animal models (35). In humans, it is stated the importance of uric acid as a contributing metabolic factor to cardiovascular disease (36).

Antioxidant Status in Serum and Cecum. The antioxidant system is constituted by enzymatic and non-enzymatic antioxidants, and it is difficult to evaluate each non-enzymatic antioxidant separately. Moreover, an isolated measure of one type of antioxidant does not take into account the potential synergic effects among antioxidants. In consequence in this work two biological compartments, serum and cecum, using a global antioxidant measure such as the reduction power (RP) towards Fe(III) were evaluated. These compartments were chosen as a systemic one and as a partial representation of the gut environment.

Reactive oxygen species are generated in the human being at physiological conditions as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. The need to protect cells from oxidation stress, since an unbalanced oxidative stress could lead to apoptotic death or necrosis in cells, is well documented. It is suggested that a redox state, which involves gluthatione, could protect the cell from oxidative damage. Specifically, in the gut environment, the high exposure to exogenous reactive oxygen species is described. Thus, it is likely that an increase of the RP in this compartment would have a positive health influence (37).

Apparently, the intake of the brown algae *Saccharina latissima* did not lead to an increase in the RP value in serum (Table 4) neither at 4 min nor at 30 min. Since the major determinant of RP in serum is uric acid (UrA) concentration (27) (35), the specific UrA value in serum was measured. Remarkably, UrA was depleted in the seaweed-fed group *versus* the control group. When the RP was adjusted in serum UrA-value, there were

significant differences between seaweed-fed and control rats in $RP_{ser\text{-}UrA}$ at 30 min (Table 4). In the case of UrA, the relationship of FRAP reaction at 4 and 30 min is absolutely lineal (slope 1.0197, $P < 0.0001$). This means that UrA reacts completely with the ferric system immediately after the reaction is started, whereas other antioxidants react more slowly (35). In the present work, seaweed feeding leads in serum compartment to on one hand an UrA decrease and on the other hand to an increase in certain slow-reacting antioxidants in serum, which compensated and elevated the total $RP_{ser\text{-}UrA}$ at 30 min in the seaweed-treated group.

In the case of the animals fed the seaweed diet, the value of RP in the cecum was significantly higher (36.6%) than those fed the control diet (Table 4). A similar effect has been found previously in artichoke and okara, a Mediterranean edible vegetable and a by-product from the soybean processing industry respectively, which are both rich in DF (35, 38). In order to tentatively elucidate the bioactive compounds responsible for the antioxidant activity in the cecum, it is worth to mention that our group has found a relatively high value of different phytochemicals in *Saccharina latissima*, mainly sulfated polysaccharides and polyphenols, which are responsible for the *in vitro* antioxidant capacity assayed (21). These compounds are described as co-travelers of the DF component through the digestive tract, reaching as a non-digestible fraction the final bowel (6, 8) in which are metabolized by the microbiota. As a consequence, active metabolites may exert its action in the body after its absorption at end-part of the bowel (39, 40). This is partly evidenced in this work by the measuring of uronic acids from alginate, which are the main sulfated polysaccharides in brown seaweeds, in the cecal compartment of the seaweed-fed rats (Table 3).

Apart from that, it is worth to mention that the RP value is influenced by the redox state of the system (41). A highly reduced environment in the cecum of the rats fed the seaweed diet was found, as compared to the control group. However we cannot rule out that the redox state of the cecum was likely to influence the increase in the formation of specific reduced products such as propionic acid. Thus, these results could suggest the use of *Saccharina latissima* as a new source of antioxidant DF which tentatively would promote a beneficial reduced environment inside the cecum.

Prebiotic Effect. *pH Value, SCFA and Lactate in Cecum.* Colonic fermentation resulted in a slight but significant increase of cecal pH value (6.41 ± 0.16) in the seaweed-fed group, compared to the control (5.95 ± 0.15). As expected, the mean values of total SCFA in cecum were significantly higher in the seaweed-fed group, than in the control (Table 3). It is interesting to note that, the molar proportions of both acetic and propionic were significantly higher (29.8 ± 5.7% and 15.8 ± 2.4%, respectively) in the seaweed-fed group than in the control (10.6 ± 1.6% and 19.9 ± 1.6%, respectively), whereas that of butyrate was significantly lower (54.4 ± 7.6 7%) in the seaweed-fed group than in the control (69.6 ± 2.3%). Regarding iso-butyric, iso-valeric and valeric acids, values were less than 3% of SCFA in both groups of animals. Lactate values in cecal content did not significantly differ between groups (data not shown).

It is known that several factors in the gut environment, such as local conditions of pH, available dietary substrate, oxygen and hydrogen and gut transit time could lead to shifts in the species composition of the colonic microbiota, since bacterial metabolism and competition are strongly influenced by the gut environment (9). Apart from that, the degree of digestibility of a DF source from a brown seaweed increases with feeding period time in

a rat model (42). Regarding, fermentable substrate factor, it is described that, in comparison to a casein-enriched diet, resistant protein increases pH and improves the productivity of total SCFA and cecal fermentability in healthy rats (43).

Studies dealing with the fermentation of brown seaweeds are controversial. It is stated (44) that laminarin does not exhibit prebiotic effect like fructooligosaccharides (FOS), but its fermentation leads to high proportions of propionate and butyrate as compared with glucose, which mainly produces acetate. Michel and his collaborators (45) showed that laminarin is used by human intestinal bacteria with the production of propionate and butyrate. In their study, this polysaccharide does not promote the growth of lactobacilli and bifidobacteria. Thus, more research concerning butyrate and propionate production by fermentation of brown seaweeds is needed.

Evidence had been shown that the seaweed diet modulated the distribution of SCFA, through promotion of a relatively increased level of acetic and propionic acids in the cecum. These results suggest the potential use of the brown seaweed *Saccharina latissima* as a prebiotic, which modifies large intestinal fermentation. It is the first time that both, the fermentation production of propionic acid and the evaluation of overall antioxidant status in the cecum, have been linked in an animal model.

Uronic Acids in Cecum. Colonic fermentation resulted also in a higher uronic acids content (10.2 ± 2.7%) in the cecum of treated group, compared to the control (3.9 ± 0.6%) (Table 3). *Saccharina latissima* is a brown seaweed composed mainly by the polysaccharide alginate (46), which is a copolymer or mannuronic and guluronic acid. The two-fold higher uronic acid amount in the cecum of seaweed-fed rats was presumably produced by the colonic fermentation of alginate which escaped digestion in the small

intestine. It is the first time that uronic acid from alginate fermentation of a brown seaweed has been measured in the rat cecum. Alginate digestibility tends to the increase during the feeding period of *Laminaria angustata* as source of DF in an animal model. Specifically, its measure in the feces of rats indicates that molecular weight of alginate is depleted 60%, being mannuronic acid more digestible in comparison with guluronic acid (42).

Fecal Weight, Water Content and Bacterial Fecal Count. The weight and water content of feces was higher for the treated than for control group after the 28-days experimental period, showing statistically significant differences for moisture (Table 2). Differences may be explained by the different composition of the diets, which lead to different hydration grade of the DF components. In this sense, in *S. latissima* relatively high water retention and swelling capacities of brown seaweeds in comparison to other vegetable materials have been reported by our group (30). In the case of the seaweed-based diet the relatively content in soluble DF fraction was around 56.23% of total DF, whereas in control diet the DF component was 100% insoluble DF fraction (cellulose) (Table 1).

No significant difference was observed in bacterial count on feces between both groups of rats (data not shown) at the end of the experiment. However, a tendency to the increase in the total amount of bacteria was observed (control group, 25.40 log CFU/g; treated group, 27.01 log CFU/g; $P < 0.3011$). Fermentation of DF from seaweeds can affect the composition of colonic microbiota and modify the fermentative activity of bacteria (47). However, our results showed that the intake of Sugar Kombu for 28 days was not apparently capable to change significantly the composition of colonic microbiota in a healthy rat.

Mineral Balance Related to Prebiotic Effect. Although a high intake of DF may retain divalent cations such as calcium and magnesium in the small intestine, some reports have demonstrated that the cecum and colon have a large capacity for calcium and magnesium absorption (48). Carbohydrates that escape digestion in the small intestine are substrates for the formation of short chain fatty acids (SCFA) in the large intestine which enhance mineral bioavailability (12). Cecal fermentation varies according to the type and amount of DF(49). Edible seaweeds are particularly rich in the soluble fraction of DF and could induce different fermentative patterns (50, 51) In this work we evaluated the metabolism of calcium and magnesium in rats feeding diets rich in algae during the four weeks of assay (Table 5). Positive effects in calcium bioavailability were shown in seaweed-fed rats, with significantly higher apparent absorption ($P < 0.001$) and true retention ($P < 0.001$).

In this study total SCFA content derived from cecal microbiota fermentation was significantly higher in the treated group than in the control one. Also higher cecum weight in the treated group was evidenced. These findings can influence mineral absorption in the large intestine. SCFA can directly improve mineral absorption by forming complexes with calcium, thereby increasing their uptake by the intestinal cells (52). SCFA also stimulate epithelial proliferation (53). SCFA originates a hypertrophy in mucose cells inducing an enlargement of the intestine and favoring calcium absorption (54). It is suggested (55) that the concentration of propionic acid is a more important factor in calcium absorption in the large intestine than other factors. In the present study we found that proportion of propionic acid to total SCFA in the seaweed-fed group was twice higher than in the control one.

In this research magnesium absorption was unaffected by alga diets as compared to the control. These findings are similar to those obtained feeding rats different brown seaweeds such as Kombu (23), and they also are in agreement with the literature results in the

evaluation of Mg apparent retention in diets rich in Wakame seaweed (51). Among SCFA butyrate is the most effective in stimulating magnesium flux in relation to acetate or propionate (56). In this study, in contrast to the other two acids, butyric acid did not proportionally increase in the seaweed-fed group with respect to the control. Some authors (12) detect that high levels of dietary Ca produce an inhibition of Mg absorption in the cecum and contribute to reduce Mg digestibility. Coudray and collaborators (52) reported that the soluble cecal Mg levels were inversely related to Ca intake. These facts could explain the lack of effect on magnesium absorption and retention in the rats feeding the seaweed diet in the current work.

In conclusion, intake of *Saccharina latissima,* a brown seaweed rich in DF and associated compounds, could promote health benefits on antioxidant status in a rat model. Additionally, prebiotic effect leading to a significant increase in total SCFA and molar proportions of acetic and propionic acids, and a potential decrease of cardiovascular risk factors, especially serum UrA and TGL, were evidenced. Sulfated polysaccharides and polyphenols, which are an essential part of the healthy seaweed DF-complex and consequently reach the large intestine undigested, seem to be responsible for the nutritional benefits of the seaweed diet. Further research on human trials need to be undertaken before any health claims for functional ingredients based on seaweed DF-complex can be proposed.

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ABBREVIATIONS USED

AMA, Apparent mineral absorption; AMB, apparent mineral balance; ANOVA, one-way analysis of variance; DF, dietary fiber; EMR, efficiency of mineral retention; FE, feeding efficiency; FRAP, ferric-reducing antioxidant power; GLC, gas-liquid chromatography; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein cholesterol; RP, reduction power towards Fe(III); SCFA, short chain fatty acids; TC, total cholesterol; TGL, triglycerides; TE, Trolox equivalent; TPTZ, (2,4,6-tri(2-pyridyl)-s-triazine); Trolox, 6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid; UrA, uric acid; UA, uronic acids

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TABLE 1

Composition of Test Diets Used in the Feeding Experiment (g/kg)

	Control	Treated
Moisture	120	108
Protein	154	139
Fat and Oil	29	26
Carbohydrates	605	545
Starch	443	399
Total sugar	25	23
DF (cellulose)	39	35
Saccharina latissima[a]	0	100
Vit A (U/kg)	15000	13500
Vit D_3 (U/kg)	1500	1350
Vit E	0.020	0.018
Minerals	48	47
Calcium	9.26	9.09
Magnesium	1.68	1.63
Zinc	0.096	0.095
Phosphorus	5.90	5.31
Copper sulfate	0.012	0.011
Energy (Kcal/kg)	3224	3107

[a]Proximate composition of *Saccharina latissima* (g/100 g, dry weight): moisture, 6.64 ± 0.06; DF, total dietary fiber, 30.23 ± 0.85; soluble fiber, 17.12 ± 0.84; insoluble fiber, 13.11 ± 0.56; protein 25.70 ± 0.11; oil 0.79 ± 0.07; ash 34.78 ± 0.08 (30). Main inorganic Anions of Ash (g/100 g): chloride 34.8 ± 0.05; sulfate 5.55 ± 0.05 (57). Low-molecular weight carbohydrates, 9.46 ± 0.61

TABLE 2

Effect of Sugar Kombu Intake on Feeding Efficiency[a], and Kidney and Cecal Weight[b] in Healthy Rats

	Control		Sugar Kombu	
	Mean	SD	Mean	SD
Food Intake	471	20.0	511	48.7
Feeding efficiency	0.07	0.02	0.05	0.02
Kidney weight	1.46	0.03*	1.65	0.19*
Fecal moisture	49.73	2.86*	58.88	5.70*
Cecal weight	3.15	0.57*	4.82	0.82*

Values are means ± SD for each group of animals

[a]Feeding efficiency = body weight gain x food intake^{-1}

[b]Values are expressed as g fresh weight

*Statistically significant difference between both groups of animals ($P < 0.05$)

TABLE 3

Effect of Sugar Kombu Intake on Cecal and Serum Parameters[a] in Healthy Rats

	Control		Sugar Kombu	
	Mean	SD	Mean	SD
Serum compartment				
TGL (mg/dL)	148	16*	75	17*
Uric acid (mg/dL)	4.01	0.74*	2.72	0.56*
Cecal compartment				
pH	5.95	0.15*	6.41	0.16*
Uronic acids (g/100 g dw)	3.9	0.6*	10.2	2.7*
Total SCFAs (μmol/g dw)[a]	144	8.4*	198	28.9*
Molar Proportion				
Acetic	10.19	0.7*	15.07	2.4*
Propionic	19.12	1.6*	28.43	5.7*
Butyric	66.95	2.3	51.97	7.6

Values are expressed as the mean value ± SD for each group of animals

[a]Serum and cecal samples from each animal were recorded at the end of the trial.

[b]SCFA = Short chain fatty acids. A trace of isovaleric, isobutyric and valeric acid was detected (< 3% of the total SCFAs value)

*Statistically significant difference between both groups of animals ($P < 0.05$)

TABLE 4

Effect Sugar Kombu Intake on Antioxidant Status[a] in Cecal and Serum Compartments in Healthy Rats

	Control		Sugar Kombu	
	Mean	SD	Mean	SD
RP_{ser} (μmol of TE/L)	417	112	577	121
$RP_{ser\ -UrA}$ (μmol of TE/L)	317	107*	505	131*
RP_{cec} (μmol of TE/g dw)	69.0	12.8*	94.3	14.9*

Values are expressed as the mean value ± SD for each group of animals

[a]Serum and cecal samples from each animal were recorded at the end of the trial. RP analysis by FRAP assay was made on each sample in triplicate.

