INTERNATIONAL DOCTORAL SCHOOL
OF THE USC

MAURO
GISBERT VERDÚ

PhD Thesis

Ultrasound–Assisted Extraction of Biopolymers from A*scophyllum nodosum* Brown Seaweeds: Bioactivity and Enzyme Inhibition of Phlorotannins Assessment

Santiago de Compostela, 2022

Doctoral Programme in Chemical and Environmental Engineering

DOCTORAL THESIS

ULTRASOUND–ASSISTED EXTRACTION OF BIOPOLYMERS FROM *ASCOPHYLLUM NODOSUM* BROWN SEAWEEDS: BIOACTIVITY AND ENZYME INHIBITION OF PHLOROTANNINS ASSESSMENT

MAURO GISBERT VERDÚ

INTERNATIONAL PHD SCHOOL

OF

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

PHD PROGRAMME IN

CHEMICAL AND ENVIRONMENTAL ENGINEERING

SANTIAGO DE COMPOSTELA

2022

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DECLARACIÓN DEL AUTOR/A DE LA TESIS

D./Dña. Mauro Gisbert Verdú

Título de la tesis: Ultrasound–Assisted extraction of biopolymers from Ascophyllum nodosum brown seaweeds: Bioactivity and enzyme inhibition of phlorotannins assessment

Presento mi tesis, siguiendo el procedimiento adecuado al Reglamento y declaro que:

1) La tesis abarca los resultados de la elaboración de mi trabajo.
2) De ser el caso, en la tesis se hace referencia a las colaboraciones que tuvo este trabajo.
3) Confirmo que la tesis no incurre en ningún tipo de plagio de otros autores ni de trabajos presentados por mí para la obtención de otros títulos.
4) La tesis es la versión definitiva presentada para su defensa y coincide la versión impresa con la presentada en formato electrónico.

Y me comprometo a presentar el Compromiso Documental de Supervisión en el caso que el original no esté depositado en la Escuela.

En Santiago de Compostela, **24 de octubre de 2022**.

Firma electrónica

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DEL DIRECTOR / TUTOR DE LA TESIS

Ultrasound–Assisted extraction of biopolymers from *Ascophyllum nodosum* brown seaweeds: Bioactivity and enzyme inhibition of phlorotannins assessment

D. Ramón F. Moreira Martínez y D. Jorge Sineiro Torres
En condición de Tutor y Director En condición de Director

INFORMAN:

Que la presente tesis, se corresponde con el trabajo realizado por D/Dª. Mauro Gisbert Verdú, bajo nuestra dirección/tutorización, y autorizamos su presentación, considerando que reúne los requisitos exigidos en el Reglamento de Estudios de Doctorado de la USC, y que como directores de esta no incurre en las causas de abstención establecidas en la Ley 40/2015.

De acuerdo con lo indicado en el Reglamento de Estudios de Doctorado, declaramos también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de Monográfica con reproducción de publicaciones, en los que la participación del doctorando fue decisiva para su elaboración y las publicaciones se ajustan al Plan de Investigación.

En Santiago de Compostela, 24 de octubre de 2022

Fdo. Ramón F. Moreira Martínez Fdo. Jorge Sineiro Torres

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Als meus pares **Maria José** i **Victor**

La meua inspiració, la meua força, el meu suport i per damunt de tot la meua tempança

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ACKNOWLEDGEMENTS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A los profesores Ramón (**Moncho**) Moreira y Jorge (**Xurxo**) Sineiro, mis directores de Tesis, por formarme como investigador, ayudarme en el análisis científico y el espíritu crítico de los resultados. Ha sido un privilegio formar parte de este grupo de investigación estos tres años tanto que casi se ha convertido en mi segundo hogar.

A aquellos que me precedieron, Santiago (**Santi**) Arufe y Dolores (**Loli**) Torres que siempre fueron, son y serán una inspiración. Y a aquellos que se quedan, Daniel (**Dani**) Franco, suerte en tu nueva andadura, estoy seguro de que serás un profesor que dejará huella en sus alumnos. A **Andrea** Aleixandre cuyo compañerismo, profesionalidad y actitud han sido uno de los aspectos más destacables de esta tesis. A **Cristina** M. Rosell por acogerme en su laboratorio.

A **Leticia** (Leti) Montes por esas extensas charlas y salseos que echábamos antes de empezar cada día. Espero tener la suerte que mis próximos/as compañeros/as de trabajo sean al menos la mitad de encantadores, profesionales y divertidos que tú porque a lo bueno se aficiona uno rápido, y a lo excepcional más. Ánimo en tu último tramo, contamos contigo para obtener unos panecillos ricos, antioxidantes y que no sepan a marea baja.

A **Iria** Domínguez y **Oscar** López mis sin cuya adopción no creo que hubiera podido resistir estos 6 años en Galicia. Suerte en vuestra andadura madrileña y espero pronto tener noticias vuestras con campanas ceremoniales sean del tipo que sean.

A Cristina (**Cris**) Iglesias quien realmente me enseñó a hacer “poyata”. Que me decía todo lo que debe hacer un buen investigador y como buena doctoranda jamás aplicó y por extensión yo tampoco. Siempre nos quedará el *Seahorse*. Mi agradecimiento infinito a una de las mejores investigadoras que he conocido y que espero que consiga su sueño de ser profesora para que más personas puedan disfrutar de su infinita paciencia, devoción y capacidades para la enseñanza. También a **Gloria**, **Cintia**, **Vanesa**, **Angela**, **Nerea**, **Blanca**, **Alfonso**, **Juan**, **Samuel**, **Anxo**, **Miriam**, **Mar** y **Luz** gracias por hacer de mi primera toma de contacto con la investigación una experiencia tan sobresaliente.

A **Alejandra** Valle una de las personas más maravillosas que he conocido, espero que allá donde te lleve tu camino sea lo que buscabas y que este a la altura de la mejor “evisceradora de rodaballos” del mundo mundial.

A Manuel (**Manu**) Barcala con el que tengo el honor de compartir mi primera publicación. Espero que todo te vaya genial allá donde pares y espero que siempre mantengas esa actitud y positivismo que te caracterizan. ¡He hablado!

A **Jose Manuel** Sánchez el mejor técnico de la USC y cuyas amables palabras y constantes ánimos eran el estímulo que me hacía seguir adelante. A **Iria**, la becerra, la "boomer", porque sus caras con cada uno de mis "salseos" no tenían precio, espero que "Never Gonna Give You Up" en todo aquello que te propongas.

A los "jefes", por esas charletas con **Sonia** Freire, por las risas y las barbaridades del señor secretario **Diego** Gómez, por las entrañables visitas de Francisco **Chenlo**, por los "Holiiis" con **Ana** Soto, por las dudillas informáticas de Alberto **Arce**, por las tertulias de la hora de la comida con **Oscar** Rodríguez. A **Isabel** Vidal por trasmitirme su pasión por la seguridad en clase y en los laboratorios. A **Angeles** Val del Rio por esos buenos consejos mientras compartíamos una bolsa de chucherías. A **Mónica** Dosil y a Maria del **Mar** Orge por siempre echarme una mano y los buenos consejos. A **Anuska** Mosquera por ser la primera profesora de prácticas que podía desear para empezar a investigar aquí en Galicia. A **Julia** González la mejor directora de la ETSE de la que se tiene constancia, por esas palabras de ánimo de **Pastora** Bello. A **Eva** Rodil, la mejor coordinadora de doctorado en Ingeniería Química y Medioambiental que he tenido, a la "señorita" **Charo** ahora te quedas sola ante el peligro, tú ya me entiendes. Y, por último, a **Catarina**, **Lidia**, **Clara**, **Federica**, **Bahid**, **Bochra**, **Adela**, **Andrea**, **Alba**, **Cristina** y **Carlos** gracias por esos cafés difíciles de olvidar.

Al personal de la ETSE, **Agus**, **Noelia**, **Susan**, **Helena**, **Montse**, **Vanesa**, **Alejandro**, **Manuel**, **Javier** y **Suso** siempre de buen humor. Al personal del Cactus sin los cuales ni esta, ni otras tesis spodrían llevarse a cabo: **Raquel** Antón (SEM), **Manuel** Martín (NMR), **Esteban** Guitián (MALD–TOF–MS) **Ezequiel** Vázquez y Javier **José** López (FT–IR).

A la **Universidade de Santiago de Compostela** (USC) e igualmente al **Departamento de Ingeniería Química y Medioambiental** por acogerme estos últimos 6 años (máster y doctorado). Y, por último, pero no menos importante, gracias al **Ministerio de Ciencia e Innovación de España**, al **Fondo Europeo de Desarrollo Regional** (FEDER) de la Unión Europea por el proyecto de investigación (RTI2018–095919–B–C2) y la **Xunta de Galicia**, España (Proyecto de Consolidación ED431B 2019/01) ayudas sin las cuales esta Tesis, podrían haber sido llevadas a cabo.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

"Life would't be complete without all its challenges" **Stan Lee** (1922–2018)

RESUMO

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Unha dieta baseada en produtos sen glute implica un maior risco de sufrir problemas de diabetes, debido ao seu alto índice glicémico. Os alimentos de panificación sen glute foron deseñados inicialmente como unha necesidade imperiosa e apresurada para producir alimentos aptos para os celíacos, pero tiñan unha baixa calidade nutricional cun contido proteico baixo en relación cos de azucres e/o graxas. Por esta razón, o desenvolvemento de novos produtos sen glute converteuse nun auténtico reto, xa que debe contemplar tanto os aspectos nutricionais como sensoriais. Na actualidade, a aparición de novos produtos sen glute para satisfacer unha demanda crecente fai que este subsector do mercado alimentario sexa o que está a experimentar un maior crecemento nos últimos anos.

A ausencia de glute fai obrigatorio buscar axentes estruturantes alternativos que o substitúan. Estes substitutos do glute deben emular as súas propiedades viscoelásticas e non modificar na medida do posible a aparencia e a estrutura dos produtos de panadería convencionais, sen alterar as características sensoriais. O uso de fariñas de provenientes de cereais e pseudocereais sen glute como millo, arroz, pataca, garavanzo coa adición de hidrocoloides, fibra e/ou proteínas é unha das estratexias económicas máis estendida no deseño de novos produtos sen glute. Algúns dos hidrocoloides utilizados no sector alimentario son a hidroxipropilmetilcelulosa, a goma xantana, a goma tragacanto o carraxenato, o agar e o alxinato (estes últimos obtidos de diferentes especies de algas), entre outros.

A resposta glicémica postprandial está a atraer moita atención debido á súa relación directa cun alto risco de desenvolver enfermidades metabólicas coma o déficit de crecemento, anemia, deficiencias vitamínicas, enfermidades da tiroides, trastornos mentais, fatiga e/ou infertilidade. O consumo de carbohidratos ten un alto impacto nesta resposta xa que se trata de compostos esenciais para a dieta humana e son inxeridos en forma de amidón, sacarosa, glicosa, fructosa e oligosacáridos. Unha estratexia para controlar os niveis altos de glicosa en sangue é a inhibición das encimas dixestivas involucradas na descomposición do amidón, oligosacáridos e disacáridos en glicosa. Algúns fármacos antidiabéticos coma a acarbosa son inhibidores destas encimas permitindo o control da glicosa postprandial en pacientes diabéticos. Con todo, a investigación atópase máis centrada no descubrimento de inhibidores naturais das encimas α–amilasa e α–glucosidasa como os polifenois e as súas características beneficiosas para a saúde.

Deste xeito proponse a reformulación deste tipo de produtos mediante o uso de aditivos alimentarios baseados en florotaninos (polifenois de algas mariñas) que poden actuar como

antioxidantes e tamén como axentes inhibidores de encimas dixestivos, concretamente a α–amilasa e a α–glucosidasa, e así controlar parcialmente o índice glicémico destes produtos amiláceos sen glute de nova xeración. O alto contido de polisacáridos, ácidos graxos poliinsaturados, minerais, fibra, vitaminas e moléculas bioactivas converte ás algas en candidatas ideais para seren utilizadas en diversas aplicacións médicas, farmacéuticas, cosméticas, fertilizantes, e, fundamentalmente, alimentarias. De feito, a demanda mundial de produtos derivados de algas mariñas xera beneficios socioeconómicos relevantes, estimándose que aumenten ata os 26 millóns de euros antes do 2025.

Os florotaninos son metabolitos secundarios exclusivos de algas pardas. Particularmente, nesta Tese Doutoral emprégase como fonte destes aditivos o alga parda *Ascophyllum nodosum* presente nas costas galegas. Este tipo de polifenois son biopolímeros que derivan exclusivamente da reacción de polimerización do floroglucinol (1,3,5–trihidroxibenceno). O contido en polifenois das algas depende de diversos parámetros ambientais como as mareas, correntes, salinidade, dispoñibilidade de luz, radiación UV, entre outros. De feito, estas moléculas actúan coma defensas químicas contra os herbívoros e a radiación UV. Os florotaninos tamén teñen funcións estruturais que forman parte das paredes celulares xunto co alxinato.

Estes polifenois clasifícanse en seis tipos diferentes, atendendo ás variacións da súa ensamblaxe e distribución de grupos hidroxilo, a saber, eckoles, fuhaloles, fucofloretoles, floretoles, fucoles e carmaloles. Os floretoles e fuhaloles caracterízanse por ter ligazóns aril–éter, os fucoles ligazóns aril–aril, os fucofloretoles unha mestura de ligazóns éter e fenilo. Os eckoles presentan unha unidade de 1,4–dibenzodioxina. Os carmaloles presentan unha unidade de 4–dibenzodioxina na terceira e sétima posición. Os eckoles diferéncianse dos carmaloles polo seu menor peso molecular e pola presenza dun grupo OH substituído no cuarto carbono. Os fuhaloles difiren dos floretoles pola súa secuencia regular de ligazóns para– e orto–éter, pola presenza de grupos OH adicionais en cada terceiro anel e pola falta dun ou máis grupos OH en todas as moléculas.

A composición dos florotaninos segue sendo pouco coñecida, existindo moi poucos estándares comerciais dispoñibles que permitan a súa identificación, ao contrario que nos polifenois de plantas, onde o número de compostos de referencia non deixou de aumentar nos últimos anos. Numerosos métodos de illamento de florotaninos pódense encontrar na literatura,

pero estes procesos adoitan ser complexos, custosos e ademais moi pouco eficientes polo que a identificación dos florotaninos de algas segue sendo un reto. Os florotaninos foron avaliados inicial e principalmente polas súas propiedades antioxidantes dada a súa alta capacidade para doar protóns debido aos seus múltiples grupos hidroxilo xunto coa súa capacidade para deslocalizar o radical antioxidante resultante dentro da estrutura. Nas últimas décadas, numerosas propiedades beneficiosas para a saúde destes compostos foron descubertas: antioxidantes, anticoagulantes, antitrombóticas, antidiabéticas, reguladoras da actividade encimática, bactericidas, antitumorais, antiinflamatorias, antihipertensivas e antivirais, entre outras. Tamén, encontráronse resultados prometedores no seu uso en tratamentos do Alzheimer, o Parkinson e a artrite.

Os alxinatos son copolímeros binarios lineais unidos por ligazóns 1–4–glucosídicos. Os monómeros de alxinato son α–L–guluronato (G) e β–D–manuronato (M). O valor da relación M/G mide a distribución dos bloques manuronato e guluronato ao longo das moléculas de alxinato mentres que os valores de $N_{G>1}$ serven para medir a lonxitude media do bloque. Os valores de M/G e $N_{G>1}$ calculados mediante a norma estandarizada "Método de ensaio estándar para determinar a composición química e a secuencia en alxinato mediante espectroscopía de resonancia magnética nuclear de protóns" pódese utilizar como un índice para as propiedades físicas dos xeles de alxinato. Estas propiedades dependen da especie de alga empregada como fonte de alxinato e modificarse en función dos métodos e condicións de extracción e procesado empregadas polo que, deste xeito, pódese cubrir un amplo espectro de aplicacións industriais.

A extracción asistida por ultrasóns é considerada unha técnica de extracción ecolóxica e de alta eficacia. Ofrece unha alta reproducibilidade con tempos de extracción curtos, opera a baixa temperatura, con equipos simples e de baixo custo, reduce o consumo de solventes e de enerxía e ademais é altamente efectiva na produción de extractos enriquecidos en antioxidantes. Esta técnica promove a penetración dos disolventes nos materiais celulares, mellorando a transferencia de materia e rompendo as paredes celulares vexetais, facilitando a liberación do contido celular. A selección do método de extracción e as condicións de operación son aspectos críticos que se discuten ao longo desta Tese de Doutoramento. Así mesmo, o disolvente utilizado para a extracción de compostos é un dos aspectos clave no rendemento da extracción.

A auga é o disolvente máis seguro e empregado en extraccións industriais xa que ademais de ser económico respecta os principios da química verde, á conta dunha menor eficiencia de

extracción en comparación cos disolventes orgánicos. Demostrouse que as mesturas acuosas de metanol, etanol, acetona ou ácidos son disolventes máis eficaces para a extracción de florotaninos de algas pola polaridade dos mesmos e a súa afinidade por disolventes menos polares ca a auga. Con todo, estes disolventes son volátiles, inflamables e tóxicos. O uso da auga reduce as etapas posteriores de purificación e a xeración de residuos residuais. Deste xeito, nesta Tese Doutoral explorase principalmente o uso de auga como disolvente durante as extraccións de biopolímeros.

O obxectivo principal desta Tese Doutoral é a obtención de biopolímeros con alta capacidade antioxidante e inhibición encimática, a partires da alga parda *Ascophyllum nodosum* para seren utilizados como aditivos funcionais e controladores do índice glucémico.

Para cumprir cos obxectivos e metas propostas nesta Tese Doutoral, en primeiro lugar, revisouse a literatura actual sobre a extracción de florotaninos de alga *Ascophyllum nodosum* mediante extracción sólido–líquido convencional e asistida por ultrasóns. Revisáronse as variables principais empregadas na operación de extracción, coma o tipo de disolvente de extracción (auga-acetona, auga de mar e auga entre outros), as relacións de disolvente–alga, o tempo (t) e a temperatura (T), para avaliar o seu efecto tanto na cantidade de polifenois extraídos como na calidade destes (actividade antioxidante).

Tamén se realizou unha minuciosa procura bibliografía sobre o uso de florotaninos (e polifenois) como aditivos alimentarios, así como do seu uso inhibidores encimáticos e a metodoloxía axeitada para a avaliación das súas capacidades inhibitorias. Os resultados indicaron que nos últimos 20 anos existe unha escasa información sobre o uso de polifenois de algas tanto como inhibidores encimáticos e como aditivos alimentarios. Outro problema observado foi o gran número de variables estudadas tanto na extracción como na validación dos florotaninos como inhibidores encimáticos.

Durante a realización deste traballo empregáronse diferentes equipos tanto de laboratorio como escala planta piloto para levar a cabo distintas operacións como: secado, liofilización, moenda, peneirado, extracción, cromatografía e diálise, entre outras. Tamén se empregaron diversas técnicas experimentais para análises químicos (contido de: polifenois, carbohidratos, ácidos urónicos, proteínas, sales minerais, lípidos e humidade), bioactividade (actividade antioxidante determinada por ensaios de análises da capacidade antirradicalaria e/ou de doazón de electróns), inhibicións de reaccións encimáticas, análise de imaxe (microscopía electrónica

de varrido ou SEM), métodos espectroscópicos: infravermellos por transformada de Fourier (FT–IR), resonancia magnética nuclear (NMR) e espectroscopia de masas por de desorción/ionización láser asistida por matriz e tempo de voo (MALDI–TOF–MS) que permitiron a obtención e análise dos resultados.

A materia prima, a alga parda *Ascophyllum nodosum*, secouse por convección forzada con aire quente (temperatura de 50 ºC; humidade relativa de 30%, velocidade de aire de 2 m/s e densidade de carga de 2 kg/m^2) durante 8,5 h ata que a humidade foi constante. Posteriormente, a alga seca moeuse e caracterizouse por medio de peneiras con distintas luces de malla, así como da súa capacidade para reter auga. As condicións empregadas no secado, moenda, empaquetado, selado a baleiro e conservación a 4 ºC baixo refrixeración foron axeitadas para manter as propiedades bioactivas das mostras de alga ao longo do período de realización desta Tese Doutoral.

Ensaiáronse distintas condiciones experimentais para a extracción de florotaninos. Empregando un deseño experimental Box-Behnken avaliáronse os efectos da potencia de sonicación (de 70 a 90 W/cm^2), tempo de sonicación (de 2 a 6 min) e relación líquido-sólido (de 20 a 40 g_{auga}/g_{alga}) para obter mediante superficie de resposta as condición óptimas de extracción. Estas condicións foron utilizadas con outros disolventes coma auga salgada e acetona-auga (70/30 v/v). Simultaneamente, determináronse as cinéticas de extracción sen sonicación (de 2 a 60 min) empregando auga destilada e auga salgada como disolventes con fins comparativos para avaliación do efecto da sonicación. Todos os extractos caracterizáronse quimicamente determinando o seu contido en polifenois, carbohidratos, ácidos urónicos e as súas capacidades antioxidantes mediante os métodos de DPPH, ABTS, y FRAP. Os extractos con mellores propiedades foron purificados mediante cromatografía en xel e estes extractos purificados foron novamente caracterizados química, bioactivamente e a súa actividade inhibitoria fronte as ancimas dixestivas α–amilasa e α–glucosidasa. Nos extractos purificados avaliouse o efecto de diferentes tipos de oxidación (con convección natural e forzada de aire e con peróxido de hidróxeno cunha relación de 50% v/v co extracto) e a súa caracterización por tamaño despois da separación por diálise (de 2 a 20 kDa) para determinar as fraccións máis bioactiva. Así mesmo, estudiáronse as interaccións entre os polifenois das algas e o amidón de millo baixo diferentes condicións de mestura e procesos térmicos (ata 100 ºC) promovendo a xelatinización do amidón. Análises químicos, espectroscópicos e de análise de imaxe foron empregados para determinar o tipo de interacción moleculares e os cambios estruturais. Por

último, co fin dunha valorización máis integral da alga, unha vez extraídos os polifenois, avaliouse a posibilidades de extraer por sonicación os alxinatos no refugallo sólido (secado a temperaturas < 90 ºC). As características do alxinato obtido determináronse mediante técnicas espectróscopicas (RMN y FT-IR) y viscosimétricas.

A extracción acuosa e continúa asistida por ultrasóns aumentou todos os parámetros fitoquímicos dos extractos, especialmente o contido de polifenois (aprox. 1,4 veces máis alto) así como da súa actividade antioxidante (DPPH aumentou ata 2,1 veces) respecto a aqueles extractos obtidos sen sonicación. Dentro dos rangos de tempo de sonicación estudados (desde 2 a 15 min) non se produciron cambios significativos, evidenciando a alta capacidade da tecnoloxía do extracción asistida por ultrasóns para a extracción de compostos bioactivos das algas.

As cinéticas de extracción dos compostos bioactivos das algas, polifenois, carbohidratos e acedos urónicos, modelizáronse satisfactoriamente mediante o emprego da ecuación proposta por Peleg. O pseudo–equilibrio alcanzouse despois de só 15 minutos independentemente do disolvente acuoso utilizado, auga destilada ou auga salgada. A auga salgada foi un disolvente adecuado co fin de obter extractos bioactivos de *Ascophyllum nodosum.*

A modelización das cinéticas de extracción de *Ascophyllum nodosum* demostrou que o contido de florotaninos nos extractos dependeu principalmente da potencia de sonicación (SP) e a relación líquido–sólido (LS) e, en menor medida, do tempo de sonicación (t_R). O máximo contido de florotaninos nos extractos, ademais do menor consumo específico de enerxía, acadouse nas seguintes condicións: SP de 90 W/cm^2, LS de 20 g/g e t_R de 2 min. Os modelos propostos permitiron establecer as condicións óptimas de extracción, que foron 15 min iniciais de SLE, axitando a mestura (170 rpm) e a continuación un curto período de operación (2 min). Estas condicións foron utilizadas ó longo da Tese Doutoral para a obtención dos diferentes extractos. Ademais o contido de polifenois nos extractos sempre foi satisfactoriamente relacionado coas diferentes actividades antioxidantes, medidas a traveso dos métodos de DPPH, ABTS e FRAP, evidenciando que a principal bioactividade dos extractos era debida o seu contido en florotaninos.

Os extractos ricos en florotaninos purificáronse axeitadamente empregando cromatografía de filtración en xel de Amberlite XAD16 que permitiu a diminución do contido de carbohidratos dos extractos (ata 1,7 veces menor) sen cambios significativos do contido de florotaninos.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Como resultado, as capacidades antioxidantes e de inhibición encimática aumentaron polo menos 1,2 veces nos extractos purificados en comparación cos crús. Estas diferenzas atopadas entre os extractos asociáronse coa existencia de complexos florotanino–carbohidrato que coa purificación foron parcialmente eliminados. Doutra banda, tamén se concluíu que os extractos obtidos mediante extracción asistida por ultrasóns contiñan pequenas cantidades de florotaninos oxidados, principalmente quinonas, que afectaban á capacidade antioxidante e de inhibición encimática dos mesmos, pero que a purificación eliminaba parte deles. En consecuencia, a purificación é un tratamento altamente eficaz para aumentar a bioactividade xunto coa capacidade de inhibición encimática dos extractos da alga *Ascophyllum nodosum.*

A oxidación dos extractos purificados ricos en florotaninos estudouse a través de tratamentos de distinta intensidade: por unha parte unha oxidación con convección natural de aire, aplicando aireación forzada e pola adición de peróxido de hidróxeno. Todos os tratamentos foron levados a cabo deixando a mostra 120h a temperatura ambiente. A única oxidación que significativamente afectou ás bioactividades medidas dos extractos foi o tratamento con peróxido de hidróxeno. Estes resultados puxeron en evidencia as altas capacidades antioxidantes dos extractos e a súa resistencia fronte ó emprego de condicións severas de oxidación.

Paralelamente, analizáronse as diferenzas de bioactividade dos florotaninos en función do seu tamaño molecular, onde se atopou que a principal fracción bioactiva estaba no rango de 10 a 20 kDa, con contido de polifenois e valores de DPPH superiores ao 50% respecto de extractos non dializados. En canto aos extractos con menor tamaño de florotaninos (< 2 kDa) obtivéronse baixa bioactividades, que mesmo mostraron resultados similares aos extractos oxidados. Estes resultados foron corroborados empregando técnicas NMR, FT–IR e MALDI–TOF–MS. De feito, a técnica de MALDI–TOF–MS permitiu a identificación dos graos de polimerización (número de unidades de floroglucinol ou PGUs) dos florotaninos do alga (4–16 PGUs). Tamén se obtiveron os patróns de fragmentación dos florotaninos de *Ascophyllum nodosum* e a presenza de diversas sinais que evidenciaban a presenza de diferentes grupos de florotaninos.

Cando se estudaron as interaccións e mecanismos de entrecruzamento de sistemas amidón (de millo) e fariña de alga concluíuse que a composición e as transicións de fase (xelatinización e xelificación do amidón) promovidas polas operacións de quecemento modificaban tanto as características fitoquímicas, coma a natureza e a cantidade das interaccións formadas, o cal á

súa vez redundou en cambios das morfoloxías transversais das mostras. O amidón mostrou diferentes retencións de polifenois dependendo do seu estado (granular ou xel). As interaccións entre os florotaninos e o amidón debéronse á fisisorción coma mecanismo principal cando as algas se puxeron en contacto con amidón. Así mesmo, a cantidade adsorbida dependeu do área superficial dispoñible, sendo superior nas mostras de xel de amidón. Con todo, a retención de polifenois aumentou notoriamente cando a xelatinización do amidón se realizou en presenza da fariña de alga, porque os florotaninos se adsorberon fisicamente na superficie do xel de amidón e tamén quedaron atrapados dentro das paredes do mesmo. Os procedementos empregados serviron para cuantificar de forma sinxela o modo de retención de moléculas bioactivas das algas en alimentos amiláceos.

Os extractos acuosos de algas demostraron ser inhibidores encimáticos altamente efectivos contra os encimas dixestivos α–amilasa e α–glucosidasa, e, especialmente, con esta última. Probáronse diferentes sistemas de mesturado onde se variou a orde de adición do substrato, encima e inhibidor. Os extractos de alga foron inhibidores máis efectivos cando foron engadidos directamente ó encima antes que ó substrato. Doutra banda, a mestura de extractos cos substratos (amidón nativo ou xel, ou maltosa) antes da reacción encimática reduciu o efecto inhibitorio dos mesmos, e, particularmente na inhibición da α–amilasa. As diferenzas observadas nas capacidades de inhibición dos extractos explicáronse pola presenza de complexos alxinato–polifenois e florotaninos en estado oxidado. Os resultados obtidos comparáronse satisfactoriamente coa acarbosa, inhibidor comercial utilizado no tratamento da diabetes.

A extracción secuencial de florotaninos mediante tecnoloxía asistida por ultrasón seguido da extracción de alxinatos do refugallo sólido permitiu obter dous produtos de valor comercial. Na extracción do alxinato, estudiouse o efecto da presenza dunha etapa de secado e da súa temperatura, sobre as características fisicoquímicas do alxinato de sodio. Obtivéronse alxinatos con diversas lonxitudes media de bloque ($N_{G>1}$ dende 1,97 ata 2,15) e tamaño molecular viscosimétrico (Mv, desde 133,3 ata 257,3 kDa). A variabilidade das características obtidas segundo as condicións de secado supón que modulando as condicións pódense obter alxinatos á carta, é dicir coas propiedades desexadas en función do seu uso. O aproveitamento integral das algas mariñas implica claras avantaxes económicas e ambientais. De feito, este concepto de refinería baseada en algas empeza a ser unha realidade e seguindo os principios da química verde.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Finalmente como perspectivas de futuro, proponse a adición destes florotaninos (como aditivos bioactivos) e alxinatos (como substitutos do glute) á matrices amiláceas e estudar o seu efecto sobre as características nutricionais e sensoriais, propiedades funcionais e a súa incidencia nos índices glicémicos. Outro estudo proposto sería a análise *in vivo* de florotaninos para determinar doses efectivas e mesmo efectos a longo prazo.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

GLOSSARY

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Generalities

AF	*A. nodosum* seaweed flour
A. nodosum	*Ascophyllum nodosum*
LS	Liquid–solid
rt	Room temperature
SLE	Solid–liquid extraction
spp.	species
T	Temperature
t	Time
UAE	Ultrasound–assisted extraction

Section 1

AM	*Alaria marginata*
Aq.	Aqueous
BC	Before Christ
PNPG7	p–nitrophenyl maltoheptaoside
d.b	Dry basis
FD	*Fucus distichus*
GF	Gluten–free
GI	Glycemic index
g_{algae}	Grams of algae
$g_{d.b}$	Grams of dried extract (d.b)
g_{GAE}	Grams of gallic acid equivalents
g_{sol}	Grams of solvent
LS	Liquid–solid
mg_{PE}	Milligrams of phloroglucinol equivalents
$mg_{Extract}$	Milligrams of extract
NS	Data not specified
PF	*Pyropia fallax*
PLE	Preassurized–liquid extraction
pNPG	p–nitrophenyl–a–D–glucopyranoside
SA	*Sargasum aquifolium*
SD	*Sargasum duplicatum*
SG	*Saccharina groenlandica*
SL	*Saccharina latissima*
SP	*Sargasum polycystum*
SS	*Sargasum siliquosum*
SLE	Solid–liquid extraction
UL	*Ulva lactuta*
US$	United states dolars
X3	Process repeated 3–times

Section 3

ABTS	2,2'–azino–bis(3–ethylbenzothiazoline–6–sulfonic acid) diammonium salt
ATR	Attenuated total reflectance
C18	Octadecyl columns
CHOs	Total carbohydrate content
CLSM	Confocal laser scanning microscopy
DAD	Diode array detector
DMBA	2,4–dimethoxybenzaldehyde
DMSO	Dimethylsulfoxide
DPPH	1,1–diphenyl–2–picrylhydrazil

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ECSA	Electron spectroscopy for chemical analysis
ESI	Electrospray ionization source
FRAP	Electron donor capacity
FT–IR	Fourier transformed infrared
HILIC	Hydrophilic interaction phase
HMBC	Heteronuclear multiple–quantum correlation
HPLC	High preassure liquid chromatography
LC	Liquid chromatography
MS	Mass chromatography
NMR	Nuclear magnetic resonance
NP	Normal phase
qNMR	Quantitative NMR
QqQ	Triple quadrupole
ROESY	^{1}H–1H rotating frame nuclear overhauser effect spectroscopy
RP	Reverse phase
SAXS	Small–angle X–ray scattering
SEM	Scaning electron microscopy
SIMS	Secondary ion mass specroscopy
SLE	Solid–liquid extraction
TEM	Transmission lectron microscopy
TLC	Thin–layer chromatography
TPC	Total polyphenol content
UA	Uronic acid content
UAE	Ultrasound–assisted extraction
UHPLC	Ultra–high preassure liquid chromatography
WAXS	Wide–angle X–ray scattering
XRF	X–ray fluorescence
XPS	X–ray photoelectron spectroscopy
^{1}H–NMR	Hydrogen NMR
2DNMR	Two dimensional NMR
^{13}C–NMR	Carbon NMR

Section 4

A1	Method 1 (Enzyme + Extract) of α–amylase inhibition
A2	Method 2 (Sustrate + Extract) of α–amylase inhibition
A3	Method 3 (Starch gelatinized with Extract) of α–Amylase inhibition
ABTS	ABTS scavenging activity
$ABTS_{0min}$	Initial (t = 0 min) absorbance of ABTS solution
$ABTS_{15min}$	Final (t = 15 min) absorbance of ABTS solution
AF_{dried}	*A. nodosum* seaweed flour dried
AF_{wet}	*A. nodosum* seaweed flour wet
ASTM	American Society for Testing and Materials
BB	Box–Behnken
PNPG7	p–nitrophenyl maltoheptaoside
C18	Octadecyl columns
CGL	Gelatinized starch in presence of AF
CHNS	Elemental analysis
CHOs	Total carbohydrate content
CLSM	Confocal laser scanning microscopy
CS	Corn starch
C^{+}	Control absorbance after inhibition protocol

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

C^-	Control blank absorbance after inhibition protocol
D	Double–distilled water
d.b	Dry basis
DMBA	2,4–dimethoxybenzaldehyde
DMSO	Dimethylsulfoxide
DMSO–d_6	Deuterated dimethyl sulfoxide
Dp_i	mean fraction diameter
DPPH	DPPH scavenging activity
$DPPH_{0min}$	Initial (t = 0 min) absorbance of DPPH solution
$DPPH_{30min}$	Final (t = 30 min) absorbance of DPPH solution
DW	The mass mean diameter
D_2O	deuterated water
Eq.	Equation
FD	freeze–dried extracts
F_G	Monad of guluronate
F_{GG}	Homopolymeric diad of guluronate–guluronate
F_{GGG}	Homopolymeric triad of guluronate–guluronate–guluronate
F_{GGM}	Homopolymeric triad of guluronate–guluronate–mannuronate
F_{GM} "or" F_{MG}	Heteropolymeric diad of guluronate–mannuronate
F_M	Monad of mannuronate
F_{MGM}	Heteropolymeric triad of mannuronate–guluronate–mannuronate
F_{MM}	Homopolymeric diad of mannuronate–mannuronate
F_{MMM}	Homopolymeric triad of mannuronate–mannuronate–mannuronate
FRAP	Electron donor capacity
FT–IR	Fourier transformed infrared
G1	Method 1 (Enzyme + Extract) of α–Glucosidase inhibition
G2	Method 2 (Sustrate + Extract) of α– Glucosidase inhibition
g_{GE}/L	Grams of glucose equivalents per liter
GL	Gelatinized starch mixed with AF
g_{PE}/L	grams of phloroglucinol equivalents per liter
g_{TE}	Grams of trolox equivalents
g_W/g_{AF}	grams of water per grams of *A. nodosum* flour
g_W/g_{CS}	grams of water per grams of corn starch
Hr	Humidity
HPLC	High pressure liquid chromatography
IC_{50}	Minimal concentration value to achieve 50% of inhibition
K_1	related to initial extraction rate (min L/g)
K_2	related to equilibrium content (L/g)
LC	Liquid chromatography
L/h	Liters per hour
LS	Liquid–solid
M/G ratio	Glucoronate–mannuronate ratio
M1	Method 1 (Enzyme + Extract) inhibition
M2	Method 2 (Sustrate + Extract) inhibition