[b]RP_{ser} = Reduction power in serum; $RP_{ser\ -UrA}$ = Reduction power in serum without the contribution of uric acid; RP_{cec} = Reduction power in cecal compartment

*Statistically significant difference between both groups of animals ($P < 0.05$)

TABLE 5

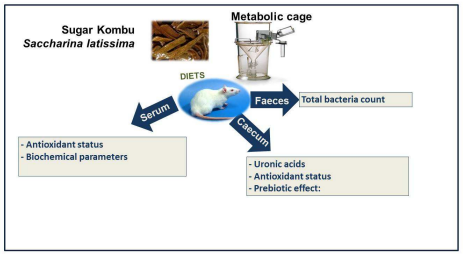
Effect of Four-Week Sugar Kombu Intake on Mineral Metabolism in Healthy Rats[a]

Mineral	Week	Apparent Mineral Absorption[b]				Apparent Mineral Balance[c]				Efficiency Mineral Retention[d]			
		Control		Treated		Control		Treated		Control		Treated	
		Mean	DS	Mean	DS	Mean	DS	Mean	DS	Mean	DS	Mean	DS
Calcium	1	25.67	9.63	34.49	4.22*	15.29	5.95	20.42	3.04*	25.08	9.25	33.45	4.09*
	4	16.75	6.71	27.80	5.55**	10.29	4.11	18.01	6.62**	16.14	6.43	26.13	7.08**
Magnesium	1	64.83	18.76	55.46	6.56	13.18	3.40	13.44	1.74	54.21	13.85	48.27	6.17
	4	43.64	12.39	53.54	14.52	9.96	3.87	14.22	2.61	39.64	13.05	49.52	11.12

[a]Values are expressed as mean values ± SD for each group of animals

[b]Apparent mineral absorption (AMA) (%) = [(mineral intake – fecal excretion)/mineral intake]*100, [c]Apparent mineral balance (AMB) (mg/day) = [(mineral intake – fecal mineral excretion) – urinary mineral excretion] and [d]Efficiency of mineral retention (EMR) (%) = 100*(AMB (mg/day)/mineral intake)

[e]Values differing significantly (*$P < 0.05$; **$P < 0.01$) from the control group


Sugar Kombu
Saccharina latissima
Metabolic cage
DIETS
Serum
Faeces
Total bacteria count
Caecum
- Antioxidant status
- Biochemical parameters
- Uronic acids
- Antioxidant status
- Prebiotic effect:

TOC GRAPHIC

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Article Type: Research Article (max 7,500 words)

Keywords: Seaweed; dietary fibre; carrageenan; sulfated-galactan; antioxidant; anticoagulant; cholesterol lowering

Corresponding Author: Dr. PILAR RUPEREZ, PhD

Corresponding Author's Institution: Instituto del Frío, CSIC

First Author: Eva Gómez-Ordóñez

Order of Authors: Eva Gómez-Ordóñez; Antonio Jiménez-Escrig; PILAR RUPEREZ, PhD

Abstract: Health-promoting effect of dietary supplementation with the red seaweed Mastocarpus stellatus was studied. Its major component is dietary fibre (31.7g/100g dry weight), 72% as soluble fibre mainly formed by carrageenans, sulfated-galactans of red seaweeds. Thus, rats were fed either a basal- or an algal-supplemented diet (10%). Then, lipid metabolism was assessed in serum, and reducing power measured in serum and caecum by FRAP method. Also, caecal pH was monitored and short chain fatty acids analysed by gas-liquid chromatography. Seaweed intake reduced significantly triglycerides and total cholesterol in healthy rats but not atherogenic index. Also a significant increase in caecal moisture and proportion of acetic and propionic acids was obtained but no clear prebiotic effect was shown. Sulfated-galactans seemed to be related to the antioxidant status improvement in caecum and also to the 1.7-fold increase in anticoagulant capacity of plasma. Therefore, Mastocarpus could be regarded as a source of functional ingredients but its health benefits need to be further explored depending on specific use.

Highlights: <Beneficial effect of *Mastocarpus stellatus* on lipid metabolism and antioxidant status> <Significant reduction of triglycerides and total cholesterol in serum> <Significant increase of reducing power in caecum> <Carrageenans role on lipid lowering, antioxidant and anticoagulant capacity increase> <Potentially useful for the prevention of hyperlipidemia and thrombosis in rats>

Effect of the red seaweed *Mastocarpus stellatus* intake on lipid metabolism and antioxidant status in healthy Wistar rats

Eva Gómez-Ordóñez, Antonio Jiménez-Escrig and Pilar Rupérez[1]

Metabolism and Nutrition Department, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, ES-28040 Madrid, Spain

[1]Corresponding author: Phone: +34915445607; Fax: +34915493627

E-mail address: pruperez@ictan.csic.es (P. Rupérez)

8 **Abstract**

9 Health-promoting effect of dietary supplementation with the red seaweed *Mastocarpus*
10 *stellatus* was studied. Its major component is dietary fibre (31.7g/100g dry weight),
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22 depending on specific use.

23

24 **Key words**: Seaweed; dietary fibre; carrageenan; sulfated-galactan; antioxidant;
25 anticoagulant; cholesterol lowering

26

Abbreviations: AI = atherogenic index (TC/HDL-cholesterol); ANOVA = one-way analysis of variance; APTT = activated partial thromboplastin time; CFU = colony forming units; dw = dry weight; FE, feeding efficiency; FRAP = ferric-reducing antioxidant power; GLC = gas-liquid chromatography; HDL = high-density lipoprotein; HDL-C = high-density lipoprotein-cholesterol; IC = ion chromatography; LDL = low-density lipoprotein; LDL-C = low-density lipoprotein-cholesterol; RP = reduction power towards Fe(III); SCFAs = short chain fatty acids; TC = total cholesterol; TE = Trolox equivalent; TGL = triglycerides; TPTZ = (2,4,6-tri(2-pyridyl)-s-triazine); Trolox = 6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid; UrA = uric acid; VLDL = very low-density lipoprotein

Highlights: <Beneficial effect of *Mastocarpus stellatus* on lipid metabolism and antioxidant status> <Significant reduction of triglycerides and total cholesterol in serum> <Significant increase of reducing power in caecum> <Carrageenans role on lipid lowering, antioxidant and anticoagulant capacity increase> <Potentially useful for the prevention of hyperlipidemia and thrombosis in rats>

1. Introduction

Several studies have demonstrated over the past few decades that seaweeds, due to their tremendous diversity of compounds, are promising organisms for providing both, novel biologically active substances and essential compounds for human nutrition, with high potentially economical impact in food and pharmaceutical industry and public health (MacArtain, Gill, Brooks, Campbell & Rowland, 2007; Cardozo et al., 2007; Smit, 2004).

Since ancient times, seaweeds have been part of the oriental diet in Asian countries, especially China, Japan and Korea (Nisizawa, Noda, Kikuchi & Watanabe, 1987). Nowadays the consumption of seaweeds is on the increase, even in Western societies, due to their nutritional and health benefits (Rupérez, 2002). The high content of polysaccharides, undigested by man, in the algal cell wall contributes to its high dietary fibre content (33-50g/100g dry weight, dw) (Rupérez & Saura-Calixto, 2001; Gómez-Ordóñez, Jiménez-Escrig & Rupérez, 2010). These polysaccharides are also considered as potential prebiotics, since they escape digestion in the small intestine, but undergo bacterial fermentation in the large intestine thus beneficially affecting the intestinal microbiota (Louis, Scott, Duncan & Flint, 2007; Macfarlane, Macfarlane & Cummings, 2006; Blaut, 2002). Moreover, seaweeds are an excellent source of bioactive substances like sulfated polysaccharides, peptides and polyphenols with biological activities such as antioxidant, anticoagulant, antiproliferative, antitumoral, anti-inflammatory, antibacterial, antiviral, antipeptic and antiadhesive activities (Rupérez, Ahrazem & Leal, 2002; Jiao, Yu, Zhang & Ewart., 2011; Jiménez-Escrig, Gómez-Ordóñez & Rupérez,

66 2011a; Costa et al., 2010; Tierney, Croft & Hayes, 2010; Kang et al., 2008; Cumashi et
67 al., 2007).

68 Among sulfated-polysaccharides, carrageenans are isolated from the cell walls of
69 some red seaweeds (Rhodophyta) (Rupérez & Saura-Calixto, 2001; Gómez-Ordóñez et
70 al., 2010) and their economical value is high because of their gelling properties, which
71 make them of current use in the food, cosmetic and medical/pharmaceutical industries.
72 These polysaccharides are made up of repeating α-(1,3)-galactose and β-(1,4,3,6)-
73 anhydro-D-galactose alternating units (Pereira & Van De Velde, 2011; Tuvikene et al.,
74 2009; Li, Lu, Wei & Zhao, 2008). Carrageenans are classified into three industrially
75 relevant types of families, identified by Greek letters: kappa- (κ), lambda- (λ) and iota-
76 (ι) carrageenans. Each family is in turn sub-divided into different types which differ in
77 the amount and position of their ester sulfate substitutes and (3,6)-anhydro-galactose
78 content.

79 *Mastocarpus stellatus* is a red macroalga belonging to the Gigartinaceae family. This
80 underexploited seaweed is a known source of dietary fibre (31.7 g/100 g dw), protein
81 (21.3 g/100 g dw) and minerals (25.0 g/100 g dw) (Gómez-Ordóñez et al., 2010). It also
82 presents antioxidant activity *in vitro* due to the sulfated polysaccharides of its soluble
83 dietary fibre (72% of total dietary fibre), mainly composed of kappa-/iota- hybrid
84 carrageenan (Gómez-Ordóñez & Rupérez, 2011). In this context, a screening study for
85 the potential antioxidant activity of several brown and red edible seaweeds commonly
86 collected from the Northwestern Atlantic coast of Spain has been made by our research
87 group (Jiménez-Escrig, Gómez-Ordóñez & Rupérez, 2011b).

88 Several reports have dealt with the health-promoting effects of edible seaweeds in
89 animal models, specifically regarding the effects on lipid metabolism in

90 hypercholesterolemic rat models. For example, Amano, Kakinuma, Coury, Ohno &
91 Hara (2005) have found that when rats are fed a cholesterol-rich diet containing a
92 mixture of seaweeds (9-10%) for 28 days, serum total cholesterol, LDL-cholesterol,
93 free-cholesterol, and triglyceride levels decline significantly compared to the control
94 (Amano et al., 2005). And it has been shown recently (Bocanegra, Bastida, Benedí,
95 Nus, Sánchez-Montero & Sánchez-Muniz, 2009) in a hypercholesterolemic rat model
96 that Nori fed-animals (red seaweed) for three weeks had lower postprandial
97 cholesterolaemia and better lipoprotein profile (lower LDL-C and a tendency towards
98 higher HDL-C and lower cholesterol-enriched VLDL-C) than control rats or than those
99 ingesting Kombu (brown seaweed), suggesting that Nori would be the alga of choice in
100 the dietary treatment of hypercholesterolemia.

101 Other studies deal with the effect of seaweed intake on antioxidant status in healthy
102 rats (Bocanegra, Benedí & Sánchez-Muniz, 2006), concluding that total antioxidant
103 activity adjusted in plasma cholesterol-value after the intake of Kombu was
104 significantly lower than that of control- and Nori-fed rats, respectively.

105 Therefore, in order to assess for the potential of edible seaweeds as a food ingredient,
106 our aim was to evaluate the health-promoting effects of a rich-in-dietary fibre algal
107 (*Mastocarpus stellatus*) supplemented diet on prebiotic effect, biochemical parameters
108 (total protein, albumin, uric acid, glucose), lipid metabolism (triglycerides, total
109 cholesterol, LDL- and HDL-cholesterol, atherogenic index), antioxidant status and
110 anticoagulant capacity in healthy rats.

111 **2. Material and Methods**

112 *2.1. Algal material*

The red seaweed *Mastocarpus stellatus* (Stackhouse) Guiry (Rhodophyta, Gigartinaceae) was obtained from a local supplier (Porto-Muiños, Cambre, Coruña, Spain). In the supplier industry it was cleaned from epiphytes and sand, washed with tap running water, air-dried at 50 ^{o}C and milled to less than 1.0 mm particle size. The milled seaweed samples were stored in sealed plastic bags at 2 ºC until analysis.

2.2. Animals, Maintenance and Experimental Design

Female Wistar Hannover rats (196-206 g weight) were provided by the feeding animal centre at the Faculty of Biology (Universidad Complutense de Madrid, Madrid, Spain). The use of animals was conducted in compliance with the Laboratory European guidelines for the care and use of laboratory animals and approved by the Experimental Animals Committee at Universidad Complutense de Madrid and by the Bioethical Committee from CSIC (Spanish National Research Council). Rats (n = 12) were housed individually in metabolic cages in a room at 22 ± 1 ºC and 60-66% humidity with a controlled 12h light-dark cycle. Rats were fed a basal diet (Panlab S.L., ref A04, Barcelona, Spain) for a one week adaptation period (week 0), then they were randomly divided into two groups: control group (n = 6) and treated group (n = 6). The treated group was fed the basal diet plus 10% of the milled algal product during the four experimental weeks. Diets and water were provided *ad libitum*. The composition of the diets is shown in Table 1.

2.3. General Procedure

Body weight, food intake, faecal weight, and urine volume were recorded at the end of each week, including the adaptation week. The feeding efficiency (FE), a parameter which reflects the relation between body weight gain and food intake, was also

136 calculated. Faecal and urine samples from each animal were collected separately on
137 alternate days, three times per week. Immediately after collection the samples were
138 frozen at -20 ºC, and at the end of each week the samples from each animal were
139 pooled; as a result, a pooled sample per animal and per week was analysed in triplicate.
140 After the experimental period, animals were anesthetized with carbonic anhydride and
141 killed by blood extraction via carotid puncture. Several organs (heart, kidneys, spleen
142 and liver) were rapidly removed and weighed. The gastrointestinal tract was removed
143 and its longitudinal measure recorded; also the caecum, including its content was
144 weighed, recorded and stored at -80 ºC until used for analysis.

145 *2.4. Blood Sampling and Preparation*

146 Blood samples were consecutively collected in two different ways, depending on the
147 analyses to be performed: (1) to obtain plasma for anticoagulant assays, blood was
148 collected into citrated tubes, slowly mixed by inversion and centrifuged (1500 x *g*, 4 ºC,
149 15 min). Plasma was immediately tested for anticoagulant capacity; (2) to obtain serum,
150 blood was collected into test tubes and after clotting the blood at room temperature, it
151 was centrifuged (1500 x *g*, 4 ºC, 10 min), then the serum was collected and stored at -80
152 ºC for further use.

153 *2.5. Determination of pH, SCFA and Lactate in Caecum*

154 A portion of the caecal content was diluted 1:3 in water immediately after sampling;
155 the pH was measured using a microelectrode (Crison, micro pH 2001) and the diluted
156 sample was stored at -80 ºC until analysis of Short Chain Fatty Acids (SCFAs) by gas-
157 liquid chromatography (GLC) was accomplished (Jiménez-Escrig et al., 2008). The

158 remaining caecal sample was also stored at -80 ºC until analysis of the antioxidant status
159 in the caecum was performed.

160 *2.5.1. SCFAs Determination*

161 To measure SCFAs, diluted caecal samples were defrozen and centrifuged (9000 x *g*,
162 4 ºC, 15 min) and supernatants utilized for GLC. A 0.4 mL sample with 0.5 mL internal
163 standard in 12% formic acid (4-methyl valeric acid, 2 μmol/mL) and made up to 1 mL
164 with water, was centrifuged as above, and 1 μL of supernatant was injected into a GLC
165 (5890 Hewlett-Packard) equipped with a flame ionization detector and a fused silica
166 column (Carbowax 20 M, 10 m × 0.53 mm × 1.33 μm film thickness). The carrier gas
167 was nitrogen with a flow rate of 15 mL/min. The injector and detector temperature was
168 250 ºC and the column temperature was isothermal at 120 ºC (Jiménez-Escrig et al.,
169 2008).

170 *2.5.2. Lactate Determination by Ion Chromatography (IC)*

171 Diluted samples were centrifuged (9000 x *g*, 15 min, 4 ºC) and supernatants utilized
172 for lactate determination by IC. Supernatants were filtered through 0.45 μm filters just
173 before injection. A Metrohm Advanced compact ion chromatographic instrument (IC-
174 861 model, Metrohm AG, Switzerland) controlled using Metrodata IC Net 2.3 software
175 and attached to an Advance Sample Processor (IC-838) with an injection Valve Unit
176 (IC-812) with a 20 μL sample loop was used in lactate analyses. The instrument was
177 also equipped with a Pump (IC-818), an Eluent Degasser (IC-837) and a Liquid
178 Handling Unit (IC-833) with a 0.45 μm filter. Detection was performed with an
179 Advanced Conductivity Detector (IC-819) from Metrohm. Separation was performed in
180 a Metrosep Organic Acids column (250 x 7.8 mm, 5 μm particle size). The carrier

181 material was a polystyrene-divinylbenzene copolymer with sulphonic acid groups. All
182 measurements were carried out at 20 ºC (column temperature) under the following
183 elution conditions: 0.5 mmol/L H_2SO_4/acetone (85:15; v/v) as mobile phase at 0.50 mL
184 min^{-1}. Results were expressed as gram of lactate per 100 g of caecal dry weight.

185 *2.6. Biochemical Parameters in Serum*

186 Total protein, albumin and glucose were measured in the rat serum using an
187 Autoanalyzer (RA-500, Bayer, Spain). Diagnostic kits (Spinreact, Girona, Spain) were
188 used for determination of uric acid (UrA), triglycerides (TGL), total cholesterol (TC),
189 and fractions in the rat serum with a Beckman Spectrophotometric equipment according
190 to manufacturer's specifications (Spinreact, Girona, Spain). HDL-cholesterol (HDL-C)
191 was measured after selective precipitation of low (LDL) and very low-density (VLDL)
192 serum lipoproteins with phosphotungstate in the presence of magnesium ions and
193 further removal by centrifugation. The atherogenic index (AI) was calculated as: AI =
194 TC/HDL-C (Jiménez-Escrig, Tenorio, Espinosa-Martos & Rupérez, 2008).

195 *2.7. Total Bacteria Count on Faeces*

196 Faeces were collected in aseptic conditions into sterile glass tubes with 4.5 mL
197 buffered peptone water (Cultimed). Samples were vortexed and dilution series from 10^{-1}
198 to 10^{-7} were performed. Three bacterial culture media were used: (1), for enterobacteria
199 a VRBG (Violet Red Bile Glucose) agar medium was prepared according to
200 manufacturer's specifications (Cultimed) and tempered at 47 ± 2 ºC; (2), for faecal
201 coliform and *E. coli β-glucoronidase positive* a chromogenic chromID Coli (COLI ID-
202 F, Biomérieux) medium was prepared and tempered at 47 ± 2 ºC; (3), for lactic bacteria

203 a MRS agar medium (De Man, Rogosa & Sharpe, 1960) was prepared according to
204 manufacturer's specifications (Merck) and tempered at 47 ± 2 ºC.
205 Enterobacteria, lactic bacteria, faecal coliform and *E. coli* were harvested by adding 1
206 mL of each dilution series and 15 mL of fresh medium in a Petri dish until solidification
207 and then 10 mL of fresh medium was again added to allow bacterial growth in
208 anaerobic conditions.
209 Incubation conditions were as follows: 30 ºC during 72 h for enterobacteria and lactic
210 bacteria, and 37 ºC during 24 h for faecal coliform and *E. coli β-glucoronidase positive*.
211 Afterwards colony count was performed in Petri dishes with a growth range of 0-300
212 colony forming units (CFU) and results were expressed as log CFU/g faecal dry weight.

213 *2.8. Antioxidant Status in Rats*

214 *2.8.1. Preparation of Extracts from Caecal Content*

215 An aliquot of fresh test sample (0.300 g) was placed in a centrifuge tube; 40 mL of
216 methanol/water (50:50; v/v) were added and thoroughly shaken at room temperature for
217 1h. The tube was then centrifuged (2500 x *g,* 10 min), and the supernatant was
218 recovered. Insoluble residue was extracted with 40 mL of acetone/water (70:30; v/v)
219 and shaking and centrifugation steps were repeated. Both extracts were mixed and made
220 up to 100 mL with water. Extracts were produced in triplicate and used to measure the
221 *ex vitro* antioxidant activity (Jiménez-Escrig, Jiménez-Jiménez, Pulido & Saura-Calixto,
222 2001).

223 *2.8.2. Reduction Power (RP) in Serum and Caecum*

224 Antioxidant capacity, as reduction power (RP) towards Fe(III), of aqueous-methanol-
225 acetone extracts from the caecum of *Mastocarpus*-fed rats was evaluated (Jiménez-

226 Escrig et al., 2001). Briefly, 900 μL of FRAP reagent, freshly prepared and warmed at
227 37 ºC, was mixed with 90 μL of water and either 30 μL (caecum)/10 μL (serum) of test
228 sample or standard or appropriate reagent blank. The final dilution of the test sample in
229 the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of a 10 mmol/L
230 TPTZ solution in 40 mmol/L HCl, plus 2.5 mL of 20 mmol/L $FeCl_3$ $6H_2O$, plus 25 mL
231 0.3 mol/L acetate buffer pH 3.6. Readings at the absorption maximum (595 nm) were
232 taken every 15 s using a Beckman DU-640 spectrophotometer thermostatized at 37 ºC.
233 The readings at 4 and 30 min were selected for calculation of RP values. Methanolic
234 solutions of known Trolox concentrations were used for calibration and results
235 expressed as Trolox equivalents (TE).

236 *2.9. Anticoagulant Capacity in Plasma*

237 Anticoagulant capacity was measured in plasma of control and treated rats using the
238 activated partial thromboplastin time (APTT) biological assay. Briefly, 100 μL of
239 citrated rat plasma was incubated for 3 min (37 ºC) with 100 μL APTT reagent. Then,
240 100 μL of 0.02 M calcium chloride was added and the clotting time was recorded in a
241 Biobas10 coagulometer (Spinreact, Girona, Spain).

242 *2.10. Statistical Analysis*

243 Results were expressed as mean values ± standard deviations. One-way analysis of
244 variance (ANOVA) was carried out to assess for any significant differences between the
245 means. Differences between means at the 5% ($p < 0.05$) level were considered
246 significant.

247

248 **3. Results and discussion**

249 *3.1. Weight Gain and Feed Consumption*

250 All animals treated with the algal-supplemented diet were in good health throughout
251 the experiment and no side effect, such as diarrhoea, was apparent. In addition, the rats
252 appeared to behave normal throughout the study. Both diets –control and treated–
253 showed similar energy values (Table 1).

254 At the end of the experimental period (4 weeks), the treated group had consumed a
255 higher feed amount than the control group (445 *vs* 420 g, $p < 0.0363$) (Table 2), and
256 body weight gain was also slightly higher in treated than in control group (Table 2),
257 although the difference was not significant (34.0 *vs* 31.67 g, $p < 0.5189$). The present
258 results are in line with those reported by other authors (Bocanegra et al., 2006; Amano
259 et al., 2005), who found that body weight gains of rats fed seaweed-based diets were
260 similar to those of others given basal diets. Also there was no statistically significant
261 difference in the feeding efficiency between both groups.

262 *3.2. Effect of Seaweed Intake on Internal Organs, and Longitudinal Measure of*
263 *Gastrointestinal Tract*

264 There was no difference in the weight of internal organs between both groups (data not
265 shown), except for the liver with a lower value in the treated group (Table 2, $p <$
266 0.0276). No difference on colour or aspect in the liver of control and treated group was
267 found, but the significant weight decrease might indicate a benefit from seaweed intake
268 on liver health that could be related to lipid metabolism (Amano et al., 2005).
269 Longitudinal measure of gastrointestinal tract did not significantly differ between both
270 groups (0.42 ± 0.06 *vs* 0.41 ± 0.04 cm/g of body weight, $p < 0.6694$).

271 *3.3. Effect of Seaweed Intake on Caecal Parameters and SCFAs*

272 Intake of *Mastocarpus* supplemented diet caused a significant increase in caecal
273 weight mainly due to the amount of water held by the caecum in comparison with the
274 control diet (Table 3). The red seaweed *Mastocarpus* exhibits considerable *in vitro*
275 water retention and swelling capacity (Gómez-Ordóñez et al., 2010), which may be
276 responsible for the significant bulking effect caused by the algal supplement, with
277 higher weight and moisture of caecal contents in treated than in control rats. These
278 hydrating properties are associated to dietary fibre content in *Mastocarpus* (31.7g/100g
279 dw; Gómez-Ordóñez et al., 2010), of which 72% is soluble fibre and is mainly
280 composed by kappa-/iota- hybrid carrageenans, a kind of sulfated-galactans from red
281 seaweeds. Carrageenans are hydrocolloids with bulking effect and also indigestible
282 carbohydrates susceptible of being fermented by the colonic microbiota. Similar effects
283 in caecal parameters are reported in Wistar rats fed different diets supplemented with
284 seaweeds such as red Nori (*Porphyra tenera*) and brown Wakame (*Undaria pinnatifida*)
285 (Gudiel-Urbano & Goñi, 2002).

286 Besides, caecal pH was slighty but significantly higher in the treated than in the
287 control group (Table 3). Caecal pH is not only influenced by the accumulation of
288 fermentation products such as SCFAs (acetate, propionate, butyrate, etc.) and organic
289 acids, but also by the composition of the non-digested material in the gut. Thus, the
290 slight pH increase observed in the treated group could be related to the total SCFAs
291 decrease, but also to the lower algal fermentability observed. Also available data from
292 brown and red seaweeds show a high resistance of alginates and carrageenans to
293 intestinal degradation (Gudiel-Urbano & Goñi, 2002), which could be related to the
294 higher pH and SCFAs values found in the treated group. On the other hand, colonic

295 fermentation resulted in a significant decrease in total SCFAs in the caecum of the
296 treated group (Table 3). Yet it is interesting to note that the molar proportion of acetate
297 and propionate was significantly higher in the treated group, whereas that of butyrate
298 was significantly lower. Values of isobutyric, isovaleric, and valeric acids were less
299 than 3% of total SCFAs in both groups of animals (Table 3). This SCFAs composition
300 could affect the metabolic activity of colonocytes as well as lipid metabolism in serum
301 and liver. Specifically, propionate plays an important role in liver as inhibitor of
302 cholesterol biosynthesis and in the decrease of serum and hepatic cholesterol levels
303 (Martí del Moral, Moreno-Aliaga & Martínez, 2002).

304 Lactate values in caecal content did not differed significantly between both groups
305 (data not shown).

306 Thus, intake of the red seaweed *Mastocarpus stellatus,* rich in hybrid carrageenans,
307 was related to an increase in caecal moisture and molar proportion of acetic and
308 propionic acids, but no clear prebiotic effect was shown in rats. Therefore, the effect of
309 *Mastocarpus* intake on mineral balance in faeces and urine of rats was not further
310 reported in this study.

311 A slight but significant decrease in total protein and albumin content was observed in
312 the treated group, compared to the control (Table 4). No difference was observed in
313 glucose level, but a tendency to a lower value (Table 4, $p < 0.666$) and no significant
314 difference in uric acid was observed between groups. A significant decrease of TGL and
315 TC was found (Table 4), however, no significant difference in the atherogenic index
316 (TC/HDL-C) was observed (Table 4, $p < 0.111$).

317 Research concerning the effect of seaweeds on cholesterol levels and lipid metabolism
318 has been limited to hypercholesterolemic rat models (Bocanegra et al., 2006; Amano et

al., 2005; Wang, Yoshie & Suzuki, 2002). Regarding the effect on liver, it has been reported that seaweed intake is beneficial to liver health in hypercholesterolemic rats (Amano et al., 2005), since the liver of control animals were pale and with many white spots, while those of the test group turned a dark red, indicating recovery from a fatty liver condition. Besides, in another research with hypercholesterolemic rats, a significant increase in liver weight is related to cholesterol feeding and higher cholesterol levels (Bocanegra et al., 2006). In our study, healthy rats were fed a seaweed-supplemented diet and no colour or aspect differences in the liver of control and treated group was found, but in comparison with hypercholesterolemic rats, the significant weight decrease on liver weight in the treated group (Table 2) could also be related to lipid metabolism and to the significant decrease in TGL and TC. Moreover, in our study TC and TGL levels were only measured in the serum, but the algal diet used could possibly lower TC level in the liver.

Our results showed that the intake of *Mastocarpus* for 28 days was capable to produce a beneficial effect on lipid metabolism in healthy rats, most probably *via* propionate synthesis by colonic fermentation. Nevertheless, more research work is needed in order to elucidate the mechanism of lipid lowering by this red seaweed.

3.5. Effect of Seaweed Intake on Faecal Parameters and Bacterial Faecal Count

The intake of *Mastocarpus* supplemented diet caused a significant increase in faecal weight (48.1 ± 4.7 g *vs* 95.0 ± 15.1 g, $p < 0.000$) and faecal moisture (55.9 ± 1.9% *vs* 68.1 ± 3.7%, $p < 0.000$) at the end of the experimental period. The significant bulking effect caused by the algal supplement would be responsible for the significant amount of water retained in faeces in comparison with the control diet.

342 No significant difference was observed in bacterial count on faeces between both
343 groups of rats (data not shown) at the end of the experiment. However, a tendency to the
344 increase in the total amount of bacteria was observed (control group, 27.72 log CFU/g;
345 treated group, 29.26 log CFU/g; $p < 0.1868$). Fermentation of dietary fibre can affect
346 the composition of colonic microbiota and modify the fermentative activity of bacteria
347 (Gudiel-Urbano & Goñi, 2002). However, our results showed that the intake of
348 *Mastocarpus* for four weeks was not apparently capable to change significantly the
349 composition of colonic microbiota in a healthy rat. Nevertheless, it is interesting to note
350 that the population of lactic bacteria (32.9%, $p < 0.000$) was predominant in both groups
351 of rats, compared to the rest of bacteria (enterobacteria, 25.9%; *E. coli β-glucuronidase*
352 *positive*, 24.7%; and faecal coliform, 25.9%).

353 *3.6. Effect of Seaweed Intake on Antioxidant Status in Serum and Caecum*

354 Two biological compartments were evaluated, serum and caecum, using a global
355 antioxidant measure, such as the reducing power by FRAP method. These
356 compartments were chosen as a systemic one and as a partial representation of the gut
357 environment.

358 Intake of *Mastocarpus* did not lead to an increase in RP values in serum, neither at 4
359 min (control group, 306.34 ± 63.65 μmol TE/L; treated group, 314.62 ± 40.02 μmol
360 TE/L) nor at 30 min (Table 5). Since the major determinant of RP is uric acid
361 concentration (Jiménez-Escrig et al., 2008), the specific uric acid content in serum was
362 measured (Table 4). Thus, no significant difference appeared in RP of serum, either
363 with or without the contribution of uric acid (Table 5). Therefore, the algal-
364 supplemented diet did not alter the RP measurement.

365 In contrast, RP value in caecum was significantly higher ($p < 0.0007$ at 4 min; $p <$
366 0.0005 at 30 min) in the treated group as compared to the control (Table 5). A similar
367 effect has been found previously in okara, a byproduct of the soymilk industry which is
368 rich in dietary fibre and protein (Jiménez-Escrig et al., 2008). Also, it has been reported
369 previously (Jiménez-Escrig et al., 2011b) a significant relation between sulfate content
370 and multifunctional antioxidant capacity in red seaweeds. Thus, the antioxidant capacity
371 found in caecum could be related to sulfated polysaccharides, more specifically to
372 kappa-/iota- hybrid carrageenan produced by the red seaweed *Mastocarpus*, since they
373 escape digestion in the small intestine showing their effect in the caecal compartment.

374 *3.7. Effect of Seaweed Intake on Anticoagulant Capacity in Plasma*

375 Anticoagulant capacity in plasma was evaluated by the APTT assay, which evaluates
376 the intrinsic coagulation pathway. With the presence of an anticoagulant, the normal
377 time of coagulation (usually between 29-30 seconds) is delayed according to the
378 anticoagulant capacity of the substance. In the case of animals fed the algal-
379 supplemented diet, the value of APPT in plasma was significantly longer ($p < 0.0001$),
380 approximately 1.7-fold, than that in the control group (Figure 1). Also, it has been
381 studied by our group that carrageenans from *Mastocarpus* are potent anticoagulants *in*
382 *vitro* with a high correlation between anticoagulant capacity and sulfate content
383 (unpublished data). The mechanism of APTT prolongation suggests inhibition of the
384 intrinsic factors such as VIII, IX, XI and XII and/or common coagulation pathway
385 (Wang, Zhang, Zhang, Song & Li, 2010). The main basis for the anticoagulant activity
386 of carrageenans appears to be an anti-thrombic property (Shanmugam & Mody, 2000)
387 and the presence of sulfate groups is an essential requirement for the anticoagulant

activity (Zhao, Guan, Yue, Zhang & Li, 2007). Our results showed that the intake of *Mastocarpus* for four weeks was apparently capable to elicit a significant increase of the anticoagulant capacity in the plasma of healthy Wistar rats.

4. Conclusions

Intake of *Mastocarpus stellatus,* a red seaweed rich in total and soluble dietary fibre, mainly composed by hybrid carrageenans (a type of sulfated-galactans), could promote an improved lipid profile in the serum and a better antioxidant status in the caecum of healthy rats. A significant increase in caecal moisture and molar proportions of short chain fatty acids, especially acetic and propionic, was obtained but no clear prebiotic effect could be observed under the experimental conditions used. Moreover, sulfated-galactans of carrageenans seemed to be responsible for the significant reducing power increase in caecum and also for the anticoagulant capacity increase in serum. *Mastocarpus stellatus* intake might be useful for the prevention of hyperlipidemia and thrombosis in rats. Further research work focused on specific applications for this particular red seaweed is needed before any health claims can be made.

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510 **Figure captions**

511 Figure 1. Effect of seaweed intake on anticoagulant capacity by APTT assay in healthy

512 rats ($p < 0.0001$)

513

514 Table 1

515 Composition of test diets used in the feeding experiment (g/Kg)

	Control	Treated
Moisture	120	108
Protein	154	139
Fat and oil	29	26
Carbohydrates	605	545
Starch	443	399
Total sugar	25	23
Dietary fibre (cellulose)	39	35
Red alga [a]	0	100
Minerals	48	47
Calcium	9.10	8.19
Phosphorus	5.90	5.31
Copper	0.012	0.011
Vit A (U/kg)	15000	13500
Vit D (U/kg)	1500	1350
Vit E	0.020	0.018
Energy (kcal/g)	3224	3080

516 [a]Proximate composition of *Mastocarpus stellatus* (% dw): moisture, 8.86 ± 0.04; total
517 dietary fibre, 31.70 ± 0.23; soluble fibre, 22.85 ± 0.19; insoluble fibre, 8.85 ± 0.67;
518 protein, 21.30 ± 0.18; oil, 0.39 ± 0.02; ash, 24.99 ± 0.12; sulfate, 57.1 ± 0.8 (% ash dw);
519 low molecular weight carbohydrates, 6.89 ± 0.53 (Gómez-Ordóñez et al., 2010).