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

M3	Method 3 (Starch gelatinized with Extract) inhibition
m_{AF}	Mass of *A. nodosum* flour
m_{Alg}	Mass of sodium alginate
mg_{PE}/g_{CS}	Milligrams of phloroglucinol equivalents per grams of CS
mg_{PE}/L	Milligrams of phloroglucinol equivalents per liter
mg_{TE}/mg_{DE}	Milligrams of trolox equivalents per milligram of dried extract
m_{res}	Mass of UAE residue
MS	Mass spectrometry
mM_{TE}/mg_{FD}	Millimolar of trolox equivalents per miligram of freeze–dried extract
Mv	Molecular viscosimetric weight
m_Y	Experimentally determined phytochemical concentration
ND	Alginate from UAE residue not dried
$N_{G>1}$	average block length
NMR	Nuclear magnetic resonance
NT	Granular (Native) starch mixed with AF
OH	Carboxylic group or alcohol group
q	TPC sorption value
R^2	Regression coefficient
RMSE	Root mean squared error
RP–HPLC	Reverse phase high pressure liquid chromatography
rt	Room temperature (19 ± 1 ºC)
S	Saline water
SEM	Scanning electron microscopy
SLE	Solid–liquid extraction
SP	Sonication power
spp.	Species
TPC	Total polyphenol content
TPC_{AF}	Total polyphenol content of AF sample
$TPC_{AF–CS}$	Total polyphenol content of AF–CS sample
TPC_{CS}	Total polyphenol content of CS sample
tR	Time residence
U/mL	Unities per milliliter
UA	Uronic acid content
UAE	Ultrasound–assisted extraction
UHPLC	Ultra–high pressure liquid chromatography
UV	Ultraviolet
V	Volume
v_a	Air velocity
W	Watts
WRC	Water retention capacity
x_i	Weight fraction
X_t	Content at time t (min)
X_0	Initial content (assumed as zero)
X^+	Sample absorbance after inhibition protocol
X^+	Sample blank absorbance after inhibition protocol

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Y_P	Polyphenol sorption yield
ρ	Load density
$\mu g_{TE}/mg_{FD}$	Micrograms of trolox equivalents per milligram of freeze–dried extract
μM_{TE}	Micromolar of Trolox equivalents
^{1}H–NMR	Hydrogen NMR
50D	Alginate from UAE residue dried at 50 ºC
90D	Alginate from UAE residue dried at 90 ºC

Section 7

ABTS	ABTS method
A–region	Defined between 10 and 20 min of retention time
B–region	Defined between 28 and 40 min of retention time
CHOs	Total carbohydrate content
C–region	Defined between 45 and 70 min of retention time
D	Double distilled water
D20	Extraction at 20 g_W/g_{AF} with D–solvent
D30	Extraction at 30 g_W/g_{AF} with D–solvent
D40	Extraction at 40 g_W/g_{AF} with D–solvent
DPPH	DPPH method
Eq.	Equation
FRAP	Electron donor capacity
g_{PE}/L	Grams of phloroglucinol equivalents per liter
g_{GE}/L	Grams of glucose equivalents per liter
g_W/g_{AF}	Grams of water per grams of *A. nodosum* flour
K_1	related to initial extraction rate (min L/g)
K_2	related to equilibrium content (L/g)
LS	Liquid–solid
LS20	Liquid–solid ratio of 20 g_W/g_{AF}
LS30	Liquid–solid ratio of 30 g_W/g_{AF}
LS40	Liquid–solid ratio of 40 g_W/g_{AF}
mg_{PE}/L	Miligramns of phlroglucinol equivalents per liter
mM_{TE}	Milimolar of Trolox equivalents
RMSE	Root mean squared error
RP–HPLC	Reverse phase high preasure liquid chromatography
S	Saline water
S20	Extraction at 20 g_W/g_{AF} with S–solvent
S30	Extraction at 30 g_W/g_{AF} with S–solvent
S40	Extraction at 40 g_W/g_{AF} with S–solvent
SLE	Solid–liquid extraction
SOL	Solvent
TPC	Total polyphenol content
UA	Uronic acid content
UAE	Ultrasound–assisted extraction
UAE20–90	Sonicated extract of LS = 20 g_W/g_{AF} and SP = 90 W/cm^2
UAE20–80	Sonicated extract of LS = 20 g_W/g_{AF} and SP = 80 W/cm^2
UAE20–70	Sonicated extract of LS = 20 g_W/g_{AF} and SP = 70 W/cm^2
UAE30–90	Sonicated extract of LS = 30 g_W/g_{AF} and SP = 90 W/cm^2
UAE40–90	Sonicated extract of LS = 40 g_W/g_{AF} and SP = 90 W/cm^2
UV	Ultraviolet
X_0	Initial content (assumed zero)
X_t	Content at time *t* (min)
μM_{TE}	Micromolar of Trolox equivalents

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Section 8

ABTS	ABTS method
BB	Box–Behnken
CHOs	Total carbohydrate content
DPPH	DPPH method
Eq.	Equation
FRAP	Electron donor capacity
g_{GAE}/L	Grams of galic acid equivalents per liter
g_{GE}/L	Grams of glucose equivalents per liter
g_{PE}/L	Grams of phloroglucinol equivalents per liter
g_W/g_{AF}	Grams of water per grams of *A. nodosum* flour
IC_{50}	Concentration to achieve 50% of decayment
LS	Liquid–solid
mg_{PE}/L	Miligramns of phlroglucinol equivalents per liter
mg_{TE}/mg_{DE}	Miligrams of Tolox equivalents per miligram of dried extract
$mmol_{DPPH}$	Milimols of DPPH compound
mM_{TE}	Milimolar of Trolox equivalents
p	Significance
RSM	Response surface model
rt	Room temperature (19 ± 1 ºC)
S	Saline water
SP	Sonication power
tR	Time residence
TPC	Total polyphenol content
UA	Uronic acid content
UAE	Ultrasound–assisted extraction
UV	Ultraviolet
μM_{TE}	Micromolar of Trolox equivalents
*	Significance level $p < 0.05$
**	Significance level $p < 0.01$
**	Significance level $p < 0.001$

Section 9

DPPH	2,2–diphenyl–1–picrylhydrazyl
d.b	Dry basis
EA	UAE carried with 70% (v/v) acetone:water
EW	UAE using double–distilled water
FD	freeze–dried extracts
FT–IR	Fourier transform infrared
GF	Gluten–Free
g_{PE}/L	grams of phloroglucinol equivalents per litre
GRAS	Generally Recognised As Safe
g_S/g_{AF}	Grams of solvent per gram of AF
g_{TE}/L	Grams of trolox equivalents per litre
GAE	Galic acid equivalents
GE	Glucose equivalents
^{1}H–NMR	Hydrogen nuclear magnetic resonance
LS	Liquid–solid
MALDI	Matrix–Assisted Laser Desorption Ionization
TOF	Time–Of–Flight
MS	Mass Spectrometry
OH	Carboxylic group or alcohol group
Peak 1	Peak area from chromatograms at 16.5 min of retention time;
Peak 2	Peak area from chromatograms at 53.0 min of retention time;
Peak 3	Peak area from chromatograms at 90.5 min of retention time
PBS	Podium phosphate buffer

PD20	PW dialysated throughout 20 kDa
PD10	PW dialysated throughout 10 kDa
PD3	PW fraction dialysated throughout 3.5 kDa
PD2	PW dialysated throughout 2 kDa
PE	Phloroglucinol equivalents
POA	PW air oxidated (120 h rt)
PON	PW naturally oxidated (120 h rt)
POP	PW chemically oxidated with peroxide (120 h rt)
PW	EW purified extract
RP–HPLC	Reverse phase high performance liquid chromatography
SLE	Solid–liquid extraction
spp.	Species
SP	Sonication power
tR	Time residence
T	Temperature
V	volume

Section 10

ABTS	ABTS method
CGL	Gelatinized starch in presence of AF
CHOs	Total carbohydrate content
CS	Corn starch
DPPH	DPPH method
Eq.	Equation
FRAP	Electron donor capacity
FT–IR	Fourier transformed infrared
GRAS	Generally recognised as safe
g_W/g_{AF}	Grams of water per grams of *AF*
g_W/g_{AF}	Grams of water per grams of *CS*
GL	Gelatinized starch mixed with AF
IC_{50}	Concentration to achieve 50% of decayment
LS	Liquid–solid
mg_{PE}/g_{CS}	Miligramns of phlroglucinol equivalents per grams of CS
mg_{PE}/L	Miligramns of phlroglucinol equivalents per liter
NT	Granular (Native) starch mixed with AF
q	TPC sorption value
RMSE	Root mean squared error
SEM	Scaning electron microscopy
TPC_{AF}	Total polyphenol content of AF sample
TPC_{CS}	Total polyphenol content of CS sample
$TPC_{AF–CS}$	Total polyphenol content of AF–CS sample
Y_P	Polyphenol sorption yield
μM_{TE}	Micromolar of Trolox equivalents

Section 11

A1	Method 1 (Enzyme + Extract) of α–amylase inhibition
A2	Method 2 (Sustrate + Extract) of α–amylase inhibition
A3	Method 3 (Starch gelatinized with Extract) of α–amylase inhibition
A–region	Region comprised between 5.20 and 5.40 ppm
B–region	Region comprised between 5.75 and 6.40 ppm
CHOs	Total carbohydrate content
C–region	Region comprised between 6.40 and 6.55 ppm
d.b	Dry basis
E70	*A. nodosum* extract sonicated at 70 W/cm^2

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

E80	*A. nodosum* extract sonicated at 80 W/cm^2
E90	*A. nodosum* extract sonicated at 90 W/cm^2
FRAP	Electron donor capacity
FT–IR	Fourier transformed infrared
G1	Method 1 (Enzyme + Extract) of α–glucosidase inhibition
G2	Method 2 (Sustrate + Extract) of α–glucosidase inhibition
IC_{50}	Minimal concentration value to achieve 50% of inhibition
M1	Method 1 (Enzyme + Extract) inhibition
M2	Method 2 (Sustrate + Extract) inhibition
M3	Method 3 (Starch gelatinized with Extract) inhibition
mg_{FD}	Miligrams of freeze–dried extract
mg_{GE}	Miligrams of glucose equivalents
mg_{PE}	Miligrams of phloroglucinol equivalents
mM_{TE}/mg_{FD}	Miliolar of trolox equivalents per miligram of freeze dried extract
P90	*A. nodosum* extract sonicated at 90 W/cm^2 and Amberlite purified
RP–HPLC	Reverse phase high preasure liquid chromatography
SEM	Scaning electron microscopy
TPC	Total polyphenol content
^{1}H–NMR	Hydrogen nuclear magnetic resonance
$\mu g_{TE}/mg_{FD}$	Micrograms of trolox equivalents per miligram of freeze dried extract

Section 12

AF	*Ascophyllum nodosum* flour
A. nodosum	*Ascophyllum nodosum*
ASTM	American Society for Testing and Materials
CHNS	Elemental analysis
CS	Comercial aglinate from Sigma
F_M	Monad of mannuronate
F_G	Monad of guluronate
F_{GG}	Homopolymeric diad of guluronate–guluronate
F_{GGG}	Homopolymeric triad of guluronate–guluronate–guluronate
F_{GGM}	Homopolymeric triad of guluronate–guluronate– mannuronate
F_{GM} "or" F_{MG}	Heteropolymeric diad of guluronate– mannuronate
F_{MGM}	Heteropolymeric triad of mannuronate– guluronate–mannuronate
F_{MM}	Homopolymeric diad of mannuronate–mannuronate
F_{MMM}	Homopolymeric triad of mannuronate–mannuronate–mannuronate
FT–IR	Fourier transformed infrared
G	Guluronate
M	Mannuronate
M/G ratio	Glucoronate – mannuronate ratio
M_v	Molecular viscosimetric weight
ND	Alginate from UAE residue not dried
$N_{G>1}$	average block length
UAE	Ultrasound–assisted extraction
^{1}H–NMR	Hydrogen nuclear magnetic resonance
50D	Alginate from UAE residue dried at 50 ºC
90D	Alginate from UAE residue dried at 50 ºC

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CONTENT

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Acknowledgements I

Resumo V

Glossary XVII

Content XXVII

1. Introduction 35

1.1. Generalities 37

1.1.1. Green algae 38

1.1.2. Red algae 38

1.2. Brown algae 38

1.2.1. Carbohydrates 39

1.2.2. Phlorotannins 40

1.2.3. Alginates 43

1.3. *Ascophyllum nodosum* seaweed 44

1.4. Market and applications 46

1.5. Antioxidant capacities 49

1.6. Antidiabetic capacities 55

1.7. State of art 60

1.7.1. Phlorotannins extraction 60

1.7.2. Seaweed biopolymers in food 66

1.7.3. Phlorotannins as enzyme inhibitors 67

2. Objectives & Hypothesis 77

3. Theoretical fundamentals 81

3.1. Extraction 83

3.1.1. Generalities 83

3.1.2. Ultrasound–assisted extraction 86

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.1.3. Fractionation–isolation of seaweed extracts 88
3.2. Characterization of seaweed biopolymers 89
3.2.1. Antioxidant activity determinations 90
3.2.2. Chromatographic studies 95
3.2.3. Fourier transformed infrared spectroscopy (FT–IR) 97
3.2.4. Nuclear magnetic resonance (NMR) 98
3.2.5. Optical analysis 98
3.2.6. X–ray diffraction (XRD) 99
3.3. Seaweed biopolymers in food 100
3.3.1. Generalities 100
3.3.2. Starchy–based systems 102
3.3.3. Phlorotannins as enzyme inhibitors 105
4. Experimental & Methodology 107
4.1. Materials 109
4.2. Drying & Milling & Sieving 109
4.3. Particle size and water retention capacity 111
4.4. Biopolymers extractions 112
4.4.1. Solid–liquid extraction 113
4.4.2. Ultrasound–assisted extraction 114
4.4.3. Alginate extraction 117
4.4.4. Extraction yields 118
4.5. Extracts purification 118
4.6. Raw and prurified extracts characterization 119
4.6.1. Total polyphenol content 120
4.6.2. Total carbohydrate content 122

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.6.3. Uronic acid content 124
4.6.4. DPPH scavenging activity 126
4.6.5. ABTS scavenging activity 128
4.6.6. Electron donor capacity 130
4.6.7. Other analyses 132
4.7. Extracts oxidation treatments 132
4.8. Extracts cut–off dialysis 133
4.9. Extracts chromatography 134
4.10. Extracts fragmentation patterns 135
4.11. Fourier transform infrared spectroscopy 136
4.12. Average viscosimetric molecular weight 136
4.13. Nuclear magnetic resonance 137
4.13.1. Sodium alginate 137
4.13.2. Phlorotannin-eriched extracts 139
4.14. Seaweed–starch interactions 139
4.15. Inhibitory capacities of extracts 141
4.16. Scanning electron microscopy 144
4.17. Statistical analysis 144
5. Results & Discussion 145
6. *Ascophyllum nodosum* flour obtention 149
6.0. Generalities 151
6.1. Assayed systems 153
6.2. Drying kinetics 154
6.3. Particle size distribution 154
6.4. Water retention capacity 155

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Highlights 155

7. Extraction solvent and method assessment 157

7.1. Generalities 159

7.2. Assayed systems 160

7.3. Solid–Liquid Extraction (SLE) 162

7.3.1. Solid–Liquid extraction kinetics modelling 165

7.4. Ultrasound–assisted extraction (UAE) 165

7.4.1. Analysis of composition and activity ratios 171

7.5. Chromatography profiling: SLE *vs* UAE 175

7.5.1. Relationship HPLC areas–antioxidant capacities 178

Highlights 180

8. Ultrasound-assisted extraction optimization 181

8.1. Generalities 183

8.2. Assayed systems 185

8.3. Extracts chemical characterization 187

8.4. Response surface modelling 189

8.5. Analysis of TPC ratios 192

8.6. Antioxidant activity of extracts 195

Highlights 197

9. Influence of oxidation and dialysis on bioactivity 199

9.1. Generalities 201

9.2. Assayed systems 202

9.3. Bioactivity characterization 204

9.4 Chromatographic characterization 209

9.5. Spectroscopic characterization 212

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

9.5.1. Fourier transformed infrared (FT–IR) 212
9.5.2. Hydrogen nuclear magnetic resonance (^{1}H–NMR) 215
9.5.3. MALDI–TOF–MS 217
Highlights 221
10. A*scophyllum nodosum* & corn starch Interactions 223
10.1. Generalities 225
10.2. Assayed systems 227
10.3. Aqueous phase chemical characterization 229
10.3.1. Total polyphenolics content (TPC) 230
10.3.2. Total carbohydrate content (CHOs) 234
10.3.3. Antioxidant activities (DPPH, ABTS and FRAP) 235
10.4. Fourier transformed infrared (FT–IR) 237
10.5. Scanning Electron microscopy (SEM) 239
Highlights 241
11. Enzyme-inhibiting activity of A*scophyllum nodosum* extracts 243
11.1. Generalities 245
11.2. Assayed systems 246
11.3. Seaweed extracts chemical composition 248
11.4. Inhibition effect of extracts against digestive enzymes 250
11.5. Chromatography and spectroscopic analysis 254
Highlights 257
12. Alginate obtained from phlorotannins extraction solid residue 259
12.1. Generalities 261
12.2. Assayed systems 263
12.3. Extraction yield 265

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

12.4. Fourier transformed infrared 265

12.5. Nuclear magnetic resonance 267

12.6. Average viscosimetric molecular weight 270

Highlights 270

13. Conclusions 271

14. List of Figures 277

15. List of Tables 285

16. Bibliography 289

17. Diffusion of results derived from the Thesis 341

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. INTRODUCTION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1.1. GENERALITIES

Algae are a wide and heterogeneous group of autotroph organisms with more than 10 million estimated species (*spp.*) (Graham et al., 2009). Their classification can be carried out according to their body types, size or color. Regarding body type, seaweeds can be classified according to their complexity between unicellular and pluricellular organisms and can be distinguished (Graham et al., 2009) among: Coccoid unicellular organisms (e.g., *Chlorococcum acidum*), Coccoid colonies (e.g., *Microcystis wesenbergii),* Coenobial colonies (e.g., *Pediastrum boryanum),* Flagellate colonies (e.g., *Platydorina caudata),* Uniseriate unbranched filament (e.g., *Mougeotia viridis),* branched filament (e.g., *Stigeoclonium aestivale),* Coenocytic bodies (e.g., *Vaucheria disperma),* Parenchymatous bodies (e.g., *Macrocystis pyrifera).* Size classification of algae discerns between microalgae (microscope is necessary to observe them) and macroalgae (seen with the unaided eye), also known as seaweeds (Boyd et al., 2005). Focusing on seaweeds, the widespread classification is based on their color (Figure 1.1), green, red, or brown (Lobban & Harrison, 1994; Li et al., 2011). Indeed, the Cambridge Dictionary defines seaweed as "*a green, brown, or dark red plant that grows in the sea or on land very close to the sea*".

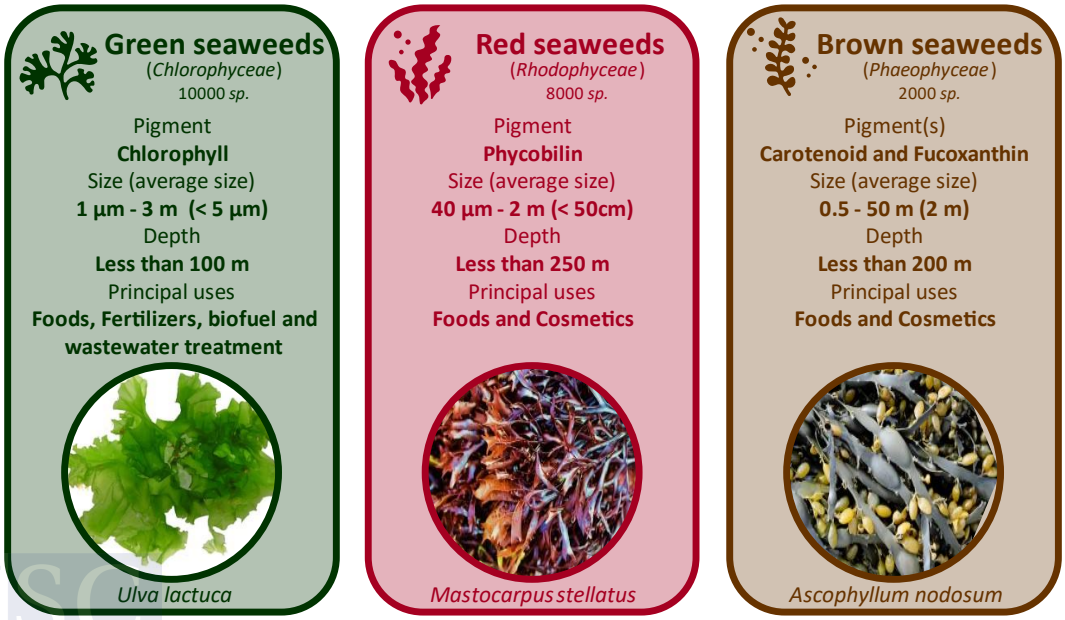


Figure 1.1. Seaweed color classification, main characteristics and representative species.

1.1.1. GREEN ALGAE

Green algae (*Chlorophyceae*) owe their color to chlorophyll (a and b) pigments. This group is the most diverse of all algae with more than 10000 different identified species. The size of green algae varies from 1 μm up to 3 m long, however, average size range of these species is around 5 μm in size (García–Pichel & Belnap, 2021). Green algae are found on both sandy and rocky beaches, closer to the surface because they require high levels of sunlight. Some green edible seaweeds are *Ulva spp.*, *Enteromorpha spp.*, *Monostroma spp.*, *Caulerpa spp.*, and *Codium spp.* (Kilinç et al., 2013; Kim, 2011).

1.1.2. RED ALGAE

Red seaweeds, also named as *Rhodophyceae*, that can vary between blue and red depending on their phycobilin pigments (Kilinç et al., 2013). It has been identified more than 8000 species of red algae with different shapes and measures from 40 μm until 2 m, however, average species size is less than 50 cm (Lee et al., 2018). These seaweeds are found in intertidal and subtidal zones to depths from 40 to 250 m (Kim, 2011). Their cell walls contain cellulose, agar and carrageenan, making them an interesting and profitable resource for food and cosmetic industries (McHugh, 2003; Kilinç et al., 2013). Main red and edible seaweeds are *Palmaria spp.*, *Chondrus spp.*, and *Mastocarpus spp*.

1.2. BROWN ALGAE

Brown algae or *Phaeophyceae*, owe their color to carotenoid (yellowness) and fucoxanthin (brownish) pigments. The size of the brown seaweeds ranges from 0.5 to 50 m. These seaweeds can be found until 200 m of depth. The cell walls of these algae contain alginic acid (Kim, 2011). Some examples of brown edible seaweeds are: *Fucus spp.*, *Macrocistys spp.*, *Undaria spp.*, *Bifucrcaria spp.* and *Ascophyllum spp.* Hence, *Ascophyllum nodosum* (*A. nodosum*) is one of the most studied brown edible seaweed by their high content of alginate and bioactive compounds (Kadam et al., 2015c; Catarino et al., 2019).

USC UNIVERSIDADE DE COMPOSTELA

Seaweeds biochemical composition is affected by botanical sources, season, geographic origin, contaminants, and their natural individual variability (Tabassum et al., 2016). Brown seaweeds are rich in nutraceuticals such as polysaccharides, polyphenols and some minerals (sodium, calcium, magnesium, potassium, chloride, sulphates, phosphorous, and fluoride). Brown seaweeds show low content of lipids (< 5% d.b) and variable content of proteins from 5 up to 24% d.b (Gómez–Ordóñez & Rupérez, 2011; Kilinç et al., 2013; Tabassum et al., 2016). These bioactive compounds have recently gained notorious relevance by their beneficial health properties that will be seen in Sections 1.4, 1.5, 1.6 and 1.7.

1.2.1. CARBOHYDRATES

Seaweeds can be classified by their structural and reserve polysaccharide composition. Seaweed polysaccharides are polymeric molecules linked by glycosidic bonds, that are receiving increasing attention, together with phenolic compounds, due to their bio–functional (i.e., health benefits and biological activities) and physicochemical (e.g., emulsifiers, thickeners, or gelling agents) features (López–Hortas et al., 2021). Polysaccharide features depend on their molecular weight and conformation (Hahn et al., 2012).

Hydrocolloids from seaweeds have extended industrial interest (Kim, 2011) and are used as thickener, gel formation or stabilizer ingredients. Seaweeds as a source of these hydrocolloids dates back to 1658, when the gelling properties of agar, from red seaweeds, were first discovered in Japan (Kilinç et al., 2013). Some examples of red seaweeds' hydrocolloids are, agar, carrageenan and porphyrin, while the main hydrocolloids of brown seaweeds are laminarins, fucoidans and alginates (Holdt & Kraan, 2011).

Laminarins are the main reserve of polysaccharides in brown algae, and they could be found in high quantities in *Laminaria spp.*, *Undaria spp.*and *Fucus spp.* Laminarins are mainly dietary fiber, however, have shown promising bioactive properties (Kadam et al., 2015a). Fucoidans are sulphated polysaccharides found in the cell wall and are mainly be obtained from *Cladosiphon okamuranus* (ozuku) and *Laminaria japonica* (kombu) edible seaweeds (Gómez–Ordóñez & Rupérez, 2011). Researchers have reported promising anti–tumoral results using seaweed fucoidans (An et al., 2022).

USC UNIVERSIDADE DE SANTIAGO

1.2.2. PHLOROTANNINS

Polyphenols are secondary metabolites from plants (terrestrial and marine) and lichens (Koivikko et al., 2007). Generally, they act as cell walls structural components and shelters against environmental stress (Tierney et al., 2013a). Polyphenols are aromatic compounds with more than one hydroxylic groups (Balboa et al., 2013). Polyphenols could be divided into three categories, phenolic acids, flavonoids and non–flavonoids (Figure 1.3).

Phlorotannins have achieved notorious research, industrial and medical interest due to their bioactive capacities (Figure 1.2) since have showed antioxidant, anticoagulant, antithrombotic, antidiabetic, enzymatic regulatory, bactericidal, antitumoral, anti–inflammatory, antihypertensive, antiviral, among others, properties. Besides, they have shown promising results in Alzheimer, Parkinson, and arthritis treatment (Li et al., 2011; Wang et al., 2012; Balboa et al., 2013; Harnedy et al., 2013; Kumar et al., 2020, Tavakoli et al., 2021; Cebrián–Lloret et al., 2022).

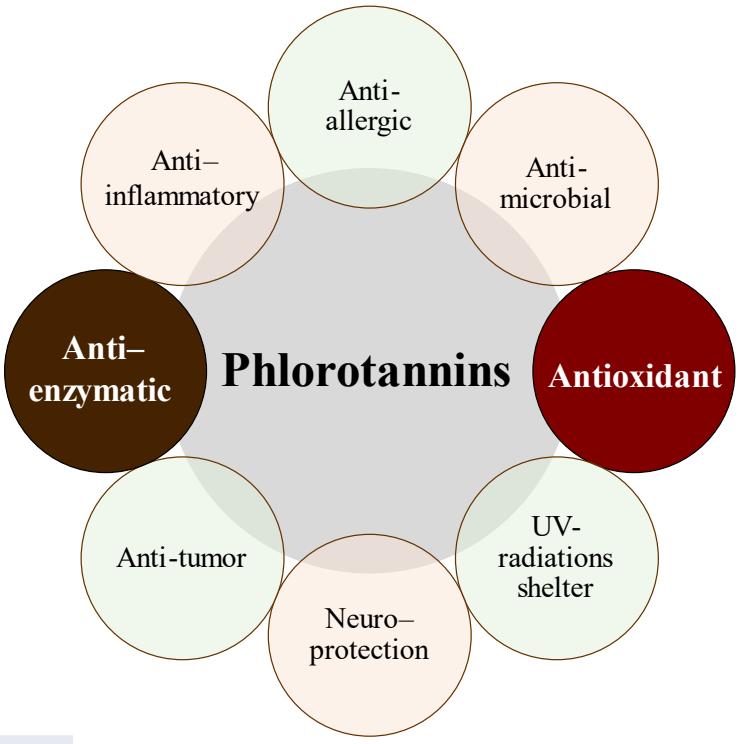


Figure 1.2. Phlorotannins bioactive capacities.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Seaweeds are gaining recognition as a source of bioactive compounds, suitable to be used as health promotors and disease preventors. Phlorotannins are a group of tannins that only can be obtained from brown seaweeds (Figure 1.3) and have recently gained such a huge relevance due to their bioactive properties together with the low time– and economic– consumption that suppose the seaweed growth. Brown algae have higher contents of these compounds, with notorious content in *Fucus vesiculosus* and *Ascophyllum nodosum* species (Gómez–Ordóñez & Rupérez, 2011).

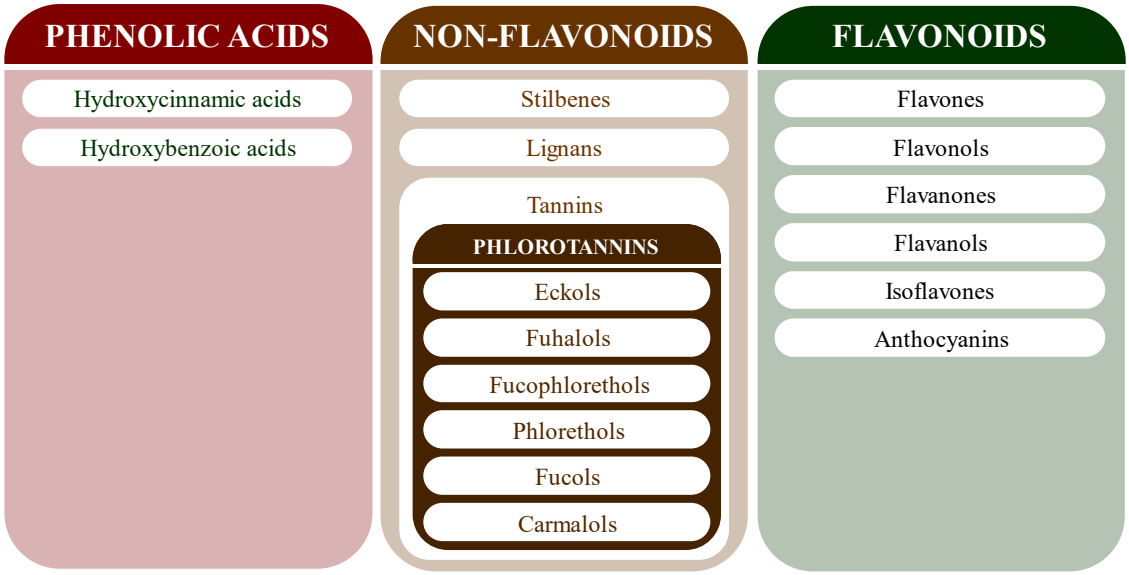


Figure 1.3. Polyphenol's classification.

Phlorotannins are produced entirely by polymerization of the phloroglucinol molecule in the polyketide pathway reaction (Koivikko et al., 2007) and stored in physodes and/or cell–wall (Figure 1.4) forming complexes (Agregán et al., 2017). Phlorotannins content of seaweeds depends on environmental conditions, such as tides, salinity, light availability, UV radiation and herbivory intensity (Tabassum et al. 2016). Phlorotannins have structural roles forming part of cell walls (Figure 1.4) together with alginic molecules (Koivikko et al. 2005). These compounds are hydrophilic components with a wide range of molecular sizes ranging between 126 and 650 kDa (Ragan & Glombitza, 1986).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

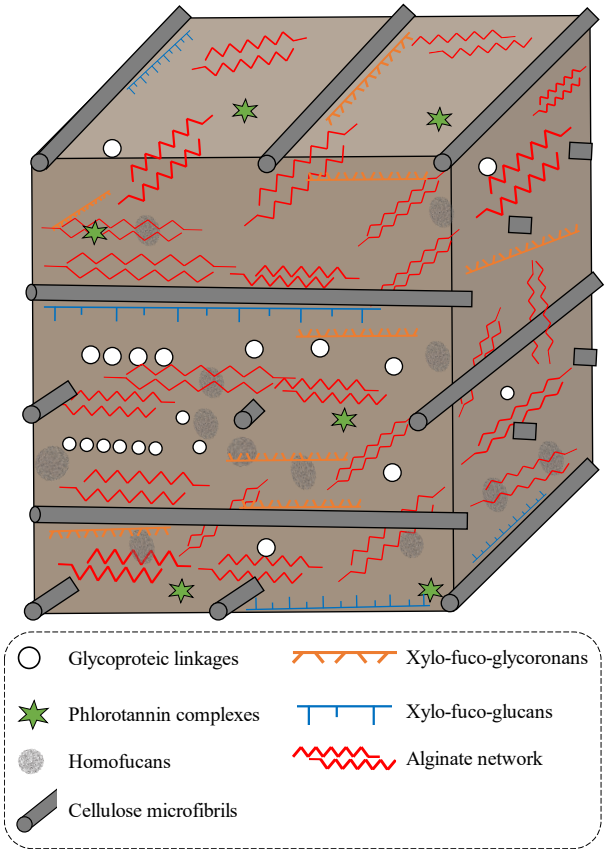


Figure 1.4. Proposed matrix of brown seaweed cell–walls.

Phlorotannins are divided into six different classes, eckols, fuhalols, fucophlorethols, phlorethols, fucols and carmalols (Figure 1.5), based upon variations in their assemblage, and distribution of hydroxyl groups (Li et al., 2011). Phlorethols and fuhalols present aryl–ether linkages, fucols aryl–aryl bonds, fucophlorethols a mixture of ether and phenyl bonds. Eckols present a 1,4–dibenzodioxin unit (Thomas & Kim, 2011). Carmalols present a 4–dibenzodioxin unit at the third and seventh position (Kumar et al., 2022). Eckols differ from carmalols by their lower molecular weight and by the presence of a OH group substituted at fourth carbon (Li et al., 2009). And fuhalols differ from phlorethols by their regular sequence of para– and ortho–ether bonds, by the presence of additional OH groups in every third ring and by the lack of one or more OH groups in the whole molecule (Ford et al 2019).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

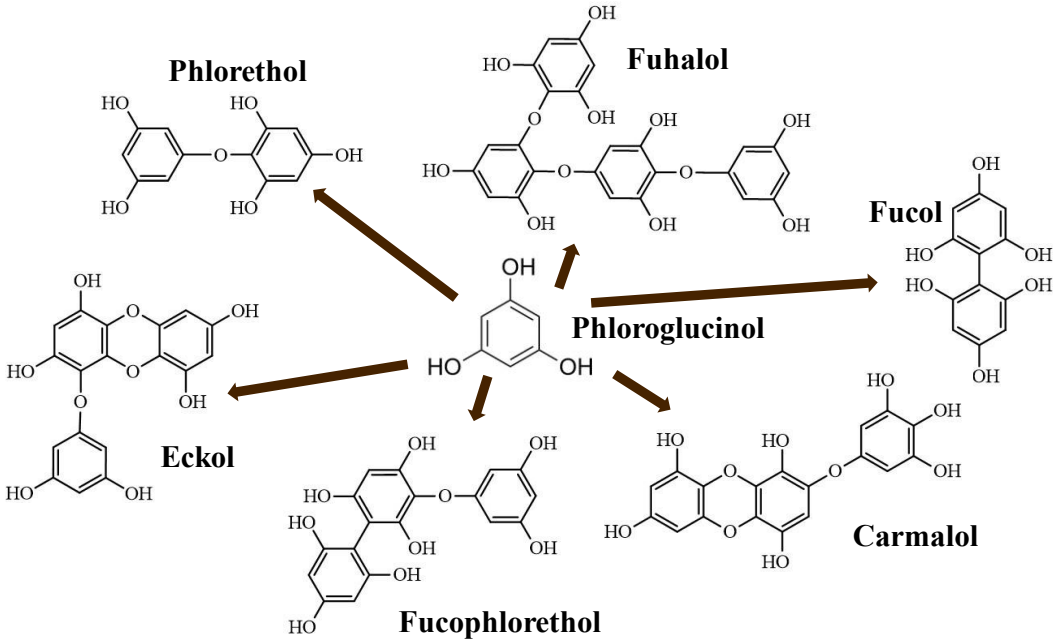


Figure 1.5. Phlorotannin main groups, all derived from phloroglucinol polyketide pathway reaction.

1.2.3. ALGINATES

Alginate is a polyuronan isolated from some seaweeds (McHugh, 2003) or produced by some bacterial species (Govan et al., 1981) and their content can reach 55% of dry basis (d.b) in some species (Pereira, 2011). It is found in around all brown seaweeds species, but its exploitation is mainly focused on 38 species. Worldwide production of alginate comes from following species: *Ascophyllum nodosum* (12–16% d.b), *Durvillaea potatorum* (45–55% d.b), *Ecklonia arborea* (24–28% d.b), *Laminaria hyperborea* (14–21% d.b), *Laminaria digitata* (16–36% d.b), *Lessonia trabeculata* (13–29% d.b), *Macrocystis pyrifera* (18–45% d.b) and *Saccharina latissima* (16–34% d.b) (Peteiro et al., 2018). They can reach up to 40–47% of the seaweed dried weight, varying between seasons, reaching maximums in spring (Tabassum et al., 2016). Alginate isoforms have shown antihypertensive and anti–inflammatory activities, as well as the capacity to reduce cholesterol levels (Gómez–Ordóñez & Rupérez 2011).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Alginate is a structural component of the multicomponent algae matrix cell wall as can be seen in Figure 1.4 according to model proposed by Lobban & Harrison (1994). Alginates are responsible of flexibility and mechanical resistance of seaweeds (Braccini, et al., 1999; Stephen et al., 2006). In fact, alginate yield extraction is improved when seaweeds are harvested from turbulent waters. Alginate also plays a role into seaweed ion–exchange equilibrium retarding desiccation when seaweeds are air–exposed during low tide periods (Donati & Paoletti, 2009).

Alginates have been widespread industrial used by their thickeners, emulsifiers, and stabilizers properties. Food industry adds alginate in sauces, jams, marmalades or mayonnaise (Jensen, 1993; Mancini et al., 2002) to improve their organoleptic qualities and the final product appealing (Paraskevopoulou et al., 2006). It is also used in ice cream production since inhibit the formation of large ice crystals producing smoother textures (Kilinç et al., 2013). It is used in dye industry, biomaterials dressing, medical and pharmaceutical sectors for microencapsulation, dental impressions, drug delivery vectors or anti–reflux therapies (Ertesvåg, et al., 1998; Ingram et al., 1998; Skaugrud et al., 1999; Mandel et al., 2000; Uludag et al., 2000).

1.3. *ASCOPHYLLUM NODOSUM* SEAWEED

Ascophyllum nodosum (Linnaeus) Le Jolis (Figure 1.6), also known as rockweed or *A. nodosum*, is one of the most studied brown edible seaweeds containing 14% (polyphenol) and 28 % (d.b) of fucoidan content (Holdt & Kraan 2011; Liu & Gu, 2012; Kadam et al., 2015c; Catarino et al., 2019). Thus, together with their availability along Galicia's coasts and the experience of the group GI–1618 with this alga (Arufe, 2017), *A. nodosum* was selected as main source of bioactive compounds, mainly phlorotannins and sodium alginates of the current Thesis.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



Figure 1.6. *Ascophyllum nodosum* (*Linnaeus*) *Le Jolis* seaweed (A), fronds (B) and flotation vessels (C) from Algaebase database.

A. nodosum is a dark brown seaweed (Figure 1.6A) common in the North–Western coast of Europe (from Norway to Portugal), and the North–Eastern coast of North America. *A. nodosum* seaweed has long olive–brown fronds (Figure 1.6B) with large egg–shaped airbladders (Figure 1.6C). The fronds can reach 2 m in length and are attached by a holdfast to rocks and boulders. *A. nodosum* seaweed is used as a raw material for industrial production of fucoidans, alginates, ascophyllan and laminarins and as a fertilizer in the agrochemical industry (Khan et al., 2012). This seaweed is found on the northwest coast of Europe and northeastern of America (Bertness et al., 2014), Figure 1.7.

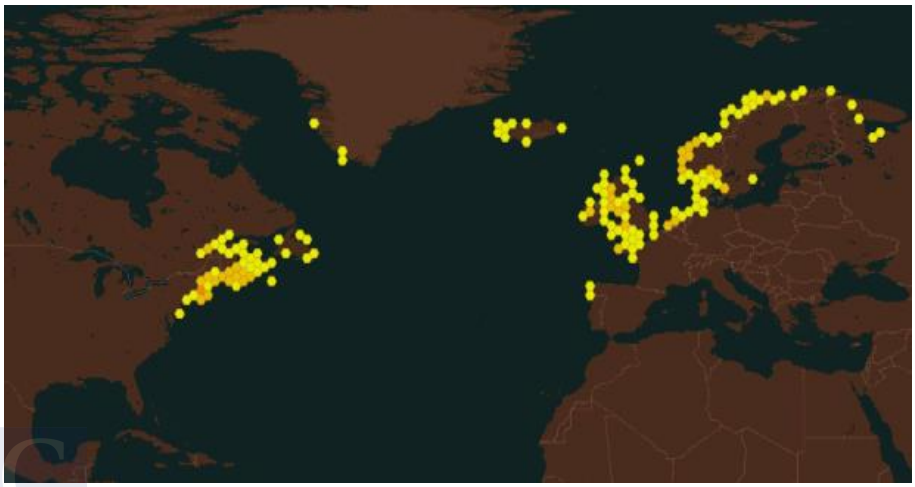


Figure 1.7. Geographical distribution of *A. nodosum* seaweed from GBIF database, (2022).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The chemical composition of this seaweed showed in Figure 1.8 of average moisture, protein, ash (mineral content), lipid, phenolic and carbohydrate content (Yuan & Macquarrie (2015).

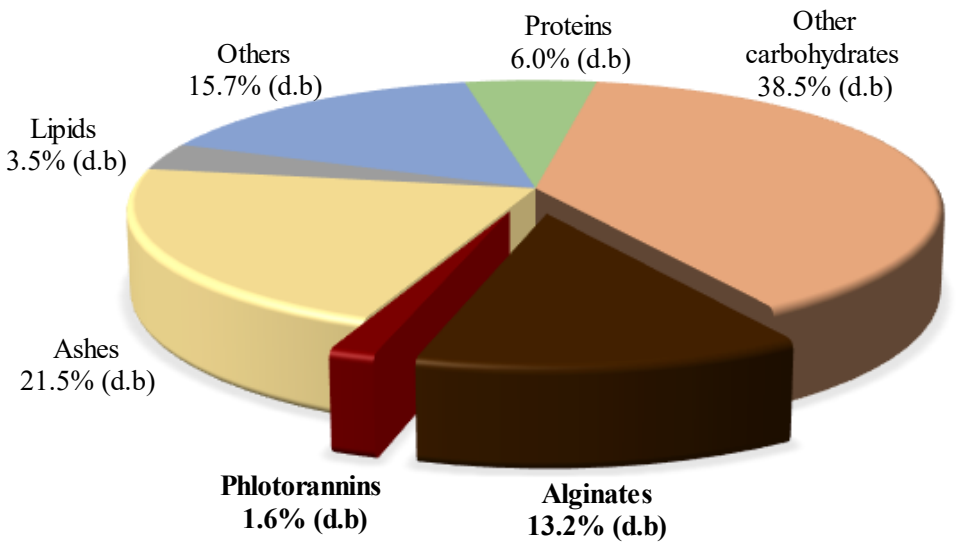


Figure 1.8. Average composition of *A. nodosum* brown seaweed.

1.4. MARKET AND APPLICATIONS

Macroalgae, also called seaweeds, are becoming an interesting and demanded crop due to their fast growth (Kerrison et al., 2015; Wood et al., 2017) and high content in bioactive compounds. Marine organisms comprising around half of the total global biodiversity, then oceans offer a prominent resource for novel compounds (Aneiros & Garateix, 2004; Barrow & Shahidi, 2007). The benefits of macroalgae as a source of novel bioactive products are being globally revealed by the scientific community with the increasing interest on their different biological activities and the consumers are getting more attracted to marine algae–derived foods (Kim & Wijesekara, 2010; Wijesekara et al., 2010). Seaweeds provide promising and exclusive compounds, such as bioactive phenolic compounds (Isaza et al., 2013; Belanche et al., 2016), unsaturated fatty acids, fucoidans, alginate and biopolymers (Schmid et al., 2014; Angell et al., 2016) with notorious features (Ford et al., 2019; Gimpel et al., 2015a,b) and usages (Figure 1.9).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

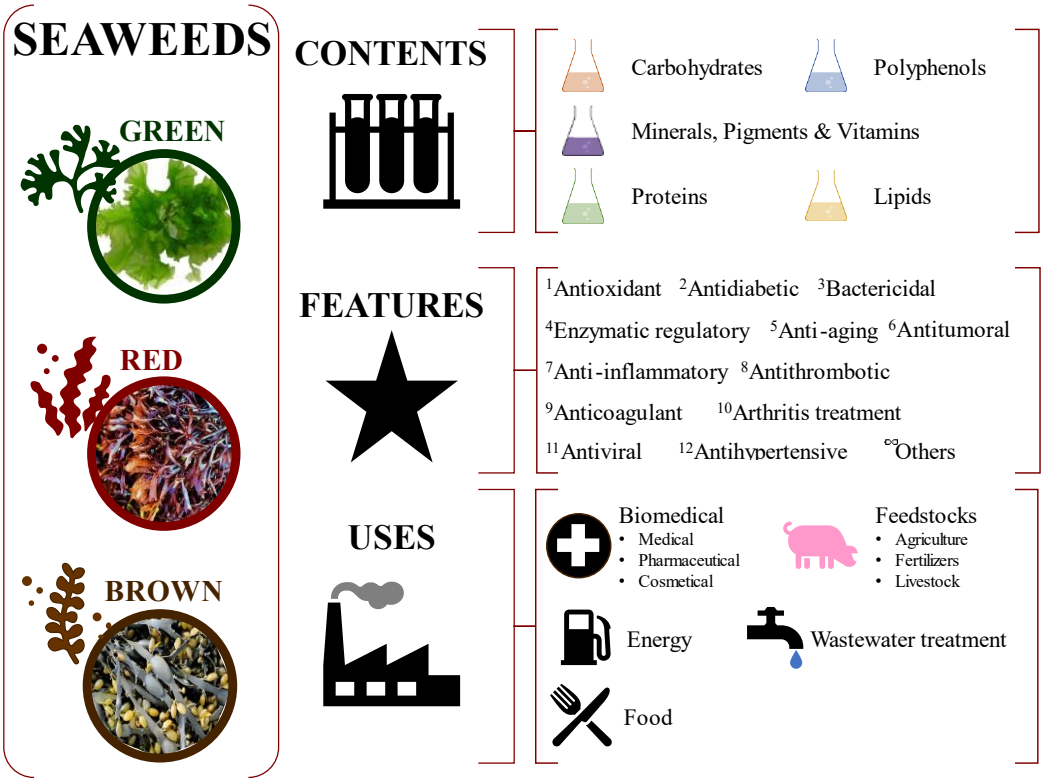


Figure 1.9. Industrial potential of seaweeds.

Bioactive compounds of seaweeds with relevant health benefits (Cumashi et al., 2007;) are exploited by several industrial sectors such as: biomedical (Holdt & Kraan, 2011; Valderrama, 2012), feedstocks (Craigie, 2011), biofuel production (Graham et al., 2009), wastewater treatment (Gómez–Ordóñez & Rupérez, 2011; Lawton, 2017) and food industry (Leandro et al., 2020). Hence, this rising global consumption of seaweeds and their derived products is generating notorious demand with socio–economic benefits, that can generate higher revenue streams through the processing of high value products (Ford et al., 2019), increasing up to US$ 26 million by 2025 (Ferdouse et al., 2018) that according to FAO (2018) trends to increase up to 10% in the coming years.

Biomedical (medical, pharmaceutical and cosmetical): The number of cosmetic products that include seaweed compounds are increasing in recent years. It is common to see product labels with "marine extract", "extract of algae", "seaweeds extract" or similar. For example, alginate increases skin moisture retention properties of some lotions (Wijesinghe & Jeon, 2011). Seaweed–derived pastes are commonly used in thalassotherapy together with hydrotherapy to partially relief rheumatism and osteoporosis problems (Kilinç et al., 2013).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Seaweeds can provide antiaging, photoprotective, moisturizing, antioxidant, anti–melanogenic, anti–allergic, anti–inflammatory, antimicrobial, anti–acne, anti–wrinkling, whitening, low cytotoxicity and allergen content (López–Hortas et al., 2021). The use of seaweed as food supplement for the control of the glycemic index has been also investigated (Apostolidis & Lee, 2010; Kim et al 2014) and will be deeply explored in this Thesis.

Feedstocks (agriculture, fertilizers and livestock): Seaweeds have been used as fertilizer since 19th century by coastal dwellers. Seaweeds enhance soil moisture retention, and their mineral content is source of trace essential elements (McHugh, 2003). Seaweeds have fast growing rates, do not require costs associated to planting, fertilization or irrigation (Ketheesan & Nirmalakhandan, 2011; Rhoades, 2012). Hence, seaweed supplemented animal feeds offer nutritionally renewable feedstocks with several positive health features for livestock (Evans & Critchley 2014). For instance, Huang et al. (2018) and Silva et al. (2018) demonstrated that pigs fed with diets supplemented with seaweeds showed a lower need of antibiotics. Additionally, Belanche et al., 2016 demonstrated that ruminants fed with seaweed diets reduced their methane emissions.

Energy production: Direct combustion of algae biomass is a traditional method of generating heat or steam. However, it is not a suitable method since produce emissions together with a low efficient energy production (Milledge & Harvey, 2018). Seaweeds could be used to produce syngas throughout a gasification reaction however it is a process that requires high temperatures (Rauch et al., 2014). The production of biofuel from seaweeds can be carried out in presence of catalysts and hydrogen at high pressure and lower temperatures with hydrothermal liquefaction process (Milledge et al., 2014).

Wastewater treatment: Some compounds from seaweeds have demonstrated to be efficient chelators to remove hazardous pollutants as heavy metal from industrial downstreaming (Gómez–Ordóñez & Rupérez, 2011). Food industry: Use of seaweeds as food has strong roots in Asian countries such as China, Japan, and Korea, being notoriously less consumed in occidental countries (Ali et al., 2000). Seaweeds are considered the food supplement for 21st century due to their high vitamins, minerals and dietary fibers content together with a low caloric content (Gómez–Ordóñez & Rupérez, 2011; Kilinç et al., 2013). Thus, seaweeds are being studied to be employed as food additives (Zhang et al., 2018a), to replace chemical

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

preservatives, to open up new prospects in food preparing, appealing and health beneficial aspects.

Phlorotannins have been reported as potential food additives that act as competitive inhibitors of digestive α–amylase and α–glucosidase enzymes, inhibiting carbohydrates hydrolysis that cause a reduction of the sugar intake levels (Rodrigues et al., 2015) acting as glycemic index regulators. Last aspects are studied in this Thesis. Finally, hydrocolloids from seaweeds have been widespread used in several applications (Chenlo et al., 2009).

1.5. ANTIOXIDANT CAPACITIES

"Antioxidant" is a widespread used term, but surprisingly difficult to define clearly. Food technologists use antioxidants to inhibit lipid peroxidation and consequent rancidity in foods (Prior et al., 2005). Museum curators use them to preserve their exhibits. In polymer research, they are used to control polymerization in the manufacture of rubber plastics and paints. In energy fields, they are extensively used in the design of better fuels and lubricating oils. These are some examples of points of view regarding antioxidants concept (Halliwell & Gutteridge, 2007). Several plants have high content of interesting antioxidant compounds to be used in different treatments of preeclampsia, atherosclerosis, acute renal failure, Alzheimer's and/or Parkinson's diseases (Munteanu & Apetrei, 2021). The chemical structure of antioxidants determines its antioxidant mechanisms and reactivities. Efficiency is highly related to antioxidant concentration, localization of molecules in the system, also named bioaccessibility (Shahidi et al., 2011) and their reaction kinetics (Antolovich et al., 2002). All these parameters should be considered during selection of an antioxidant source. In this Thesis, antioxidants obtained from seaweeds and used as food additives (Halliwell et al., 1992).

The antioxidant relevance is due to their function as shelter against all species of dangerous oxidants (RS) like the oxygen derivative and other reactive species (Prior et al., 2005). RS is considered any atom or molecule that contains one or more unpaired electrons that alter molecule chemical reactivity (Halliwell, 1994), it is a collective term that includes oxygen radicals and non–radical derivatives of O2 (Table 1.1), hence, all oxygen radicals are RS, but not all RS are oxygen radicals. This term has been expanded to include reactive nitrogen,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

chlorine and bromine, sulphur, selenium, carbonyl species among other oxidant compounds (Halliwell & Gutteridge, 2007).

Table 1.1. Reactive species.

Radicals		Non–radicals	
Superoxide	$O_2^{\bullet -}$	Hydrogen peroxide	H_2O_2
Hydroperoxyl	$HO_2^{\bullet}$	Peroxynitrite	$ONOO^-$
Hydroxyl	$OH^{\bullet}$	Peroxynitrous acid	$ONOOH$
Peroxy	$RO_2^{\bullet}$	Nitrosoperoxycarbonate	$ONOOCO_2^-$
Alkoxyl	$RO^{\bullet}$	Hypochlorous acid	$HOCl$
Carbonate[d]	$CO_3^{\bullet -}$	Hypobromous acid	$HOBr$
Protein radical	$P^{\bullet}$	Nitroxyl anion	NO^-
Thiyl radical	$RS^{\bullet}$	Nitrosyl cation	NO^+
Carbon dioxide	$CO_2^{\bullet -}$	Ozone	O_3

RS are highly volatile compounds capable of damaging several cellular functions such as deactivation of enzymes and protein damage, gene alteration and lipid oxidation (Halliwell, 1994). Approximately 98% of O_2 consumed by cells enters the mitochondria in the electron transport system, however, sometimes is inappropriately metabolized becoming an RS (Hancock et al., 2001). Indeed, it is reported that every person produces over 2 kg of superoxide per year (Stadler, 1998). Not all RS are hazardous, some superoxides are produced deliberately by phagocytes (neutrophils, monocytes, macrophages, eosinophils) during immune reactions (Halliwell, 1992). An RS lifetime *in vivo* is vanishingly small because it attacks whatever it is next to. For instance, attack of RS on cell membranes or lipoproteins produces lipid peroxidation that is particularly related with atherosclerosis. When RS attack DNA molecules alter guanine bases that are converted into 8–hydroxyguanine producing mutations that could end up developing cancer (Halliwell, 1994). There is growing evidence that free–radical damage is involved in highest global deathly diseases such as cardiovascular, cancer, diabetes, sclerosis, among others (Sies, 2019).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

In general, RS are kept under control by antioxidant defenses, these antioxidants could be classified according to (Halliwell & Gutteridge, 2007):

- Agents that catalytically remove RS (e.g., superoxide dismutase, superoxide reductase, catalase, or peroxidase enzymes).
- Agents that decrease RS formation (e.g., pro–oxidant proteins such as transferrin, albumin, haptoglobin, haemopexin, haemoxygenase, metallothionein, polyamine and/or caeruloplasmin).
- Proteins that protect biomolecules against oxidative damage by other mechanisms (e.g., chaperones).
- Agents that promote the physical "quenching" of RS (e.g., singlet O_2 by carotenoids).
- The replacement of molecules sensitive to oxidative damage by molecules resistant to it (e.g., fumarase C of *Escherichia coli*).
- Sacrificial agents that are preferentially oxidized by RS to preserve other biomolecules (e.g., α–tocopherol, bilirubin, ascorbate, urate, albumin and/or plasmalogens).

On the other hand, antioxidants can also be divided between endogenous and exogenous (Figure 1.10) depending on their synthesis site (Halliwell & Gutteridge, 2007). Endogenous antioxidants are comprised by intracellular organelles such as peroxisomes and mitochondria and extracellular components as macrophages, eosinophils, and neutrophils (Gunathilake et al., 2022; Niki, 1996). The levels and composition of antioxidant defenses differ among tissues, cell types and organisms. Indeed, it is reported that embryonic stem cells are exceptionally rich in antioxidant defenses, which decrease with cell differentiation (Bors et al., 1990; Dernbach et al., 2004). Superoxide dismutase (SOD) found in mitochondria and cytosol, is one of the main protective mechanisms of cells against RS since convert superoxide anion into hydrogen peroxide. Hence, amyotrophic lateral sclerosis (ALS) patients present a gene defect that decreases activity of this enzyme up to 40% (Di Matteo & Esposito, 2003).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

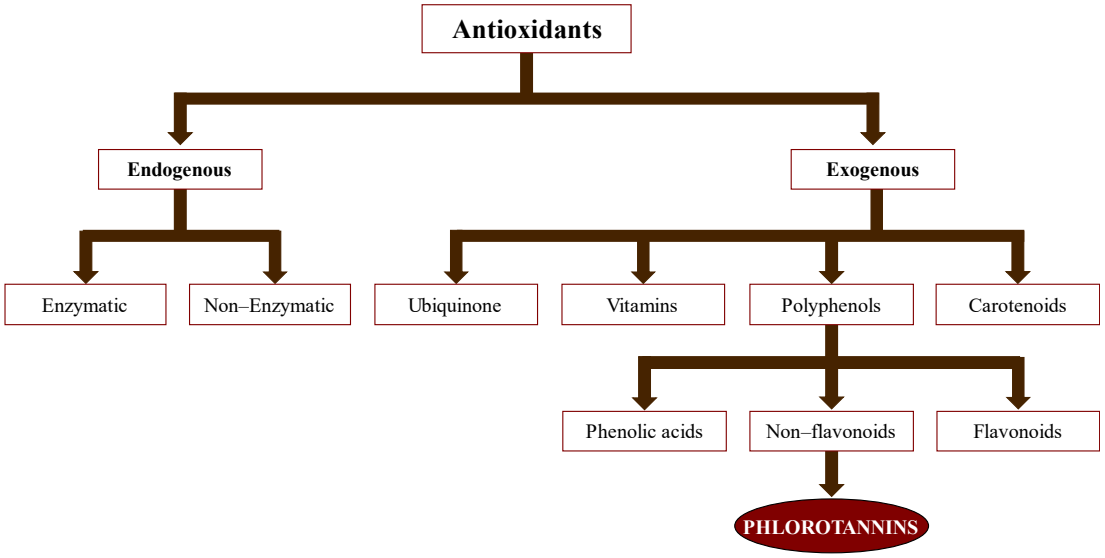


Figure 1.10. Classification of antioxidants.

Exogenous antioxidants are comprised by extracellular compounds such as plasma transferrin, lactoferrin, hemopexin, haptoglobin, albumin or caeruloplasmin. Some alternative defenses could be found in both, intra– and extra–cellularly, as α–tocopherol that blocks the RS reaction in cell–membranes and lipoproteins (Bruettner, 1993; Jacobsen et al., 2019). A diet rich in natural products such as fruits, nuts, and vegetables (terrestrial and marine) seems to be protective against several human diseases due to their high content of antioxidants (Halliwell, 1994; Jovanovic et al., 1994; Paganga et al., 1996).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroxylquinone (TBHQ) and ethoxyquin (EQ) have been considered over las decades, (Shahidi & Ambigaipalan, 2015) but their safety in target animals, humans and the environment has been questioned (Gunathilake et al., 2022; Agregán et al., 2018). Consequently, researchers and consumers are particularly focused on natural antioxidants sources, being seaweeds one of the widespread current explored candidates in the last decades (Holdt & Kraan, 2011; Agregán et al., 2017; Maheswari et al., 2022).

Several studies have been conducted on the high antioxidant potential of phlorotannins from marine brown algae (Ahn et al., 2007). However, in contrast to the polyphenols from terrestrial plants, there is a lack of knowledge about phlorotannins *in vivo* applications and their potential health benefits (Steevensz et al., 2011; Jacobsen et al., 2019).

USC
UNIVERSIDADE DE COMPOSTELA

The screening of phlorotannins and other polyphenols for their uses as antioxidants is assessed by determining their direct free radical scavenging activity as hydrogen/electron donating capacities, their ability to chelate transition metal ions, their efficacy as chain–breaking oxidation reactions and the scavenging of nitrogen reactive species, which may involve nitration, hydroxylation, or oxidation of the antioxidant molecule (Bors et al., 1990; Zakaria et al., 2011; Tanniou, et al., 2014; Aklakur, 2018). Phlorotannins are efficient hydrogen–donating antioxidant due to their multiple hydroxyl groups together with their abilities to delocalize the resulting antioxidant radical within the structure (Rice–Evans et al., 1996).

Sathya et al. (2017) reported that phlorotannins from brown algae are more powerful free radical scavengers than polyphenols derived from terrestrial plants since have up to eight interconnected aromatic rings whereas terrestrial phenols have three to four rings. Liu et al. (2017) studied the antioxidant activity of phlorotannin-enriched extracts from *Saccharina latissima*, *Alaria esculenta*, *Laminaria digitata*, *Fucus vesiculosus*, and *A. nodosum* comparing them with commercial antioxidants (butylated hydroxytoluene and ascorbic acid). Then, phlorotannin showed outstanding antioxidant capacities specially with *Fucus vesiculosus* and *A. nodosum* extracts demonstrating that these seaweeds are proficient sources of antioxidant compounds (Prior et al., 2005).

Currently, there are not known limits or possible related harmful effects for either animal or human health respect to the consumption of seaweeds, excepting those referring to the high iodine levels and consequently the derived recommendation of avoiding an excessive frequent ingestion. For centuries, seaweeds have generally been considered safe to consume in relatively large quantities, especially in oriental countries. It was France, in 1990, the first country that established certain rules and regulations for seaweeds consumption (Roleda et al., 2019). The presence of high levels of toxic contaminants, such as residual pesticides, minerals, and heavy metals (e.g., cadmium, mercury, tin, lead, among others) limit their applications as feedstocks (human and animal) (Gunathilake et al., 2022). Nowadays, seaweeds are considered a “novel food” under European regulations (Regulation (EU) 2017/2470) and the current brown edible seaweed species could be seen in Table 1.2.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 1.2. Current recognized as safe and edible brown edible seaweeds (FAO, 2018).

Scientific name	Traditional name
Alaria esculenta	Badderlocks
Ascophyllum nodosum	Rockweed
Cladosiphon okamuranus	Mozuku
Durvillaea antarctica	Cochayuyo
Ecklonia cava	
Eisenia bicyclis	Arame
Fucus spiralis	Spiral wrack
Fucus vesiculosus	Bladderwrack
Himanthalia elongata	Thongweed
Laminaria digitata	Oarweed
Nereocystis luetkeana	Bull kelp
Pelvetia canaliculata	Channelled wrack
Postelsia palmaeformis	Sea palm
Saccharina japonica	Kombu
Saccharina latissima	Sugar kelp
Sargassum cinetum	
Sargassum echinocarpum	Limu Kala
Sargassum fusiforme	Hijiki/Hiziki
Sargassum myriocysum	
Sargassum swartzii	
Sargassum vulgare	
Undaria pinnatifida	Wakame
Undaria undarioides	Hiromi

Antioxidants from seaweeds are becoming a research target in food, medicinal and cosmetical fields (see Section 1.4) due to their protective roles in food products against oxidative deterioration and in the body against oxidative stress–mediated pathological processes (Munteanu & Apetrei, 2021). This Thesis was focused on increasing the current knowledge about the health benefits and technological applications of phlorotannins, from *Ascophyllum nodosum* brown edible seaweed and their several bioactivity features, mainly their antioxidant and antidiabetic capacities.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1.6. ANTIDIABETIC CAPACITIES

Celiac disease is the intolerance to gluten protein present in grains such as wheat, barley, rye and oatmeal. Celiac disease is an immune–based disease that cause the mucosal inflammation of the small intestine altering the nutrient absorption and subsequently other problems as growth deficit, anemia, vitamin deficiencies, thyroid diseases, mental disorders, fatigue and/or infertility (Patterson et al., 2009; Carstensen et al., 2014). First of celiac disease concepts were reported by Arateus of Capadocia (II century, BC) who named patients with intestinal problems as *koliakos* (celiacs') that means "those who suffer from intestine". However, it was around 1900 when Dr. Willem Karel Dicke discovered that gluten was the origin of these intestinal problems and designed the first gluten–free (GF) diet (Stoop, 1991).

The estimated prevalence of the celiac disease is around 1% and it is expected that it increases (Singh et al., 2018; Cozzi et al., 2022). Celiac disease shows a variety of clinical manifestations. The incidence and different pathologies could be explained by the celiac iceberg (Figure 1.11). The well–diagnosed patients comprise the visible part of the iceberg and represent around 10% of the whole group. The last group are the silent cases, around of 75% of incidence, that suffer un–diagnosticated intestinal mucosa disorders (Maki & Collin, 1997). Finally, there is a latent group with predisposition to suffer this disease. This group is formed by childhood diagnosed patients that could recover themselves after the onset of the GF diet and were maintained in subclinical state and/or populations without previous reported gluten problems that end up developing the disease spontaneously (Nenna et al., 2013).

Gluten is an amorphous protein, that represents 80% of wheat protein content. It is the responsible for the elasticity and spongy consistency of average bakery products. The unique suitable treatment for celiac disease is a GF diet during the patient's entire life. This causes a clinical and functional digestive normalization permitting the reparation of intestinal vellosities injuries (Gallagher et al., 2004). Celiac disease and their treatment in recent years, is a trending research, industrial and economical interest. The increasing consumption is correlated with the increasing incidence of gluten related disorders, and the population misconception of these products' beneficial healthy properties.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

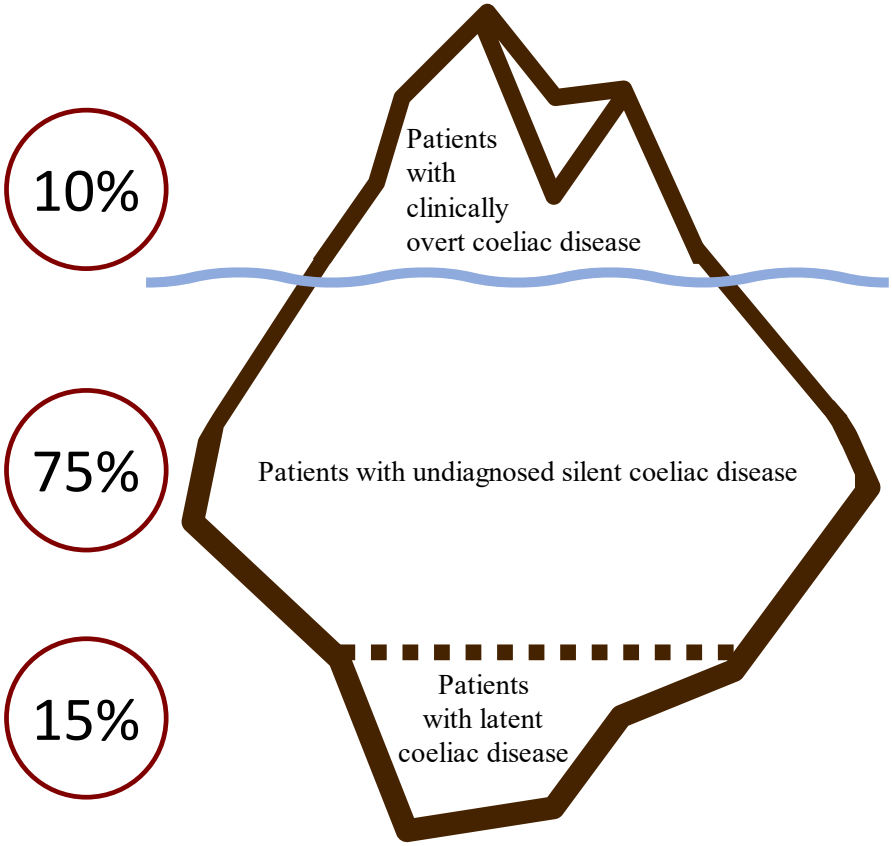


Figure 1.11. Diagram of celiac disease iceberg.

Nevertheless, the adherence to GF diet frequently leads to overweight and obesity disorders (Kabbani et al., 2012). With regards to this topic, the relationship between celiac disease and diabetes mellitus has been reported by Zong et al., (2018). Diabetes mellitus is a metabolic disorder, that consists in a chronic hyperglycemia (high levels of sugar in blood), that also produces alteration in carbohydrates, lipids, proteins and mineral salts homeostasis (Khan et al., 2024).

The global diabetes prevalence in 2019 was estimated around 9.3% (463 million people) and it is foreseen to reach values until 10.9% in 2050 (Saeedi et al., 2019). The fight against this disease must be priority for global health systems since it responsible for millions of death per year. Indeed, it shows an increasing trend of 3% annually in children and adolescents (Carstensen et al 2014; Patterson et al 2009).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The treatment of diabetes is limited to insulin intake by intramuscular injection or oral ingestion of agents or antidiabetic drugs, that could produce side effects like stomach disorders. Currently, the non–pharmacological interventions are limited to apply changes in dietary lifestyle promoting the consumption of low glycemic index (GI) foods. Actually, current GF products have high GI values, since they are low in proteins and high in fat and sugar content, whose consumption quickly increase glucose levels in bloodstream. Hence, consumption of these products prone to suffer diabetes disease.

GF foods were initially designed as a mandatory and rushed need to produce foods suitable for celiac patients but with a low nutritional quality (Miranda et al., 2014). Then, the development of new GF products becomes a challenge, since it must be carried out attending both nutritional (GI control) and sensorial appealing (i.e., flavor, quality and sensory features) points of view (Sivaramakrishnan et al., 2004).

The absence of gluten makes mandatory to seek alternative structuring agents. These gluten counterparts must be suitable to emulate its viscoelastic properties, appearance and crumb structure of conventional bakery products (Lazaridou et al., 2007) without altering products sensorial featuring. The use of gluten–free flours from alternative origin such as corn, rice, potatoes, chickpea combined with the addition of hydrocolloids (food gums), fiber and/or proteins are a suitable strategy to obtain economic, nutritional and functional GF products (Gambus et al., 2001; Behall & Hallfrisch, 2002; Novotni et al., 2012; Wolter et al., 2013;). Some of the widespread used gums in food sector are hydroxyethyl cellulose, xanthan gum, carrageenan, tragacanth gum, agar and alginate (Chenlo et al., 2009; Silva et al., 2017; Arufe et al., 2018a and b;). Some of these hydrocolloids are obtained from seaweeds, that in recent years are being in the spotlight by their high content of bioactive molecules such as polyphenols and fucoidans (Koivikko et al., 2007). To replace gluten with gums together with polyphenols from seaweed is proposed as economic, health–beneficial and novel manner of producing GF functional foods (Arufe et al., 2019a and b).

Algae have become a resource for the discovery and development of ingredients and food products due to their bioactive compounds, also called nutraceuticals (Vaz et al., 2016). Nutraceuticals defined as “a food or a part of a food with demonstrated safety and health benefits beyond the basic nutritional functions to supplement the diet, that contain specific compounds in a quantity that exceeds those that could be obtained from normal foods” (Prasad

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

et al., 2010). Seaweed nutraceuticals, polylactide, proteins, secondary metabolites (phlorotannins) and lipids among others, have demonstrated their beneficial health applications (Cumashi et al., 2007; Damonte et al., 2012). In addition, they have demonstrated to be promising digestive enzyme inhibitors to use them as GI regulators (Ademiluyi et al., 2012).

These approaches show the potential of algae–based platforms (Scranton et al., 2015). Indeed, seaweeds are proposed as multiple resource to develop biorefineries for production of valuable compounds as unique raw material (Herrero & Ibañez 2015). European Union approved in 2017 the project MIRACLES (*Multiproduct Integrated bioRefinery of Algae: from Carbon–dioxide and Light Energy to high–value Specialties)* based on this concept. The multi–extraction component from seaweed has several advantages based on the 13 principles of green chemistry, since the extraction solvent could be efficiently carried out with water, the processes do not require excessive temperatures and the successive extraction of multiple components minimizes the residues generated.

The current strategies to manage high glycemic levels are divided between oral ingestion of anti–diabetic agents and intramuscular injection of insulin. However, anti–diabetic drugs present undesirable side effects, such as risk of hypoglycemia, flatulence, abdominal discomfort and, sometimes, diarrhea are the main responsible for therapy discontinuation (Krentz & Bailey, 2005; Lopes et al., 2017). It is being studied the use of natural compounds as inhibitors of digestive enzymes (α–amylase and α–glucosidase) involving plant– and seaweed–derived compounds such as polyphenols due to their health beneficial features (Kan et al., 2020).

Polyphenols from terrestrial origin, as catechins, quercetin, ferulic acids epicatechins, chlorogenic acids, naringenin, tannic acid, and caffeic acid have been used to control small intestinal glucose absorption via inhibition of sodium glucose co–transporters (Kobayashi et al., 2000; Johnston et al., 2005;). Johnston et al. (2003) and Dao et al. (2011) demonstrated that the polyphenols are able to mediate hyperglycemia and impaired glucose tolerance by an aided insulin response and constricted synthesis of glucagonlike polypeptide–1 (GLP–1).

Waltner–Law et al. (2002) used epicatechins and catechins to moderate hyperglycemia and hepatic glucose production through regulating the expression of hepatic glucokinase and phosphoenolpyruvate carboxy–kinase. Zhou et al. (2001) and Towler & Hardie (2007) revealed that epigallocatechin gallate could trigger AMP–activated protein kinase pathway for inhibiting the expression of enzymes involved in gluconeogenesis. There are several literature that studies

USC UNIVERSIDADE DE COMPOSTELA

the effect of polyphenols on metabolism reactions, evidencing of the health beneficial properties of these compounds.

Phlorotannins have demonstrated to reduce diabetes–related complications, showing that seaweeds compounds are promising anti–diabetic compound. Yang et al., (2019) reported IC_{50} values of phlorotannins isolated from *Ishige okamurae* to inhibit amylase (0.53 mM) and glucosidase (0.16 mM) enzymes. Sugiura et al. (2017) studied anti–diabetic activity of different phlorotannins isolated from *Ecklonia cava* (i.e., phlorofucofuroeckol–A, eckol, phloroglucinol, fucofuroeckol A, dieckol, and 8,8'–bieckol) and reported that fucofuroeckol A and dieckol were the most effective with IC_{50} value of 7.4 μM. Additionally, phlorotannins have also demonstrated their capacities to inhibit several diabetic enzymes such as amylase, glucosidase, aldose reductase, protein tyrosine phosphatase and/or dipeptidyl peptidase (Gunathilaka et al., 2020). Lee & Jeon, (2013) showed that phlorotannins isolated from *Ecklonia stolonifera* (i.e., eckol, dieckol, 6,6'–bieckol, phlorofucofuroeckol–A, phloroglucinol, and 7–phloroeckol) inhibited α–glucosidase showing IC_{50} values of 10.7 μM. Similarly, Gotama et al. (2018) found that with a dose of 300 mg/kg of phenolic compounds from *Sargassum hystrix* were enough to reduce blood glucose levels. There are several studies about the use of seaweeds and their derivative compounds (i.e., phlorotannins, fucoidans, laminarins, etc.) as an anti–diabetic and antioxidant agents. In the current Thesis the study was focused on the integration of seaweed derivatives into starchy based products and their subsequent capacities to inhibit diabetic enzymes.