520

521 Table 2

522 Effect of seaweed intake on body, liver weight and feeding efficiency in healthy rats

	Control (n = 6)		Treated (n = 6)	
Body weight (g)	mean	SD	mean	SD
Initial weight	199.67	4.93	199.33	2.73
Final weight	231.33	7.92	233.33	6.71
Weight gain	31.67	6.02	34.00	6.07
Food intake[a]	420	17.37*	445	18.45*
Feeding efficiency[b]	0.08	0.01	0.08	0.01
Liver weight[c]	35.14	2.84*	30.93	2.82*

523 Values are expressed as the mean value ± SD of each group of animals

524 * Statistically significant difference between both groups of animals ($P < 0.05$)

525 [a] Values are expressed as gram in total experimental period

526 [b] Feeding efficiency = weight gain x food intake^{-1}

527 [c] Values are expressed as mg/g body weight

528

529 Table 3

530 Effect of seaweed intake on caecal[a] parameters and SCFAs[b] in healthy rats

	Control (n =6)		Treated (n =6)	
	mean	SD	mean	SD
Caecal weight (mg/g bw[c])	15.06	1.48*	24.24	5.24*
Caecal moisture (%)	81.70	0.87*	88.70	1.86*
Caecal pH	5.95	0.17*	6.62	0.25*
Total SCFAs (μmol/g dw[d])	203.87	32.66*	126.83	26.41*
Molar proportion:				
Acetic	12.85	1.89*	25.38	3.83*
Propionic	19.16	2.99*	34.02	6.52*
Butyric	67.99	4.60*	40.59	6.37*

531 Values are expressed as the mean value ± SD of each group of animals
532 * Statistically significant difference between both groups of animals ($P < 0.05$)
533 [a] Caecum samples from each animal were recorded at the end of the trial. SCFAs
534 analysis was done on each sample in triplicate
535 [b] SCFAs = short chain fatty acids
536 [c] bw = body weight
537 [d] dw = dry weight
538 A trace of isovaleric, isobutyric and valeric acids was detected (< 3% of the total
539 SCFAs value)
540

541 Table 4

542 Effect of seaweed intake on biochemical parameters in the serum[a] of healthy rats

	Control (n =6)		Treated (n =6)	
	mean	SD	mean	SD
Total protein (g/dL)	6.98	0.38*	6.04	0.48*
Albumin (g/dL)	4.93	0.27*	4.27	0.34*
Glucose (mg/dL)	148.33	9.14	144.17	21.05
UrA (mg/dL)[b]	1.48	0.27	1.54	0.33
TGL (mg/dL)[b]	104.42	35.38*	69.19	25.16*
TC (mg/dL)[b]	55.60	8.54*	46.87	10.60*
AI (TC/HDL-C)[b]	1.25	0.18	1.11	0.07

543 Values are expressed as the mean value ± SD of each group of animals
544 * Statistically significant difference between both groups of animals ($p < 0.05$)
545 [a] Serum samples from each animal were recorded at the end of the trial
546 [b] Determined colorimetrically according to manufacturer's specifications (Spinreact)
547 UrA = uric acid; TGL = triglycerides; TC = total cholesterol; AI = atherogenic index;
548 HDL-C = HDL cholesterol
549

550 Table 5

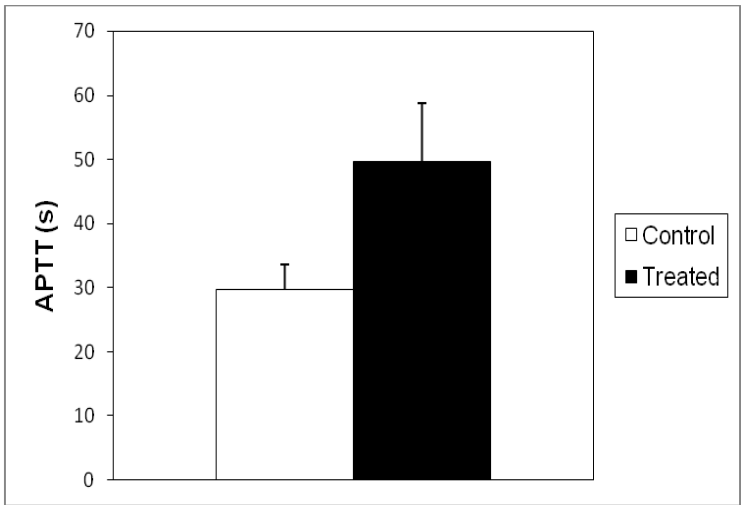
551 Effect of seaweed intake on antioxidant status[a] of caecum and serum in healthy rats

	Control (n = 6)		Treated (n = 6)	
Reduction power (RP[b])	mean	SD	mean	SD
RP_{cec} (µmol TE/g dw)	75.08	12.05*	108.53	25.22*
RP_{ser} (µmol TE/L)	483.93	93.83	494.26	71.09
$RP_{-UrA\ ser}$ (µmol TE/L)	434.61	95.06	448.22	68.51

552 Values are expressed as mean value ± SD
553 * Statistically significant difference between both groups of animals ($p < 0.05$)
554 [a] Serum and caecum samples from each animal were recorded at the end of the trial
555 [b]RP towards Fe(III), FRAP value at 30 min; RP_{cec} = Reduction power in caecum; RP_{ser}
556 = Reduction power in serum; $RP_{-UrA\ ser}$ = Reduction power in serum without the
557 contribution of uric acid
558

559 Figure 1

560



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CAPÍTULO 5

DISCUSIÓN GENERAL

1. Caracterización y evaluación nutricional de las algas *in vitro*

En este apartado se estudia la evaluación nutricional de un total de cinco algas, tres algas pardas (*Himanthalia elongata*, *Bifurcaria bifurcata* y *Saccharina latissima* (anteriormente *Laminaria saccharina*) y dos algas rojas (*Mastocarpus stellatus* y *Gigartina pistillata*) en relación a la composición centesimal (fibra alimentaria, cenizas, proteína, contenido lipídico y humedad) (Publicación 1 (P1)) y en segundo lugar a la metodología que se ha puesto a punto con el fin de llegar a una mejor caracterización de las algas estudiadas (P2, P3 y P5).

También se incluye la caracterización de los extractos acuosos y orgánicos en nueve algas (P4) y el fraccionamiento secuencial de los polisacáridos realizado en el alga parda *Saccharina latissima* y en el alga roja *Mastocarpus stellatus* (P5).

1.1. COMPOSICIÓN CENTESIMAL

La ***fibra alimentaria*** es el objetivo principal de este estudio siendo una fracción mayoritaria tanto en las algas pardas (30,23-37,42 g/100 g peso seco) como en las rojas (29,31-31,7 g/100 g). Estos valores de fibra total provienen de la suma de la fibra insoluble y fibra soluble obtenidos mediante la aplicación del método de la AOAC modificado (P1-Table 2). Valores similares de fibra total se han obtenido en otras especies de algas pardas como Wakame (33,58 g/100 g) y *Laminaria* spp. (36,12 g/100 g) (Rupérez & Saura-Calixto, 2001) e incluso valores mayores de hasta un 50% en *Fucus* spp. (Rupérez & Saura-Calixto, 2001) o *Himanthalia* spp. (50,3%) (Cofrades et al., 2008) y de un 60,0% en el alga roja *Grateloupia turuturu* (Denis et al., 2010).

El interés de la fibra de algas radica por un lado en su contenido, siendo éstos valores de fibra mayores que los valores de fibra encontrados en la mayoría de vegetales terrestres comestibles (MacArtain et al., 2007; Mabeau & Fleurence, 1993). Por otro lado, cabe destacar la diferencia en las fracciones soluble (SDF) e insoluble de la fibra (IDF), siendo la fracción soluble la de mayor importancia

en las algas (14,6-23,6 g/100 g) (P1-Table 2), lo contrario a lo que normalmente ocurre en la mayoría de plantas terrestres (Anderson & Bridges, 1988).

Esta diferencia en cuanto a las fracciones soluble e insoluble de la fibra se debe principalmente a la naturaleza de los polisacáridos que componen la pared celular de las algas y a sus propiedades de hidrosolubilidad que son consecuencia del ambiente en el que viven estos organismos acuáticos. No obstante, se aprecian diferencias en los valores de SDF e IDF entre ambos grupos de algas (pardas y rojas), debidas a la diferente naturaleza de sus polisacáridos (Rupérez & Saura-Calixto, 2001).

A partir de los resultados de composición de la fibra (P1-Table 3) en las cinco algas estudiadas se ha podido corroborar la presencia de los principales polisacáridos que componen la pared celular de estas algas. En la figura 5.1 se muestra las diferencias en composición entre algas pardas y rojas en la fracción soluble de la fibra. Mientras que en las **algas pardas**, el contenido en ácidos urónicos (UA) es mayoritario constituyendo hasta el 56% del total en *Saccharina latissima* en las algas rojas el contenido mayoritario es en azúcares neutros (NS) hasta un 88,9% del total en *Mastocarpus stellatus*.

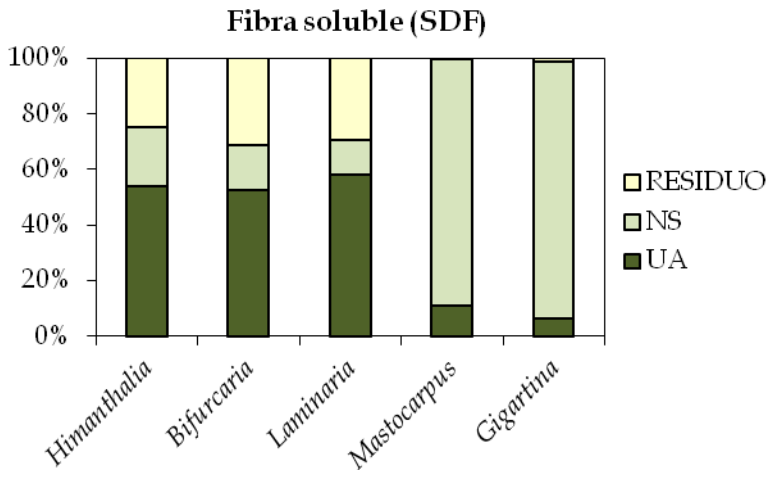


Figura 5.1. Composición de la fracción de fibra soluble (SDF) en las algas estudiadas (NS: azúcares neutros; UA: ácidos urónicos)

El contenido de ácidos urónicos en las algas pardas revela la presencia del polisacárido alginato, constituido por unidades de ácido manurónico y ácido gulurónico. Además sólo en las algas pardas aparece un residuo insoluble (24-

31% peso seco total en SDF) durante la determinación de fibra por el método de la AOAC, concretamente después del tratamiento con ácido sulfúrico. El análisis del residuo liofilizado por espectroscopía infrarroja (FTIR) reveló la presencia de bandas de absorción características (808 cm^{-1} y 787cm^{-1}) que nos permitieron identificarlo como ácido algínico (Mackie, 1971; Leal et al., 2008). Los alginatos son la sal del ácido algínico y son estables a pH 6-9, pero a pH ácido (3-4) se vuelven insolubles y precipitan en la forma de ácido. Así, sumando ambas fracciones (UA y residuo) obtenemos una fracción mayoritaria de 78-85%, siendo el alginato el principal polisacárido estructural de estas algas bien en forma de sal o en forma de ácido.

Por otro lado el análisis por cromatografía de gases (GLC) de los azúcares neutros en SDF (P1-Table 4) indica que los NS mayoritarios en las algas pardas son fucosa (46-54%), glucosa (12-35%) y galactosa (10-22%) respectivamente, pudiendo estar relacionados con la presencia de los polisacáridos fucoidano y laminarina.

Con respecto a las **algas rojas** el principal azúcar neutro es la galactosa (88-91% del total de NS), relacionado con el principal polisacárido de las algas rojas, el carragenano, constituido por unidades de galactosa y anhidrogalactosa sulfatadas. Además se ha identificado por GLC (P1-Table 4) en el alga *Mastocarpus* una pequeña cantidad de 3,6-anhidro-galactosa, confirmando su composición.

En la fracción insoluble de la fibra (IDF) las diferencias en cuanto a composición no son tan importantes como en la fracción soluble, solo hay que destacar el mayor porcentaje de lignina Klason (KL) en las pardas, especialmente en *Himanthalia* (70,6% del total de IDF) respecto de las rojas y el mayor contenido de azúcares neutros en las rojas (40% del total de IDF). Otros componentes como proteína resistente y polifenoles en las algas pardas (Jiménez-Escrig et al., 2001) suelen estar asociadas a la lignina Klason (Pinelo et al., 2006), que pueden ser responsables del elevado contenido en KL.

Además el análisis de los azúcares neutros en la fracción insoluble de la fibra revela que en las algas pardas el azúcar mayoritario es la glucosa (hasta un 90% del total de NS), que se relaciona con la presencia del polisacárido celulosa, el principal polisacárido que constituye la fibra insoluble en las algas pardas. Aunque las algas rojas también contienen celulosa, el principal NS en la fracción insoluble es la galactosa, al igual que como ocurre con la fibra soluble.

El contenido de ***cenizas totales*** en las algas estudiadas es muy elevado debido a su gran riqueza mineral y constituye la segunda fracción mayoritaria después de la fibra alimentaria (25-36% peso seco; P1-Table 1). Otros vegetales terrestres tienen un contenido en cenizas que no supera el 10% en peso seco (USDA, 2001). Valores similares se han encontrado en otros grupos y especies de algas marinas comestibles (Mabeau & Fleurence, 1993; Cofrades et al., 2008; Sánchez-Machado et al., 2004), como por ejemplo en Wakame (39,8%) o Nori (21%) (Rupérez, 2002). El elevado contenido en cenizas en las algas marinas es debido a su gran riqueza mineral. La presencia tanto de cationes (sodio, potasio, calcio, magnesio), como de aniones (cloruro, sulfato, fósforo, etc.) les permiten crecer en un medio con una elevada fuerza iónica, como es el medio marino. Los procesos de osmorregulación en las algas marinas se deben principalmente al intercambio iónico de sodio, potasio y cloro.

El análisis de las cenizas por cromatografía iónica (IC) (P2) ha mostrado la diferente composición en aniones entre las algas pardas y rojas (P2-Table 5), de forma que en las algas pardas encontramos un mayor contenido en cloruros (70-85% del total de aniones), mientras que en las algas rojas el anión mayoritario es el sulfato (80-90% del total de aniones) (Figura 5.2). El sulfato es un anión común que forma parte de los polisacáridos sulfatados de las algas, como en el fucoidano en las algas pardas y el carragenano en las rojas, el cual permanece unido al polisacárido mediante enlaces tipo éster ($O\text{-}SO_3^-$). Sin embargo, no hay muchos estudios que relacionen el papel del cloruro con los polisacáridos de las algas. Por un lado, se cree que el contenido en cloruro en las algas está relacionado con la salinidad del medio marino en el que viven (Nguyen &

Rossbach, 1993) y como las algas rojas suelen vivir a mayor profundidad, donde el nivel de salinidad es menor, el nivel de cloruros es también menor.

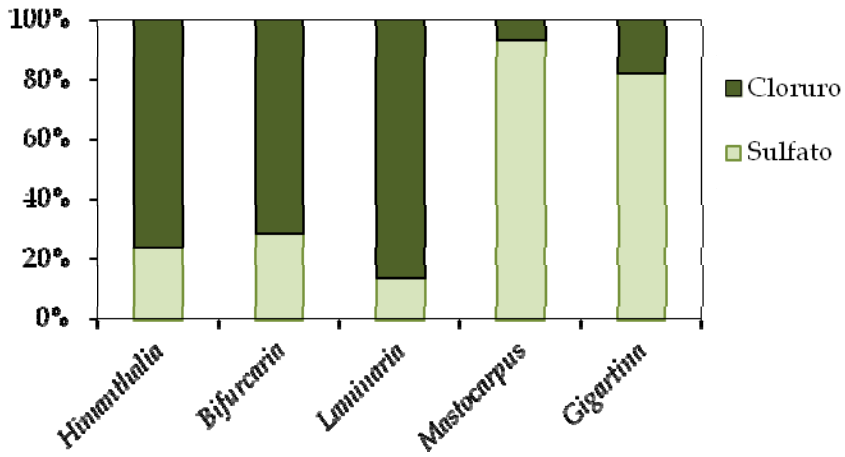


Figura 5.2. Composición de aniones: cloruro y sulfato por cromatografía iónica (IC) en las algas estudiadas

Por otro lado, diversos estudios han encontrado fucoles, terpenos y polifenoles clorados en las algas pardas (LaBarre et al., 2010). No obstante, se sabe que el contenido mineral de las algas puede sufrir variaciones no solo entre especies, sino también en función de factores medioambientales, fisiológicos y estacionales (Mabeau & Fleurence, 1993; Nisizawa et al., 1987).

El ***sulfato*** es un componente típico de los polisacáridos de las algas marinas y parece que juega un papel fundamental en la regulación iónica y en las propiedades biológicas atribuidas a los polisacáridos sulfatados (Jiao et al., 2011; Wijesekara et al., 2011; Costa et al., 2010). Dada su importancia no sólo se ha determinado el sulfato a partir de las cenizas en estas cinco algas sino también en los extractos acuosos obtenidos del total de las nueve algas estudiadas (P4-Fig. 4a) y en las fracciones obtenidas del fraccionamiento secuencial de polisacáridos de *Saccharina* y *Mastocarpus*. Más adelante se discutirá la implicación del sulfato en las propiedades biológicas de las algas estudiadas y sus polisacáridos.

La ***proteína*** en las algas es también un componente importante en las algas estudiadas (10,9-25,7% del peso seco) (P1-Table 1), especialmente en *Saccharina* (25,7%) y *Mastocarpus* (21,3%). Generalmente los valores de proteína son más

elevados en las algas rojas que en las pardas, siendo consideradas como una fuente importante para el consumo humano. Tal es el caso de Nori, un alga roja, que tiene hasta un 30% proteína (Rupérez & Saura-Calixto, 2001), alcanzando valores similares a la soja (38-40%) (Mateos-Aparicio, 2008) y a otras legumbres (Liu, 1997) de gran riqueza proteica (20-25%). En este sentido cabe destacar los valores de proteína encontrados para el alga parda cultivada *Saccharina latissima* que pueden estar relacionados con las condiciones más favorables del cultivo.

Los ***polifenoles*** constituyen un componente minoritario en las algas en general, aunque las algas pardas tienen un mayor contenido en polifenoles respecto de las rojas. En las nueve algas objeto de estudio se ha determinado el contenido en polifenoles en extractos acuosos y orgánicos con unos valores inferiores al 3,5% del peso seco (P4-Fig. 2). En este caso, en general también las algas pardas, presentan un mayor contenido de polifenoles, de hasta 4,6 veces el contenido en las algas rojas. Aunque los polifenoles constituyen un componente minoritario en las algas, los florotaninos que lo componen parece que suponen una contribución importante a la actividad antioxidante de algunas algas pardas como *Fucus* (Díaz-Rubio, Pérez-Jiménez & Saura-Calixto, 2009).

El ***contenido lipídico*** es también bajo, no llegando a superar el 1% en peso seco en la mayoría de las algas estudiadas (P1-Table 1), a excepción del alga parda *Bifurcaria bifurcata* con un 5,6%. Aún así estos valores están apoyados por otros autores (Rupérez & Saura-Calixto, 2001; Dawczynski et al., 2007; Cofrades et al., 2010) y se puede decir que las algas marinas constituyen un alimento de bajo aporte calórico. Aunque el perfil de ácidos grasos no se ha determinado en esta ocasión, cabe destacar que aunque el contenido lipídico en las algas es bajo, éste está constituido por valores elevados de ácidos grasos poliinsaturados (MUFA), en especies de *Himanthalia*, *Undaria* y *Porphyra*, también recolectadas en Galicia (Cofrades et al., 2010) y contienen lo que se considera una proporción ideal desde el punto de vista nutritivo de ácidos grasos omega n-6/n-3 (Dawczynski et al., 2007).

Con el fin de lograr una caracterización más amplia de las algas estudiadas, se han desarrollado tres métodos cuyos resultados se discuten a continuación:

1.2. FTIR COMO HERRAMIENTA PARA IDENTIFICAR LOS POLISACÁRIDOS DE ALGAS

Fundamento del método. La técnica de espectroscopía de absorción molecular en el infrarrojo (FTIR) es una herramienta muy útil que permite la caracterización preliminar de un compuesto orgánico, a partir de ciertas bandas específicas de absorción que aparecen en el rango de frecuencias de 4000-650 cm^{-1} (número de onda) del espectro infrarrojo, debido a que no existen, teóricamente, dos compuestos que absorban exactamente en las mismas frecuencias (Kakuráková & Wilson, 2001).

La identificación de un polisacárido se hace a partir del estudio sistemático del espectro correspondiente en dos pasos sucesivos: (1) Se empieza por identificar los grupos funcionales o enlaces químicos de la molécula. Para ello es muy útil elaborar una tabla de grupos funcionales donde aparecen las frecuencias en cm^{-1} a las que es previsible la aparición de las bandas de absorción de cada uno de los grupos funcionales. Con esta tabla se puede saber que si un espectro no contiene la absorción típica de cierto grupo funcional, la molécula no contiene dicho grupo; (2) Seguidamente, se procede al análisis de la región de la huella dactilar (frecuencias 950-700 cm^{-1}) (Mathlouthi & Koenig, 1987), que es característica de cada compuesto. De manera adicional también se puede usar la herramienta proporcionada por el equipo FTIR y obtener espectros de absorción referidos a la segunda derivada, donde aparecen bandas y picos que permiten una más clara identificación de los grupos funcionales (Matsuhiro & Rivas, 1993).

Aplicación del método. La técnica FTIR ha permitido la identificación de los principales polisacáridos asociados a la pared celular y a los espacios intercelulares de algas pardas y rojas como el alginato y el carragenano en un total de ocho algas estudiadas (P3). Estos polisacáridos se utilizan como fuente

de ficocoloides, como agentes gelificantes y espesantes por muchas industrias hoy día y la demanda en la utilización de estos productos es cada día mayor, por lo que la espectroscopía FTIR es una herramienta muy útil que permite determinar la calidad y composición de polisacáridos a partir de una muestra de un alga desconocida. En este caso, partimos de unas muestras de algas secas y molidas sin necesidad de ningún tratamiento previo, ya que el equipo FTIR permite analizar estas muestras directamente.

Algas pardas. Con el fin de identificar los grupos funcionales de los polisacáridos de algas pardas, se ha recurrido: (1) a la información que ya está publicada en la bibliografía (Kakuráková & Wilson, 2001; Rupérez et al., 2002; Leal et al., 2008; Rioux et al., 2010; Synytsya et al., 2010), (2) al análisis de los espectros de patrones comerciales de algas pardas, como son el alginato sódico, el fucoidano, la laminarina y la celulosa (Sigma-Aldrich), y (3) al análisis de las frecuencias de los espectros en la segunda derivada. En la Tabla 5.1 se resumen tanto los grupos funcionales y sus frecuencias (cm^{-1}), como la presencia o ausencia de estos grupos funcionales en los polisacáridos comerciales analizados. En la publicación 3 también se muestran los resultados de las frecuencias de los espectros en la segunda derivada (P3-Table 2).

Una vez recopilada esta información, no hay más que obtener los espectros de las muestras de las algas objeto de estudio (P3-Fig. 1) y verificar la ausencia y/o presencia de las bandas que se corresponden con los polisacáridos. Así, en las algas pardas (*Himanthalia*, *Saccharina* y *Bifurcaria*) encontramos que el principal polisacárido que forma parte de las paredes celulares es el alginato, lo cual concuerda con los resultados obtenidos de la composición centesimal de fibra. También concuerda la presencia de estos alginatos tanto en forma de sal como en forma de ácido, éste último debido a la presencia de un pequeño hombro a 1730 cm^{-1}.

Además, no sólo es posible identificar los principales polisacáridos de algas, sino que la técnica FTIR proporciona información sobre el contenido en sulfato

(banda de absorción a 1260-1225 cm^{-1}) o proteína (bandas amida-I y amida-II a 1650 y 1550 cm^{-1}, respectivamente) de una muestra. En las tres algas pardas estudiadas puede observarse tanto la banda de sulfato como las de proteína, siendo aún más evidente cuando se obtienen los espectros de la segunda derivada que proporcionan una mejor resolución de los picos. Estos resultados también concuerdan con los resultados previos de composición centesimal.

Tabla 5.1. Tabla [a] de grupos funcionales e identificación de los principales polisacáridos de algas pardas (alginato, fucoidano, laminarina y celulosa) por espectroscopía FTIR.

Frecuencia (cm^{-1})	Grupo funcional	Polisacárido[b]			
		A	L	F	C
1750-1735	Grupo éster ácido carboxílico (C=O)	-	-	+	-
1650-1640	Enlace Amida tipo I (N-H_2)	-	-	-	-
1637-1615	Anión carboxilato (COO^-)	+	+	+	+
1600-1500	Enlace Amida tipo II (N-H) (aminoazúcares y proteínas)	-	-	-	-
1425	Enlace tipo -β de glucosa	-	-	-	+
1415	Grupo carboxilato (O-C-O)	+	+	-	-
1260-1225	Grupo éster sulfato (S=O)	+	+	+	-
960-969	Residuo (CH_3) –Fucosa, Acético, (COS)	-	-	+	-
948	Residuo ácido urónico (C-O)	+	-	-	-
883	Residuo ácido β-manurónico (C1-H)	+	-	-	-
890	Enlace tipo –β	-	+	+	+
845	Grupo sulfato en posición C-4 axial	-	-	+	-
820	Grupo sulfato en posición ecuatorial	+	-	+	-
663	Enlace tipo –β de glucosa	-	-	-	+

(+), Presencia; (-), Ausencia; (A), alginato; (L), laminarina; (F), fucoidano; (C), cellulosa.
[a] Adaptado de Rupérez, Gómez-Ordóñez & Jiménez-Escrig, 2011; [b] Patrones de Sigma-Aldrich chemicals

Relación M/G en alginatos de algas pardas mediante la técnica FTIR. La técnica FTIR también es una herramienta útil para la estimación cuantitativa de la relación de ácido manurónico y ácido gulurónico (M/G) a partir de los valores de absorción de ciertas bandas del espectro: 808 y 1030 cm^{-1} para el ácido manurónico y 787 y 1080 cm^{-1} para el ácido gulurónico (Mackie, 1971;

Sakugawa et al., 2004). La relación M/G calculada de esta forma para las tres algas pardas estudiadas permiten conocer la naturaleza del gel de alginato que se forma en cada una de ellas, y que tiene gran interés en la industria alimentaria y cosmética. Alginatos con una relación M/G superior a 1, forman geles más elásticos y blandos que los alginatos con una relación M/G inferior a 1 (Draget et al., 2006). En nuestro estudio, las tres algas pardas estudiadas presentan alginatos con una relación M/G superior a 1 (valores entre 1,5 y 2,4).

Algas rojas. En este caso, con el fin de identificar los grupos funcionales de los polisacáridos de algas rojas (carragenano y agar), se ha recurrido: (1) a la información que ya está publicada en la bibliografía (Chopin et al., 1999; Van de Velde et al., 2002; Pereira & Mesquita, 2004; Pereira et al., 2009), (2) al análisis de los espectros de patrones comerciales de los tres principales tipos de carragenanos (kappa, iota y lamba) y del agar (Sigma-Aldrich) y (3) al análisis de las frecuencias de los espectros en la segunda derivada (P3-Table 3). En la Tabla 5.2 se resumen tanto los grupos funcionales y sus frecuencias (cm^{-1}) como la presencia o ausencia de estos grupos funcionales en los principales tipos de carragenanos de algas rojas, tomado de la bibliografía.

Tabla 5.2. Tabla [a] de grupos funcionales e identificación de los principales tipos de carragenanos de algas rojas por espectroscopía FTIR.

Frecuencia (cm^{-1})	Grupo funcional	Tipo de carragenano						
		κ	ι	λ	μ	ν	θ	ξ
1210-1260	Grupo éster sulfato (S=O)	+	+	+	+	+	+	+
928-933, 1070	3,6-anhidro-D-galactosa	+	-	-	-	-	-	-
840-850	D-galactosa-4-sulfato	+	+	-	+	+	-	-
830	D-galactosa-2-sulfato	-	-	+	-	-	+	+
820, 825	D-galactosa-2,6-disulfato	-	-	+	-	+	-	-
810-820, 867	D-galactosa-6-sulfato	-	-	-	+	-	-	-
800-805, 905	3,6-anhidro-D-galactosa-2-sulfato	-	+	-	-	-	+	-

(+), Presencia; (-), Ausencia; (κ): Kappa; (ι): Iota; (λ): Lambda; (μ): Mu; (ν): Nu; (θ): Theta; (ξ): Xi.
[a] Adaptado de Rupérez et al., 2011

De esta forma, el análisis de los espectros IR (P3, Fig.2B) de las ocho algas estudiadas nos ha dado información sobre la presencia o ausencia de ciertas bandas relacionadas con la presencia o ausencia de un tipo u otro de carragenano y con la ausencia de bandas correspondientes al agar. Por tanto, se puede decir que todas las algas rojas estudiadas son mayoritariamente productoras de carragenanos, con algunas modificaciones. Las tres algas rojas que pertenecen a la familia Gigartinaceae: *Mastocarpus*, *Gigartina* y *Chondracanthus*, presentan la variación típica estacional de esta familia. Durante la fase gametofítica producen carragenanos de la familia kappa-/iota-; mientras que durante la fase esporofítica producen carragenanos de la familia lambda-/theta-. De acuerdo con sus espectros infrarrojos, se puede decir, que *Mastocarpus* produce carragenanos del tipo kappa-/iota- (fase gametofitica), mientras que *Gigartina* y *Chondracanthus* producen carragenanos del tipo lambda-/theta- (fase esporofítica). Esta diferencia en la composición coincide con la fase de recolección de estas algas, mientras que *Gigartina* y *Chondracanthus* fueron recolectadas durante el mes de junio de 2009, en plena fase reproductora (esporofítica), *Mastocarpus* sin embargo fue recolectada a principios de la primavera o finales del invierno de ese mismo año (fase gametofítica), presentando esa diferencia en la composición del carragenano.

La técnica FTIR ha permitido identificar de una manera más clara y exhaustiva los principales polisacáridos de la pared celular de estas algas proporcionando información adicional complementaria a la obtenida a través del análisis de la composición centesimal y fibra alimentaria.

1.3. VALIDACIÓN DE MÉTODOS ANALÍTICOS: IC Y HPSEC

Durante la realización de esta tesis doctoral, se han desarrollado y validado dos métodos para su aplicación en la caracterización de las algas: (1) Determinación de aniones por cromatografía iónica (IC) (P2) y (2) Determinación del peso molecular de los polisacáridos por cromatografía líquida de exclusión molecular (HPSEC) (P5).

Actualmente existen muchos procedimientos analíticos que permiten establecer los parámetros necesarios para llevar a cabo la validación de un método químico. Para la validación de estos dos métodos se tuvieron en cuenta los siguientes parámetros de validación: *Linealidad, Sensibilidad, Precisión* y *Exactitud*.

La *linealidad* de un método analítico es la habilidad (dentro de un rango) de obtener una respuesta (o señal) que sea directamente proporcional a la concentración del analito en la muestra (EMEA, 2006).

La *sensibilidad* es un parámetro que permite discernir pequeñas variaciones en la concentración de un analito y hace referencia a la pendiente de la recta de calibración. Este parámetro está relacionado con los parámetros: *límite de detección (LOD)* y *límite de cuantificación (LOQ)*. El límite de detección es la cantidad mínima (o concentración) de un analito que proporciona una respuesta detectable con un valor exacto; mientras que el límite de cuantificación es la cantidad mínima (o concentración) que proporciona una respuesta que se puede determinar cuantitativamente con los parámetros de precisión y exactitud (EMEA, 2006).

La *precisión* es la medida de la dispersión de los datos y puede considerarse en tres niveles: (1) repetibilidad, (2) reproducibilidad intralaboratorio (diferentes días, diferentes analistas, etc.), también llamada precisión intermedia (EMEA, 2006) y (3) reproducibilidad interlaboratorio. Para este estudio se tuvieron en cuenta tanto la repetibilidad como la llamada precisión

intermedia o entre días. La forma más apropiada de cuantificar la precisión es usando el coeficiente de variación (CV) o la desviación estándar relativa (RSD).

La *exactitud* expresa la diferencia o el grado de concordancia entre el valor analítico y el valor real de un compuesto. El valor real lo da un determinado material de referencia o patrón. La exactitud se puede cuantificar como la medida de la recuperación.

Los resultados así obtenidos (P2 y P5) han demostrado la buena fiabilidad de los métodos que nos han permitido su aplicación, no sólo en nuestro estudio de caracterización de algas, sino también su potencialidad de aplicación en otras muestras desconocidas de interés.