Then, it is proposed the implementation of biopolymers extracted from *A. nodosum* brown seaweeds in the next generation of GF products, focused on bakery products. It is a challenging task with multiple objectives, such as preventing problems derived from the short–term consumption of GF products, reduce long–term health problems of the habitual consumption of products with high GI and improving the bioactive and healthy beneficial properties of these new bakery products. The wide diversity of seaweeds put them in the forefront of research to obtain bioactive molecules with anti–diabetic properties (Barde et al., 2015). As sources of novel, structurally diverse and exclusive bioactive compounds is a promising study field (Sharifuddin et al., 2015; Lopes et al., 2017).

Current researchers are focused on adding extracted, purified and fractionated seaweed bioactive compounds to food products. The use of purified fractions had the main advantage

that phlorotannins added will be more efficient to increase their bioactivity features using minimal amount. Nevertheless, the production of these purified additive involves economic– and time–consuming processes. The addition of raw seaweed, as ingredient could be a quick and low–cost method. Furthermore, the use of raw algae would involve the addition of other bioactive components from algae as fucoidans and other carbohydrates improving, even more, the healthy beneficial characteristics (Koivikko et al., 2005) of these new algae–based foods (Sections 1.7.2 and 1.7.3). Therefore, as a compromise solution between these both factors, crude extracts were used in this Thesis. However, it implies the determination of chemical composition of biopolymers in the extracts and the enzymatic inhibition features related to starch digestion.

Conversely, algae are prominent quelators and often retain some pollutants hazardous for humans. Thus, brown seaweeds as raw ingredient must be carefully studied taking some considerations such as chemical characterization or a detailed tracking of their origin, growth, and harvest conditions. All these aspects will be explored in Section 3.2. Then, the selection of either the raw seaweeds or the extracted/purified phlorotannin fractions is a key aspect in industrial applications and will be explored together with the optimal method to produce these fractions with acceptable antioxidant features and polyphenols content carried out with an easy– and low–cost procedure. Finally, it will be tentatively analyzed the potential anti–diabetic capacities of these phlorotannins extracted from *A. nodosum* brown edible seaweed, comparing the results with the reference drugs and the available literature.

1.7. STATE OF ART

1.7.1. PHLOROTANNINS EXTRACTION

In the last twenty years, several researchers have studied different extraction characteristics, such as method, focusing on solid–liquid extractions (SLE) and ultrasound–assisted extractions (UAE), solvent type, liquid–solid ratio, time, and temperature as critical aspects of phlorotannin extraction from *Ascophyllum nodosum* brown edible seaweed (Table 1.3).

Table 1.3. Overview extraction conditions (method, extractant, liquid–solid ratio, time, and temperature) to obtain phlorotannins enriched extracts from *Ascophyllum nodosum*.

Reference	Method	Extractant	LS (g_{sol}/g_{algae})	Time	Temperature	TPC
Liu et al. (2021a)	SLE	Ethanol	90	30 min	30 ºC	0.7 $mg_{PE}/mg_{extract}$
Sardari et al. (2021)	SLE	Water	100	1 h	65 ºC	7.3 $mg_{PE}/g_{extract}$
		Ethanol (30% v/v)	5	30 min	25 ºC	4.1 $mg_{PE}/g_{extract}$
		Ethanol (80% v/v)	10	20 (+ 5) h	25 (+ 65) ºC	3.4 $mg_{PE}/g_{extract}$
Ford et al. (2020)	SLE	Acetone (70% v/v)	20	3 h	rt	24.5 $mg_{PE}/g_{extract.}$
Ummat et al. (2020)	SLE	Ethanol (50% v/v)	15	4 h	20 ºC	0.2 $g_{PE}/g_{extract}$
	UAE		10	30 min	NS (35 kHz)	0.4 $g_{PE}/g_{extract}$
Liu et al. (2019b)	UAE	Ethanol (40% v/v)	50	30 min	60 ºC	0.5 $g_{PE}/g_{extract}$
Poole et al. (2019)	SLE	Water	10	24 h	4 ºC	52 $mg_{PE}/g_{extract}$
		Methanol (50% v/v)				77 mg_{PE} /$g_{extract}$
		Ethanol (75% v/v)				95.4 mg_{PE} /$g_{extract}$
		Dioxolane (75% v/v)				90 mg_{PE} /$g_{extract}$
		1,3–propanediol				98.5 $mg_{PE}/g_{extract}$
Agregán et al. (2018)	UAE	Ethanol (50% v/v)	10	30 min	rt	46.6 $mg/g_{extract}$
Cikoš et al. (2018)	SLE	Methanol (70% v/v)	10	4 h	rt	0.5 $g_{GAE}/g_{extract}$
	MAE (2.45 GHz)			15 min	110 ºC	1.4 $g_{GAE}/g_{extract}$
Yuan et al. (2018)	SLE	Methanol (70% v/v)	10	4 h	rt	0.5 $mg_{GAE}/g_{d.b}$
	MAE (2.45 GHz)			15 min	110 ºC	1.4 $mg_{GAE}/g_{d.b}$
Moreira et al. (2017)	UAE (80 W/cm^2)	Acetone (70% v/v)	30	4 min	<35 ºC	31.8 $mg_{PE}/g_{d.b}$
Kadam et al. (2015a)	SLE	Water	20	150 min	70 ºC	0.17 $mg_{PE}/g_{d.b}$
		HCl (0.1 M)				0.11 $mg_{PE}/g_{d.b}$
	UAE (35.6 W/cm^2)	Water		15 min	<35 ºC	0.16 $mg_{PE}/g_{d.b}$
		HCl (0.1 M)				0.13 $mg_{PE}/g_{d.b}$

continue.→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Reference	Method	Extractant	LS (g_{sol}/g_{algae})	Time	Temperature	TPC
Kadam et al. (2015b)	UAE (75.8 W/cm^2) + SLE	HCl (0.03 M)	10	10 min + 22 h	<35 ºC	135.7 $mg_{GAE}/g_{d.b}$
Kadam et al. (2015c)	UAE (75.8 W/cm^2)	HCl (0.03 M)	10	25 min	<35 ºC	143.1 $mg_{GAE}/g_{d.b}$
Pantidos et al. (2014)	SLE	Water	10	60 min	20 ºC	178.0 mg_{GAE}/mL
		HCl (5 mM)				210.0 mg_{GAE}/mL
Tierney et al. (2013a)	SLE	Water	20	24 h	rt	70.5 $mg_{PE}/g_{extract}$
		Ethanol (80% v/v)	10			66.3 $mg_{PE}/g_{extract}$
		Acetone (80% v/v)				155.9 $mg_{PE}/g_{extract}$
	PLE	Water	2	NS	120 ºC (1500 psi)	93.4 $mg_{PE}/g_{extract}$
		Ethanol (80% v/v)			100 ºC (1000 psi)	101.3 $mg_{PE}/g_{extract}$
		Acetone (80% v/v)			60 ºC (1000 psi)	127.4 $mg_{PE}/g_{extract}$
O'Sullivan et al. (2011)	SLE	Methanol (60% v/v)	15	3 h	40 ºC	4.5 $mg_{GAE}/g_{d.b}$
Zhang et al. (2007)	SLE	Ethanol (50% v/v)	12.5	90 min	80 ºC	38.8 $mg_{PE}/g_{d.b}$
Wang et al. (2009)	SLE	Water	20	24 h	rt	138.0 $mg_{PE}/g_{extract}$
		Acetone (70% v/v)				159.0 $mg_{PE}/g_{extract}$

where, g_{algae}, is grams of algae; $g_{d.b}$, is grams of algae in dry basis (d.b); g_{sol}, is grams of solvent MAE, is microwave–assisted extraction; $mg_{extract}$, is milligrams of extract; mg_{GAE}, is milligrams of gallic acid equivalents; mg_{PE}, is milligrams of phloroglucinol equivalents; NS, is "non–specified" in the study; rt, is room temperature; SLE, is solid–liquid extraction; UAE, is ultrasound–assisted extraction; PLE, is pressurized–liquid extraction.

According to literature showed in Table 1.3 it can be clearly observed the relevance of TPC values obtained from the different extracts. TPC values ranged from 0.7 $mg_{PE}/mg_{extract}$ (Liu et al., 2021a) up to 0.5 $g_{PE}/g_{extract}$ (Liu et al., 2019b) both studies carried out extractions using ethanol but at different concentrations, 96% and 40% v/v, respectively. Lowest TPC values was obtained using conventional solid-liquid extraction, SLE, with one of the highest liquid–solid ratio values (LS = 90 $g_{solvent}/g_{AF}$), during intermediate operational time (t = 30 min) and temperature (T = 30 ºC). As it was expected the highest extraction was achieved using UAE, carried out with lower LS value (50 $g_{solvent}/g_{d.s}$), similar time (30 min) and higher temperature (60 ºC).

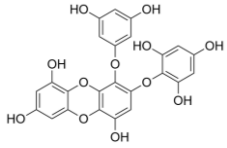
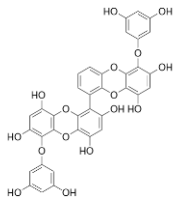
USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Briefly, from these results, organo–solvents were more common employed in phlorotannin extractions, new methods as UAE, MAE or PLE increased extraction yields and low temperatures were usually employed to avoid thermal degradation of phytochemicals, except for MAE treatment. However, it is mandatory to note that expression units as well as other parameters such as temperature and/or time differ among different authors making the comparison often difficult. A suitable solution to reduce mistakes associated to data comparison could be to show in detail the experimental designs in which wide ranges of conditions were proved and their trends could be suitable used to determine the better extractive conditions.

Additional publications of UAE applied to *A. nodosum* were found, Kadam et al. (2017) used them to extract protein from seaweed. Okolie et al. (2019) evaluated UAE influence on *in vitro* prebiotic assays of fucoidans enriched extracts. Garcia–Vaquero et al. (2018 & 2019) optimized the extraction of fucose and glucans using UAE technology and subsequently combining it with thermal–assisted extraction. Okolie et al. (2020) determined the impact of UAE on the structure–function relationship of sodium alginate.

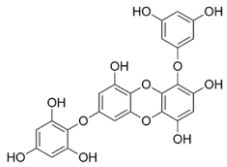
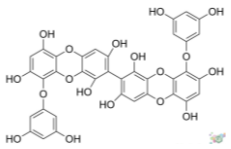
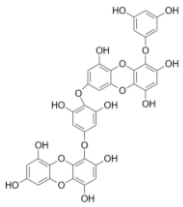
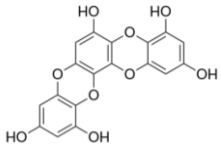
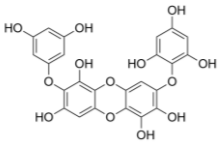
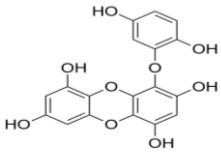
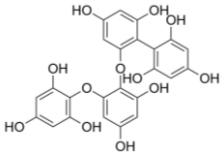
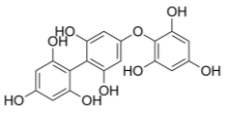
Several chromatographic techniques have been employed for separation, preparative isolation, purification, identification, and quantification of individual phenolic compounds from various plant materials, but still scarce studies deal with the individual phenolic compounds from brown algae (Table 1.4). These are essential research to assess and understand the phlorotannin chemical and physical structure effects on their bioactivities.

Table 1.4. State of art of current phlorotannins isolated from brown seaweeds.

Phlorotannin		Seaweed	References
2–Phloroeckol		*Eisenia. bicyclis*	Okada et al. (2004)
		Euphorbia stolonifera	Jung et al. (2008)
6,6'–Bieckol		*Ecklonia cava*	Artan et al. (2008)
		Ishige okamurae and *Euphorbia stolonifera*	Yoon et al. (2009)

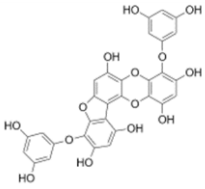
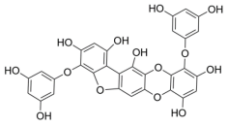
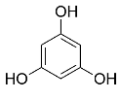
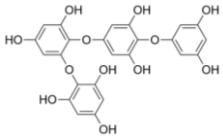
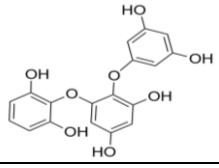
continue.→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Phlorotannin		Seaweed	References
7–Phloroeckol		*Euphorbia stolonifera*	Jung et al. (2008)
		Ascophyllum nodosum	Nwosu et al. (2011)
8,8'–Bieckol		*Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome*	Shibata et al. (2008)
Dieckol		*Eisenia bicyclis*	Nakamura et al. (1996)
Dioxinodehydroeckol		*Ephorbia stolonifera*	Jung et al. (2008)
Diphlorethohydroxycarmalol		*Ishige okamurae*	Heo et al. (2009)
Eckol		*Ecklonia kurome*	Fukuyama et al. (1989)
Fucodiphloroethol G		*Ecklonia cava*	Ham et al. (2007)
Fucophlorethols		*Cystophora retroflexa*	Sailler & Glombitza (1999)
		Cupressus torulosa	Glombitza et al. (1997)
		Sargassum spinuligerum	

continue.→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Phlorotannin		Seaweed	References
Phlorofucofuroeckol A		*Ecklonia kurome*	Fukuyama et al. (1990)
		Euphorbia stolonifera	Iwai (2008)
Phlorofucofuroeckol B		*Erica arborea*	Sugiura et al. (2006)
Phloroglucinol		*Ecklonia cava*	Glombitza et al. (1973)
Tetraphlorethols E		*Cystophora retroflexa*	Sailler & Glombitza (1999)
Triphlorethol		*Ecklonia cava*	Kang et al. (2005)

Authors (Table 1.4) have achieved the isolation of several phlorotannins from different seaweed however these processes involve a lot of steps, solvent amounts, long time, and energy making the process complicated and expensive. This fact explains the scarce number of standards and current commercial availability of phlorotannins. Nevertheless, the isolation of these compounds is required to understand their bioactivity features to be further use in real applications. Several methods for extraction of phenolic compounds from *A. nodosum* seaweed have been investigated, however, there are limited data on the effect of using UAE to produce phenolic compounds enriched extract (Ummat et al., 2020).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Among the available literature (Table 1.3) it is clear that the studies conducted on *A. nodosum* vary in the extraction methods, solvents, parameters, pre– and post–treatments and expression of the results, which makes the comparison of the results from different studies a challenging task. Additionally, different standards were used for quantification such as gallic acid, phloroglucinol, pyrocatechol (PCE), raw phlorotannin and catechin (Mekinic et al., 2019). The establishment of a standardized protocol to quantify algal bioactive extracts would be convenient.

1.7.2. SEAWEED BIOPOLYMERS IN FOOD

As before mentioned in Sections 1.4, 1.5 and 1.6, polyphenols are candidates to be food additives due to their health beneficial characteristics. Hence, the interest to replace synthetic additives by these natural antioxidants in foods is increasing. There are many publications about the potential use of polyphenols in foods. In example, 1337 results were found after a bibliographic search focused on the polyphenols implementation in starchy systems. It is difficult to summarize all this information, however some interesting studies and reviews are commented below. Qiu et al. (2013) studied the kinetics and equilibrium adsorption of three polyphenols (procyanidins, epicatechins and catechins) from terrestrial plants on starchy matrixes.

Diez–Sánchez et al. (2021) studied the interactions between polyphenols from blackcurrant pomace and the main macronutrients in foods, and the changes that occurred during *in vitro* digestion, using a model that combined systems with water, wheat starch, olive oil, and/or whey protein. Wang et al. (2021) studied the effects of mixing orders of tannic acid, starch, and on the α–amylase enzyme inhibition. Zhu (2017) reviewed the functional ingredients encapsulation onto various materials for controlling their release during food digestions. Deladino, et al. (2015) evaluated corn starch's ability to bind and carry a yerba mate polyphenolic enriched extract.

Nevertheless, very few publications report the effect of addition seaweed and/or their nutraceuticals among starchy based foodstuffs. Parada et al. (2019) reviewed aspects of a functional design of seaweed enriched food systems for glycemic control. Mamat et al. (2014) reported the effect of *Kappaphycus alvarezii* seaweed addition on dough rheological properties

and the quality of bread. Allsopp et al. (2016) investigated the effect of consuming bread with red seaweed *Palmaria palmata* incorporated. Menezes et al. (2015) assessed the effect on nutrient composition, caloric value, technological and sensory evaluation of the addition of *Cladophora spp.* and *Ulva spp.* green seaweeds breads. Hall et al. (2012) investigated energy intake and nutrient absorption of *A. nodosum* seaweed enriched breads. Rózylo et al. (2017) assessed the effect of this brown algae addition on the physical, antioxidant and appealing properties of bread. Considering the current state of art of the implementation of algal nutraceuticals, considering their beneficial heal properties, it is expected that the extension of these studies will be higher in the coming years.

1.7.3. PHLOROTANNINS AS ENZYME INHIBITORS

The available literature on the use of brown seaweed extracts or isolated phlorotannin fractions, regarding their ability to suppress carbohydrate digestion has been explored for the last two decades. α–amylase and α–glucosidase are two key enzymes in carbohydrate digestion (Lebovitz, 1997), then the inhibitory capacities of polyphenols against these digestive enzymes is being studied. However, it is difficult to handle, since several methodologies, reagents and their origin of the assayed materials are used. A summary of published studies of polyphenols obtained from *A. nodosum* (Table 1.5) other seaweeds (Table 1.6) and terrestrial plants (Table 1.7) are proposed, where experimental conditions used to analyze the *in vitro* inhibition against α–amylase and α–glucosidases are showed.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 1.5. State of art of inhibitory capacities against α–amylase and α–glucosidase digestive enzymes of polyphenols from *A. nodosum* seaweed.

Reference	Extraction conditions (Method, repetitions LS, t, Sol, T)	Inhibitor	Substrate	Enzyme	Reaction conditions	IC_{50}
Yuan et al. (2018)	SLE x3 10 g_{sol}/g_{algae} 4 h Methanol (70% v/v) rt	Raw extract	Potato starch (1% w/w)	α–amylase (1 U/mL)	t = 10 min T = 37 ºC	0.052 mg/mL
			p–NPG (6 mM)	α–glucosidase (0.2 mg/mL)	t = 10 min T = 37 ºC	0.52 mg/mL
Liu et al. (2016b)	SLE 5 g_{sol}/g_{algae} overnight Chloroform rt	Raw extracts	p–NPG (10 mM)	α–glucosidase (2 U/mL)	t = 10 min T = 28 ºC	194.2 µg/mL
	SLE 5 g_{sol}/g_{algae} overnight EtOH rt					36.3 µg/mL
	SLE 5 g_{sol}/g_{algae} overnight Acetone (70% v/v)rt					13.0 µg/mL
Lordan et al. (2013)	SLE (x3) 40 g_{sol}/g_{algae} 3 h Water 25 ºC	Raw extract (10 mg/mL)	Wheat starch (1% w/v)	α–amylase (NS)	t = 10 min T = 25 ºC	*10%
			p–NPG (5 mM)	α–glucosidase (0.1U/mL)	t = 5 min T = 37 ºC	*8%
Nwosu et al (2011)	SLE (x3) 10 g_{sol}/g_{algae} overnight MetOH NS	Raw extract	Potato starch (1% w/v)	α–amylase (0.4 mg/mL)	t = 2h T = 37ºC	0.1 µg/mL
			p–NPG (2 mM)	α–glucosidase (10 mg/mL)	t = 2h T = 37ºC	19.0 µg/mL
Apostolidis & Lee, (2010)	SLE 20 g_{sol}/g_{algae} 30 min Water 80 ºC	Raw extract	Wheat starch (1% w/w)	α–amylase (13 U/mL)	t = 10 min T = 25 ºC	1.34 µg
			p–NPG (5 mM)	α–glucosidase (NS)	t = 20 min T = 37 ºC	0.24 µg
Roy et al (2011)	NS NS NS NS rt	Comercial raw extract	Corn starch (1% w/w)	α–amylase (0.2 U/mL)	t = 5 min T = 20 ºC	2.8 µg/mL
			p–NPG (NS)	α–glucosidase (NS)	t = 20 min T = 37 ºC	5.0 µg/mL
Zhang et al. (2007)	SLE 12.5 g_{sol}/g_{algae} 90 min EtOH (50% v/v) 80 ºC	Raw extract	p–NPG (0.7 mM)	α–glucosidase (0.1 mg/mL)	t = 15 min T = 37 ºC	38.0 µg/mL

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

where g_{algae}, is grams of algae; g_{sol}, is grams of solvent; IC_{50}, is concentration to achieve the 50% of enzyme inhibition; LS, is liquid–solid ratio; NS, is "non–specified" in the study; p–NPG, is p–nitrophenyl–a–D–glucopyranoside; rt, is room temperature; SLE, is solid–liquid extraction; Sol, is solvent type; t, is time of extraction; T, is temperature of extraction; *, results expressed in relation to the control (acarbose).

As it is shown in Table 1.5 there are a low number of publications that study the antidiabetic or anti–enzymatic capacities of *A. nodosum*. Surprisingly, all authors used conventional SLE to produce bioactive extracts despite other techniques are more efficient to extract bioactive compounds. The application of those techniques supposes a promising field of study to demonstrate that seaweeds are a good option to produce antidiabetic products when they are obtained by a more efficient and less-resource consuming extractive technique. Additionally, phlorotannins from other seaweeds were also studied whereas

Table 1.6 includes the capacities of some isolated polyphenolic compounds from seaweeds to inhibit carbohydrates digesting enzymes. Indeed, in the last decade, *Ecklonia spp.* and *Eisenia spp.* are, by far, the most widely explored.

Table 1.6. State of the art of inhibitory capacities against α–amylase and α–glucosidase digestive enzymes of polyphenols from alternative seaweeds.

Seaweed *spp.*	Extraction (Method, repetitions, LS, t, Sol and T)	Inhibitor	Substrate	Enzyme	Reaction conditions (t and T)	IC_{50}	Reference
AM FD SG SL PF UL	SLE x2 $10g_{sol}/g_{algae}$ 3 h MetOH (80% v/v) NS	Raw extracts (4 mg/mL)	Wheat starch 1% w/v	α–amylase NS	10 min 37 ºC	AM 6.4% FD 3.4% SG 76.1% SL 75.1% PF 6.6% UL 88.0%	Kellogg et al. (2014)
			p–NPG 1 mM	α–glucosidase 75 U/mg	5 min 30 ºC	AM 7.9% FD 18.4% SG 65.5% SL 56.3% PF 62.2% UL 82.4%	

continue.→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Seaweed *spp.*	Extraction (Method, repetitions, LS, t, Sol and T)	Inhibitor	Substrate	Enzyme	Reaction conditions (t and T)	IC_{50}	Reference
Ecklonia stolonifera	SLE x0 4g_{sol}/g_{algae} 30 min Water NS	Raw extract	Wheat starch 20 mg/mL	α–glucosidase 10 U/mL	5 min 37 ºC	0.026 mg/mL	Iwai (2008)
Ecklonia cava	SLE x3 0.5g_{sol}/g_{algae} 10 days MetOH rt	Fucodiphloroethol G Dieckol 6,6'–Bieckol 7–Phloroeckol Phlorofucofuroeckol A	p–NPG 3 mM	α–amylase 1 U/mL	5 min 37.5 ºC	> 0.5 mM 0.1 mM > 0.5 mM 0.3 mM > 0.5 mM	Lee et al (2009)
				α–glucosidase 2 U/mL	20 min 37.5 ºC	19.5 µM 10.8 µM 22.2 µM 49.5 µM 19.7 µM	
Ecklonia cava	SLE x3 NS 10 days MetOH 80% v/v rt	Dieckol	PNPG7 3 mM	α–amylase 0.7 U	5 min 37.5 ºC	0.66 mM	Lee et al. (2010)
				α–glucosidase 1 U/mL		0.24 mM	
Ecklonia cava	SLE x3 50g_{sol}/g_{algae} 3 h EtOH 70% v/v rt	Phlorofucofuroeckol A	PNPG7 5 mM	α–amylase 100 U	t5 min rt	6.3 µM	You et al. (2015)
			p–NPG 5 mM	α–glucosidase 0.7 U		19.5 µM	
Ecklonia stolonifera *Eisenia bicyclis*	SLE x3 20 g_{sol}/g_{algae} 1 h MetOH 40 ºC	Phloroglucinol	p–NPG 2.5 mM	α–glucosidase 0.2 U/mL	15 min 37 ºC	141.2 µM	Moon et al (2011)
		Dioxinodehydroeckol				34.6 µM	
		Eckol				11.8 µM	
		Phlorofucofuroecko–A				1.4 µM	
		Dieckol				1.6 µM	
		7–Phloroeckol				6.1 µM	

continue→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Seaweed *spp.*	Extraction (Method, repetitions, LS, t, Sol and T)	Inhibitor	Substrate	Enzyme	Reaction conditions (t and T)	IC_{50}	Reference
Eisenia bicyclis	SLE x3 2 g_{sol}/g_{algae} 3 h MetOH rt	Compound 1 (1 mM)	Wheat starch 1% w/v	α–amylase NS	15 min 37 ºC	96.2%	Okada et al. (2004)
		Compound 2 (1 mM)				86.7%	
		Compound 3 (1 mM)				76.0%	
Eisenia bicyclis	SLE x3 40 g_{sol}/g_{algae} 3 h EtOH 25 ºC	Methanolic extract n–Hexane fraction Dichloromethane fraction Ethyl acetate fraction n–Butanol fraction Water fraction Fucofuroeckol A Dioxinodehydroeckol	Potato starch 1 % w/v	α–amylase 0.4 mg/mL	2 h 37.5 ºC	0.5 µg/mL 3.5 µg/mL 0.3 µg/mL 48.1 ng/mL 0.2 µg/mL 1.9 µM 51.6 nM 93.3 nM	Eom et al. (2012)
			p–NPG 3 mM	α–glucosidase 0.4 mg/mL	20 min 37.5 ºC	>500 µg/mL >500 µg/mL 39.98 g/mL 2.9 µg/mL 4.6 µg/mL >500 µg/mL 42.9 µM 0.47 mM	
Ishige foliacea	SLE x3 40 g_{sol}/g_{algae} 3 h MetOH 80% v/v 25 ºC	Octaphlorethol A	p–NPG 5 mM	α–glucosidase 0.7 U/mL	5min rt	0.11 mM	Lee et al. (2014)

continue→

Seaweed *spp.*	Extraction (Method, repetitions, LS, t, Sol and T)	Inhibitor	Substrate	Enzyme	Reaction conditions (t and T)	IC_{50}	Reference
Ishige okamurae	SLE NS NS MetOH 80% v/v rt	Diphlorethohydroxycarmalol	p–NPG 5 mM	α–amylase 0.7 U	5 min rt	0.53 mM	Heo et al (2009)
				α–glucosidase 0.7 U/mL		0.16 mM	
Sargassum ringgoldianum	SLE x3 NS NS MetOH 80% v/v NS	Raw extract	PNPG7 5 mM	α–amylase 100 U	5 min rt	0.18 mg/dL	Lee & Han (2012)
			p–NPG 5 mM	α–glucosidase 0.7 U		0.12 mg/dL	
			p–NPG 5 mM	α–glucosidase NS	20 min 37 ºC	0.24 µg	
SF SA SS SP SD	SLE x3 40 g_{sol}/g_{algae} 30 min EtAc 90 ºC	Raw extracts	Wheat starch 1% w/v)	α–amylase 13 U/mL	10 min 25 ºC	SF 27.0% SA 55.0% SS 30.0% SP 42.0% SD 36.0%	Firdaus & Prihanto (2014)
			p–NPG 5 mM	α–glucosidase 0.7 U	5 min 25 ºC	SF 37.0% SA 65.0% SS 40.0% SP 52.0% SD 46.0%	
Triticum aestivum	SLE x2 40 g_{sol}/g_{algae} 1 h EtOH 25 ºC	(2–(4–(3,5–dihydroxyphenoxy)–3,5–dihydroxyphenoxy) benzene–1,3,5–triol)	Amylopectin 1% w/v	α–amylase 1 µM	5 min 37 ºC	3.2 µg/mL	Kawamura–Konishi et al. (2012)
			Maltose 100 mM	α–glucosidase 1 µM	30 min 37 ºC	114.0 µg/mL	
			Sucrose 100 mM			25.4 µg/mL	

where, AM, is *Alaria marginata* seaweed; EtOH, is ethanol; EtAc, is ethyl acetate, FD, is *Fucus distichus* seaweed; g_{algae}, is grams of algae; g_{sol}, is grams of solvent; LS, is liquid–solid ratio; MetOH, is methanol; NS, is "non–specified" in the study; PF, is *Pyropia fallax* seaweed; p–NPG is p–nitrophenyl–a–D–glucopyranoside; PNPG7 is p–nitrophenyl maltoheptaoside; SA, is *Sargasum aquifolium* seaweed; SD, is *Sargasum duplicatum* seaweed; SF, is *Sargasum filipendula* seaweed; SG, is *Saccharina groenlandica* seaweed; SL, is *Saccharina latissima* seaweed; SLE, is solid–liquid extraction; Sol, is solvent type; SP, is *Sargasum polycystum* seaweed; SS, is *Sargasum siliquosum* seaweed; t, is time; T, is temperature; UL, is *Ulva lactuta* seaweed; x0, is process not repeated; x2, is process repeated twice; x3, is process repeated three times.

The isolation of polyphenols from brown seaweeds has been scarcely studied until last decade, perhaps due to it is a time and cost consuming process. Then available literature of phlorotannins as digestive enzyme inhibitors is scarce, furthermore the purification and isolation of phlorotannins is another aspect with very few bibliographic sources. Additional studies where polyphenols from terrestrial plant origin (Table 1.7) isolated and identified, have been added to complement this section of polyphenols as enzyme inhibitors.

Table 1.7 State of art of inhibitory capacities against α–amylase and α–glucosidase digestive enzymes of polyphenols from terrestrial plants.

Polyphenol	**Substrate**	**Enzyme**	**Reaction conditions (t and T)**	**IC_{50}**	**Reference**
3–O–galloylepicatechin	Wheat starch NS	α–amylase NS	10 min 37 ºC	739.0 µM	Bhandari et al. (2008)
3–O–galloylcatechin				401.0 µM	
Apigenin	PNPG7 NS	α–amylase 0.03 mg/mL	10 min 37 ºC	>0.5 mM	Lo Piparo et al. (2008)
Luteolin	Potato starch NS	α–amylase 0.2 µM	10 min rt	18.4 µM	
Apigenin	p–NPG NS	α–glucosidase NS	5 min 37 ºC	82.0 µM	Proenca et al. (2017)
Apigenin	p–NPG 0.6 mM	α–glucosidase 0.05 U/mL	15 min 37 ºC	21.9 µM	Li et al. (2009b)
Luteolin				17.0 µM	
Caffeic acid	Wheat starch 0.25 mg/mL	α–amylase 5.0 mg/mL	10 min 25 ºC	3.7 µM	Oboh et al. (2015 a and b)
Theaflavin	Wheat starch 3.3 mg/mL	α–amylase 0.2 mg/mL	5 min 25 ºC	0.4 mg/mL	
Rutin				0.04 µM	
Quercetin	p–NPG 3.8 mM	α–glucosidase 0.2 U/mL		0.04 µM	
Caffeic acid	Wheat starch 2.5 mg/mL	α–amylase 7.5 U/mL	3 min 25 ºC	6.7 mM	Tan et al. (2017)
	p–NPG 2.0 mM	α–glucosidase 0.1 U/mL	10 min 37 ºC	21202 µM	

continue→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Polyphenol	Substrate	Enzyme	Reaction conditions (t and T)	IC_{50}	Reference
Chlorogenic acid	p–NPG 2 mM	α–glucosidase 0.2 U/mL	10 min 37 ºC	4 mg/mL	Venditti et al. (2015)
Apigenin				0.1 mg/mL	
Luteolin				0.3 4 mg/mL	
Cyanidin–3 glucoside	Corn starch 0.1 mg/mL	α–amylase 0.2 mg/mL	15 min 37 ºC	0.02 mM	Sui et al. (2016)
(+)–catechin		α–amylase 0.04 U/mL	NS 37 ºC	9.3 mg/mL	
Cyanidin–3 glucoside	Wheat starch 1.0 mg/mL	α–amylase 3.0 U/mL	10 min NS	0.3 mM	Akkara-chiyasit et al. (2010)
Cyanidin–3.5 diglucoside				> 1.0 mM	
Daidzein	Wheat starch 1.0 mg/mL	α–amylase 0.1 U/mL	10 min 37 ºC	> 400.0 µM	Zhang et al. (2017)
Genistein				> 400.0 µM	
Luteolin				>400.0 µM	
Epicatechin–3–gallate	Rice starch 16.0 mg/mL	α–amylase NS	12 min 37 ºC	1.5 mM	Koh et al. (2010)
	p–NPG 2.0 mM	α–glucosidase 0.6 mg/mL		1.4 mM	
Ferulic acid	Potato starch 0.2 mg/mL	α–amylase NS	20 min 37 ºC	0.6 mM	Zheng et al. (2020)
	p–NPG 0.18 mM	α–glucosidase 0.2 mg/mL		4.5 mM	
Gallic acid	Wheat starch 5.0 mg/mL	α–amylase 0.3 mg/mL	10 min 25 ºC	1.2 µM	Adefegha et al. (2015)
	p–NPG 1.3 mM	α–glucosidase 0.5 U/mL		7.2 µM	
Gallic acid	p–NPG 1.3 mM	α–glucosidase 0.5 U/mL	5 min 25 ºC	0.04 µM	Oboh et al. (2016)
Gallic acid	Wheat starch NS	α–amylase NS	3 min NS	57.0 mM	Cirillo et al. (2010)
Genistein	p–NPG NS	α–glucosidase NS	30 min 37 ºC	50 µM	Lee & Lee (2001)
Hesperidin	Potato starch 5.0 mg/mL	α–amylase 5.0 U/mL	t30 min 37 ºC	6 µM	Sahnoun et al. (2017)
Naringin	p–NPG NS	α–glucosidase 0.04 U/mL		0.55 µM	
Luteolin	PNPG7 NS	α–amylase 0.03 mg/mL	10 min 37 ºC	0.4 mM	Tadera et al. (2006)
Quercetin	p–NPG 1.3 mM	α–glucosidase 5 µg/mL	5 min 30 ºC	7 µM	

continue→

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Polyphenol	Substrate	Enzyme	Reaction conditions (t and T)	IC_{50}	Reference
Luteolin	p–NPG 0.5 mM	α–glucosidase 1.0 mM	NS 37 ºC	172.0 µM	Yan et al. (2014)
Quercetin	p–NPG 4.2 mM	α–glucosidase NS	5 min 37 ºC	0.1 µM	De Souza et al. (2010)
Theaflavin				67.0 µM	
Quercetin	Potato starch 2.5 mg/mL	α–amylase 1.0 U/mL	3 min 25 ºC	0.9 µM	
Rutin				1.2 µM	
Theaflavin	p–NPG NS	α–glucosidase NS	15 min 37 ºC	54 µM	Jeon et al. (2013)

where, NS, is "non–specified" in the study p–NPG is p–nitrophenyl–α–D–glucopyranoside; PNPG7 is p–nitrophenyl maltoheptaoside; t, is reaction time; T, is reaction temperature.

To optimize and expand the use of algae and their bioproducts will be the next step to explore innovative applications in food, medical, pharmaceutical, and/or cosmetic industries. The state of art previously showed in this Thesis about extraction methods (Section 1.7.1) and the use of seaweed biopolymers as food additives in starchy–based systems (Section 1.7.2) and as digestive enzyme regulators (Section 1.7.3), evidence that seaweeds are potentially an extensive resource in the future.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

2. OBJECTIVES & HYPOTHESIS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The general objective of this Thesis is the extraction of biopolymers with high antioxidant and anti–enzymatic activities from Galician native brown seaweeds with great local socioeconomic interest. These biopolymers can be included in food formulations to improve their functional properties. Specifically, *Ascophyllum nodosum* (*A. nodosum*) was selected as a valuable and suitable source of phlorotannins (antioxidant and digestive enzyme inhibitor agent) and alginates (thickener and gelling agent) as replacements of current synthetic compounds. Antioxidant and enzyme inhibitor additives may be active controllers for the management of glycemic control over time, for the alleviation and prevention of diabetes, particularly critical in celiac people. To achieve this general objective several specific objectives were established:

1. Reviewing in–depth bibliography on the characterization, operational conditions, ranges of interest for key variables, food application, among others, for the biopolymers extracted along the Thesis (Sections 1 up to 12).

2. Set–up and management of experimental equipment and techniques employed for different operations such as drying, milling, sieving, compositional analysisis, *in vitro* enzymatic, chromatography, spectroscopic and image methods (Sections 6 up to 12).

3. Physicochemical characterization of dried *A. nodosum* flour (Section 6).

4. Comparison of conventional solid–liquid (SLE) and novel ultrasound–assisted extraction (UAE) that were discussed in Section 7.

5. Experimental determination and modelling of polyphenol extraction kinetics from *A. nodosum* seaweed by means of different procedures to establish adequate extraction conditions and, on the other hand, to measure the ionic strength effect by using and salty water on aqueous extracts bioactivity (Sections 7, 8 and 9)

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

6 To determine the optimal conditions of aqueous UAE of phlorotannins following a Box-Behnken experimental design to evaluate the effects of sonication power, sonication time and the liquid-solid ratio (Section 8).

7 Characterization and purification of phlorotannins in extracts of *A. nodosum* for the isolation of the most bioactive (antioxidant and enzyme-inhibiting) fractions (Sections 9 and 11).

8 To obtain phlorotannin–enriched extracts from *A. nodosum* UAE extracts using methods based on both size (using different cut-off dialysis membranes) and oxidation state to assess the resistance to oxidation, the bioactivity distribution and the identification of some phlorotannins (Section 9).

9 To study the interactions between *A. nodosum* bioactive phlorotannins and corn starch (employed in gluten–free and non–gluten–free food formulations) by several blending methods and proportions (Section 10).

10 To analyze and identify the *in vitro* inhibitory capacities of phlorotannin–enriched extracts from *A. nodosum* using UAE against digestive enzymes (α–amylase and α–glucosidase) by several blending methods (Section 11).

11 Integral valorization of brown seaweeds, by means of the use the solid residue, after the UAE of phlorotannins from *A. nodosum* seaweed, that still contains other valuable compounds, such as alginate (Section 12).

12 To determine the effect of the process conditions, specifically for drying operation, on physicochemical properties of alginates by means of spectroscopic and viscometry capillary techniques (Section 12)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3. THEORETICAL FUNDAMENTALS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3.1. EXTRACTION

3.1.1. GENERALITIES

The bioactivities and characteristics of phytochemical compounds are influenced by extraction methods and their conditions. Nowadays, extraction technologies and strategies are a prolific researching field that pretends to determine the optimal extraction conditions (Ummat et al., 2020). An insight into nutraceuticals extraction would help to obtain adequately highly bioactive compounds and even could be useful to obtain phlorotannins or other seaweed compounds with tunable featuring and characteristics depending on their pretended use. According to the consulted literature aspects such as seaweed species and extraction conditions (operation mode, solvent, method, solid–liquid ratio, time, temperature and pre– and prost–treatments) are crucial in polyphenols/phlorotannins extraction (Kim et al., 2013; Leyton et al., 2016; Li et al., 2017; Mekinic et al., 2019).

Operation mode selection is a key aspect in nutraceuticals industrial production. Batch extractions require interruptions for charging and discharging and high amounts of solvents. These problems are reduced with semi–continuous extraction that consists in several batch extractors operated in series. Continuous extraction, solute and extractant are continuously charged and discharged (Eaggers & Jaeger 2003).

The solvent used for the extraction is of major importance to enhance process yield and to minimize coextraction of undesirable substances (Koivikko et al., 2005; Oreopoulou, 2003). The solubility of seaweed nutraceuticals is influenced by the type of extractant, degree of polymerization and their interactions with other food constituents (Shahidi & Naczk, 2014; Catarino et al., 2019). Indeed, extraction of phlorotannins is a challenge since they are embedded deeply among the seaweed components forming complexes (Soria & Villamiel, 2010; Wang et al., 2016). For the extraction of antioxidants from plants and seaweeds organic solvents are largely recommended (Koivikko et al., 2007; Singh & Sidana, 2013; Júnior et al., 2015; Lopes et al., 2017; Sathy et al., 2017; Hermund et al., 2018; Zhang et al. 2018b; Mekinic et al., 2019). Nevertheless, these solvents are volatile, inflammable, and toxic (Chemat & Vian,

2014; Tavakoli et al., 2021;). Thus, water is being proposed as an efficient and green–label solvent for polyphenols extraction from seaweed (Machu et al., 2015; Leyton et al., 2016;).

There are several extraction methods (Figure 3.1) developed, studied and applied for bioactive compound extraction from algal material in order to achieve new, safe, effective and affordable extraction technologies with minimum presence of residues that also considers health concern aspects and environmental issues (Tiwari, 2015; Mekinic et al., 2019; Kumar et al., 2022;).

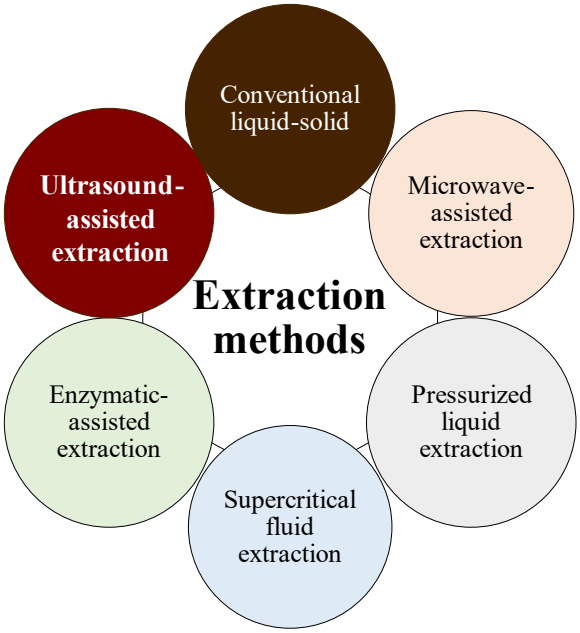


Figure 3.1. Phlorotannins extraction methods.

Recently, several greener and cleaner extraction technologies have being studied (Tierney et al., 2013a). Pressurized liquid extraction utilizes high pressures (up to 15 MPa) and temperatures (until 200 ºC) together with low extractant volumes and short extraction times. In microwave–assisted extraction, electromagnetic waves induce and facilitate the compounds extraction. However, these methods promote the partial degradation of thermolabile compounds. Supercritical fluid extraction uses fluids with temperature and pressure above their critical point to increase mass transfer lowering viscosity to increase internal diffusion (Chemat & Vian, 2014). It is rarely used since each extractant must be optimally selected for each solute, especially for the extraction of phenolics, due to its dependency of compound chemical characteristics. Enzyme–assisted extraction use enzymes to disrupt the cell wall during the extraction however it depends on the algal biochemical composition (Marathe et al., 2017).

In the current Thesis, two main extraction methods were studied, the novel and ecofriendly ultrasound–assisted extraction (UAE) and the conventional solid–liquid extraction (SLE). SLE, also named leaching, is a complex process due to is a multicomponent, multiphase and un–steady state mass transfer operation. Overall, SLE process (Figure 3.2) is composed of several coetaneous processes (Aguilera 2003; Lee et al., 2006; Moreira et al 2008; Chenlo et al., 2018;):

A. Solvent entrance into the solid matrix.

B. Solubilization/breakdown of components.

C. Solute transport of the to the solid matrix surface.

D. Extracted solute migration from the solid surface through solvent bulk.

E. Movement of the extract with respect to the solid.

F. Separation and discharge of the extract and the solid.

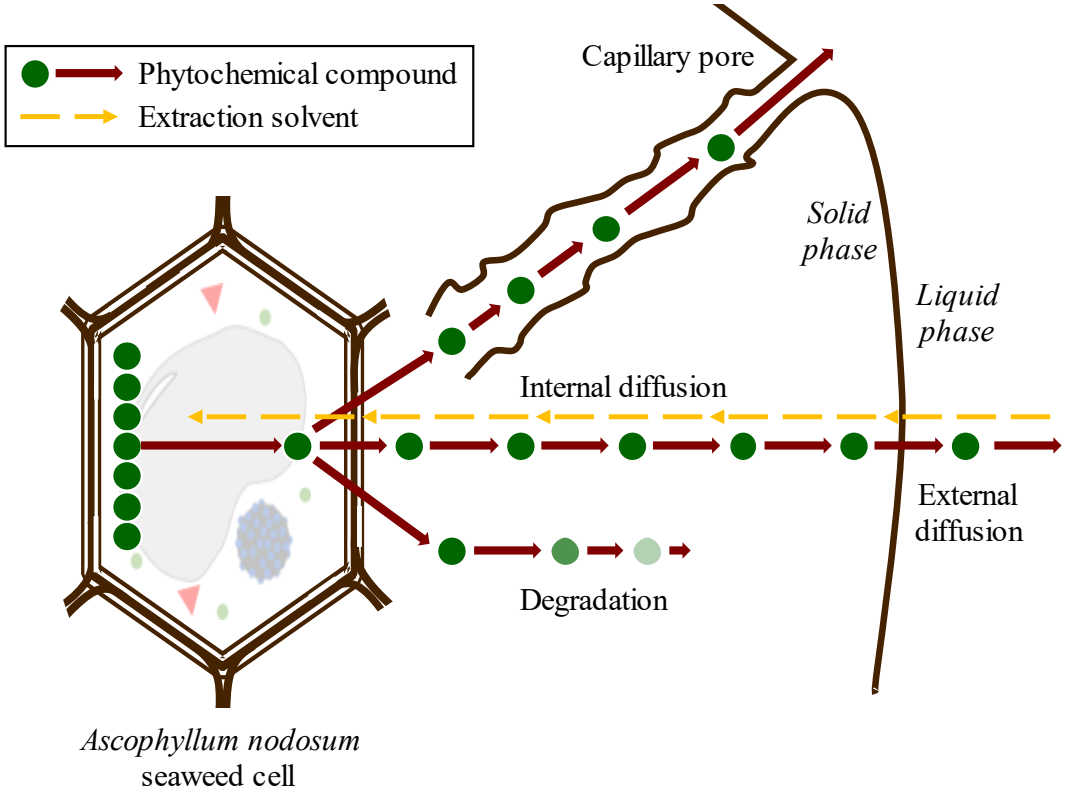


Figure 3.2. Scheme of the coetaneous processes in SLE of compounds from seaweed cell.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

SLE is a widespread extraction method used in food industry to extract sucrose, lipids, proteins, phenolic compounds and hydrocolloids among others (Chemat & Vian 2014). In this process, the main transport mechanism is the molecular diffusion, where compounds are transported by a concentration gradient in the solid phase (Tzia, 2003). The intricate microstructure of solids plays a major role in the rate and the quality of the extraction process. In addition, SLE, as a multicomponent extraction, is also affected by the relative rate at which different compounds migrate through the solid. The above–mentioned elementary steps (Figure 3.2) occur at their characteristic rate and in some cases, sequentially.

Compound transport through the solid matrix is usually the rate–controlling step resulting in an energy– and time–consumption process that require potentially high amounts of solvents to produce acceptable extraction yields (Koivikko et al 2007; Sánchez–Camargo et al., 2016; Wang et al., 2016; Ummat et al., 2020;). Extraction of bioactive compounds at a commercial scale requires high extraction yields and the conservation of their bioactivities, which are difficult to achieve using conventional SLE method (Bordoloi & Goosen, 2019).

3.1.2. ULTRASOUND–ASSISTED EXTRACTION

Ultrasound–assisted extraction (UAE) utilizes soundwaves to disintegrate cell structure (Kadam et al 2015b; Topuz et al., 2016). The use of ultrasound technology is widely extended in the food industry (Mekinic et al., 2019), since it can be used either as a pre–treatment, can be combined with different type of solvents (Tierney et al., 2013a; Flores–Fernandez & Gonzalez Munoz, 2017) and with other methods. UAE can be used to reduce or remove the use of toxic chemicals, decrease levels of solvent consumption, has minimal impact on the stability of the target compounds (Oh et al., 2011; Dang et al. (2017a), reduces time and operational costs (Tiwari, 2015), does not require high temperatures (Dang et al., 2017a) and globally ensures better extractabilities increasing process efficiency and extraction yields (Duarte et al., 2014; Chemat et al., 2017).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Ultrasound comprises mechanical waves that are above human hearing, ranged from 20 kHz up to 10 MHz (Picó, 2013). Ultrasound involves various phenomena including shear forces, compression pressure gradients, agitation, rarefaction, vibration, microjets, radical formation and cavitation (Tiwari, 2015; Rodrigues et al. 2015), promoting cell walls disruption and the subsequent release of compounds (Hahn et al., 2012; Agregán et al., 2018;). Cavitation is the main driving force for ultrasound extraction: it is a hydrodynamic effect that occurs when vapor cavities are created within liquid and different pressure forces are present (Picó, 2013). These processes produce expansion and implosive collapse of micro bubbles, formed due to a series of compressions and rarefactions in molecules generated by ultrasound waves improving heat and mass transfers along the system improving solvent penetration and cell walls breaking (Tiwari, 2015). The UAE method improves solvent penetration and cell walls disruption, increasing the extraction yields respect to conventional extraction methods (Soria & Villamiel, 2010; Roselló–Soto et al., 2015;). Figure 3.3 shows the general scheme of ultrasound-assisted extraction from seaweed tissues.

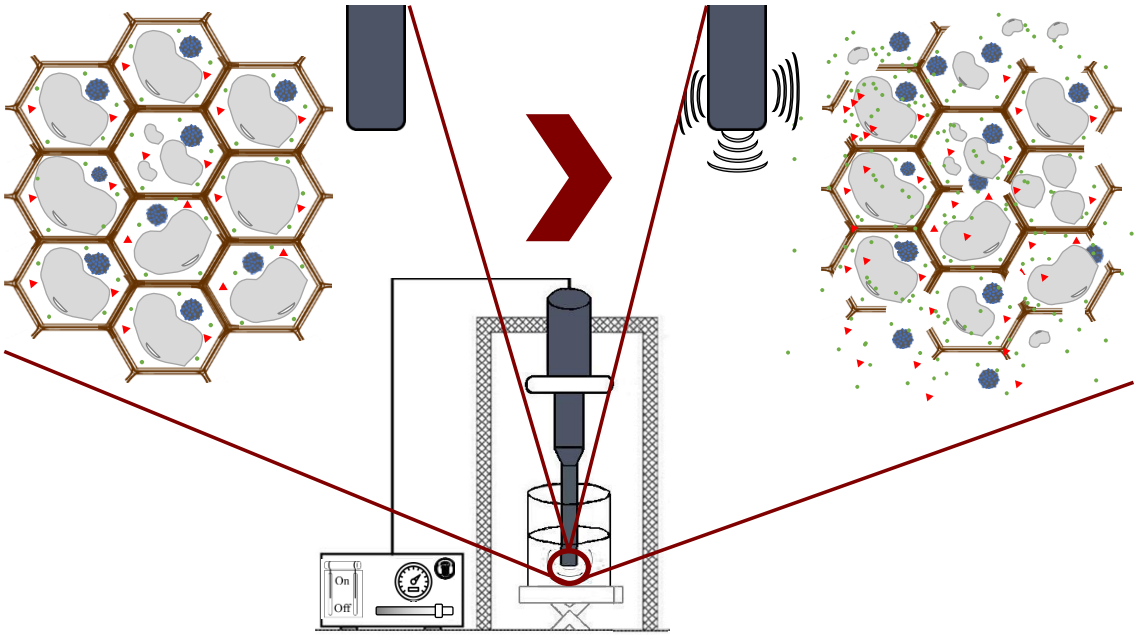


Figure 3.3. Ultrasound–assisted extraction process.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

UAE is a simple, cost–effective and efficient replacement for traditional technologies largely employed to extract bioactive compounds (Section 1.7.1). Indeed, UAE extraction technology is being employed in both laboratory and large–scale industrial applications (Kadam et al., 2015c) such as emulsification, homogenization, extraction, crystallization, dewatering, low–temperature pasteurization, degassing, defoaming, activation and inactivation of enzymes, among others (Cikoš et al., 2018).

3.1.3. FRACTIONATION–ISOLATION OF SEAWEED EXTRACTS

During the extraction of phenolic compounds, other molecules are co–extracted, mainly polysaccharides and proteins. The study of phenolic compounds from seaweeds makes mandatory their separation and purification for the fractionation and/or isolation of the compounds to reduce extracts complexities. Briefly, fractionation of the extracts consists in separation based on molecular weights, charges, chemical affinities and/or solubilities (Houchi et al., 2019; Vissers et al., 2017).

Adsorption–based separations are emerging due to their simplicity and the potential for scale–up and higher specificity compared to other primary fractionation techniques (Soto, et al., 2011). Separation is achieved by contact of seaweed extracts with a solid matrix with different affinities for phlorotannins and the remaining compounds. Then, phlorotannins can be recovered by separating the solid and liquid phases (McInnes, et al., 1985).

Solid phase extraction is other adsorption–based technique, where sorbent is immobilized in a cartridge or column, allowing the sequential elution of compounds with gradient of solvents (Santos et al., 2019). Liquid–liquid extraction is a solubility–based separation where the broad range of polarities of phenolic compounds prone partition of the distinct compound to obtain polarity–segregated fractions enriched of phlorotannins (Santos et al., 2019). Other separations that could be performed are ultrafiltration and/or molecular–weight cut–off dialysis. Ultrafiltration and dialysis are techniques that separate compounds by their molecular weight with minor instrumental requirements, time, expertise, indeed both techniques allow separations of discrete fractions over a wide range of molecular weights with few combinations of membranes/filters (Jégou, et al., 2015; Lin, et al., 2018).

USC UNIVERSIDADE DE SANTIAGO

3.2. CHARACTERIZATION OF SEAWEED BIOPOLYMERS

The objective of this section was to summarize the set of techniques currently available for the characterization of extracts and biopolymers extracted from seaweeds and the information provided by each technique. Seaweeds extracts are composed by a large, diverse, and complex mixtures of compounds, whereas there are phlorotannins together with polysaccharides, proteins, and other metabolites. Chemical characterization of crude and purified compounds from seaweeds is essential to understand their bioactivities and beneficial health properties. The study of these compounds is relevant food engineering, medicine, and pharmacy areas (Munteanu & Apetrei, 2021). Nevertheless, the isolation is often difficult due to their diverse molecular weights, structural similarities and their rapid reactivities (Parys et al., 2009; Tanninou et al., 2014; Vissers et al., 2017).

Initially, raw characterization is commonly carried out by spectrophotometric assays that are simple, cheap, rapid, and have a feasible comparison with other studies (Pulido et al., 2000; Munteanu & Apetrei, 2021;). Nevertheless, nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FT–IR), Matrix–assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOF–MS) and chromatographic (HPLC and/or UPLC) have been reported as more reliable techniques for seaweed extracts characterization (Santos et al., 2019). These techniques allow the qualitative and quantitative estimation of extracts composition. Furthermore, it is largely recommended that antioxidant activities must be tested by more than one method (Moharram & Youssed, 2014). However, the number of parameters considered in each determination hinders the comparison of results, as it was also mentioned in Section 1.7.1 for phlorotannins extractions.

The current section provides a comprehensive overview of characterization techniques suitable to be applied on seaweed extracts and their isolated compounds. Some widespread techniques, also used in this Thesis, will be explained to give general background of their mechanisms, procedures and data obtained from each one (Figure 3.4). This knowledge of analytical methods available will improve the understanding of seaweed bioactive compounds and their potential uses (Sun et al., 2022).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

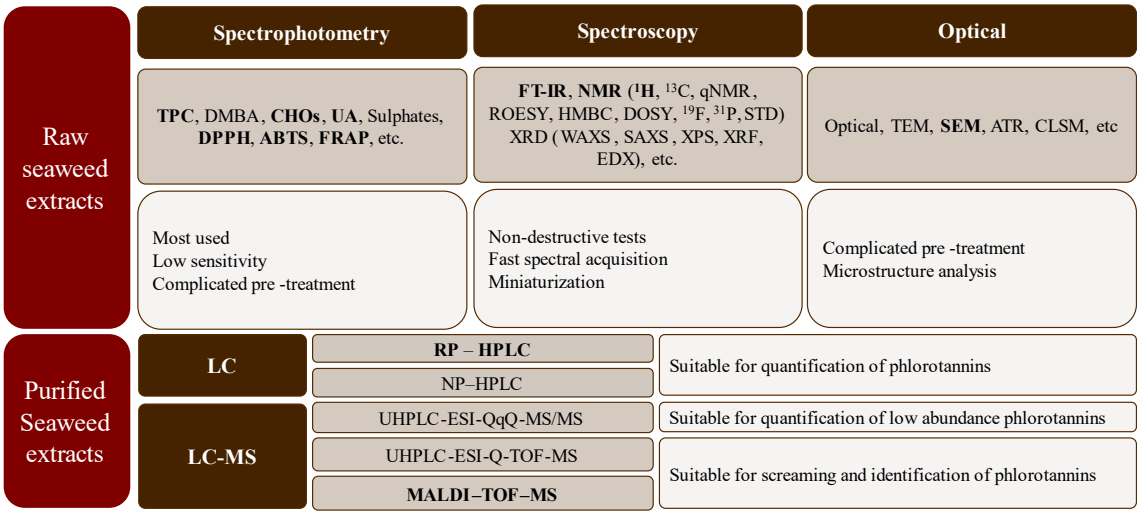


Figure 3.4. Summary of analytical methods often used for characterization of seaweed biopolymers whereas bold techniques were applied in this Thesis.

3.2.1. ANTIOXIDANT ACTIVITY DETERMINATIONS

This section provides the background of the methods to understand the reaction mechanisms of antioxidants, together with the advantages and limitations of the different assaying tests. Spectrophotometric methods rely on the linear relationship between absorbance and concentration whereas the appearance of colors or the discoloration of the solutions at specific wavelength is measured (Ford et al., 2019). Current available methods could be divided based on transfer of hydrogen atoms ([H^+]), electron ([e^-]) and/or mixture of both (Figure 3.5) such as: Hydroxyl Radical Antioxidant Capacity (HORAC), Total Peroxyl Radical Trapping Antioxidant Parameter (TRAP), Total Oxyradical Scavenging Capacity (TOSC), Oxygen Radical Absorption Capacity (ORAC), 2,20–AzinoBis–(3–Thylbenzothiazoline–6–Sulfonic) acid (ABTS), 2,2–Di(4–tert–octylPhenyl)–1–PicrylHydrazyl) (DPPH), Cupric Reducing Antioxidant Power (CUPRAC), Ferric Reducing Antioxidant Power (FRAP) and finally the total polyphenol content (TPC) determinations (Prior et al., 2005; Everette et al., 2010; Apak, et al., 2018; Munteanu & Apetrei, 2021).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

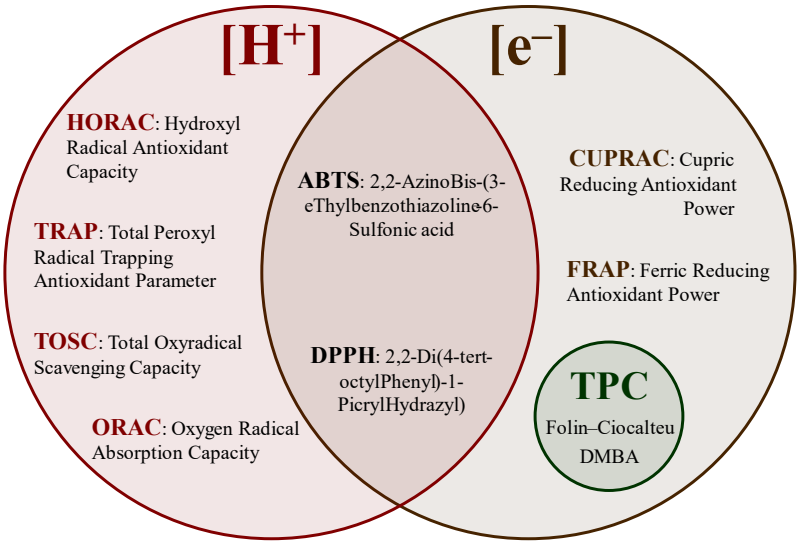


Figure 3.5. Antioxidant activity determination methods.

3.2.1.1. OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC)

ORAC test is used to determine the scavenging activity through the inhibition of the oxidation of peroxyl radicals. Peroxyl radicals, seen in Section 1.5, are free radicals that predominate in lipid oxidation in biological systems (Apak, et al., 2018). This test is based in the emission of peroxyl radicals by a generator (e.g., azo–compounds) that reacts with a fluorescent (e.g., fluorescein) sample that leads to loss of fluorescence due to the antioxidant effect (Becker et al., 2013).

3.2.1.2. HYDROXYL RADICAL ABSORBANCE CAPACITY (HORAC)

In the HORAC method it is measured the ability of antioxidants to avoid complexation reaction between hydroxyl radical complexation and cobalt ions (Co^{2+}). Fluorescein as fluorescence source, is incubated with the assayed antioxidant samples, then a generator of hydroxyl radicals is added (e.g., Fenton mixture). The decay of fluorescence provides a direct measurement of antioxidant capacity (Cíž et al., 2010; Munteanu & Apetrei, 2021).

USC
UNIVERSIDADE DE COMPOSTELA

3.2.1.3. TOTAL RADICAL-TRAPPING ANTIOXIDANT PARAMETER (TRAP)

TRAP method measures the assayed compound capacity to inhibit the reaction between peroxyl radicals and target molecules by means of the oxygen consumption during the peroxidation process of 2,20 azobis(2–amidinopropane) dihydrochloride. The elapsed time for the oxygen absorption (i.e., the induction time) is used to determine the total antioxidant capacity of the samples (Apak et al., 2018).

3.2.1.4. TOTAL OXYRADICAL SCAVENGING CAPACITY (TOSC)

The TOSC test is based on the inhibition of the formation of ethylene in presence of antioxidant compounds that compete with α–keto–γ–bethiolbutiric acid (KMBA) for oxidants. This test uses the relationship between area under the ethylene concentration curve (obtained by a gas chromatography) and the reaction time between KMBA and oxidants (Regoli & Winston, 1999).

3.2.1.5. CUPRIC REDUCING ANTIOXIDANT CAPACITY (CUPRAC)

The CUPRAC test determine the total antioxidant capacity based on the reduction of cupper ions (Cu^{2+} and Cu^{+}). Like molybdenum in the TPC method and iron in FRAP method (Bartosz, 2010) this method is based in a ligand reaction mechanism with the neocuproine (2,9–dimethyl–1,10–phenanthroline) whose color is determined at 450 nm and provides the antioxidant activity of the samples (Munteanu & Apetrei, 2021).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3.2.1.6. FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

The FRAP test is based on the reduction of iron (Fe^{3+} to Fe^{2+}) as ligand by effect of antioxidants. Antioxidant activity is determined as an increase color measured at 593 nm (Antolovich et al., 2002; Prior et al., 2005). FRAP analysis is used on a large scale, providing results for a variety of purposes, including the estimation of the antioxidant content in foods and their contribution to the supply of antioxidants, to investigate the effect of storage, growth, draught, solar radiation, processing, genetic modification in foods, medicines, traditional medicines, herbs, spices, teas and wines as a standardized quality and control test (Munteanu & Apetrei, 2021).

3.2.1.7. TOTAL POLYPHENOL CONTENT (TPC)

Total polyphenols content (TPC) test based on Folin–Ciocalteu method is the most common assay used with polyphenol (terrestrial and marine) enriched products (Parys et al., 2009; Tanninou et al., 2014; Vissers et al., 2017; Wootton et al., 2001). It was originally designed to analyze proteins, but it was later adopted by Singleton et al. (1999) to analyze the phenolic components in wine, after which it became a routine test for the antioxidant evaluation of food and plant extracts (Apak et al., 2018; Wright et al., 2001). TPC test detects the compounds suitable to transfer electrons from reductant compounds to molybdenum complexes of Folin-Ciocalteau reagent (Ainsworth & Gillespie, 2007; Everette et al., 2010) that promotes a color change, detected at 765 nm (Singleton & Rossi, 1965).

TPC value is determined with a calibration curve carried out with a polyphenol standard compound (e.g., gallic acid or phloroglucinol). The disadvantage of this method is the possible interferences gave by non–phenolic substances such as pigments, sugars, proteins, amines, organic acids, or inorganic substances (Blasco et al., 2005; Magalhaes et al., 2008). Additionally, there is a lack of standardized methodology in what concerns the volume, incubation time and concentration of extract and reagents (Heffernan et al., 2015). Although this method has some defects it is largely applied due to the economical and convenient experimental conditions (Ignat, et al., 2011; Sun et al.,2022).

USC UNIVERSIDADE DE COMPOSTELA

Alternative methods to Folin–Ciocalteu method, are the Prussian blue and 2,4–dimethoxybenzaldehyde (DMBA) assays. In Prussian blue assay phenolic compounds reduce $Fe(CN)_6^{3-}$ anion (Price & Butler, 1977), and in DMBA assay, reagent reacts with 1,3- and 1,3,5-trihydroxybenzenes. However, both methods lead to an underestimation polyphenols content (Parys et al., 2007; Castro-Alves & Cordenunsi 2015; Ford et al., 2019).

3.2.1.8. DPPH METHOD

$DPPH^{\bullet}$ (2,2–diphenyl-1-picrylhydrazyl) is a stable radical that is soluble in different organic solvents, but not in water. (Williams, 1966). The DPPH test is based on the neutralization of $DPPH^{\bullet}$ radical by the electrons donated by antioxidant assayed (Staško et al., 2007). This reaction produces a discoloration that measured at 515 nm acts as an indicator of the scavenging activity decay of the DPPH often reported as IC_{50}, (i.e., the efficient concentration of the antioxidant necessary to reduce the initial DPPH concentration to 50%) (Foti, 2015). The use of DPPH is widely reported to produce significant and comparable results making this method suitable to be used on several optimizing and/or standardizing protocols. Besides it is a low cost, easy, reproducible and operates at room temperature (Munteanu & Apetrei, 2021).

4.2.1.9. ABTS METHOD

ABTS test is based in reaction with 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+}$) stable radical with radical scavenging molecules, which capacity is expressed as Trolox™ Equivalent Antioxidant Content (TEAC). This test measures the antioxidant capacity to neutralize the $ABTS^{\bullet}$ radical that produces a discoloration measured at 734 nm (Miller et al., 1993). ABTS test can be used over a wide pH range (Lemanska et al., 2001) and it is soluble in both, water, and organic solvents, allowing the determination of antioxidant capacity of both hydrophilic and lipophilic compounds (Cano et al., 200; Walker & Everette, 2009).

It must be noted that all assays cited in this section depend on the reaction time (i.e., time contact between antioxidant and reaction mixture), intrinsic antioxidant activity, and sample concentration that often makes difficult the bibliographic comparisons.

3.2.2. CHROMATOGRAPHIC STUDIES

Liquid chromatography (LC) is the main method for the analysis of polyphenols (Sun et al., 2022). High performance liquid chromatography (HPLC) is wide used to identify the structure, linkages position, type, and size of phlorotannins (Ford et al., 2019), even purity can be evaluated with chromatographic techniques (Kerrison et al., 2015; Vissers et al., 2017). Normal phase (NP–HPLC) involves the use of a polar stationary phase, where compounds are separated based on their polarity, it leads a difficult elution of polar phlorotannins due to their high interaction with the stationary phase (Li et al., 2009a; Yoon et al., 2013;). Reverse phase HPLC (RP–HPLC) is largely used in phlorotannins analysis due to their better reproducibility and the lower retention times. RP–HPLC involves the use of apolar stationary phase.

Thus, retention times for apolar molecules is longer, whereas polar molecules elute before. RP–HPLC can be a useful tool in separating compounds with very similar structures, as phlorotannins from *A. nodosum*, however, the rapid elution of phenols due to their high polar nature could be disadvantage (Alvarez–Rivera et al., 2019).

Ultra-performance liquid chromatography (UPLC) reduces sample and solvent usage, reducing columns size and increasing working pressures together with speed and sensitivity of analysis (Novakova et al., 2006). Size exclusion chromatography (SEC) have been used in the preparative separation of different molecular weight fractions from seaweed extract as well as to confirm the molecular size of an isolated metabolite (Kang et al., 2012b; Lee, et al., 2012;).

The stationary phase or column is the key for chromatography, being octadecylsilane columns, also known as C18, with a particle size of 5 μm the most often used, but there are available with other internal diameters and lengths. Preparative columns are used to minimize problems with complex samples, avoid damage to the HPLC equipment or to remove impurities (Santos et al., 2019).

The chromatographic detectors are also important. Phenolic compounds absorb ultraviolet radiation between 260 and 330 nm, and this absorption range is used to identify their presence. Thus, detectors based on visible and UV–vis are the simplest and widely applicable to these kind of molecules (Ford et al., 2019). This absorption is quite variable due to the similar structures and depends on pH and/or presence of other components (Shahidi & Naczk, et al., 2006). However, UV–VIS detectors provide ambiguous peak identification due to the

USC UNIVERSIDADE DE SANTIAGO

similarities between phlorotannins. Alternatively, the use of mass spectrometers directly coupled to U/HPLC UV–vis instruments minimize this problem increasing the capacity to analyze complex extracts (Santos et al., 2019) allowing the qualitative and quantitative of hundreds of polyphenolic components (Guillarme et al., 2010). Diode array detector (DAD) used for analysis of compounds with similar molecular weights but different electronic distribution in the chromophore, leading to different UV spectra (Audibert et al., 2010; Ford et al., 2019;). HPLC combined with an UV–vis or a DAD detector and C18 column is the main system used for the separation and/or quantification of phlorotannins (Ignat et al., 2011; Motilva, et al., 2013).

Mass spectrometry is an analytical method for measuring mass–charge ratio (m/z), and electrospray ionization source (ESI) is the most often used ionization procedure (Ignat et al., 2011; Devi et al., 2008). Phlorotannins throughout the ESI source is ionized in a negative ion mode to produce deprotonated molecular ions. It is worth noting that in seaweed extracts, as a complex matrix, often interferences of coeluting compounds can occur, leading to the ionization suppression or enhancement in the signals (Cladiere et al., 2018; Konermann, et al., 2013). Thus, purification is required to extract and purify the targets during sample preparation by LC–MS that could increase time and cost (Motilva, et al., 2013). In fact, the isomerization by multiple combinations of phloroglucinol units after ionization has a notorious impact on mass spectra and often it is difficult to attribute a chemical structure of the detected molecules. However, MS can easily provide the profile of degree of polymerization in a phlorotannins enriched extract (Tierney et al., 2014; Kadam et al 2015b; Lopes et al., 2017; Catarino et al., 2019; Allwood et al., 2020; Sardari et al., 2021). The main limitation of ESI is that the sample is vaporized that do not permit the analysis of higher molecular weight as well as thermally labile components (Sailler & Glombitza, 1999).

Matrix–assisted laser desorption ionization (MALDI) combined with time of flight (TOF) analyser is a particularly suitable technique for the analysis of larger oligomers, with m/z above the upper limit of ESI–MS. This technique has been also used in combination with U/HPLC–ESI–MS providing information about the size and isomeric variation of phlorotannins (Wang et al., 2013; Agregán, et al., 2017; Sun et al., 2022). Other chromatography techniques have been used for the analysis of phenolic components in macroalgae, with different specificities; thin–layer chromatography (TLC) and/or Triple quadrupole (QqQ) among others (Cho et al., 2019; Santos et al., 2019).

Although the identification of phenolic compounds on macroalgae has increased in the last years, there is still a lack of information on the structural identification of higher molecular weight compounds, particularly in the case of phlorotannins. Further, methods to fractionate these extracts and obtain phenolic compounds–enriched fractions are still insufficient in preparing the extracts for analytical platforms. It is still necessary to reduce the complexity of extract matrices for analysis purposes. It is a common way to sacrifice sensitivity by expanding the sample dilution factor. It is of crucial importance to develop novel extraction and primary fractionation methodologies that potentiate both the generation of solid scientific data, the industrial exploration of algae and the identification of seaweed polyphenols (Ford et al., 2019).

The comparison of results is hard due to different extraction conditions and result expression as was above discussed (Section 1.7). Thus, it is mandatory to stablish standard methods similar as the determination of polyphenols in in other products such as tea (e.g., ISO 14502–1:2005 and 14502–2:2005).

3.2.3. FOURIER TRANSFORMED INFRARED SPECTROSCOPY (FT–IR)

This spectroscopy provides information to identify materials and their composition, their functional groups (proximate molecular structure), also can monitor the course of reactions. It has been widely used in analytical research. Infrared spectroscopy is used to determine the molecular structure and identifies compounds based on information of the relative vibrations among the atoms (Dong et al., 2019). The absorption of infrared light by the molecule is recorded with an instrument, that cover all the infrared region (700 nm^{-1} up to 1 mm^{-1}), to obtain the infrared spectrum. Infrared spectra consist in the wave number (or wavelength) as abscissa and the transmittance (or absorbance) as the ordinate, to represent the position of the absorption peak and the absorption intensity, respectively (Hesse et al., 2005). It is a nondestructive and fast acquisition technique in which chemical reagents are not necessary. Hence, FT–IR has been a widespread method used in the characterization of phenolic extracts. The presence of phenolic groups in macroalgae extracts has been assessed by the simultaneous occurrence of bands of hydroxyl groups at 3000 to 3500 cm^{-1} and aromatic rings at 1200 to 1700 cm^{-1} and also at 2850–3000 cm^{-1} (Lim et al., 2002; Wang, et al., 2009; Rajauria et al., 2013; Tanniou, et al., 2014).

USC
DE SANTIAGO
DE COMPOSTELA

3.2.4. NUCLEAR MAGNETIC RESONANCE (NMR)

NMR technique provides information about the functional groups and the placement of structural moieties in the molecules, and it was successfully applied in phenolic compounds (Glombitza 1979; Glombitza & Vogels 1985; Glombitza & Schmidt 1999a and b;). NMR spectroscopy is an adequate method for identifying phlorotannins and could be used to determine their purity (Parys et al., 2009). NMR allows the analysis of complex mixtures, being suitable to be used in raw extracts analysis (Tanniou, et al., 2014). Carbohydrates can also be detected, allowing their qualitative analysis (Steinberg & Altena, 1992; Parys et al., 2009).