1.3.1. Determinación de aniones por IC

Fundamento del método. Como se ha visto, el sulfato es el principal anión presente en las algas, el cual a su vez parece que juega un papel fundamental en las propiedades biológicas atribuidas a los polisacáridos sulfatados exclusivos que componen la pared celular de las algas. Aunque son muchos y muy variados, los métodos que existen actualmente para la determinación de sulfato son principalmente colorimétricos (Rupérez, 2002; AOAC, 2005), por ello se ha desarrollado un método sencillo y rápido basado en la cromatografía iónica que no sólo permite la determinación del sulfato sino de la mayoría de los aniones presentes en una muestra.

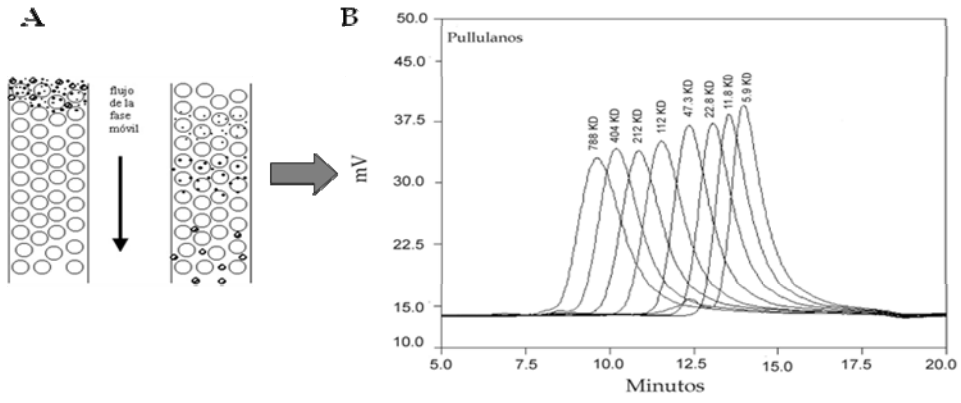
Aplicación del método. En nuestro estudio, el análisis se ha llevado a cabo a partir de una muestra de cenizas. Las cenizas tienen la ventaja de que son relativamente fáciles de obtener y permiten conocer la riqueza mineral de una muestra. Como se ha comentado se observa una diferencia en la composición de los dos aniones mayoritarios (cloruro y sulfato) entre las algas pardas y rojas (Figura 5.2). Además la aplicación del método ha permitido identificar y cuantificar otros aniones minoritarios en nuestras muestras de algas, lo cual

confirma que las algas marinas comestibles de nuestras costas pueden aportar los requerimientos de minerales necesarios para una dieta sana.

1.3.2. Determinación del peso molecular de los polisacáridos por HPSEC

Fundamento del método. La cromatografía líquida de exclusión molecular (HPSEC) es el método más común, simple y eficiente para obtener información sobre la distribución de pesos moleculares de los polímeros (Philipsen, 2004; Mori & Barth, 1999; Teresa et al., 2001). Cuando una muestra se inyecta en el sistema HPSEC ésta se separa cuando fluye junto con la fase móvil a lo largo de la columna (fase estacionaria) de un material poroso. Conforme el polímero eluye a través de la columna, las moléculas que son más grandes para penetrar los poros de la columna quedan excluidas y eluyen antes, mientras que las moléculas más pequeñas pueden penetrar los poros, recorriendo un camino más largo y por tanto, eluyendo más tarde (Figura 5.3-A).

Figura 5.3. Separación por pesos moleculares en la columna de exclusión molecular



Como es un método que permite la separación por pesos moleculares, es necesario la utilización de curvas de calibrado en un rango amplio de pesos moleculares, como es el caso de los polímeros de pullulano (Figura 5.3-B) y dextrano. Además el método HPSEC utiliza un detector de índice de refracción (RI), cuya respuesta es proporcional a la concentración del polímero proporcionando una buena estimación de la distribución de pesos moleculares.

Aplicación del método. El análisis de la distribución de pesos moleculares se ha llevado a cabo en las fracciones solubles obtenidas a partir de dos algas seleccionadas, un alga parda (*Saccharina latissima*) y una roja (*Mastocarpus stellatus*), como se detalla a continuación.

1.4. FRACCIONAMIENTO SECUENCIAL Y DETERMINACIÓN DEL PESO MOLECULAR DE LOS POLISACÁRIDOS

El tipo de fraccionamiento secuencial empleado es realmente útil para separar fracciones de acuerdo con su solubilidad (Rupérez et al., 2002). Todos los polisacáridos estructurales y de reserva de las algas son solubles, pero existen algunas diferencias entre ellos. El laminarano, el polisacárido de reserva de las algas pardas (β-glucano) es soluble en agua, pero su solubilidad depende del grado de ramificación, de forma que laminaranos poco ramificados son solubles sólo en agua caliente (60-80 ºC), mientras que laminaranos muy ramificados son solubles en agua fría (Rupérez et al., 2002), siendo esperable encontrarlos en F1 (soluble en agua 22 ºC) y F2 (soluble en agua 60 ºC). El alginato, un heteropolímero de ácido gulurónico y ácido manurónico de las algas pardas, es estable a pH entre 6 y 9 mientras que a pH ácido forma un precipitado, por lo que es esperable encontrarlo mayoritariamente en la fracción soluble en álcali (F4) y en menor proporción en las fracciones solubles en agua. El fucoidano, el polisacárido sulfatado principal de las algas pardas, es soluble tanto en agua como en ácido (F3) (Rupérez et al., 2002).

No obstante la solubilidad de estos polisacáridos puede estar relacionada con muchos factores como el pH, la concentración, iones en solución, fuerza iónica, etc (Rioux et al., 2007) por lo que de las condiciones de extracción van a depender a su vez la composición, peso molecular y por consiguiente las propiedades biológicas que se derivan de ellos.

Saccharina latissima. Es una alga parda constituida principalmente por el polisacárido alginato, tal y como se deriva de los resultados de composición de fibra y análisis de los espectros FTIR, ya comentados. En la Tabla 5.3 se

muestran los principales resultados en cuanto a composición (azúcares totales, aniones y proteína) y al rendimiento (%) de las fracciones obtenidas a partir del alga, los cuales están pendientes de publicación.

Todas las fracciones solubles (F1-F4) mostraron un mayor contenido en ácidos urónicos (72-91 %) del total de azúcares, especialmente en la fracciones F1 y F4, hecho relacionado por la presencia del alginato como principal polisacárido en este alga. La fracción soluble en álcali (F4) fue la de mayor rendimiento (25 %) y mayor contenido en ácidos urónicos Además, el análisis de los espectros FTIR obtenidos en estas fracciones también reveló la presencia de las bandas de absorción a 1616 y 1419 cm^{-1}, atribuidas al alginato (Tabla 5.1).

Tabla 5. 3. Composición química de las fracciones de los polisacáridos de *Saccharina latissima* (g/100g peso seco)

		Azúcares totales			
Fracción	**Rendimiento (%)**	**NS**	**UA**	**Aniones**	**Proteína**
Saccharina		8,0 ± 0,8	11,8 ± 0,8	14,1 ± 0,4	25,7 ± 0,1
F1	9,6	12,6 ± 0,7^{a}	50,8 ± 1,5^{a}	1,3 ± 0,3^{a}	0,5 ± 0,1^{a}
F2	5,0	12,8 ± 0,9^{a}	36,9 ± 1,4^{b}	2,1 ± 0,1^{b}	1,1 ± 0,1^{b}
F3	0,5	9,4 ± 0,6^{b}	24,5 ± 0,5^{c}	14,5 ± 0,1^{c}	1,5 ± 0,1^{c}
F4	25,0	5,2 ± 0,2^{c}	56,2 ± 2,9^{d}	0,5 ± 0,0^{d}	0,9 ± 0,1^{b}
F5	10,3	78,3 ± 1,1^{d}	12,0 ± 0,8^{e}	nd	2,3 ± 0,3^{d}

Valores expresados como la media ± SD
Letras diferentes en cada columna muestran diferencias significativas ($P < 0.05$)
NS, azúcares neutros por GLC; **UA**, ácidos urónicos (Scott); Proteína (Bradford y Leco en F5)
F1, soluble en agua a 22 ºC; **F2**, soluble en agua a 60 ºC; **F3**, soluble en 0.1 M HCl a 37 ºC; **F4**, soluble en 2M KOH a 37 ºC; **F5**, residuo insoluble. nd: sin determinar

La fracción soluble en ácido (F3) fue la de menor rendimiento (0,5 %), pero la de mayor contenido en aniones, especialmente el anión sulfato y proteína. También el análisis de su espectro FTIR reveló una mayor intensidad de las bandas amida-I y amida-II (Tabla 5.1) relacionadas con el contenido en proteína.

Por último, la fracción insoluble (F5) se caracterizó por su mayor contenido en azúcares neutros, especialmente en glucosa (98 % del total de azúcares). El análisis del espectro FTIR reveló la presencia de las bandas de absorción de la β-glucosa a 663 y 895 cm^{-1}, con lo cual se corroboró la presencia del polisacárido celulosa que forma parte de la fracción insoluble de la fibra en las algas pardas.

Con respecto a la **distribución de pesos moleculares**, todas las fracciones muestran una gran heterogeneidad (P5-Table 4). Así las fracciones F1, F2 y F4 muestran cuatro y tres picos, respectivamente de diferente peso molecular, indicando que se trata de componentes diferentes.

En las fracciones solubles en agua (F1 y F2), aparecen tres componentes mayoritarios que suman entre el 94,7- 96,5 % del área total, cuyos rangos de pesos moleculares son: 310-443 kDa, 20-27 kDa y 5-5,8 kDa, respectivamente. Los dos primeros son atribuibles a alginatos solubles en agua de alto peso molecular y alginatos de bajo peso molecular (Zvyaginsteva et al., 2005), respectivamente. Aunque el primero también puede deberse a fucoidanos solubles en agua (Rupérez et al., 2002). El tercero con peso molecular entre 5-5,8 kDa es atribuible a la presencia del polisacárido laminarano. El laminarano es un polisacárido de reserva de la pared celular de las algas pardas y sería esperable encontrarlo como componente principal en ambas fracciones solubles en agua (F1 y F2) del alga *Saccharina*. Pero en este caso, también hay que tener en cuenta la época de recolección del alga que se realizó durante las estaciones de primavera-verano, cuando la presencia de este polisacárido es minoritaria o casi nula (Holdt & Kraan, 2011).

En la fracción soluble en álcali se observa un componente mayoritario con un rango de peso molecular entre 43-49 kDa, que también es atribuible a alginatos, correlacionado con su mayor contenido en ácidos urónicos solubles en álcali (Tabla 5.3).

Mastocarpus stellatus. Es una alga roja constituida principalmente por el polisacárido carragenano híbrido tipo kappa/iota, tal y como se deriva de los

resultados de composición de fibra y análisis de los espectros FTIR, ya comentados. En la Tabla 5.4 se muestran los principales resultados en cuanto a composición (azúcares totales, aniones y proteína) y al rendimiento (%) de las fracciones obtenidas a partir del alga, los cuales están pendientes de publicación.

A diferencia de cómo ocurre con el alga parda, todas las fracciones solubles de *Mastocarpus* presentaron un mayor contenido en azúcares neutros, especialmente en galactosa (71,2-95,3 % del total de azúcares), respecto a los ácidos urónicos, lo cual coincide con la diferencia en cuanto a composición entre algas pardas y rojas ya comentada. El análisis de los espectros FTIR reveló que todas las fracciones contienen el polisacárido carragenano híbrido kappa/iota con diferentes grados de hibridación y sulfatación. En este sentido el anión mayoritario en todas las fracciones fue el sulfato. La fracción insoluble (F5) también mostró un elevado contenido en azúcares neutros, pero mayoritariamente compuesta por glucosa del polisacárido celulosa.

Tabla 5. 4. Composición química de las fracciones de los polisacáridos de *Mastocarpus stellatus* (g/100g peso seco)

		Azúcares totales			
Fracción	**Rendimiento (%)**	**NS**	**UA**	**Aniones**	**Proteína**
Mastocarpus		24,17 ± 1,4	3,0 ± 0,3	15,4 ± 0,2	21,3 ± 0,2
F1	6,7	32,9 ± 2,2^{a}	1.9 ± 0,1^{a}	4,9 ± 0,3^{a}	1,2 ± 0,1^{a}
F2	14,4	29,8 ± 3,1^{b}	0.9 ± 0,2^{b}	1,5 ± 0,3^{b}	0,6 ± 0,0^{b}
F3	25,5	34,7 ± 1,8^{a}	2.7 ± 0,1^{c}	0,8 ± 0,0^{c}	0,1 ± 0,0^{b}
F4	12,1	21,2 ± 0,8^{c}	2.5 ± 0,2^{c}	2,5 ± 0,0^{d}	3,4 ± 0,0^{c}
F5	6,0	47,3 ± 2,5^{d}	1.0 ± 0,3^{b}	nd	10,7 ± 0,5^{d}

Valores expresados como la media ± SD
Letras diferentes en cada columna difieren significativamente ($P < 0.05$)
NS, azúcares neutros por GLC; **UA**, ácidos urónicos (Scott); Proteína (Bradford y Leco en F5)
F1, soluble en agua a 22 ºC; **F2**, soluble en agua a 60 ºC; **F3**, soluble en 0.1 M HCl a 37 ºC; **F4**, soluble en 2M KOH a 37 ºC; **F5**, residuo insoluble. nd: sin determinar

Cada fracción soluble de *Mastocarpus* tiene un rango de peso molecular (P5-Table 5) que va desde valores mayores, en las fracciones solubles en agua, a

menores en la fracción soluble en álcali (F4). Lo cual sugiere que durante el proceso de extracción secuencial el grado de polimerización del polisacárido carragenano disminuye, dando como resultado pesos moleculares cada vez menores en las últimas fracciones. Es importante destacar la importancia de las condiciones de extracción en la composición y distribución de pesos moleculares en este tipo de polisacáridos de algas rojas (Tuvikene et al., 2010), muy a tener en cuenta según las aplicaciones a que se quiera destinar los polisacáridos.

En resumen, las algas estudiadas constituyen una buena fuente de fibra alimentaria, minerales y proteína. Además contienen polisacáridos específicos y diferentes entre algas pardas y rojas, por lo que es esperable encontrar propiedades biológicas diferentes atribuibles a esos polisacáridos. El grado de sulfatación de los polisacáridos es de especial importancia en las algas marinas. Llevar a cabo un fraccionamiento secuencial nos permite conocer y caracterizar mejor los polisacáridos de las algas y establecer diferencias entre ambos grupos.

2. Evaluación de las propiedades biológicas *in vitro*

Una vez caracterizadas las algas y sus polisacáridos, el siguiente paso fue evaluar las propiedades biológicas *in vitro* que se pueden atribuir a la fibra alimentaria y a los polisacáridos específicos que forman parte de la pared celular de las algas marinas.

Por lo tanto, en este apartado se estudian las siguientes propiedades biológicas *in vitro*: (1) propiedades funcionales de la fibra alimentaria de algas, (2) capacidad antioxidante en extractos acuosos y orgánicos y (3) capacidad antioxidante y anticoagulante en fracciones solubles de *Saccharina* y *Mastocarpus*, que se derivan de su composición.

2.1. PROPIEDADES FUNCIONALES DE LA FIBRA ALIMENTARIA

La fibra soluble de las algas tiene un efecto beneficioso para la salud intestinal por su capacidad de retención de agua y sus efectos sobre la disminución del tránsito intestinal (Mohamed et al., 2012), ayudando a prevenir de una manera indirecta el cáncer de colon (MacArtain et al., 2007). Las algas estudiadas constituyen una buena fuente de fibra alimentaria (29-37 g/100 g), especialmente en la fracción de fibra soluble (15-24 g/100 g) responsable en gran medida de las propiedades físico-químicas o funcionales de la fibra. Estas propiedades son de gran interés desde el punto de vista industrial y además van a predecir su comportamiento *in vivo*.

Para tal fin se han estudiado tres propiedades físico-químicas de la fibra alimentaria en las algas: (1) capacidad de hinchamiento, (2) capacidad de retención de agua y (3) capacidad de retención de aceite.

Los valores obtenidos de capacidad de retención de agua en las cinco algas estudiadas (5,4-10,2 g/g peso seco) (P1-Table 6) son comparables a los obtenidos en otras algas comestibles estudiadas anteriormente como *Fucus*, Kombu, Wakame y Nori (5,1-10,9 g/g peso seco) (Rupérez & Saura-Calixto, 2001), y con los obtenidos en otros productos vegetales de gran riqueza en fibra (Elleuch et al., 2011). En este sentido, la habilidad de las fibras alimentarias para retener agua está íntimamente relacionada con el origen de la fibra. Así, la fibra alimentaria de las algas tiene mayor afinidad por el agua que las fibras de zumos o de cereales, debido en gran parte a las diferencias en su composición química (Elleuch et al., 2011).