Because of the presence of many different hydroxyl groups, the NMR spectra of phlorotannins enriched extracts can sometimes be complicated to be interpreted. Labile protons from OH generally exhibit broad signals that can produce signals hampered. Solvent where samples are diluted and measured is critical, since their polarity may change the equilibrium of the interactions formed altering peaks size, distribution and shapes in NMR spectrum. Deuterated water (D_2O) is one of the most used in NMR literature but requires the use of high temperatures (Hesse et al., 2005). For this reason, the analysis of seaweed extracts is recommended to be carried out using deuterated DMSO (DMSO–d_6) (Igoumenidis et al., 2018). The aromatic protons of phloroglucinol are often ranged between 6.0 and 7.5 ppm (Audibert et al., 2010; Ford et al., 2019).

Hydrogen (^{1}H–) and carbon (^{13}C–) NMR are the most used for analysis and/or identifying phlorotannins. However, several NMR techniques can be employed such as two–dimensional NMR (2D–NMR), heteronuclear multiple–quantum correlation (HMQC), fluor (1^9F–NMR), phosphorus (^{31}P–NMR), heteronuclear multiple–bond correlation (HMBC), quantitative NMR (qNMR), or ^{1}H–^{1}H correlation spectroscopy (COSY), among others (Sugiura, et al., 2006).

3.2.5. OPTICAL ANALYSIS

Transmission electron microscopy (TEM) is an imaging technique for imaging the interior of materials at the atomic level with a significantly higher (thousands of times) better resolution than optic microscopes.

Scanning electron microscopy (SEM) allows the observation and surface characterization of inorganic and organic materials, delivering morphological information of the analyzed material. Can be coupled with X–ray detectors, such as energy dispersive spectrometers (EDS) or wavelength dispersive spectrometers (WDS) to semi–quantitative analyze the elemental composition of samples (André et al., 2020).

Confocal laser scanning microscopy (CLSM) is used to illuminate a sub–surface layer of the specimen. This technique can be applied in biopolymers characterization to localize phenolic compounds within seaweed tissues however very few papers have been yet published applying this technique to seaweed biopolymers (Karimi–Lotfabad & Gray, 2000).

The large depth of the light microscope produces images containing information from different focal planes in thick specimens, good–quality and high–resolution images of the internal structure of samples can only be obtained from smears, squashes, or thin sections of the samples. Procedures which apply substantial shear and compressive forces may destroy or damage structural elements and sectioning is time consuming and involves chemical processing steps that may introduce artefacts and make image interpretation difficult.

Attenuated total reflectance (ATR) is used to analyze surfaces, it is mainly used for adhesion problems, weathering plastics parts and polymer films but it can also be used, combined with IR, with organic solutes in water (Hutzler et al., 1998).

3.2.6. X–RAY DIFFRACTION (XRD)

XRD is used for the analysis of structures crystallinity in extracted biopolymers of seaweed (Shimanuki et al., 2020). Some variants of this technique are the wide–angle X–ray scattering (WAXS) and the small angle X–ray scattering (SAXS) that complement XRD providing additional information about structure of materials at the nano– and meso–scale. X–ray photoelectron spectroscopy (XPS), spectroscopy for chemical analysis (ESCA) as well as secondary ion mass spectroscopy (SIMS) provide information about elemental composition, surface chemistry and adhesion mechanisms in layers. X–ray fluorescence (XRF) also may provide information about elemental composition of assayed samples (Hesse et al., 2005; Sun et al., 2022).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.3. SEAWEED BIOPOLYMERS IN FOOD

3.3.1. GENERALITIES

In the last decades, the interest and research regarding functional foods has notoriously increased. Functional foods contain bioactive compounds with demonstrated health benefits beyond the role of basic nutrition (Doyon & Labrecque, 2008). The determination of those antioxidant capacities is therefore essential to ensuring the quality of functional foods, to prevent and treat the diseases related to oxidative stress (Munteanu & Apetrei, 2021), as it was mentioned in the Sections 1.5 and 1.7). The development and validation of functional foods is a complex process that includes natural sources chemical characterization, extraction, purification optimization, food designing, technological and appealing research and, finally, *in vivo* validation (de Boer et al., 2017).

The use of seaweeds as natural sources in new functional foods is a promising field. In fact, this should be approached by an integral and complex approach, considering different research areas (seaweeds, processing, food product, and nutrition) with multiple interactions among them (Figure 3.6).

For seaweeds used as raw materials for bioactive compounds, it is essential to increase the number of biological studies where new edible species must be identified and characterized. Engineering research on seaweed processing is necessary for the design of sustainable production systems with minimum environmental damages, costs, and time together with maximum extraction yields. The main goal is to increase current knowledge to achieve a suitable design of functional foods using seaweed as source of bioactive ingredients. This Thesis is partly focused on the production of some of these ingredients (phlorotannins and alginates) extracted from *A. nodosum* seaweeds.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

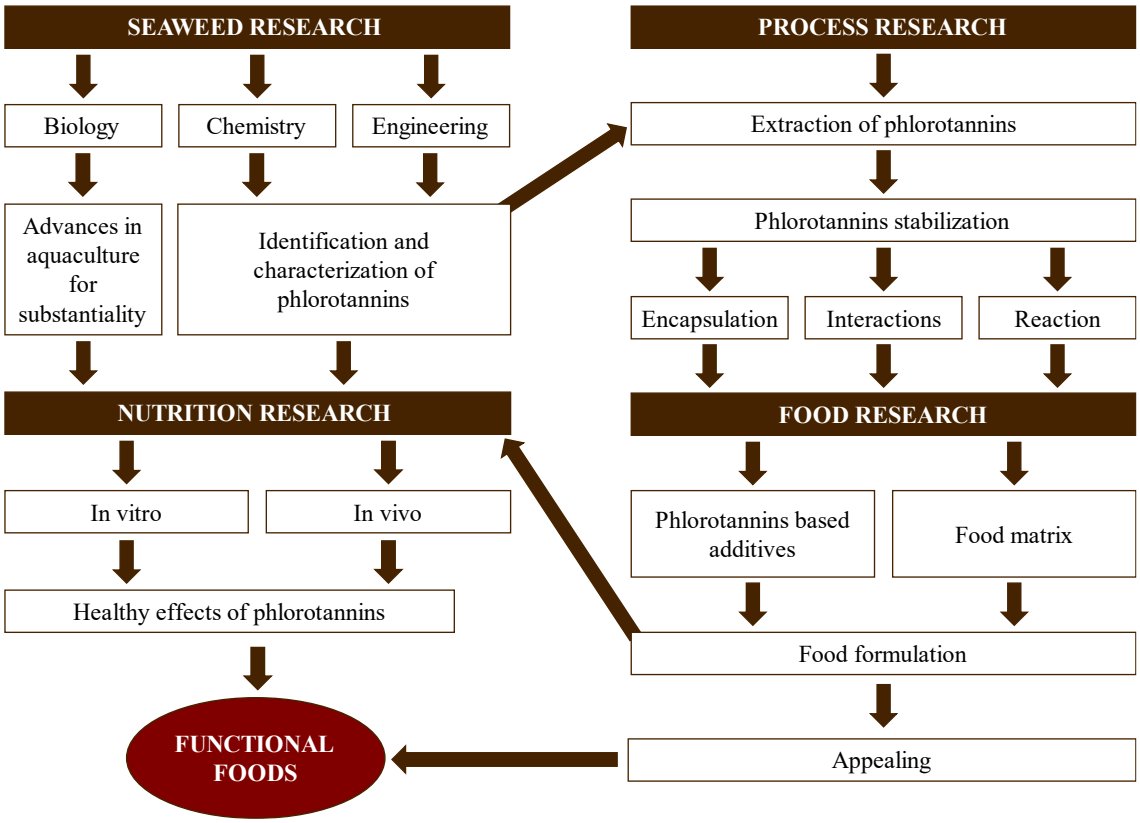


Figure 3.6. Stages in the development of functional foods using seaweed as source of phlorotannins, from a multidisciplinary point of view.

The application of seaweed extracts in real food systems is limited by both astringency and phlorotannin stability due to their high sensitivity to temperature, pH and contaminants (Koivikko et al., 2005). Encapsulation or coating polyphenols within natural or synthetic polymers (Fang & Bhandari, 2010) may also ensure the stability, bioactivity and bioavailability of phenolic compounds (Munin & Edwards–Lévy, 2011). Overall, the application of these technologies for the development of functional ingredients from seaweeds is still a pending task. In addition, high mineral content of seaweeds, such as iodine or some heavy metals is a challenge for the addition of seaweed into real food receipts.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.3.2. STARCHY–BASED SYSTEMS

Starch is the major worldwide consumed macronutrient, being the main source of energy (Björck, et al., 1994). Starch is formed by glucose molecules forming two types of bonds α–1,4, that produce a linear structure called amylose and α–1,6 bonds that form branches called amylopectin. Starch varies in granule sizes, shapes and amylose/amylopectin ratios depending on the plant origin (Sajilata et al., 2006). These granules or native starch have a compact semi–crystalline structure, with low enzymatic accessibilities that lead to both low degradation rates and low release of total sugars during digestion, making the native starch a poor source of energy (Chung et al., 2006). When starch granules are heated in the presence of abundant water, gelatinization takes place and hydrogen bonds that stabilize the starch chains are broken (Figure 3.7) appearing amorphous starch more digestible (Tester et al., 2004). After gelatinization, starch chains do not keep fully disordered and tend to reorder partially in a phenomenon called retrogradation that depends on temperature, time, and molecules size/branching (Sajilata et al., 2006). Overall, this re–crystallization partially recovers initial properties of starch (Parada & Aguilera, 2011).

Starch digestion takes places in both mouth and small intestine by the action of several enzymes. Throughout starch and other carbohydrates digestion, α–amylase and α–glucosidase are the two key enzymes involved. α–amylase is secreted by the salivary glands and the pancreas, and it catalyzes the hydrolysis of α–D–1,4 glycosidic linkages of starch generating shorter oligosaccharides (Santos et al., 2012). α–glucosidase completes the starch digestion catalyzing the hydrolysis of these oligosaccharides into smaller and absorbable sugars as glucose (Kim et al., 2010). Glucose is absorbed in the small intestine brush border and transported by the bloodstream to the tissues for its use and/or storage (Santos et al., 2012). Starch is not present as an isolate structure within the plant–cell matrix, it is often associated with other macronutrients, such as lipids or proteins; that could overall affect digestion rate (Parada & Santos, 2016).

Several starch types can be identified according to their digestion rate, slow digestible starch digested in 20 to 120 min that produce slow/moderate changes in the postprandial glycemia; quick digestible starch digested in less than 20 min that produce large changes in the glycemia even comparable with produced by simple sugars; and very rapid digestible starch

USC DE SANTIAGO DE COMPOSTELA

which is hydrolyzed in just few minutes during the first digestion stage (i.e., mastication), and its effect on the postprandial glycemia is not clear yet (Parada & Santos, 2016). An additional starch category is the resistant starch, which is not digested, and it passes to the digestion system with no effect on postprandial glycemia (Cummings & Englyst, 1995).

Furthermore, starch digestion may depend on food microstructure, which controls the accessibility to the substrate and the mobility of enzymes in the food bolus throughout the digestion process, presence of substances that could alter/inhibit the action of the digestive enzymes (e.g., natural compounds as polyphenols or drugs as acarbose), the intimate interactions of starch with other bolus components preclude the direct contact between starch and enzymes (Parada & Aguilera, 2011). The profile of each consumer, including their genetic background, metabolic status, or certain diseases presence, among others, also modify the starch digestion features. Indeed, it has been found that even particle size after mastication may affect the glycemic response, showing the complexity of this process.

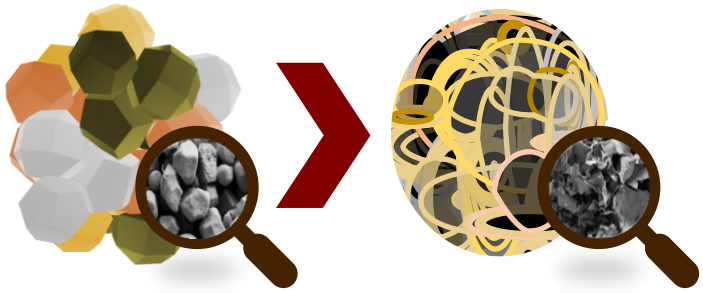


Figure 3.7. Starch gelatinization process and their microstructure changes.

Starches are being studied from multidisciplinary points of view, but the current Thesis is focused on high glycemic responses and subsequent disorders associated to the rapid digestion of gluten–free (GF) products and their derived problems to celiac patients. Glycemic response as the increasing of blood glucose levels after food consumption (Parada & Aguilera, 2011) is strongly related to starchy–based products consumption specially in traditional GF. Foods with low glycemic responses are considered favorable to health, since avoid or reduce possibilities to end up developing diabetes disease. As it was mentioned in Section 1.7 appropriate glycemic control is particularly important in celiac disease. Thus, it is mandatory to produce suitable new generation GF products to control GI responses, being the phlorotannins–enriched extracts the proposed solution in this Thesis.

Scientific evidence refutes partially the idea that a lower starch digestibility will induce a lower glycemic response (Parada & Santos, 2016), evidencing that glycemic response is a multifactorial phenomenon. Thus, deeper research, including the interdisciplinary approach, in Figure 3.6 is necessary to increase the insight of the actual relationship between starch digestion and glycemic response. Figure 3.8 shows a simplified diagram summarizing the main steps and enzymes related with food digestion and glycemic response.

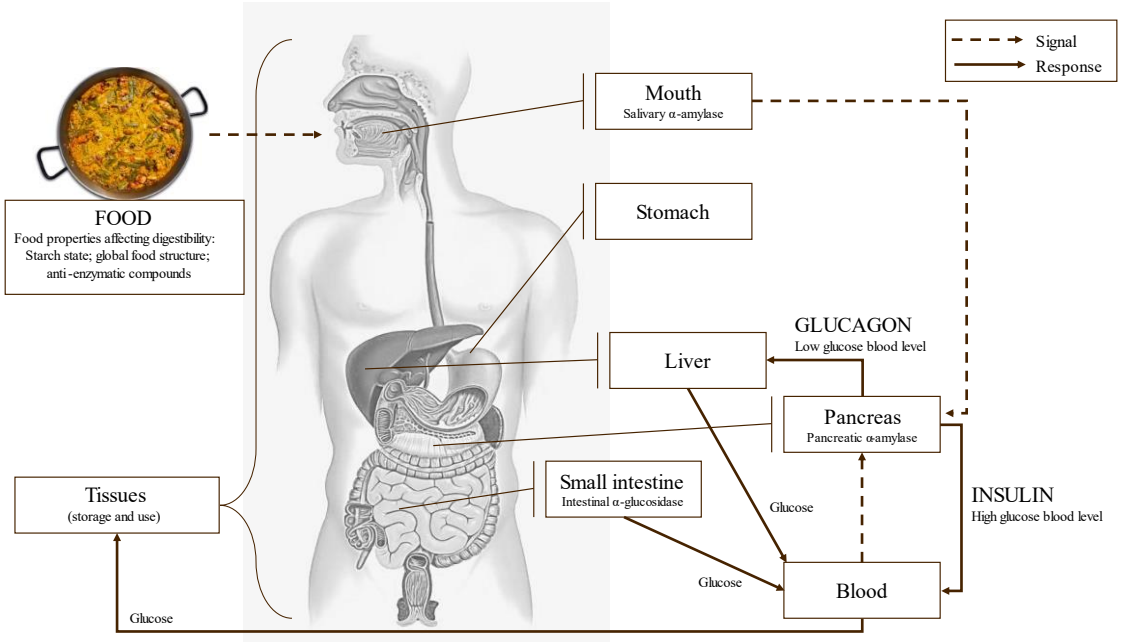


Figure 3.8. Global process of starch metabolic fate.

With the current advances in chemical and engineering technologies for extraction and identification of bioactive compounds from seaweeds, new food ingredients with potential functional activities in human health and nutrition is being achieved in the last years. Promising data from *in vitro* and animal studies have been found (Section 1.7.3), however, effects of polyphenols on glucose homeostasis in human beings are still under discussion. Further research would expand the most biologically active polyphenols screening and its derivatives and provide drug candidates for pharmaceutical purpose to the reduction or regulation of the diet–linked dysfunctions (Murugan et al., 2015).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3.3.3. PHLOROTANNINS AS ENZYME INHIBITORS

A strategy to manage the observed high levels of glucose in blood is the inhibition of α–amylase and α–glucosidase, digestive enzymes involved in the breakdown of starch and oligosaccharides (Matsui et al., 2007; Ríos et al., 2015) allowing the control of postprandial glucose in diabetic patients (Krentz & Bailey, 2005; Chen et al., 2006; Kwon et al., 2008; Zhao et al., 2021). Polyphenols are a large and heterogeneous group of phytochemicals particularly relevant as anti–enzymatic food components (Tester et al., 2004; Sajilata et al., 2006;).

Polyphenols are widely known due to their ability to associate with macromolecules that could be applied on enzymes to reduce their efficacy (Parada & Aguilera, 2011). Thus, polyphenols could be used to inhibit diverse digestive enzymes, specially α–amylase and α–glucosidase, but also maltase and/or sucrase (Williamson, 2013). Gauer et al., (2018) showed that flavonoids inhibited glucose absorption in the intestine, a promising result for the selective inhibition of pathways and to develop tailor–made treatments for every patient.

The ability of polyphenols to inhibit digestive enzymes is related to their well–documented interactions with some proteins and polysaccharides. The polyphenol–polysaccharide interactions are due to non–covalent (hydrogen bonds and hydrophobic interactions) or covalent interactions (Le Bourvellec & Renard, 2012). All these interactions, amount and type are largely influenced by food matrix structure and processing conditions. It is promising research that remains partially unexplored, but it can be a powerful tool to control the bioaccessibility, bioavailability, anti–enzymatic capacities of these new generation functional foods (Scazzocchio et al., 2015; Corona et al., 2017).

Several *in vitro* studies have reported on the enzyme inhibitory activity of polyphenols from different sources (persimmon, sorghum, rowanberry, or almond seeds, among others), on α–amylase and α–glucosidase enzymes (Flores et al., 2013). Although terrestrial polyphenols are recognized to have several bioactive functions, the literature on seaweed's polyphenols for human consumption and their disease treatment is sparse (Shibata et al., 2008) as it was previously commented (Sections 1.7.2 and 1.7.3).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Seaweeds are natural sources of enzyme–inhibiting compounds (o enzyme inhibitors), although knowledge about them is limited (Wang et al., 2005; Nyambe–Silavwe et al., 2015; Corona et al. 2017). Lordan et al. (2013) studied the enzymatic inhibition activity of phlorotannin–enriched extracts, reporting *A. nodosum* extracts as the strongest α–amylase inhibitors and *Fucus vesiculosus* extracts the best α–glucosidase inhibitors. However, diverse characteristics must be considered, such as extract manufacture, *in vitro* studies conditions, substrate used and harvesting period among others (Lee & Jeon, 2015).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4. EXPERIMENTAL & METHODOLOGY

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.1. MATERIALS

All chemical reagents used in the treatments and characterizations were analytical grade purchased from Merck[1] (Germany), Panreac[2] (Spain), Megazyme[3] (Ireland) or Sigma–Aldrich[4] (USA). Reagents used during this Thesis were 2,2'–azino–bis(3–ethylbenzothiazoline–6–sulfonic acid) diammonium salt (ABTS[4]), acarbose[1], Amberlite[4] XAD16 resin (surface area 800 m^2/g, pore diameter 10 nm), α–amylase[4] (Type VI–B, EC 3.2.1.1) from porcine pancreatic (8 U/mg), chlorohydric acid[1], corn starch[2], deuterated water[4] (D_2O), deuterated dimethyl sulfoxide (DMSO–d_6), 3,5–dinitrosalicylic acid, 2,2–diphenyl–1–picrylhydrazyl[4] (DPPH), Folin–Ciocalteu reagent[1], glacial acetic acid[1], glucose[4], D–glucose assay kit[3] (GOD/POD) , α–glucosidase[4] (Type I; EC 3.2.1.20) from *Saccharomyces cerevisiae* (11 U/mg), D(+)–guluronic acid[4], 3–hydroxybiphenyl[4], iron (III) chloride[4], iron sulphate[4], D(+)–maltose[2], methanol[1], phenol[1], phloroglucinol[4], potassium chloride[4], potassium hydroxide[2], resorcinol[4], sodium acetate[4], sodium alginate (CAS no: 9005–38–3), sodium azide[4], sodium carbonate[2], sodium chloride[4], sodium dihydrogen phosphate[2], sodium hydroxide[4], sodium phosphate[4], sodium tetraborate[4], sulfamic acid[2], sulfuric acid[2], 2,4,6–tris(2–pyridyl)–s–triazine[4], Trolox[4], and wheat starch[4].

4.2. DRYING & MILLING & SIEVING

Ascophyllum nodosum (*A. nodosum*) seaweeds harvested from the Galicia coast (Spain) during autumn season of 2019 were supplied by Mar de Ardora S.L. (Ortigueira, Spain) with a moisture content of 73.7 ± 3.8 % d.b. Samples were washed with running tap water, dried in a hot air convective dryer, Angelantoni Challenge 250, (Figure 4.1A). Optimized conditions of *A. nodosum* seaweeds were previously established by Chenlo et al., (2018). Consequently, temperature (50 ºC), relative humidity (30%), air velocity (2 m/s) and of load density (2 kg/m^2) were employed. *A. nodosum* seaweed was dried until moisture content of 4.3 ± 0.1 % d.b that was achieved after 8.5 h. Dried seaweeds were aerated for 3 days at room temperature (rt, 19 ± 1 ºC) sealed in vacuum plastic bags and conserved at 4 ºC.

USC DE COMPOSTELA

Then, dried samples were grounded in a blender (Figure 4.1B), milled in ultra–centrifugal mill (Figure 4.1C) and sieved using a vibratory sieve (Figure 4.1D) with standard meshes from 40 to 500 μm. Finally, *A. nodosum* flour (AF) was sealed in plastic bags with a vacuum packer (Figure 4.1E) and stored at 4 °C with final moisture content of 2.9 ± 0.1 % d.b.



Figure 4.1. A) Angelantoni Challenge 250 Air convective dryer (Italy), B) Laboratory blender Waring HGBTWT (USA), C) Ultra–centrifugal mill Retsch GmbH, ZM200 (Germany), D) Vibratory sieve FTL 0200, Cisa (Spain) and E) Vacuum packer Sammic V201 (Spain).

Drying kinetics were determined by monitoring samples weight. Samples were weighted in an analytical balance (Figure 4.2A), every few minutes in the first stages of drying and every hour towards the end. All experiments were performed, at least in duplicate, until moisture content was close to equilibrium. Dried weight was determined after vacuum drying (Figure 4.2B), at 70ºC and 10^4 Pa until constant weight of samples. Moisture ratio, Eq. (4.1), was employed to evaluate drying kinetics:

$$\text{Moisture ratio} = \frac{X_t - X_{eq}}{X_0 - X_{eq}} \qquad (4.1)$$

where X_t was the moisture content (d.b.) at any drying time, X_0 was the initial moisture content (d.b.) and X_{eq} was the equilibrium moisture content of the sample (d.b.) evaluated from water desorption obtained from Chenlo et al., (2018).

USC
UNIVERSIDADE
DE COMPOSTELA



A **B**

Figure 4.2. A) Vacuum oven Vacutherm VT650 (Heraeus Hanau, Germany) and B) analytical balance (Denver Instruments Spain)

4.3. PARTICLE SIZE AND WATER RETENTION CAPACITY

Chenlo et al., (2018) reported that particle size of seaweed flours was critical in biopolymers extractions. Thus, the weight mean diameter, D_w, was calculated using Eq. (4.2) assuming that the particles of AF were spherical.

$$D_w = \sum_{i=1}^{i=n} x_i D_{pi} \qquad (4.2)$$

where D_{pi} (μm) is the mean diameter for each fraction and x_i is the weight fraction calculated as the ratio between corresponding mass of each fraction and the total mass of our employed in the sieving.

Water retention capacity (WRC) measures water retained by the insoluble matrix and was determined following Robertson et al. (2000) protocol. AF was hydrated in distilled water with a liquid–solid ratio of 40 g_W/g_{AF}, for 20h in a at rt. Then, samples were centrifuged (3,000 xg. 20 min). The supernatant was decanted and conserved to be further characterized. Solids were transferred to a weighed sinter to be weighted prior to drying in a vacuum dryer. WRC was calculated by Eq. (4.3). Experimental WRC determinations were carried by triplicate.

$$\mathrm{WRC} = \frac{\mathrm{AF_{wet}} - \mathrm{AF_{dried}}}{\mathrm{AF_{dried}}} \quad (4.3)$$

where AF_{wet} is the mass hydrated and AF_{dried} is the mass dried.

4.4. BIOPOLYMERS EXTRACTIONS

Water (double distilled water) was used in all extracts as an eco–friendly and economic extraction solvent (FAO, 2018; Kadam et al., 2015a). However, to evaluate the effect of solvent type in several extraction methodologies and extraction conditions were proposed along this Thesis. To evaluate the effect of ionic strength in extractions, saline water 36 g/L NaCl, similar to seawater was studied in Section 7. Additionally, distilled and saline water were used to analyze extraction methods (solid–liquid extraction SLE and ultrasound–assisted extraction, UAE). Subsequently, using just double distilled water, the UAE and its operational conditions (sonication power, SP, time residence, tR, and liquid–solid ratio, LS) were optimized to maximize the extraction of phlorotannins (Section 8).

Finally, with the UAE conditions set up (SP = 90 W/cm^2, tR = 2 min and LS =20 $g_{solvent}/g_{algae}$), 70% (v/v) acetone:water (i.e., a better extractant of phlorotannins reported by Catarino et al., 2019) was compared with water extractions. The aim was to achieve a water–based extract that was similar in bioactivity to acetone:water. Purification throughout Amberlite XAD16 permitted to achieve an aqueous extract similar to acetone:water extract in bioactivity values. Then, this purified extract was used for the study of the influence of oxidation and dialysis into phlorotannins on bioactivity and composition (Section 9).

The nature, type, and number of interactions between those phlorotannins and corn starch was evaluated as preliminary approach of these new generation gluten–free foods (Section 10). Raw and purified UAE extracts were studied as digestive enzyme inhibitors (Section 11). Finally, isolated sodium alginate came from solid residue generated after UAE of phlorotannins (Section 12). Extractions were carried out in triplicate.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.4.1. SOLID–LIQUID EXTRACTION

Ionic strength effect on extraction was studied by using double distilled water (D) and saline solution (S) as solvents. Saline solution was prepared with 36 g/L NaCl to obtain similar saline concentration as seawater (Atkinson et al., 2010). D– and S–extractions of *A. nodosum* seaweed were carried out with 20 g of water per g of dried seaweed (g_W/g_{AF}) of liquid–solid ratio (LS) under stirring (170 rpm). Solid–liquid extraction (SLE) kinetics were studied taking aliquots at different times (2, 5, 10, 15, 30, and 60 min) at rt, determining total polyphenols (TPC), carbohydrates (CHOs), uronic acids (UA) content and antioxidant activities (DPPH, ABTS and FRAP methods) at each time. Each extraction was carried out in triplicate. SLE was applied as conventional extraction method (Section 1.7.1) to be compared with novel UAE method (Section 7).

4.4.1.1. SOLID–LIQUID EXTRACTION KINETICS MODELING

Empirical model proposed by Peleg (1988) was selected, due to its usefulness and wide extended use, to fit the evolution of TPC, CHOs and UA content together with antioxidant activities of the extracts during SLE time (Kadam et al 2015b).

$$X_t = X_0 + \frac{t}{K_1 + K_2 t} = \frac{t}{K_1 + K_2 t} \qquad (4.4)$$

where K_1 (min L/g) is inversely related to initial extraction rate (t = 0, $X(t) = 1/K_1$); K_2 (L/g) is inversely related to equilibrium content ($t \to \infty$, $X(t) = 1/K_2$), t is time, X_0 and X_t (g/L) is the initial (assumed zero in all experiments, raw solvent) and measured content at time t (min), respectively.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.4.2. ULTRASOUND–ASSISTED EXTRACTION

Continuous ultrasound assisted extraction (UAE) assays of *A. nodosum* seaweeds was carried out in the setup showed in Figure 4.3. Extracts were processed with a 1000 W ultrasound processor Hielscher, UIP–1000 hdT, (Germany), Figure 4.3A, using a 200 mL jacked chamber, Figure 4.3B, temperature was controlled with a Frigiterm circulation cryo–thermostat JP selecta (Spain), Figure 4.3C, to maintain the temperature under 30 °C during the operation. UAE operation was performed controlling the liquid flow with a peristaltic pump Cole Parmer MasterflexTM (USA), Figure 4.3D. An initial rest time (15 min) for seaweed powder rehydration with double distilled water was established (Arufe et al., 2019a) under constant stirring at 500 rpm (Figure 4.3E).

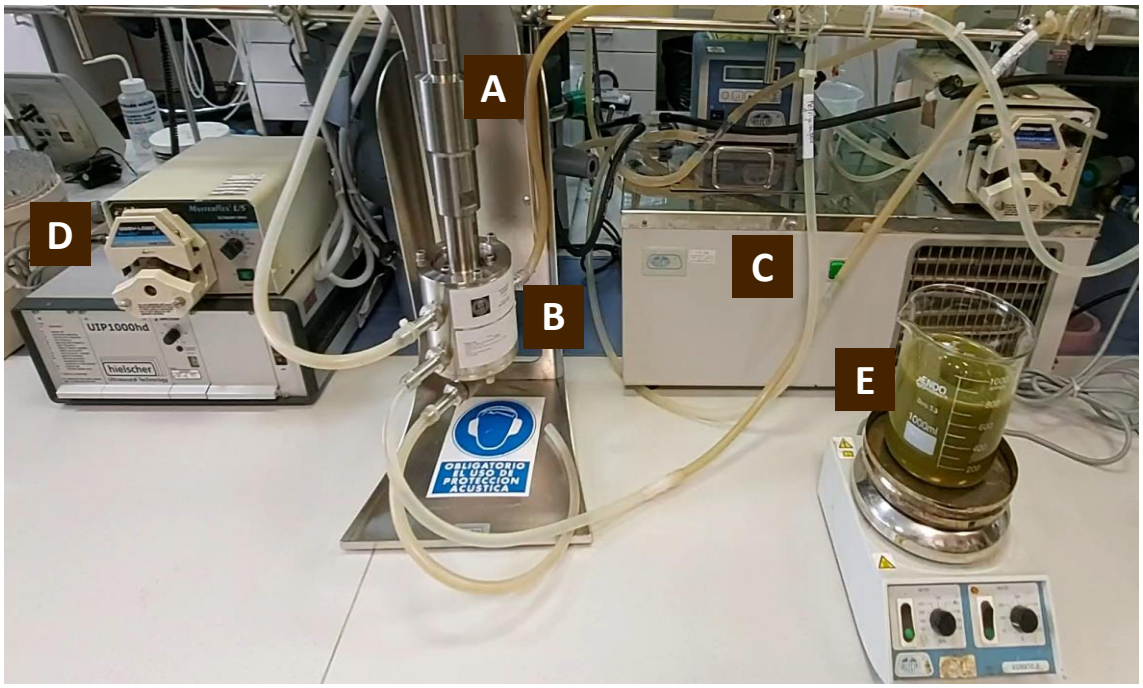


Figure 4.3. Experimental set up for the continuous aqueous ultrasound–assisted extraction of biopolymers from *A. nodosum* seaweed.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

All extracts were centrifuged at 8000 × g for 10 min (Figure 4.4A). Then, supernatant was filtered throughout 0.45 μm microfiber filter (Merck Millipore, USA) and stored at –20 °C. *A. nodosum* extracts were freeze dried during 36 h in a lyophilizer (Figure 4.4B) at –55 ºC and 0.5 Pa.

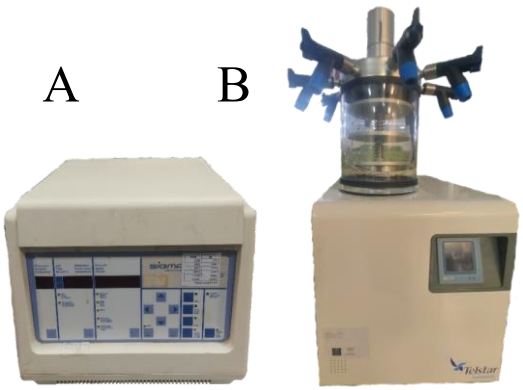


Figure 4.4. High–speed laboratory centrifuge Retsch GmbH, ZM200 (Germany, A) and Telstar LyoQuest lyophilizer –55, Telstar Technologies (Spain, B).

4.4.2.1. BOX–BEHNKEN EXPERIMENTAL DESIGN

Three sonication powers (SP) 70, 80 and 90 W/cm^2, three residence times (tR) regulated by the pump flow, 2 (1.94 mL/s), 4 (0.97) and 6 min (0.64); and three liquid–solid ratios (LS) 20, 30 and 40 g water per g of *A. nodosum* flour (g_W/g_{AF}) were evaluated. A Box–Behnken (BB) experimental design (Box & Behnken, 1960) was employed with coded variables shown in Table 4.1, generating 17 experimental runs (1–17). Reliability of the obtained models was checked preparing 6 additional extracts (A–F), included in Table 4.1. Box–Behnken (1–17) and additional (A–F) runs were characterized by means of the determination of their TPC, CHOs, UA, DPPH, ABTS and FRAP values. BB–experimental design was applied for the optimization of UAE (Section 8).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 4.1. BB experimental design of UAE carried at different sonication power (SP), residence times (t) and liquid–solid ratios (LS).

	Coded variables			Uncoded variables		
RUN	**SP**	**tR**	**LS**	**SP** (W/cm^2)	**tR** (min)	**LS** (g_W/ g_{AF})
1	–1	–1	0	70	2	30
2	–1	0	–1	70	4	20
3	–1	0	1	70	4	40
4	–1	1	0	70	6	30
5	0	–1	–1	80	2	20
6	0	–1	1	80	2	40
7	0	0	0	80	4	30
8	0	0	0	80	4	30
9	0	0	0	80	4	30
10	0	0	0	80	4	30
11	0	1	1	80	6	40
12	0	1	1	80	6	40
13	0	1	1	80	6	40
14	1	–1	–1	90	2	20
15	1	0	0	90	4	30
16	1	0	0	90	4	30
17	1	1	1	90	6	40
A	–1	0	0	70	4	30
B	–1	–1	1	70	2	40
C	0	0	–1	80	4	20
D	0	1	–1	80	6	20
E	1	1	–1	90	6	20
F	1	–1	1	90	2	40

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.4.3. ALGINATE EXTRACTION

Solid residues after UAE were centrifuged and filtered to be separated from aqueous phase (extract). Then, solids were air dried at constant relative humidity (30%), air velocity (2 m/s) at 50 ºC (50D) and 90 ºC (90D) for 9.5 h and 3 h, respectively, up to moisture content of 10.8 ± 0.4% (dry basis, d.b). Dried pellets were stored at rt until its use for sodium alginate extraction. An additional sample was obtained without forced convective drying (ND) and employed to extract alginates from the UAE wet residue. ND sample was immediately subjected to alginate extraction without additional storage time. Commercial alginate from Sigma was employed as reference.

Sodium alginates were obtained following the methodology reported by Chee et al. (2011). Briefly, the solid was soaked in $CaCl_2$ solution (1% w/w) with constant liquid–solid (LS) ratio (15 g of solvent per g of dried pellet, gs/g_{DP}). This blend was stirred at 150 rpm for 18 h at rt. The supernatant was discarded and the solid was washed 3–times with double–distilled water, maintaining the LS ratio constant and discarding all supernatants. Then, the solid particles were soaked in acid solution (HCl, 5% w/w), at the same above–mentioned conditions (LS ratio and stirring) for 1 h at rt. After acidic treatment 3 washes with double–distilled water was performed. Solid residue was decanted and blended with alkaline solution of Na_2CO_3 (3% w/w) again at the same LS and stirring for 1 h. After sodium carbonate treatment, distilled water was added to the solid (LS = 12.5 gs/g_{DR}) and rested for 8 h at rt.

The viscous blend was separated from solid residue by centrifugation at 8500 ×g for 5 min and further filtration. Sodium alginate was precipitated adding ethanol–water solution (50%, v/v) at 1:1 (v/v) liquid–liquid ratio. Sodium alginates fibers were filtered, carefully washed with ethanol (96%, v/v), aired for 24 h and finally dried at 50 ºC during 2.5 h up to 9.1 ± 0.2% (d.b) Dried alginate samples were milled and stored at rt until their use. Alginate extraction methodology was carried out to valorize the solid residue after UAE, evaluating how the presence of a drying treatment and drying temperature may affect to their physicochemical properties (Section 12). To ensure reproducibility of characterizations and produce enough alginate samples, the extractions and isolations were carried out by duplicate for each sample.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.4.4. EXTRACTION YIELDS

The extraction yield of bioactive compounds was calculated and expressed as g phytochemical per 100 g of dried seaweed (Eq. (4.5)). This yield was used in Section 7 for the characterizations and evaluation of extracts obtained using saline or distilled water together with SLE and UAE methodologies.

$$\text{Phytochemical extraction yield } = \frac{m_Y}{m_{AF}} \cdot 100 \quad (4.5)$$

where m_Y was the experimentally determined phytochemical concentration (determined by TPC, CHOs, UA, DPPH, ABTS and/or FRAP methods) and m_{AF} was de mass of *A. nodosum* flour.