Como hemos visto las algas contienen polisacáridos específicos que no se encuentran en otros vegetales de origen terrestre. La mayor parte de las fibras solubles de algas comprenden los alginatos y fucoidanos de algas pardas y los carragenanos, agar y xilanos de algas rojas (Holdt & Kraan, 2011). Estos polisacáridos son ricos en grupos hidroxilos (-OH), que les dan la habilidad de ser hidrofílicos y solubles en agua lo que les permite establecer puentes de

hidrógeno dentro de la molécula y la capacidad de interaccionar con iones externos, confiriéndoles sus propiedades como espesantes, gelificantes y emulsionantes (O'Sullivan et al., 2010).

Como consecuencia, debido a las propiedades de hidrosolubilidad de las fibras de algas, la capacidad de hinchamiento es también muy elevada y la capacidad de retención de aceite es baja (1,2-1,6 g/g peso seco) (P1-Table 6).

Estas propiedades de capacidad de hinchamiento, de retención de agua y aceite, sugieren la posibilidad de usar las fibras de algas como ingredientes en productos alimentarios. Así, las fibras con elevada capacidad de retención de agua se pueden utilizar como ingredientes funcionales que permitan modificar la viscosidad y la textura de algunos alimentos (Elleuch et al., 2011).

2.2. CAPACIDAD ANTIOXIDANTE DE ALGAS EN EXTRACTOS ACUOSOS Y ORGÁNICOS

Se ha evaluado la capacidad antioxidante multifuncional en un total de nueve algas, tres algas pardas y seis rojas. La capacidad antioxidante de los vegetales incluye varios mecanismos multifuncionales, por lo que es necesario el empleo de varios métodos para evaluar la capacidad antioxidante potencial de una muestra. En este estudio se han utilizado tres ensayos para evaluar la capacidad antioxidante en extractos acuosos y orgánicos de las algas: (1), Poder reductor (RP) por el método FRAP, basado en el potencial redox del Fe(III)/Fe(II) (0,77 V); (2), Capacidad de secuestro de radicales (RSA) por el método ABTS y (3) por el método PCL, estos dos últimos basados en la capacidad de una muestra de transferir un hidrógeno al radical sintético $ABTS^{\bullet+}$ o al radical biológico superóxido O_2^{-}, respectivamente.

Poder reductor (RP). Todas las algas estudiadas han mostrado capacidad antioxidante en el ensayo FRAP, tanto en los extractos acuosos como orgánicos (P4-Tables 3 y 4), sin embargo se ha observado un comportamiento diferente en esta capacidad entre las algas pardas y rojas.

Así, en las algas pardas se han observado valores superiores de RP en los extractos orgánicos respecto de los extractos acuosos. Se ha visto que en los extractos orgánicos el contenido en polifenoles es mayor que en los extractos acuosos (P4-Fig. 2), por lo que la capacidad antioxidante observada en los extractos orgánicos de las algas pardas puede estar correlacionada con el mayor contenido en polifenoles.

Por otro lado, en las algas rojas se han observado valores superiores de RP en los extractos acuosos respecto de los orgánicos. En este caso, se ha visto que en los extractos acuosos hay una correlación significativa entre el contenido en sulfato y el rendimiento de la extracción, es decir que los grupos sulfato unidos a los polisacáridos de las algas rojas hacen que aumente su solubilidad en agua. Por lo que la capacidad antioxidante observada en los extractos acuosos de las algas rojas está correlacionada con el mayor contenido en sulfato.

Capacidad de secuestro de radicales (RSA). En este estudio sólo las algas pardas han mostrado capacidad de secuestro de radicales por los métodos ABTS y PCL tanto en los extractos acuosos como orgánicos (P4-Table 3 y 4). Esta diferencia en la capacidad de secuestro de radicales entre algas pardas y rojas se debe fundamentalmente a la naturaleza distinta de sus polisacáridos y a las diferencias en cuanto a composición en compuestos polifenólicos entre ambos grupos.

2.3. CAPACIDAD ANTIOXIDANTE EN FRACCIONES DE POLISACÁRIDOS DE ALGAS

La capacidad antioxidante de los polisacáridos depende de muchos factores y parámetros estructurales como el peso molecular, el tipo y la posición de los grupos funcionales- hidroxilo, sulfato, amina, carboxilo-, el tipo de azúcar y el tipo de enlace (Chen et al., 2009; Barahona et al., 2011). Se ha visto que la capacidad antioxidante de polisacáridos de bajo peso molecular es mayor que la observada en polisacáridos de mayor peso molecular, lo cual puede estar relacionado con su estructura menos compacta que permite que los grupos

hidroxilo o amina puedan reaccionar con los radicales libres (Chen et al., 2009). Pero aunque se sabe la influencia de estos factores, actualmente no se conocen completamente los mecanismos reales que intervienen en la capacidad antioxidante de los polisacáridos (Chen et al., 2009).

Para ello se evaluó la capacidad antioxidante multifuncional potencial en las fracciones obtenidas del fraccionamiento secuencial en el alga parda *Saccharina latissima* y en el alga roja *Mastocarpus stellatus*, con el fin de conocer mejor los mecanismos que intervienen en dicha capacidad. De la misma forma que como se hizo para los extractos acuosos y orgánicos de algas, para evaluar la capacidad antioxidante potencial en las fracciones solubles (F1 y F2), ácida (F3) y alcalina (F4) de los polisacáridos de estas algas se emplearon tres métodos distintos: poder reductor (RP) por el método FRAP y capacidad de secuestro de radicales (RSA) por los métodos ABTS y PCL.

Los resultados más relevantes se discuten a continuación:

Saccharina latissima. La capacidad antioxidante, medida tanto como poder reductor (RP) o capacidad de secuestro de radicales (RSA) mostró la siguiente jerarquía de mayor a menor: F3>> F1 ~F2 >F4 (Figura 5.3). La fracción ácida mostró una RSA por el método ABTS de hasta diez veces mayor que el resto de fracciones. En un estudio anterior en el alga parda *Fucus* usando la misma metodología se ha visto también mayor capacidad antioxidante en la fracción ácida que se corresponde con el mayor contenido en sulfato (Rupérez et al., 2002). En el caso de *Saccharina*, también la F3 fue la fracción de mayor contenido en sulfato y se encontró una correlación positiva significativa entre el contenido en sulfato con la capacidad antioxidante medida bien por RP o RSA (RP-4, $P < 0.0001$; RP-30, $P < 0.0001$; RSA-ABTS $P < 0.0001$; and RSA-PCL $P < 0.005$).

Como hemos visto, la fracción soluble en ácido (F3) fue la de menor rendimiento (0,5 %), pero la de mayor contenido en sulfato y proteína (Tabla 5.3). Debido a su bajo rendimiento, no fue posible determinar la distribución de pesos moleculares en esta fracción (P5), pero podemos suponer dado su mayor

contenido en sulfato y bajo contenido en ácidos urónicos (Tabla 5.3) que está compuesta por fucoidano, el principal polisacárido sulfatado de las algas pardas (Wijesekara et al., 2011). Además en el espectro FTIR correspondiente a la F3 apareció una banda a 850 cm^{-1} que se atribuye al enlace C-4 de fucosa o galactosa del polisacárido comercial fucoidano (Tabla 5.1).

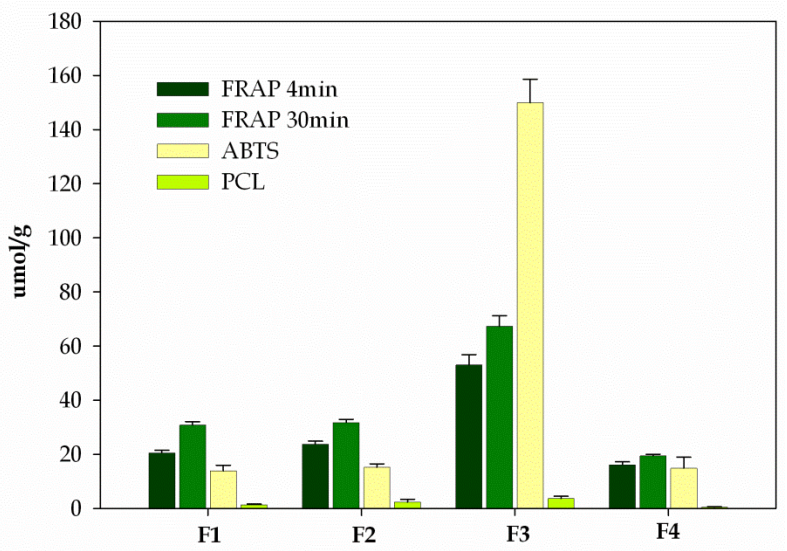


Figura 5.3. Capacidad antioxidante en fracciones de polisacáridos de *Saccharina latissima*

(**F1**, soluble en agua a 22 ºC; **F2**, soluble en agua a 60 ºC; **F3**, soluble en 0.1 M HCl a 37 ºC; **F4**, soluble en 2M KOH a 37 ºC; **RP** y **ABTS** = µmol Trolox Equivalente/g, **PCL** = µmol Vitamina C Equivalente/g)

Además, de los tres métodos empleados para determinar la capacidad antioxidante, la capacidad de secuestro de radicales por el método ABTS fue la de mayor importancia en la F3 (Figura 5.3), indicando el predominante papel de los sulfatos con el radical ABTS (coeficiente de correlación de r =0.9914). La capacidad de secuestro de radicales por el método ABTS depende de la capacidad del polisacárido sulfatado de reducir (donar un electrón) al radical catión $ABTS^{\bullet+}$. Esta capacidad además depende del efecto combinado de la densidad de las nubes electrónicas entre el radical ABTS y el sulfato, ya que la densidad de nube electrónica de un grupo funcional afecta a su capacidad de donar electrones, de forma que a mayor densidad mayor actividad donadora de electrones y por consiguiente mayor capacidad de secuestro de radicales y poder reductor. En este sentido se sabe que la densidad de la nube electrónica de los grupos sulfato es muy grande, en comparación con otros grupos funcionales presentes en los polisacáridos como los grupos amino (Chen et al., 2009) jugando por tanto, un papel fundamental en la RSA-ABTS.

Mastocarpus stellatus. En los resultados obtenidos, se observaron claras diferencias en el comportamiento antioxidante entre las fracciones de *Mastocarpus* (Figura 5.4). Los valores de RP aumentaron considerablemente de 4 a 30 minutos, en las fracciones F1 y F4 de *Mastocarpus*, al igual que como ocurrió en todas las fracciones de *Saccharina* (Figura 5.3) y como suele ocurrir en otros vegetales y algas (Jiménez-Escrig et al., 2001; Rupérez et al., 2002). Sin embargo, los valores encontrados de RP en todas las fracciones fueron mucho menores que los hallados en los extractos acuosos del alga *Mastocarpus* (P4-Table 4), excepto por la fracción F1 que mostró valores más cercanos a los hallados en el alga de partida.

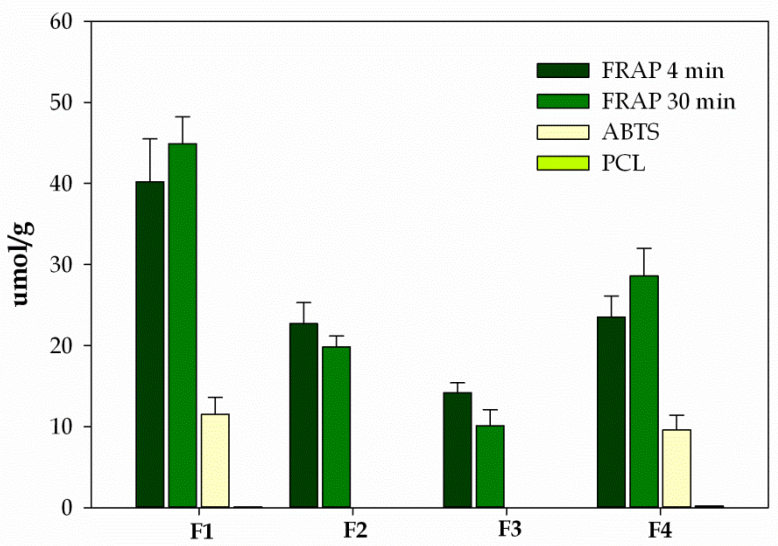


Figura 5.4. Capacidad antioxidante en fracciones de polisacáridos de *Mastocarpus stellatus*

(**F1**, soluble en agua a 22 ºC; **F2**, soluble en agua a 60 ºC; **F3**, soluble en 0.1 M HCl a 37 ºC; **F4**, soluble en 2M KOH a 37 ºC; **RP** y **ABTS** = µmol Trolox Equivalente/g, **PCL** = µmol Vitamina C Equivalente/g)

También hay que destacar la correlación entre el contenido en sulfato y RP a 4 ($P< 0.00001$, $r = 0.938654$) y 30 min ($P< 0.0001$, $r = 0.968263$). También se ha visto que el grado de sulfatación de los polisacáridos hace aumentar el poder reductor en otros vegetales (Liu et al., 2010).

Con respecto a RSA por los métodos ABTS y PCL, se encontró una correlación significativa entre el contenido en sulfato y ABTS pero no con PCL.

En general, las fracciones que mostraron mayor actividad antioxidante fueron las fracciones F1 y F4, relacionado con el mayor contenido en sulfato, pero también con la distribución de pesos moleculares. Se ha visto que las fracciones obtenidas de *Mastocarpus* tienen un rango de pesos moleculares que

va en disminución desde la F1 (mayor peso molecular) a la F4 (menor peso molecular) (P5-Table5) y otros estudios han visto que la capacidad antioxidante se correlaciona con una disminución del peso molecular (Sun et al., 2009; Zhao et al., 2011), y otros autores relacionan la protección antioxidante de los polisacáridos con las proteínas unidas a ellos (Yang et al., 2007).

En nuestro caso, la fracción de mayor contenido en sulfato (F1) mostró la mayor capacidad antioxidante, seguida por la fracción F4 de menor peso molecular (8-10 kDa, P5-Table 5) y mayor contenido proteico (Tabla 5.4), por lo que estos resultados corroboran la implicación de estos factores en la capacidad antioxidante de los polisacáridos de algas.

Además hay que destacar el diferente comportamiento de las fracciones de polisacáridos entre el alga parda y roja. Mientras que en el alga parda la actividad mayor se encontró con el método de secuestro de radicales-ABTS, en el alga roja fue mayor con el método de poder reductor, lo cual da a entender que no sólo el contenido en sulfato es importante sino que también influye en la capacidad antioxidante, el tipo de enlace, el tipo de azúcar y la composición de los polisacáridos sulfatados (Barahona et al., 2011), siendo principalmente el fucoidano en *Saccharina* y el carragenano en *Mastocarpus*.

2.4. CAPACIDAD ANTICOAGULANTE EN FRACCIONES DE POLISACÁRIDOS DE ALGAS

La capacidad anticoagulante de los polisacáridos sulfatados de las algas ha sido una de las propiedades más estudiadas (Wijesekara et al., 2011), con el fin de encontrar un sustituto de origen natural para la heparina (un polisacárido glucosaminoglucano sulfatado), que es el anticoagulante comercial más ampliamente usado desde hace más de cincuenta años en la prevención y tratamiento de enfermedades cardiovasculares. Al igual que como ocurre con la capacidad antioxidante, la capacidad anticoagulante de los polisacáridos de las algas va a depender de muchos factores como el peso molecular, la composición de azúcares, grado y distribución de grupos sulfato en la molécula, etc. (Pereira

et al., 1999; Jiao et al., 2011), siendo difícil establecer una relación clara entre estructura y propiedad biológica.

Para ello se determinó la actividad anticoagulante por los dos métodos previamente descritos (Capítulo 3, pg. 46): Tiempo de protrombina parcial activado (APTT) y tiempo de protrombina (PT) en las fracciones solubles de polisacáridos del alga parda *Saccharina* y del alga roja *Mastocarpus*. Además se determinó la actividad anticoagulante de un patrón de heparina sódica comercial a diferentes concentraciones (10, 20, 50 y 100 µg/mL) (Figura 5.5) para comparar el tiempo de retraso en la formación del coágulo en plasma humano entre la heparina y los polisacáridos sulfatados de las algas.

En el ensayo APTT, que evalúa la vía intrínseca de la coagulación, la capacidad anticoagulante de la heparina aumentó al aumentar su concentración (r =0,99368) (Figura 5.5). La actividad anticoagulante de la heparina es facilitada principalmente porque acelera la actividad de la antitrombina-III (AT-III) que a su vez inhibe los factores intrínsecos IXa, XIa y XIIa (Shanmugam & Mody, 2000) de la vía intrínseca de la coagulación.