The extraction yield of alginates was calculated and expressed as g alginates per 100 g of dried seaweed residue after sonication (Eq. (4.6)). This yield was used in Section 12 for the evaluation of extracted amount of alginate from each drying conditions assayed.

$$\text{Alginate extraction yield } = \frac{m_{Alg}}{m_{res}} \cdot 100 \quad (4.6)$$

where m_{Alg} was mass of sodium alginates extracted and m_{res} was de mass of UAE residue.

4.5. EXTRACTS PURIFICATION

Extracts purification was carried out in a (2.5 x 47 cm) Bio–Rad column (Bio–Rad, Hercules, CA, USA) filled with Amberlite XAD16 resin (Gonçalves–Fernández et al., 2019). Working flowrate was set at 110 mL/h using a peristaltic pump. 100 mL phlorotannin-eriched extract was poured into the column and washed with 300 mL of distilled water. Then, the column was flushed with 200 mL of ethanol 80% (v/v) to obtain a polyphenols enriched fraction. Then, enriched fractions were concentrated (Figure 4.5A) and freeze-dried. Sections 9 and 11 that involved purified extract included the proposed purification/freeze drying methodology. Purification of extracts was carried out in batch processes, which were characterized separately at least in triplicate to ensure the reproducibility of its bioactivity.



A B

Figure 4.5. Concentrator plus/Vacufuge plus, Eppendorf (Germany) Spectrophotometer Genesis 10S UV, Thermo Fisher Scientific (USA).

4.6. RAW AND PRURIFIED EXTRACTS CHARACTERIZATION

All extract characterizations were carried out by spectrophotometry (Figure 4.5B) at least in triplicate. Seaweeds extracts characterization includes the total polyphenol content (TPC) and the evaluation of their antioxidant activity by means of scavenging activity (DPPH and ABTS methods), and electron donor capacities (FRAP) (Mesa–Vanegas et al. 2015; Dang et al. 2017b;). Total carbohydrate content (CHOs) and total uronic acid content (UA) were also determined from the extracts. All these methods are colorimetric methods (Section 3.2.1) that were used in result sections (Sections 7 up to 12). The color change observed during reaction occurred for each method is shown in Figure 4.6.

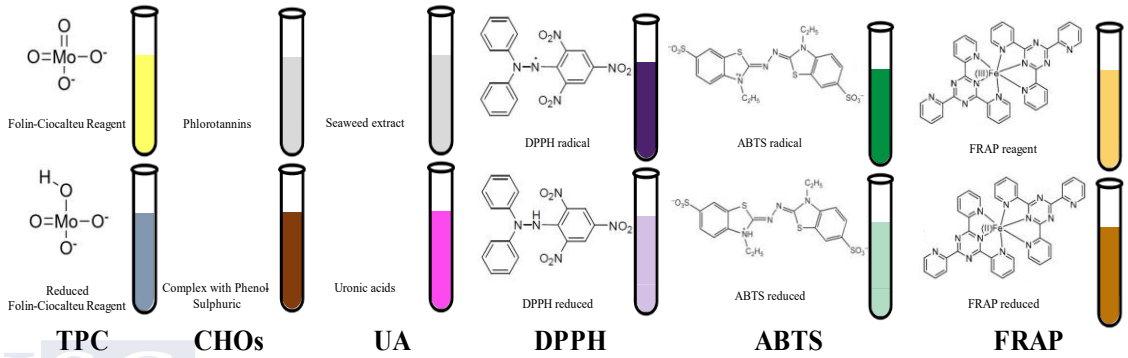


Figure 4.6. Color change occurred during colorimetric assays.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.6.1. TOTAL POLYPHENOL CONTENT

Total polyphenol content (TPC) was determined using a calibration with phloroglucinol as standard following the method proposed by Singleton & Rossi (1965). The method is based on the Folin–Ciocalteu reagent reaction with hydroxyl groups, measured spectrophotometrically at 765 nm. Aqueous seaweed extracts (0.5 mL) were mixed with 10% (v/v) Folin Ciocalteu's reagent (2.5 mL) and 7.5% (w/w) sodium carbonate solution (2 mL). The TPC values were expressed as g of phloroglucinol equivalents per liter (g_{PE}/L) using calibration curve showed in Figure 4.7.The blank was measured replacing seaweed extracts by double distilled water. Mixtures were incubated at 40 ºC for 15 min in completely darkness (Figure 4.8).

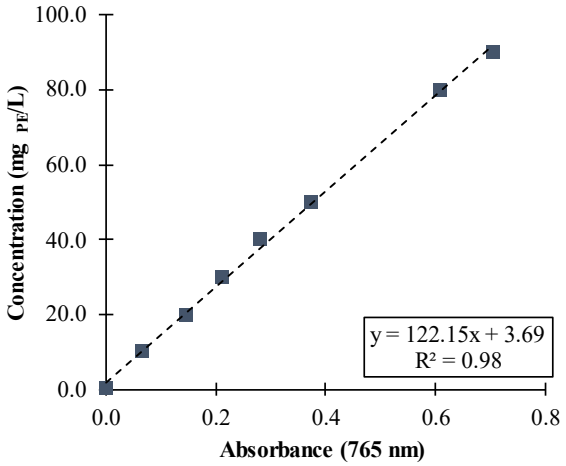


Figure 4.7. TPC calibration curve.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

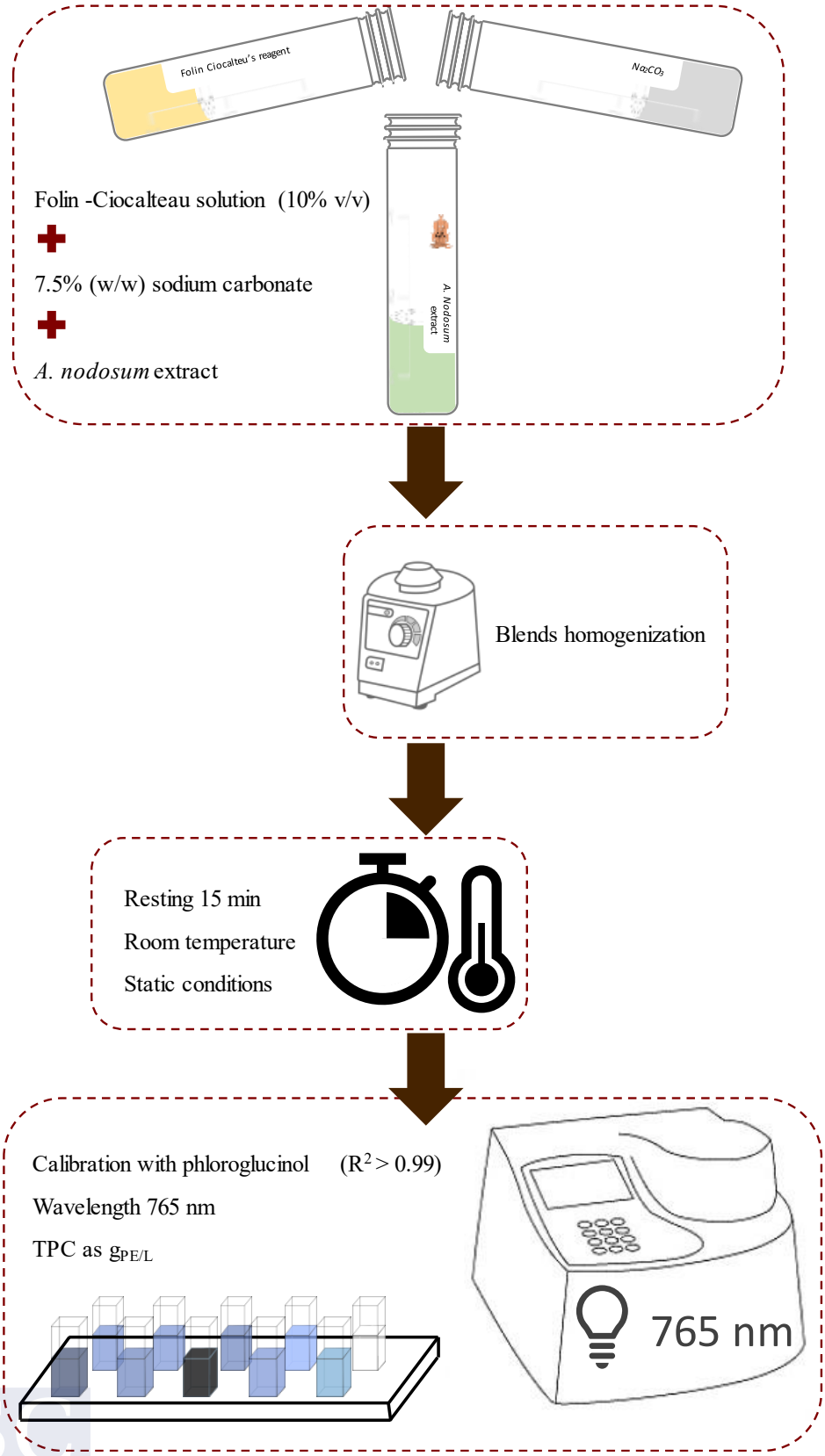


Figure 4.8. Total polyphenol content determination methodology.

4.6.2. TOTAL CARBOHYDRATE CONTENT

The total carbohydrate content (CHOs) was evaluated using a calibration with glucose as reference spectrophotometrically at 485 nm. CHOs results were given in g of glucose equivalents per liter (g_{GE}/L) using calibration curve showed in Figure 4.9. Aqueous extracts (1.0 mL) were mixed with 5% (v/v) phenol (0.5 mL) and H_2SO_4 (2.5 mL) following method proposed by Dubois et al. (1956). Mixtures were incubated at 30 ºC for 30 min (Figure 4.10).

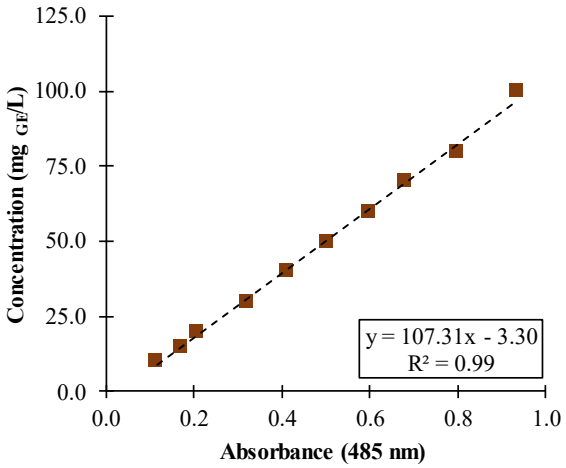


Figure 4.9. CHOs calibration curve.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

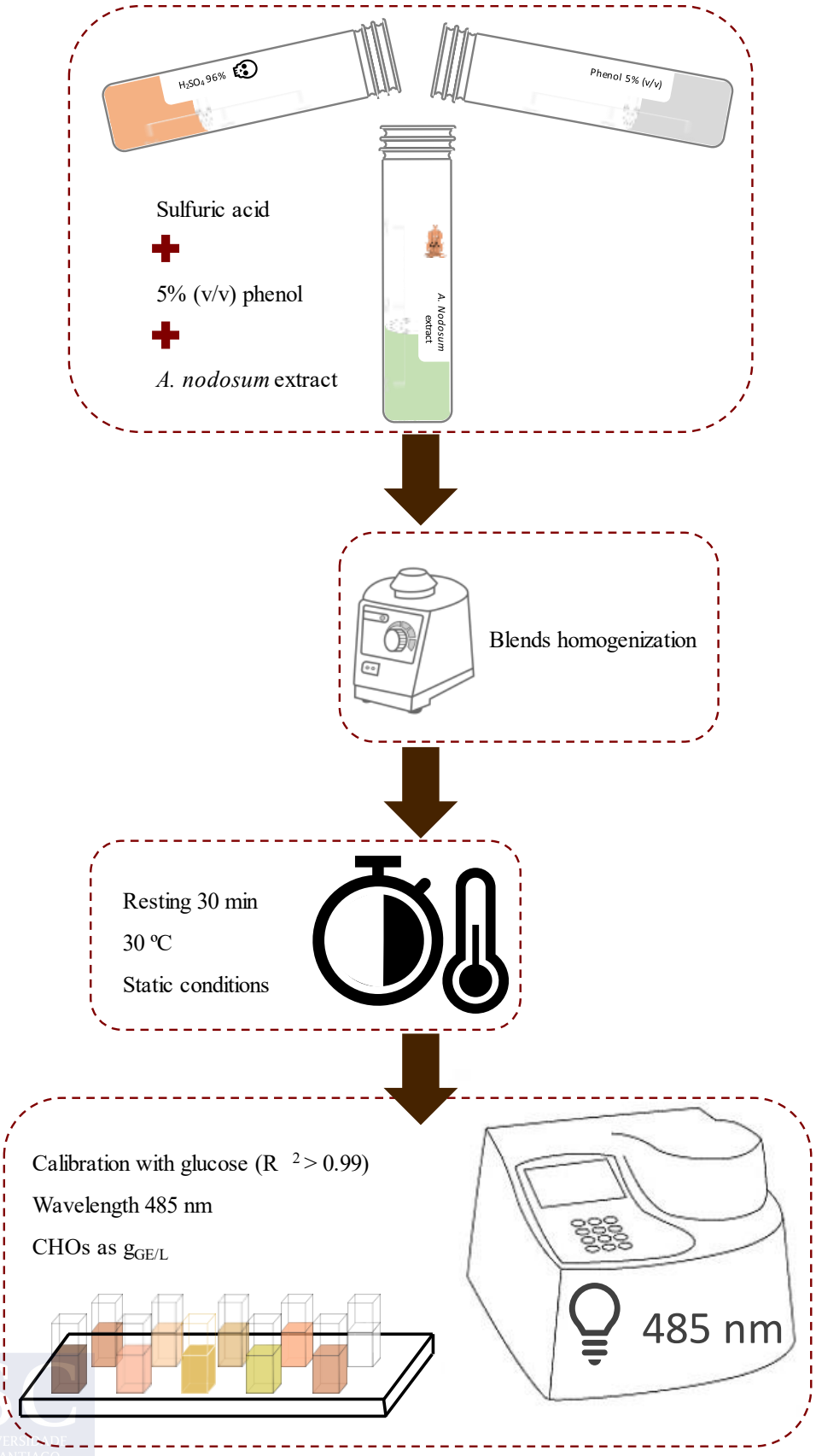


Figure 4.10. Total carbohydrate content determination methodology.

4.6.3. URONIC ACID CONTENT

Uronic acid content (UA) determination was carried mixing aqueous extracts (0.2 mL) with 4 M sulfamic acid (40 µL) and 12.5 mM sodium tetraborate solved in H_2SO_4 (1.25 mL). Mixtures were boiled at 100 ºC for 5 min and cooled for 20 min at rt. UA determinations were carried out using a calibration with glucose as reference showed in Figure 4.11 and the values are given in g of glucose equivalents per liter (g_{GE}/L). Then, after cooling samples, 0.15% (w/v) m–hydroxybiphenyl (20 µL) was added and mixture was spectrophotometrically analyzed at 520 nm following Blumenkrantz & Asboe–Hansen, 1973 protocol (Figure 4.12).

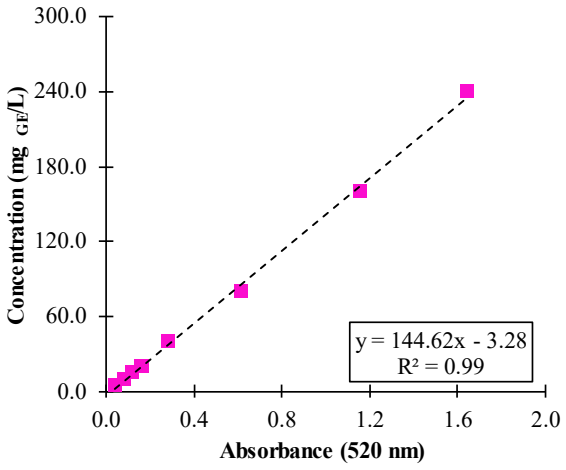


Figure 4.11. UA calibration curve.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

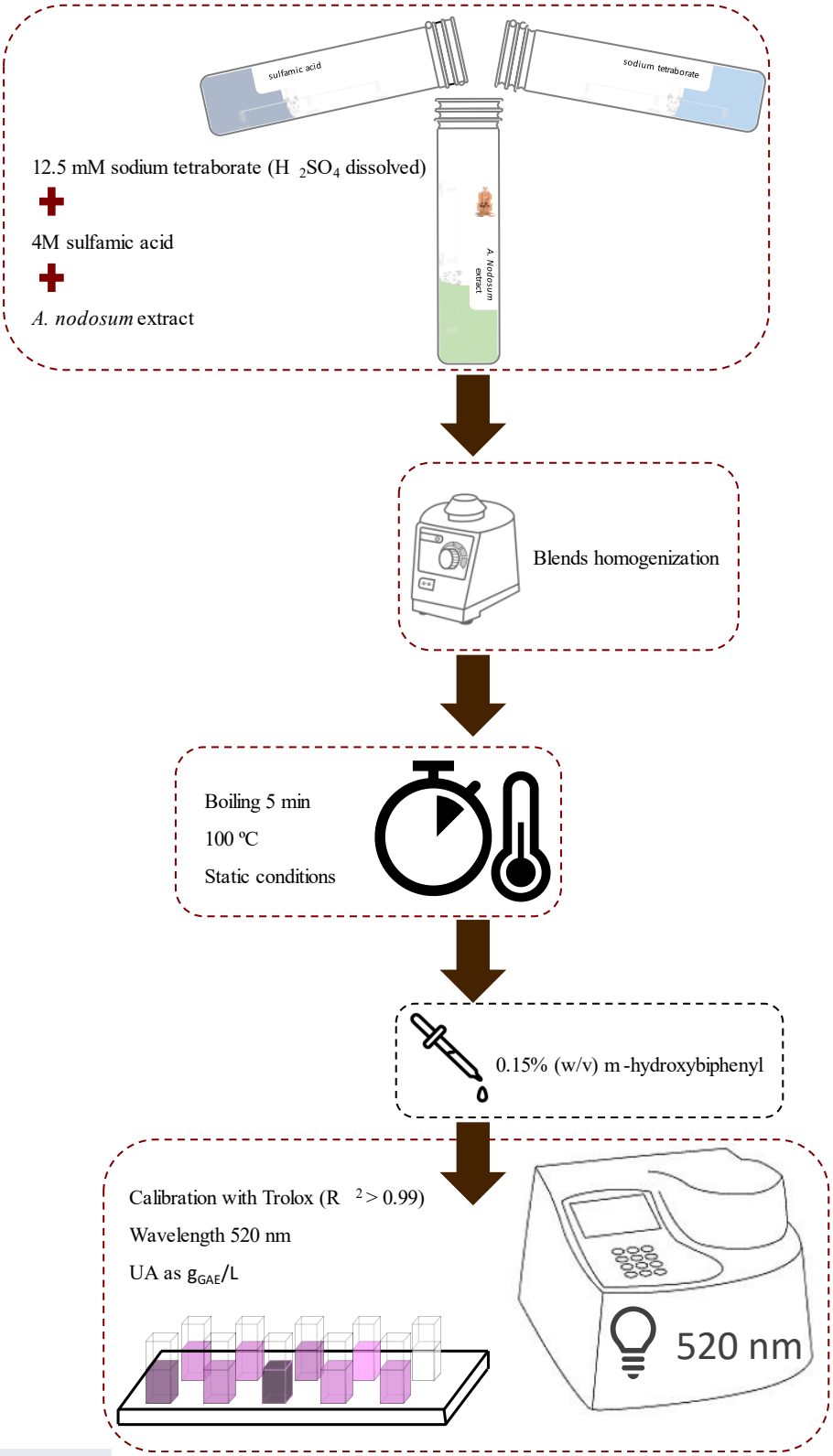


Figure 4.12. Uronic acids content determination methodology.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.6.4. DPPH SCAVENGING ACTIVITY

DPPH scavenging activity (DPPH) measures were performed following the methodology proposed by Brand–Williams et al. (1995). Extract samples (20 μL) were mixed with 60 μM DPPH methanolic solution (980 μL). Measures were taken after resting time of 30 min in completely darkness at rt. The scavenging activity of samples was determined measuring absorbance at 515 nm (Figure 4.14). DPPH scavenging activities were calculated using Eq. (4.7) and using calibration curve showed in Figure 4.13 with Trolox, to express results as millimolar of Trolox equivalents (mM_{TE}).

$$\text{DPPH} = \frac{\text{DPPH}_{0\text{min}} - \text{DPPH}_{30\text{min}}}{\text{DPPH}_{0\text{min}}} \qquad (4.7)$$

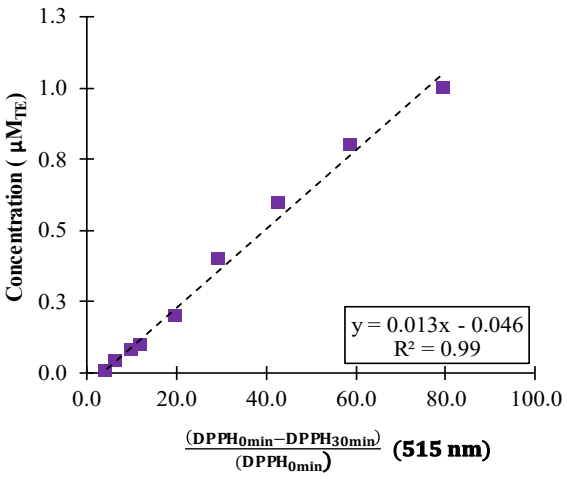


Figure 4.13. DPPH calibration curve.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

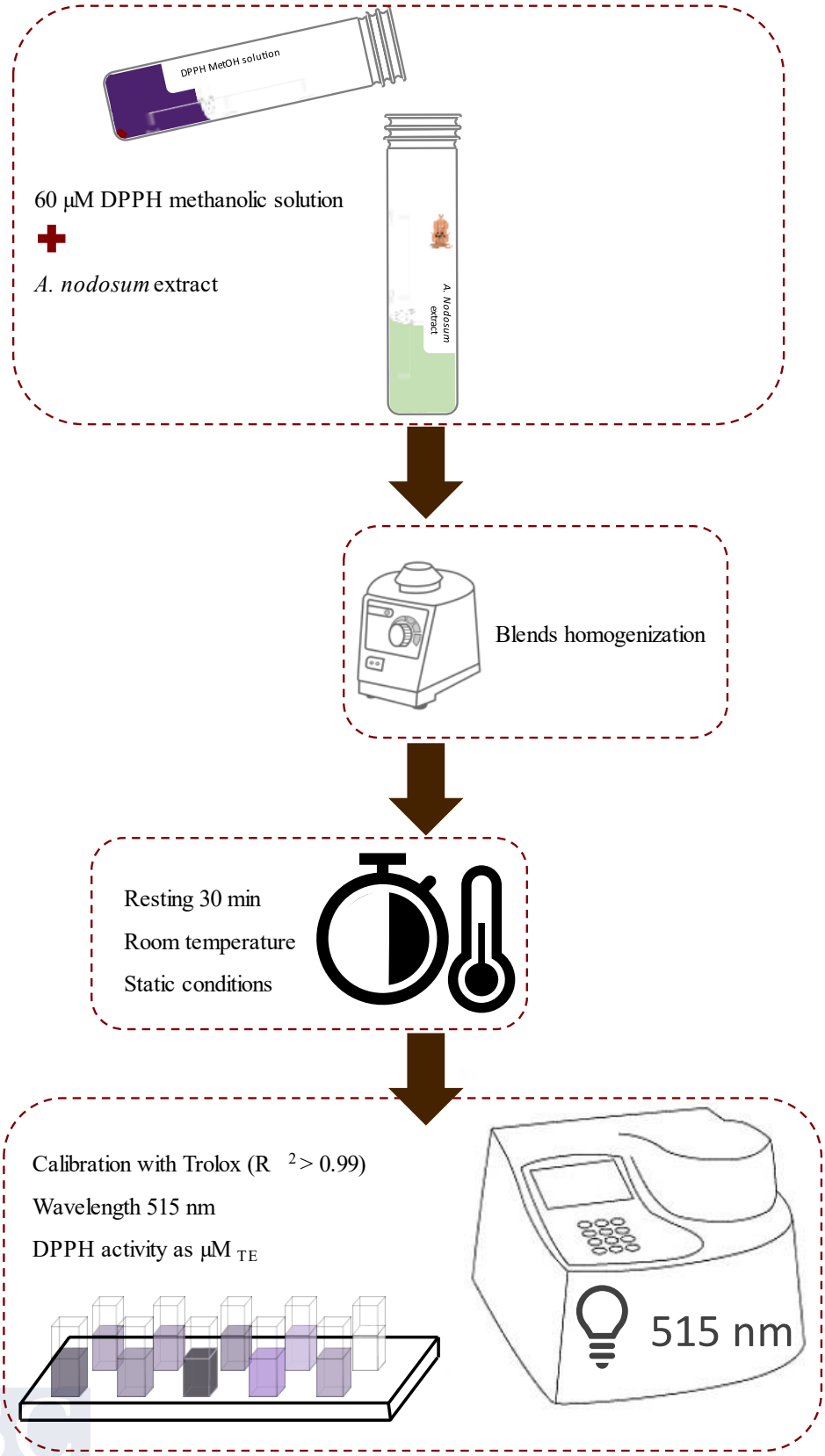


Figure 4.14. DPPH scavenging activity determination methodology.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.6.5. ABTS SCAVENGING ACTIVITY

ABTS scavenging activity (ABTS) determination was carried out following the proposed method by Re et al. (1999). ABTS reagent was prepared with 24.5 µM potassium persulfate and 15.2 mM ABTS using PBS buffer (0.14M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl and 3.1 mM sodium azide). The reagent was stirred in the dark for 16 hours at rt. ABTS solution was diluted with PBS until absorbance reach 0.70 ± 0.02 at 734 nm. Then, extracts were mixed with ABTS and incubated for 15 min at rt (Figure 4.16). ABYS scavenging activities were calculated using Eq. (4.8) and using calibration curve showed in Figure 4.15 with Trolox, to express results as millimolar of Trolox equivalents (mM_{TE}).

$$\mathrm{ABTS} = \mathrm{ABTS}_{0\mathrm{min}} - \mathrm{ABTS}_{15\mathrm{min}} \quad (4.8)$$

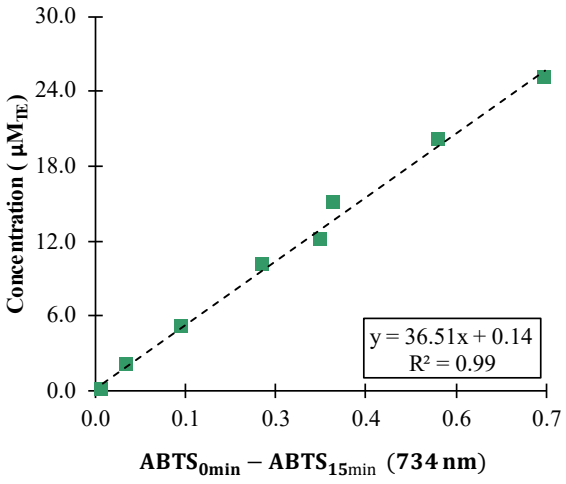


Figure 4.15. ABTS calibration curve.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

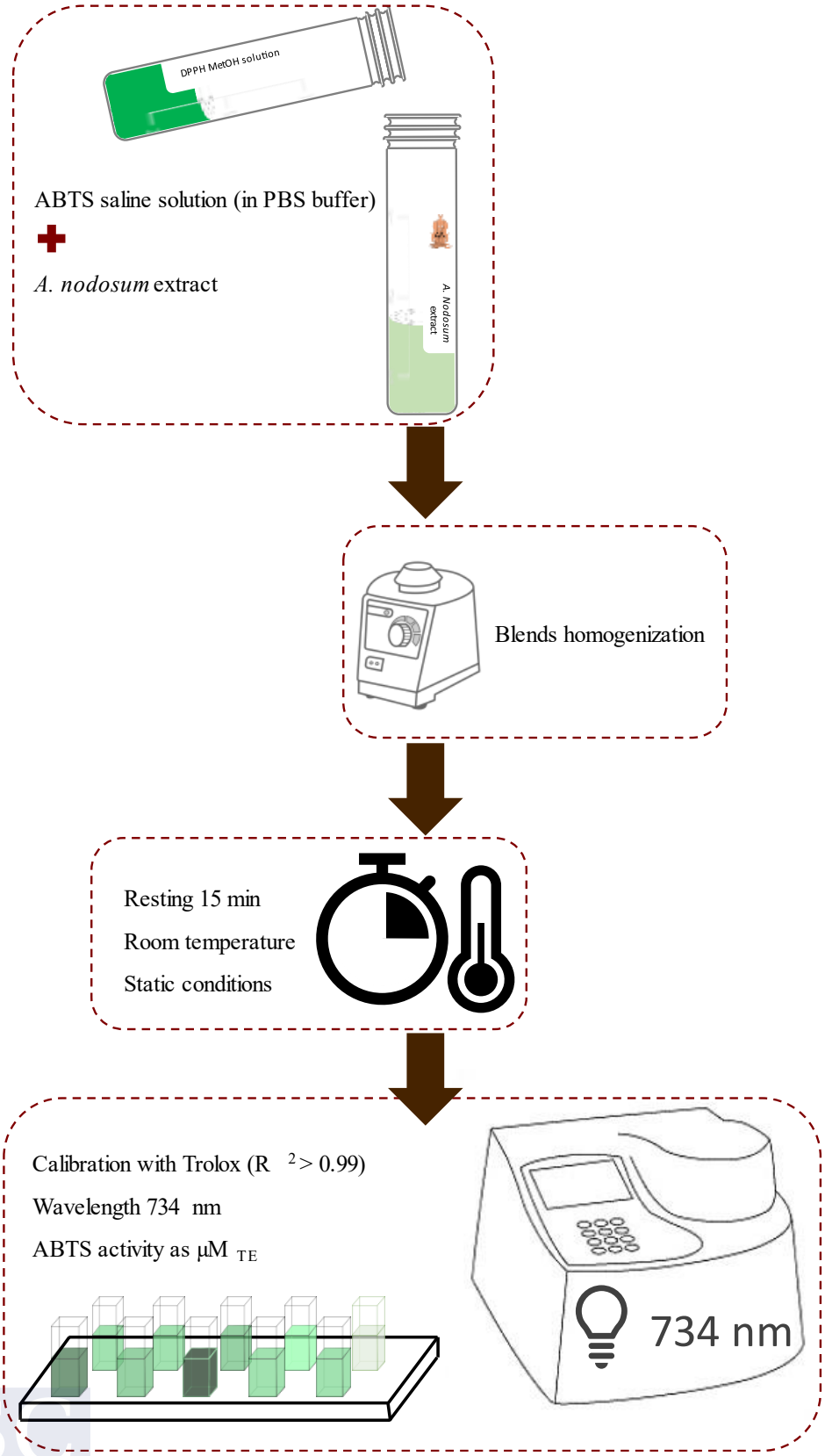


Figure 4.16. ABTS scavenging activity determination methodology.

4.6.6. ELECTRON DONOR CAPACITY

The Fe^{3+} reducing capacity or electron donor capacity (FRAP) of the extracts was evaluated according to Benzie & Strain (1996), using the calibration curve showed in Figure 4.17, and the procedure depicted in Figure 4.18. FRAP mixture was prepared with 300 mM sodium acetate buffer (pH 3.6), 10 mM of 2,4,6–tris(2–pyridyl)–s–triazine (TPTZ), and 20 mM $FeCl_3 \cdot 6H_2O$ with a ratio of 10:1:1, respectively. Extracts–FRAP mixture was incubated in darkness for 30 min at rt and absorbance was measured at 593 nm and evaluated using a calibration with Trolox™ as reference.

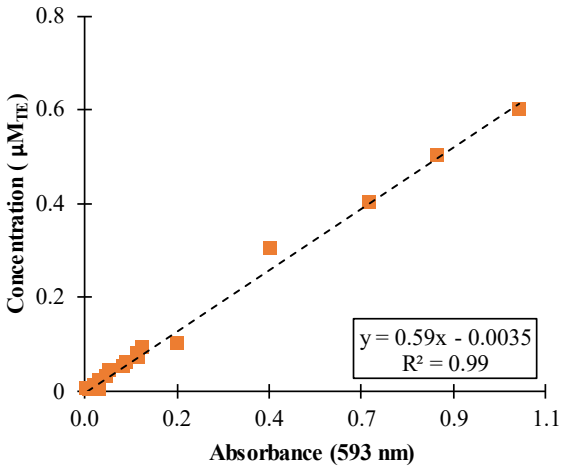


Figure 4.17. FRAP calibration curve.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

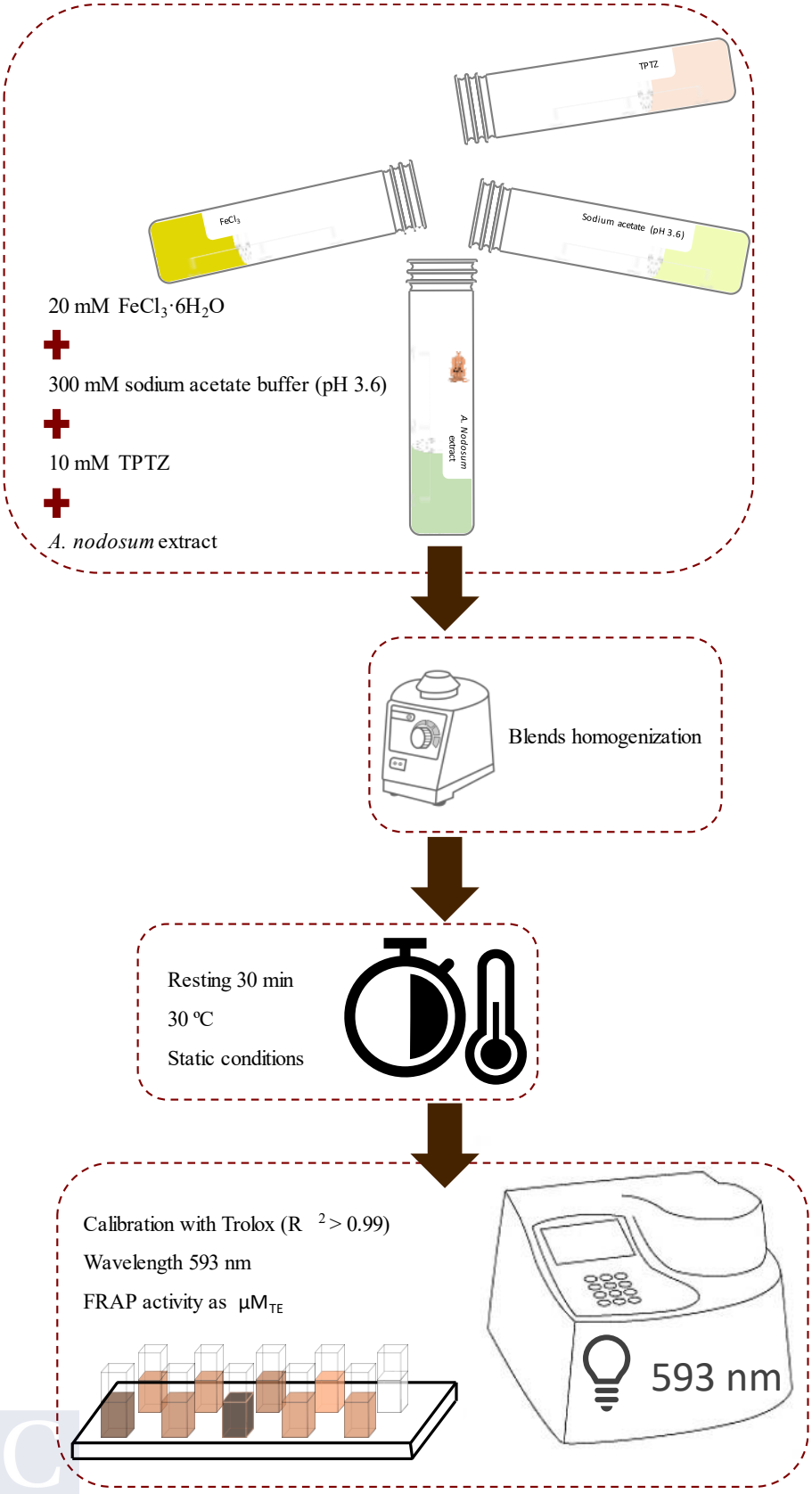


Figure 4.18. Electron donor capacity (FRAP) determination methodology.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.6.7. OTHER ANALYSES

Protein and fat content in seaweed extracts were determined by standard AOAC methods (AOAC, 2000). The fat content was determined by Soxhlet extraction and proteins were analyzed with a Elementar rapid N exceed nitrogen analyzer (Elementar, Langenselbold, Germany) using 5.0 as a conversion factor of nitrogen into proteins (Angell et al., 2016). Ash content was analyzed by burning a weighed sample in a muffle furnace at 550 ºC for 6 h. Data were expressed as percentage on a dry weight (DW). Protein, fat, and mineral content were determined for crude and purified UAE extracts in the Section 11.