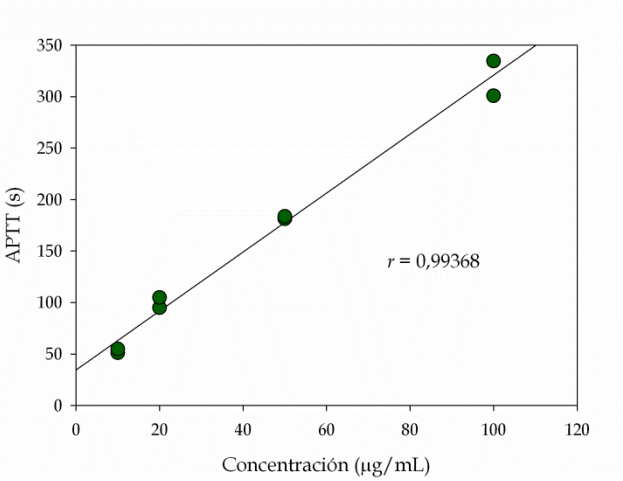


Figura 5.5. Capacidad anticoagulante de la heparina en el ensayo APTT

(Heparina sódica comercial a 10, 20, 50 y 100 µg/mL)

De la misma forma los polisacáridos sulfatados de algas, fucoidanos en algas pardas y carragenanos en algas rojas con polisacáridos polianiónicos con cargas negativas (Silva et al., 2010) presentan una estructura muy similar a la de la heparina, y el mecanismo de prolongación del APTT sugiere la inhibición de los

factores VIII, IX, XI y XII en la vía intrínseca de la coagulación (Wang et al., 2010).

En el ensayo APTT, todas las fracciones de *Saccharina* y de *Mastocarpus* aumentaron al aumentar la concentración y desarrollaron una potente capacidad anticoagulante, mayor que la del control, aunque siempre menor que la capacidad de la heparina a 100 µg/mL. Además se encontró una correlación positiva significativa entre los valores de APTT (s) y el contenido en sulfato ($P<0.0007$, $r=0.932$) en todas las fracciones. Otros estudios han corroborado que la presencia de grupos sulfato en la molécula es un requerimiento esencial para la actividad anticoagulante de los polisacáridos (Zhao et al., 2007).

En el ensayo PT, que evalúa la vía extrínseca de la coagulación, no se encontraron resultados significativamente diferentes ni en la heparina ni en las fracciones tanto del alga parda como del alga roja, respecto del valor del control. Diversos estudios han corroborado, que los polisacáridos sulfatados de algas (fucoidanos en algas pardas y carragenanos en algas rojas) intervienen mayoritariamente sobre la vía intrínseca de la coagulación y no tanto sobre la vía extrínseca (Silva et al., 2010).

En este apartado se han comentado y discutido los resultados preliminares más relevantes encontrados en las fracciones solubles de *Saccharina* y *Mastocarpus*, ya que actualmente seguimos trabajando en estos resultados para su publicación. En general, son necesarios más estudios de caracterización química y estructural de los polisacáridos sulfatados de las algas en estudio, con el fin de conseguir un mejor conocimiento acerca de los mecanismos y factores que intervienen en las propiedades biológicas *in vitro*, tanto en la capacidad antioxidante como en la capacidad anticoagulante.

3. Evaluación de las propiedades biológicas *in vivo*

Una vez realizada la evaluación nutricional de las algas y sus polisacáridos y la evaluación de las principales propiedades biológicas *in vitro*, como son la capacidad antioxidante y anticoagulante, el siguiente paso fue realizar la evaluación de las propiedades biológicas *in vivo* usando un modelo animal con ratas Wistar sanas.

Los resultados más relevantes obtenidos en los ensayo *in vivo* llevados a cabo con un grupo de ratas Wistar sanas alimentadas con dietas suplementadas con un 10% de alga seca y molida (parda *Saccharina* o roja *Mastocarpus*) frente a un grupo de animales a los que se les suministra una dieta control, se exponen a continuación.

De la observación diaria de los animales se puede deducir que la dieta ha sido bien aceptada por los mismos y que han mantenido un buen estado de salud durante las cuatro semanas que duró el ensayo. No se observó ninguna reacción adversa, como puede ser la diarrea, ni tampoco ningún síntoma derivado del consumo de la dieta base suplementada con el alga.

El tipo de alga utilizado en cada experimento ha dado lugar a diferencias en los efectos beneficiosos como consecuencia de la diferente composición de algas pardas y rojas.

3.1. *Saccharina latissima*: EFECTO SOBRE EL ESTADO ANTIOXIDANTE Y PREBIÓTICO

Saccharina latissima (Sugar Kombu) es un alga parda con un contenido en fibra alimentaria del 30,2 % (peso seco), compuesta principalmente por el polisacárido alginato y en menor medida por fucoidano y laminarano.

Efecto sobre el estado antioxidante. La ingesta del alga parda *Saccharina* ha tenido un efecto antioxidante beneficioso, especialmente en el ciego de las ratas. Así, se ha observado un aumento de la capacidad antioxidante medida como

RP en el ciego de las ratas del grupo tratado respecto del grupo control (P6-Table 4). Otros estudios con ratas sanas han encontrado este mismo efecto de aumento de RP en el ciego de las ratas, debido a la ingesta de una dieta rica en fibra alimentaria (Jiménez-Escrig et al., 2003; 2008). Además la presencia de polifenoles (P4-Fig. 2) y polisacáridos sulfatados como el fucoidano (F3, fraccionamiento *Saccharina*), junto con la correlación entre el contenido en sulfato y la capacidad antioxidante ya comentada (P4), han podido contribuir a este aumento del RP observado en el ciego, ya que los polisacáridos sulfatados y la mayoría de los polifenoles escapan a la digestión llegando intactos al colon y al ciego donde ejercen su efecto antioxidante.

Efecto prebiótico. La fermentación colónica que ha tenido lugar en el ciego de las ratas alimentadas con una dieta suplementada con fibra de algas, ha dado lugar a un aumento en la producción de ácidos grasos de cadena corta (SCFA). El contenido total de SCFA en el ciego de las ratas tratadas aumenta significativamente un 72% ($p< 0,05$) (P6-Table 3), lo cual manifiesta el efecto prebiótico de la fibra del alga *Saccharina*. Además se observa un aumento en el ciego de las ratas tratadas en el contenido de ácidos urónicos (P6-Table 3), derivados de la fermentación del polisacárido alginato presente en el alga parda estudiada. Son muchos estudios los que relacionan la producción de SCFA con el metabolismo lipídico, ya que parece que el proceso de fermentación de los prebióticos, especialmente la producción de acético y propiónico, es un marcador indicativo de las propiedades hipolipemiantes de los prebióticos y carbohidratos no digeribles (Martí del Moral et al., 2002). En este sentido, también se ha observado en las ratas tratadas una disminución significativa en el contenido de triglicéridos en el suero (P6-Table 3) respecto a las ratas control, que puede estar relacionado con la producción de SCFA en el ciego. Además, el aumento en la producción de SCFA ayuda a la biodisponibilidad de minerales en el ciego, especialmente el ácido propiónico tiene un efecto importante sobre la absorción de calcio en el intestino grueso (Lutz & Scharrer, 1991), que puede

estar implicado en el aumento de la absorción aparente del calcio encontrada en las ratas tratadas (P6-Table 5).

Por otro lado, el aumento significativo del peso y la humedad del ciego y de las heces en las ratas tratadas (P6-Table 2) es una consecuencia de la elevada capacidad de retención de agua observada en la fibra de *Saccharina* (P1-Table 6).

3.2. *Mastocarpus stellatus*: EFECTO SOBRE EL ESTADO ANTIOXIDANTE Y METABOLISMO LIPÍDICO

Mastocarpus stellatus es un alga roja con un contenido en fibra alimentaria del 31,7% en peso seco, la cual está constituida en un 72% por fibra soluble, compuesta principalmente por polisacáridos sulfatados tipo carragenano híbrido kappa/iota.

Efecto sobre el estado antioxidante. La ingesta de la fibra procedente del alga roja *Mastocarpus* ha tenido un efecto antioxidante beneficioso, especialmente en el ciego de las ratas. Se ha observado un aumento de la capacidad antioxidante medida como RP en el ciego de las ratas del grupo tratado respecto del grupo control (P7-Table 5). Este aumento en la capacidad antioxidante en el ciego está relacionado con la presencia de polisacáridos sulfatados que no son digeridos y llegan sin degradar al ciego donde pasan a ser el substrato para la microbiota. Además previamente se ha visto una correlación significativa entre el contenido en sulfato y la capacidad antioxidante, tanto en los extractos acuosos (P4) como en las fracciones solubles del alga *Mastocarpus*.

Efecto sobre el metabolismo lipídico. No se ha visto un efecto prebiótico, propiamente dicho, como resultado de la ingesta del alga roja *Mastocarpus* en ratas sanas. Sin embargo, la fibra soluble compuesta mayoritariamente por el polisacárido carragenano, presente en el alga no se absorbe a nivel del intestino delgado y es fermentada en el intestino grueso por la microbiota colónica. Como resultado de esa fermentación se ha observado un aumento, con respecto al contenido total de ácidos grasos de cadena corta, de la proporción de los

ácidos acético y propiónico en el ciego de las ratas tratadas (P7-Table 3). La producción de estos ácidos, y más concretamente la relación acético:propiónico tiene gran influencia en el metabolismo lipídico, de forma que cuanto más elevada es esta relación, mayor es el efecto hipocolesterolémico (Theuwissen & Mensink, 2008). En el presente estudio, el cociente acético:propiónico es superior en el grupo tratado (0,78) que en el grupo control (0,68), siendo mayor el efecto hipocolesterolémico. Estos resultados están relacionados con la disminución significativa en el nivel de triglicéridos y colesterol total (P7-Table 4) observado en el suero de las ratas tratadas respecto de las ratas control.

Los estudios *in vivo*, a pesar de la variabilidad que presentan por las características individuales de cada uno de los animales, son una herramienta indispensable para conocer los efectos fisiológicos positivos que se derivan de la ingesta de las algas marinas comestibles estudiadas.

CAPÍTULO 6

CONCLUSIONES

De acuerdo con los resultados obtenidos de los ensayos *in vitro* e *in vivo* realizados en las algas españolas estudiadas durante el desarrollo de esta Tesis Doctoral, se extraen las siguientes conclusiones:

De la caracterización y evaluación nutricional de las algas se deduce que:

1) El estudio de la fibra alimentaria indica que son muy ricas en esta fracción (29,3-37,4%), en la que la fibra soluble predomina sobre la insoluble, mostrando diferencias entre algas pardas y rojas en cuanto a la naturaleza de sus polisacáridos.

2) El estudio pormenorizado de la fracción soluble de fibra indica que las algas pardas contienen un 78-85% de ácidos urónicos y en menor proporción los azúcares neutros fucosa, glucosa y galactosa. En contraposición, las algas rojas contienen mayoritariamente un 82-89% de azúcares neutros, principalmente galactosa.

3) El contenido de cenizas totales (25-36%) constituye el segundo componente mayoritario después de la fibra alimentaria. El cloruro (en las algas pardas) y el sulfato (en las rojas) son los principales aniones inorgánicos. Las algas por su elevada riqueza mineral constituyen una fuente importante de minerales para el consumo humano.

4) La proteína es también un componente importante (10,9-25,7%), sobre todo en las algas rojas y en especial en el alga parda cultivada *Saccharina latissima*.

5) En cuanto a los componentes minoritarios, el contenido lipídico no supera el 1% en la mayoría de las algas estudiadas, por lo que, unido a su alto contenido en fibra dietética, constituyen un alimento potencial de bajo aporte calórico.

6) El contenido en polifenoles es mayor en las algas pardas (3%), que en las rojas estudiadas (< 1%).

De la metodología empleada en la caracterización de las algas y sus polisacáridos se puede concluir:

7) La técnica FTIR ha permitido identificar de una manera clara y exhaustiva los principales polisacáridos de la pared celular de las algas: alginatos en algas pardas y carragenanos en algas rojas, proporcionando información adicional complementaria a la obtenida a través del análisis de la composición centesimal y fibra alimentaria.

8) La cromatografía iónica (IC) es una técnica sencilla, rápida y precisa que permite analizar de forma simultánea los aniones inorgánicos presentes en las cenizas de algas y con multitud de aplicaciones potenciales en otras muestras.

9) El método de cromatografía líquida de exclusión molecular (HPSEC) desarrollado ha permitido conocer la distribución de pesos moleculares de los polisacáridos presentes en las fracciones solubles de *Saccharina latissima* y *Mastocarpus stellatus*.

La extracción secuencial de los polisacáridos de las algas estudiadas señala que:

10) En el fraccionamiento secuencial del alga parda *Saccharina latissima* se observa una fracción mayoritaria (25%) soluble en álcali, cuya composición y distribución de pesos moleculares corresponde al alginato como principal polisacárido en este alga.

11) En el fraccionamiento secuencial del alga roja *Mastocarpus stellatus* la fracción soluble en ácido es la mayoritaria (25,5%). De acuerdo con su composición, todas las fracciones contienen el polisacárido carragenano híbrido kappa/iota con diferentes grados de hibridación y sulfatación, y con un rango de pesos moleculares decreciente durante el proceso de extracción secuencial, indicando la importancia de las condiciones de extracción en la composición y actividades biológicas potenciales de estos polisacáridos.

De la evaluación de las propiedades biológicas *in vitro* se puede concluir que:

12) Las propiedades de capacidad de hinchamiento, absorción de agua y aceite en las algas estudiadas son equiparables a las encontradas en otros productos vegetales con una contribución importante en fibra alimentaria. Estas propiedades de capacidad de hinchamiento, de retención de agua y aceite, sugieren la posibilidad de usar las fibras de algas como ingredientes en productos alimentarios.

13) Las algas pardas y las rojas presentan capacidad antioxidante por un mecanismo de poder reductor debido a los polisacáridos sulfatados. Además, las algas pardas presentan actividad secuestrante de radicales libres y de poder reductor por sus compuestos polifenólicos.

14) La capacidad antioxidante en las fracciones solubles de *Saccharina latissima* fue mayor en la fracción soluble en ácido, especialmente por el método ABTS, correlacionado con la implicación del sulfato, mayor en esta fracción, en el proceso de donación de electrones al radical ABTS.

15) La capacidad antioxidante en las fracciones soluble de *Mastocarpus stellatus* fue mayor por el método FRAP, especialmente en las fracciones soluble en agua y soluble en álcali, de mayor contenido en sulfato y menor peso molecular, respectivamente.

16) Todas las fracciones de *Saccharina* y de *Mastocarpus* desarrollaron una potente capacidad anticoagulante, mayor que la del control, aunque siempre menor que la de la heparina. Además se encontró una correlación positiva significativa (p<0,01) entre los valores de APTT (s) y el contenido en sulfato en todas las fracciones. Los polisacáridos sulfatados de algas (fucoidanos en algas pardas y carragenanos en algas rojas) intervienen mayoritariamente sobre la vía intrínseca de la coagulación.

17) Las propiedades biológicas dependen en gran medida de la composición individual de las algas, por lo que son necesarios más estudios de

caracterización química y estructural para llegar a una mejor comprensión del papel de los polisacáridos sulfatados de las algas.

Los estudios *in vivo* realizados con ratas Wistar sanas concluyen que:

18) El aumento significativo del peso y la humedad del ciego y de las heces en las ratas tratadas es consecuencia de la elevada capacidad de hinchamiento y de retención de agua observada en la fibra de *Saccharina* y *Mastocarpus*.

19) La ingesta de una dieta suplementada (10%) con las algas *Saccharina* y *Mastocarpus* ha tenido un efecto beneficioso antioxidante en el ciego de las ratas, posiblemente debido a los componentes indigeribles asociados a la fibra alimentaria.

20) La fermentación colónica que ha tenido lugar en el ciego de las ratas alimentadas con una dieta suplementada con el alga *Saccharina* ha producido un aumento significativo (p<0,05) de ácidos grasos de cadena corta y de ácidos urónicos. Esto indicaría un efecto prebiótico potencial derivado de la ingesta de este alga parda.

21) La ingesta de la dieta suplementada con *Saccharina* y *Mastocarpus* produce una disminución significativa (p<0,05) de los parámetros del metabolismo lipídico en el suero de las ratas, especialmente de triglicéridos en ambas algas y de colesterol total en el caso del alga roja.

22) Conclusión general: Las algas marinas comestibles estudiadas son ricas en fibra alimentaria y polisacáridos sulfatados específicos con propiedades funcionales y biológicas muy interesantes. Los estudios llevados a cabo con animales de experimentación han puesto de manifiesto su efecto positivo en salud sobre el metabolismo lipídico, capacidad antioxidante y anticoagulante, así como efecto prebiótico potencial, lo que llevaría a pensar en la posibilidad de utilizarlas en la elaboración de alimentos funcionales para la prevención y tratamiento de la hiperlipidemia y trombosis.

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