4.7. EXTRACTS OXIDATION TREATMENTS

Extracts freeze–dried were dissolved in double distilled water (10 g_{FD}/L) and each one was exposed to different oxidation treatments. Natural oxidation (PON) was carried out resting samples for 120h at rt. Air oxidation (POA) was carried pumping air by AP80 Tetratec air pump (Tetrafish, Melle, Germany) with 80 L/h air flux for 120h at rt. Finally, peroxide oxidation was carried adding 1:1 (v/v) of hydrogen peroxide (POP) and resting samples for 120h at rt. Then, all oxidated extracts were freeze–dried. Oxidation treatments were carried out for purified (Section 4.5) and crude UAE extracts (Section 4.4.2) to evaluate the effect of phlorotannins molecular size and oxidation (Section 9) on their compositional (Section 4.6), chromatographic (Section 4.9 in on–off mode) and spectroscopic (MALDI–TOF–MS, FT–IR and ^{1}H–NMR, from Sections 4.10, 4.11 and 4.13, respectively) features.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.8. EXTRACTS CUT–OFF DIALYSIS

Prior to dialysis an aliquot of freeze–dried extracts were dissolved in double–distilled water (10 g_{FD}/L). Molecular weight cut–off dialysis was adapted from Tierney et al. (2013b) and was carried using 20, 10, 3.5 and 2 kDa Slide–A–lyzer G2 dialysis cassettes (Thermo Scientific, USA). The cassettes were clamped and immersed in a reservoir of double–distilled water and shaken (120 rpm) at 4 ºC for 120h as it is shown in Figure 4.19. Both solutions, high molecular weight (retentate) and low molecular weight (dialysate) were freeze–dried. Dialysis was carried out for purified (Section 4.5) and crude UAE extracts (Section 4.4.2) to evaluate the effect of phlorotannins molecular size (Section 9) on their composition (Section 4.6), chromatographic (Section 4.9 in on–off mode) and spectroscopic (MALDI–TOF–MS, FT–IR and ^{1}H–NMR, from Sections 4.10, 4.11 and 4.13, respectively) characteristics.

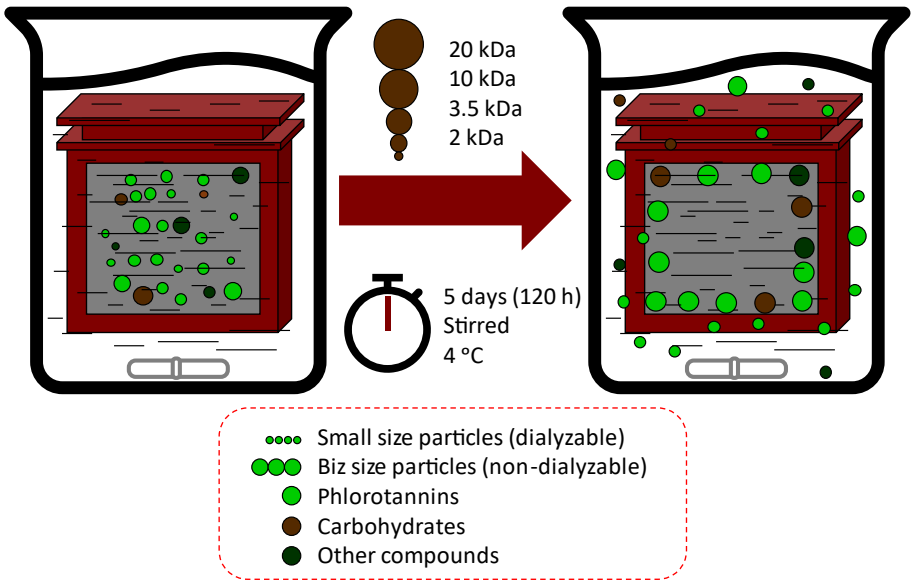


Figure 4.19. Scheme of *A. nodosum* aqueous extracts dialysis.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.9. EXTRACTS CHROMATOGRAPHY

All chromatographic profiles were obtained with a HPLC (High Performance Liquid Chromatography) system (Jasco, Japan) equipped with a PU–980 pump, an UV–1575 detector, and a degasser Populaire DP4003. Data were obtained and processed with PowerChrom 2.5 (eDaq Technologies, Australia) software. Chromatographic separation in reverse phase mode and was performed on a Kromasil C–18 semi–preparative column (8 × 250 mm) at 30 ºC. Freeze–dried samples of the extracts were diluted in EtOH 20% (aq. v/v) and filtered through 0.45 μm syringe filters. Injection volume was 25 μl, detection wavelength 266 nm, and solvent flow rate 0.4 mL/min. Polyphenols content was calculated with standard molecules of phloroglucinol and resorcinol; additionally, glucose and guluronic acid standards were injected to check for possible interference of UA and sugars (Gonçalves–Fernández et al., 2019). To ensure the reproducibility of the assays, a minimum of two injections of each extract were carried out. Chromatographic characterizations permitted, by comparison with standard commercial compounds, to corroborate the presence of phlorotannins in assayed extracts and the identification of their main families according to different peak retention time (Sections 7, 9 and 11).

Two elution gradients with water (A) and methanol (B) were used, gradient mode (0–3 min, 99% A, 1% B; 4–30 min, 5% A, 95% B; and 31–40 min 99% A, 1% B) and on–off mode (0–30 min, 100% A; 31–70 min, 100% B; and 71–100 100% A) showed in Figure 4.20A and Figure 4.20B, respectively. Gradient mode was initially used for the obtention of different fractions (isolated peaks) of phlorotannins, but their similarities did not permit to differentiate them (Sections 7 and 11). Thus, the on-off mode was used to obtain well–defined sections whereas solvent polarity was used to define the compounds obtained in each zone (Section 9).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

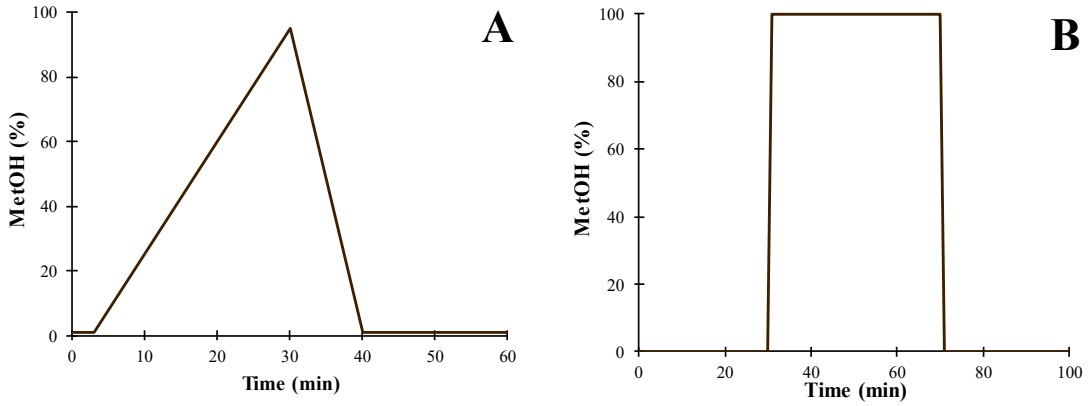


Figure 4.20. Elution gradients used during RP–HPLC analysis of *A. nodosum* extracts, based on gradient mode (A) and on–off mode (B).

4.10. EXTRACTS FRAGMENTATION PATTERNS

The mass detectors currently used chromatographic systems have a detection limit of approximately 2000 Da and become a limiting factor working with phlorotannins that are known to be larger (Catarino et al., 2019). The analysis of oligomers, above 500 Da in the extracts was carried out with a matrix–assisted laser desorption/ionization time of flight mass spectrometry (MALDI–TOF–MS). MALDI–TOF–MS spectra were recorded for samples PW, PD20 and PD2 to assess the range of mass fragmentation. The equipment used was Bruker Ultraflex III TOF/TOF equipped with a N2 laser of 337 nm and operated in positive mode. MALDI–TOF–MS spectra data treatment was carried out with Data analysis software (Bruker, USA). Fragmentation treatments were carried out for purified (Section 4.5) and crude UAE extracts (Section 4.4.1) to evaluate the effect of phlorotannins molecular size (Section 9) on their composition (Sections 4.6), chromatographic (Section 4.9 in on–off mode) and spectroscopic (MALDI–TOF–MS, FT–IR and ^{1}H–NMR, from Sections 4.10, 4.11 and 4.13, respectively) characteristics.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.11. FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier transform infrared (FT–IR) spectra were recorded with a Bruker FT–MIR model Vertex 70 V spectrometer. Wave number range was in the range 400 to 4000 cm^{-1}. Samples were blended with KBr and compressed into disks. FT–IR spectra treatment was carried out with Omnic 7.1 software (Thermo Scientific, USA). FT–IR data were supplemented by elemental analyses (CHNS) carried out with a Thermo Finnigan (Flash 1112 model) elemental analyzer. FT–IR spectroscopic analyses were carried out to analyze qualitatively the complex mixtures of extracts (Section 9), interactions between corn starch and seaweed (Section 10) and as a widespread technique used for alginate characterizations (Section 12).

4.12. AVERAGE VISCOSIMETRIC MOLECULAR WEIGHT

Molecular weight of extracted and commercial alginate was evaluated from kinematic viscosity value using a Ubbelohde viscometer (AVS 350, Schott–Geräte, GmbH, Germany) using an aqueous 0.1 M NaCl solution. For each sample, five measurements were taken at 25 ºC (±0.1 ºC). Then, the viscosity average molecular weight (M_v, g/mol) was determined by the Mark–Houwink equation following Dodero et al., (2020a & b) studies. Average viscosimetric molecular weight was used to analyze the effect of UAE residue drying temperature had on physicochemical properties of isolated sodium alginates (Section 12).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.13. NUCLEAR MAGNETIC RESONANCE

4.13.1. SODIUM ALGINATE

Hydrogen nuclear magnetic resonance (^{1}H–NMR) of alginate samples was carried out blended dried alginates with D_2O. ^{1}H–NMR spectroscopy was used to determine composition and mannuronic block (M) and guluronic block (G) distribution into monads (F_M and F_G), diads (F_{GG}, F_{MM}, F_{MG}, F_{GM}) and triads (F_{GGG}, F_{MGM}, F_{GGM}), M/G ratio and block average length ($N_{G>1}$) from sodium alginates samples, were also calculated Monads, diads, triads, M/G ratio and $N_{G>1}$ values assessment following equations from ASTM, 2012 (Eqs. (4.9) up to (4.24)) where A, B1, B2, B3, B4 and C values are obtained peak area values form each spectra following the Figure 4.21 schematic representation. ^{1}H–NMR is a commonly used method for sodium alginates characterization, that was used in this Thesis to analyze the effect of UAE residue drying had on the properties of isolated sodium alginates (Section 12).

$$G = 0.5\big(A + C + 0.5(B1 + B2 + B3)\big) \tag{4.9}$$

$$M = B4 + 0.5(B1 + B2 + B3) \tag{4.10}$$

$$GG = 0.5(A + C - 0.5(B1 + B2 + B3)) \tag{4.11}$$

$$MG = GM = 0.5(B1 + B2 + B3) \tag{4.12}$$

$$MM = B4 \tag{4.13}$$

$$GGM = MGG = (B1) \cdot 0.5(B1 + B2 + B3)/(B1 + B2) \tag{4.14}$$

$$MGM = (B2) \cdot 0.5(B1 + B2 + B3)/(B1 + B2) \tag{4.15}$$

$$GGG = GG - GGM \tag{4.16}$$

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

$$F_G = \frac{G}{M + G} \tag{4.17}$$

$$F_M = \frac{M}{M + G} \tag{4.18}$$

$$F_{GGG} = \frac{GGG}{(M + G)} \tag{4.19}$$

$$F_{MGM} = \frac{MGM}{(M + G)} \tag{4.20}$$

$$F_{GGM} = F_{MGG} = \frac{GGM}{(M + G)} \tag{4.21}$$

$$N_G = \frac{F_G}{F_{GM}} \tag{4.22}$$

$$N_{G>1} = \frac{(F_G - F_{MGM})}{F_{GGM}} \tag{4.23}$$

$$N_M = \frac{F_M}{F_{MG}} \tag{4.24}$$

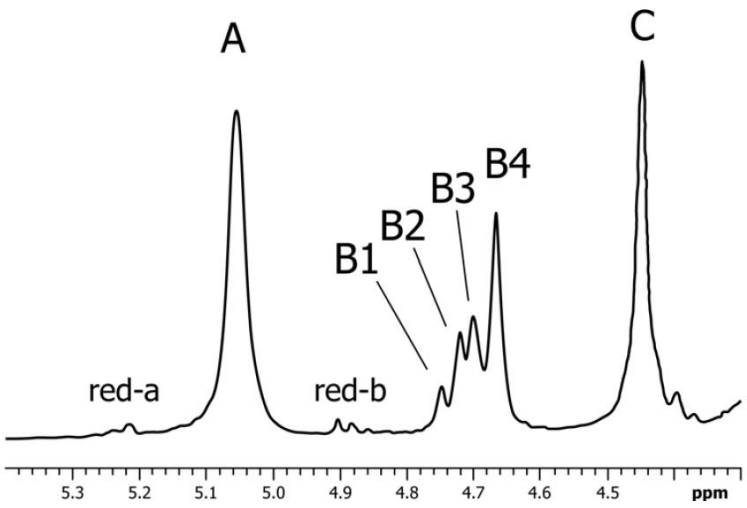


Figure 4.21. Average ^{1}H–NMR spectrum shape of alginate used for quantitative analysis.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4.13.2. PHLOROTANNIN-ERICHED EXTRACTS

^{1}H–NMR spectra were collected with Bruker NEO 750 spectrophotometer operated with a 17.61 T (750 MHz resonance ^{1}H) magnetic field strength. ^{1}H–NMR spectra from extracts was carried out using DMSO–d6 or D_2O and their treatment was carried out with MestreNova software (Mestrelab Research, Spain). NMR technique was used to analyze chemical alterations in assayed extracts due to, oxidation and dialyzation treatments (Section 9) and to evaluate UAE and purification treatments (Section 11) effects on phlorotannins features.

4.14. SEAWEED–STARCH INTERACTIONS

Three methods (NT, GL, CGL) for corn starch (CS) and AF blending were tested with slight modifications respect to protocol proposed by Wang et al. (2021). NT and GL methods were assayed to study the effect of CS structural features on the interactions with bioactive compounds from AF. Conversely, CGL method was based on two stages: first, bioactive compounds extraction from AF together with CS during gelatinization step; second, a partial leaching of bioactive molecules from gel after water addition. The objective of this last method was to simulate the behavior of bioactive compounds during the heating steps during starchy products processing. So, NT consisted in the native CS and AF blending; gelled CS was blended with AF in GL, and CS was gelatinized in the presence of AF in CGL method. AF and CS control samples were also analyzed to determine chemical characteristics promoted by both powders (Figure 4.22).

Starch gelatinization was performed according to the method reported by Wang et al. (2021). Aqueous CS (20%, w/w) was immersed in boiling water bath at 100 ºC for 20 min. The CGL gelatinization was carried at the same conditions for AF and CS blends. After gelatinization stage, the samples were cooled (20 min) at rt until gel temperature was lower than 35 ºC. Subsequently, GL and CGL samples were homogenized using a homogenizer (IKA Werke GmbH & Co., Germany) with 3 pulses of 5 s at 6500 rpm. Broken gel was blended with seaweeds and additional water in GL method, whereas only distilled water was added to obtain CGL samples.

USC DE SANTIAGO DE COMPOSTELA

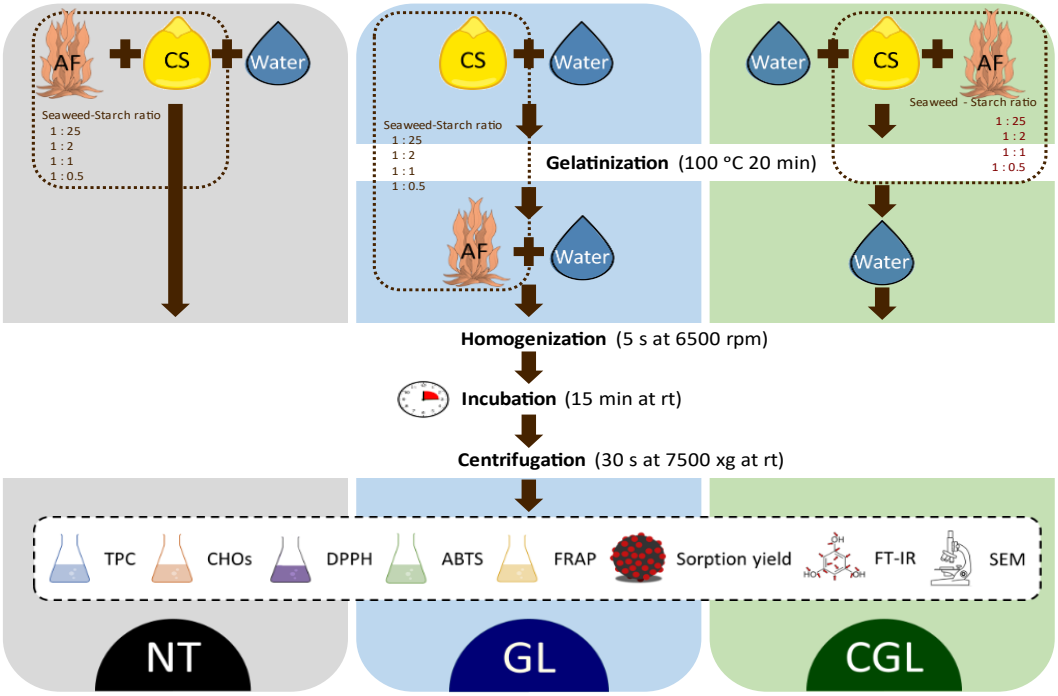


Figure 4.22. Process scheme summarizing methodology. NT method: AF (seaweeds flour) and corn starch (CS) blending; GL method: AF and CS blending, CGL method: CS gelatinized in presence of AF.

Liquid–solid ratio was set at 100 g_W/g_{AF}. AF and CS content varied to obtain different *A. nodosum* flour to corn starch blending ratios (1:25, 1:2, 1:1; and 1:0.5) corresponding to 4, 50, 100 and 200 g_W/g_{CS}, respectively. Ratio 1:25 was used as average proportion studied by bakery products (O'Shea et al., 2014), meanwhile, remaining ratios were studied to determine the molecular interactions more adequately. These AF–CS mixtures in water were homogenized and rested for 15 min at rt. Elapsed time necessary to achieve a solid/liquid pseudo–equilibrium (between water and AF) was previously determined and was shorter than 15 min. This procedure agrees to the kinetics determined between potato starch and procyanidins by Qiu et al. (2016). The phytochemicals content was analyzed after samples centrifugation at 7,500 ×g for 30 s at rt followed by filtration of the supernatant through 0.45 µm microfiber filter (Meck Milipore, USA).

Seaweed–starch interactions methodologies were carried out to evaluate the interactions of starch (native or gelled) with seaweed particles and their bioactive compounds. Interactions were evaluated by means of composition (Section 4.6), FT–IR (Section 4.11) and optical (Section 4.16) analyses and results are collected in the Section 10. Each proposed systems (NT, GL and CGL) and AF–CS ratio (1:0.5, 1:1, 1:2 and 1:25) was carried by triplicate.

TPC values provided by AF in the AF–CS blends was calculated using Eq. (4.25):

$$\mathrm{TPC_{LP}} = \mathrm{TPC_{AF-CS}} - \mathrm{TPC_{CS}} \quad (4.25)$$

where $\mathrm{TPC_{LP}}$ is the polyphenols content in the aqueous phase from AF, $\mathrm{TPC_{AF-CS}}$ is the polyphenols content measured in the liquid phase of AF–CS blends and $\mathrm{TPC_{CS}}$ the corresponding polyphenols content of CS control samples.

The polyphenols sorption yield at equilibrium, $\mathrm{Y_P}$ (%) was evaluated by Eq. (4.26) and the polyphenols adsorbed by CS, q ($\mathrm{mg_{PE}/g_{CS}}$), was determined by Eq. (4.27):

$$\mathrm{Y_P} = \left(1 - \left(\frac{\mathrm{TPC}}{\mathrm{TPC_{AF}}}\right)\right) \cdot 100 \quad (4.26)$$

$$\mathrm{q} = (\mathrm{TPC_{AF}} - \mathrm{TPC}) \left(\frac{\mathrm{V}}{\mathrm{m_{CS}}}\right) \quad (4.27)$$

where $\mathrm{TPC_{AF}}$ is the polyphenol content ($\mathrm{g_{PE}/L}$) of aqueous phase corresponding to AF control samples, V the liquid volume (L) and $\mathrm{m_{CS}}$ is the final CS mass (g), evaluated by means of Eq. (4.28):

$$\mathrm{m_{CS}} = \mathrm{m_{CSi}} - (\mathrm{TPC_{CS}} - \mathrm{CHOs})\,\mathrm{V} \quad (4.28)$$

where $\mathrm{m_{CSi}}$ is the initial CS mass (g) and CHOs the carbohydrate content ($\mathrm{g_{GE}/L}$) of liquid phase released from starch (adsorbent).

4.15. INHIBITORY CAPACITIES OF EXTRACTS

The inhibition assay of α–amylase from porcine pancreatic was adapted from recent research protocol (Zheng et al., 2020) with minor modifications. Briefly, wheat starch solution (6.25 mg/mL) was prepared in sodium phosphate buffer (0.02 M, pH 6.9 containing 6 mM NaCl), followed by starch gelatinization in a water bath at 100 ºC for 20 min (Wang et al., 2021). Reaction systems included 50 µg of freeze–dried seaweed extracts dissolved in 20% (v/v) ethanol, 50 µL of α–amylase (50 U/mL) and 400 µL of gelatinized wheat starch. Three

USC UNIVERSIDADE DE COMPOSTELA

main methodologies were carried out to evaluate extracts inhibition activities (Figure 4.23), with minor modifications depending on the enzyme ssayed.

Enzyme and polyphenol solutions were mixed and pre–incubated in an Eppendorf Thermomixer Compact at 37 ºC for 10 min at a speed of 600 rpm. Then, gelatinized starch was added, and the blend was incubated under the same conditions (A1). Gelatinized starch and polyphenolic extracts were mixed and pre–incubated at 37 ºC for 10 min before adding the enzyme, and then the mixture was incubated at 37 ºC (A2). Starch was gelatinized in the presence of the extract, and then cooled at rt for 10 min, until reaching 37 ºC. The enzyme solution was then added, and the mixture incubated at 37 ºC for 10 min (A3). To stop the reaction, 500 µL of 3,5–dinitrosalicylic acid reagent was added and the tubes placed into a boiling water bath for 10 min. Samples were diluted in distilled water (1:10), and their absorbances measured at 540 nm in a microplate reader (Epoch Biotek Instruments, Winooski, USA).

The α–glucosidase from *Saccharomyces cerevisiae* activity was measured using maltose (10 mg/mL) dissolved in sodium phosphate buffer (0.1 M, pH 6.9). The reaction system consisted of 50 µL of seaweed extracts, 50 µL of α–glucosidase (10 U/mL) and 400 µL of maltose, but two different methodologies (G1 and G2) were tested (Figure 4.23). In G1 method, enzyme and polyphenols were initially pre–incubated at 37 ºC for 10 min and then maltose was added to initiate the enzymatic reaction at 37 ºC. For G2 methodology, maltose and seaweed extract were initially pre–incubated at 37 ºC for 10 min, the enzyme was then added. In both, G1 and G2, enzymatic reaction was stopped by boiling samples in a water bath for 10 min. Absorbance was measured at 510 nm using the GOD/POD kit.

Solutions without seaweed extracts and without enzymes were analyzed as control and blank, respectively. The inhibition percentage was calculated by Eq. (4.29):

$$\text{Enzymatic activity decay} = \left[1 - \left(\frac{\mathrm{X}^{+} - \mathrm{X}^{-}}{\mathrm{C}^{+} - \mathrm{C}^{-}}\right)\right] \cdot 100 \qquad (4.29)$$

where, X^+ was the absorbance of sample with substrate and enzyme; X^- was the absorbance without enzyme; C^+ was the absorbance without extracts sample; C^- was the absorbance of substrate.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The IC_{50} value is the sample concentration required for 50% inhibition of the α–amylase or α–glucosidase activity. Acarbose was used as a positive control. Inhibition of enzymatic activities (Section 11) was evaluated for purified (Section 4.5) and crude UAE (Section 4.4.1) extracts together with chemical (Section 4.6), chromatographic (Section 4.11) and ^{1}H–NMR (Section 4.13.2) characterizations to explain the different observed activities. Each proposed method (M1, M2 and M3) and enzyme type (α–amylase and α–glucosidase) was carried by duplicate.

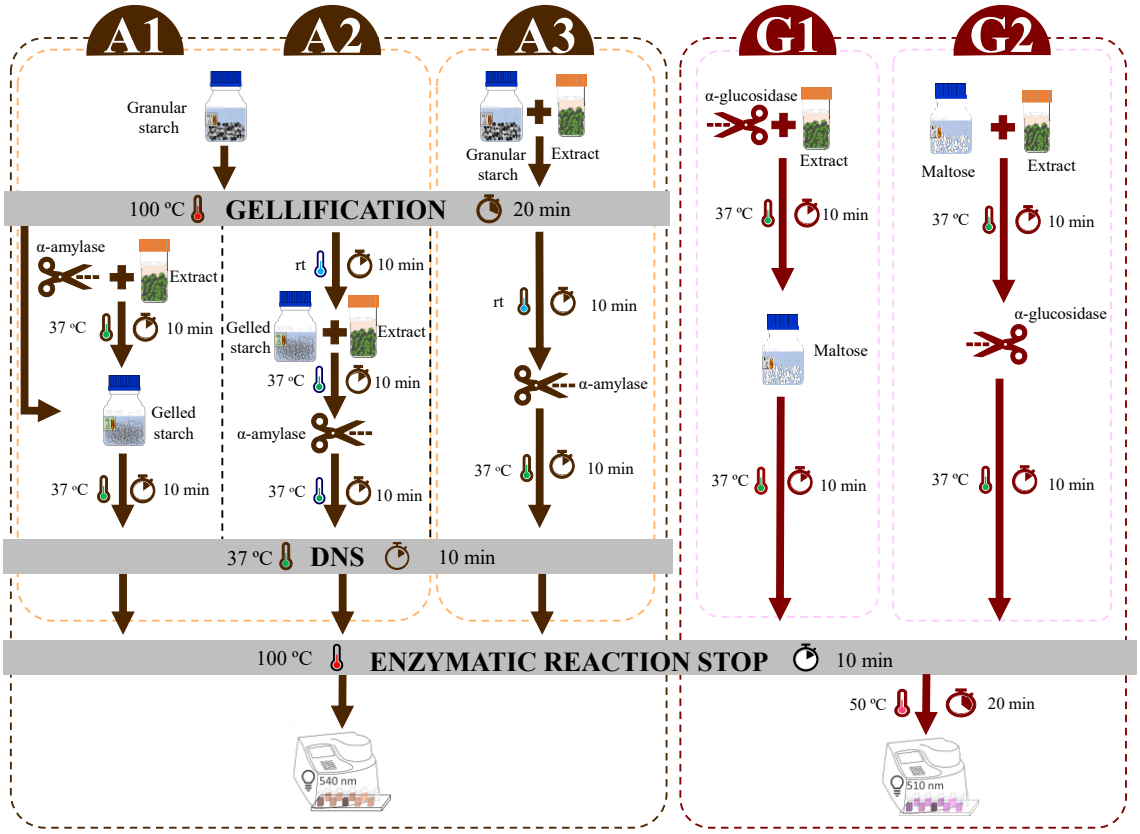


Figure 4.23. Methodology scheme of α–amylase inhibition assays (A1, A2 and A3) and α–glucosidase (G1 and G2) digestive enzymes against aqueous polyphenols extracts from *A. nodosum* seaweed.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.16. SCANNING ELECTRON MICROSCOPY

The effect of extracts addition on samples microstructure was analyzed by scanning electron microscopy (SEM). Samples were sputtered with iridium using a vacuum metallizer/shader model Q150T S (Quorum Technologies Lewes, UK) set to obtain an upper metallic layer of 5–10 nm thickness. Samples micrographs were obtained using a scanning electron microscope (Zeiss FESEM Ultra Plus with EDX, Zeiss, Germany) at 3 Kv using a SE/InLens secondary electron detector. Cross–sectional morphologies of solid phase from seaweed–starch systems allowed the evaluation of interactions and the structural effects (Section 10).

4.17. STATISTICAL ANALYSIS

Statistical analysis was carried out by IBM SPSS statistics 27 (SPSS Inc., USA) software. A one–way analysis of variance (ANOVA) was assessed based on confidence interval of 95% ($p < 0.05$) using a Duncan test. The experimental results were treated and plotted on Microsoft Excel (Microsoft Corporation, USA). All experimental results were expressed as $\bar{x} \pm \sigma$ of at least triplicate experiments (n=3). The root mean squared error (RMSE, Eq. (4.30)) was used to quantify differences between predicted and experimental values with Peleg model in Sections 7 and 10.

$$\mathrm{RMSE} = \sqrt{(\mathrm{X_i} - \mathrm{X_{Model}})^2} \qquad (4.30)$$

where x_i is the experimental value and $\mathrm{X_{Model}}$ is the predicted value.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

5. RESULTS & DISCUSSION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

This section was divided into 6 sections (6–12), Figure 5.1, according to objectives proposed in Section 2, whereas Section 6 (Seaweed flour production and characterization) started with the treatment and characterization of *Ascophyllum nodosum* seaweeds to obtain the raw material of this Thesis. The Section 7 (Extraction method, solvent type, sonication power and liquid solid ratio) and Section 8 (Ultrasound-assisted extraction: effect of operation conditions) encompass the results about the effect of the extraction methods and conditions and thus determine the best conditions for producing extracts with high polyphenols content and antioxidant capacity together with low consumption of energy, time, and solvents.

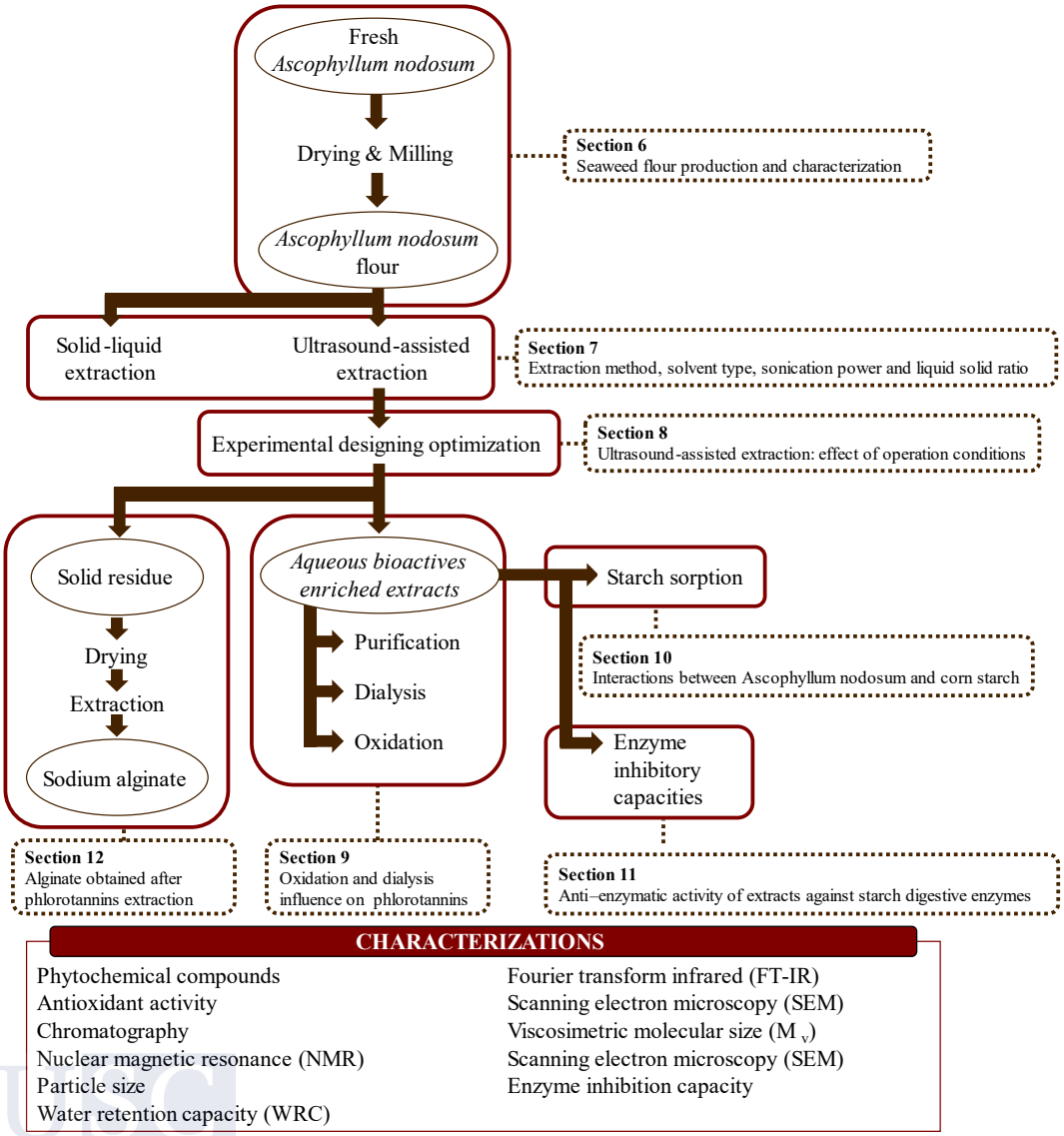


Figure 5.1. General scheme of the Results & Discussion section in the current Thesis.

In the Section 9 (Oxidation and dialysis influence on phlorotannins) it is analyzed the response of seaweeds extracts against severe oxidation conditions and the molecular size cut–off effect on phlorotannins bioactivities. The Section 10 (Interactions between *Ascophyllum nodosum* and corn starch) and Section 11 (Anti–enzymatic activity of extracts against starch digestive enzymes) show the results on the use of bioactive compounds from seaweeds in starchy products (Section 10) and the inhibitory capacities of phlorotannins against α–amylase and α–glucosidase digestive enzymes (Section 11). The potential valorization of residue, generated after phlorotannins extraction, was used to produce sodium alginate is discussed in Section 12 (Alginate obtained after phlorotannins extraction). Specific methodologies carried out in each section are defined in "Assayed systems" section.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

6. *ASCOPHYLLUM NODOSUM* FLOUR OBTENTION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

6.0. GENERALITIES

The current consumption of algae and their derived products is the cause of an increase of their production in recent years (Chenlo et al., 2018). Previously to their manufacture, algae are often preserved in different procedures, being drying/desiccation/dehydration the most common to extend their storage life and make easier their handling (Jayas, 2016). Drying is the oldest and most widely used industrial process to preserve foods with high moisture content, such as fruits and vegetables, increasing its shelf life by reducing the moisture content of the sample, which prevents the microbial growth (Moreira et al., 2016). This process involves simultaneous heat and mass transfers to remove water decreasing the sample volume and the storage requirements. Thus, drying reduces the postharvest losses and provides easiness in storage, transport and ensures product availability (Jayas, 2016). Many biological products are dried at least once for its industrial preparation (Mujumdar, 2006). Thus, drying is one of the most studied processes worldwide due to their energy consumption in manufacturing processes that accounts for around of 10–25% of the total processing energy. Seaweeds are generally sun–dried, process that requires long periods of time. A quicker drying method is the convective air drying although it has operational associated costs. The process is influenced by air temperature and material characteristics, among others that must be controlled (Chenlo et al., 2018). In the recent years, several researchers have been focused on the study of the convective drying with hot air of seaweeds.

Le Lann et al. (2008) reported on the effect of different conditioning treatments (fresh, freezing, freeze–drying, oven–drying and green house drying) on the total phenolic content (TPC) and antioxidant activities of *Sargassum muticum* and *Bifurcaria bifurcata* those with fresh and frozen ones, showing the relevance of the drying conditions in the conservation of the product and its properties. Jiménez–Escrig et al. (2001) reported on the antioxidant activities (DPPH and FRAP) and TPC from seaweeds dried with either natural air flow or forced convection (50 ºC and 48 h) on *Chondrus crispus, Laminaria ochroleuca, Fucus vesiculosus, Porphyra umbilicalis* and *Undaria pinnatifida*. Hence, antioxidant activities and TPC values decreased with processing and storage treatments.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Uribe et al. (2017) studied characteristics of hot–air dried *Durvillaea antarctica* seaweeds at different temperatures (40, 50, 60, 70, and 80 ºC). Higher drying temperatures resulted in an apparent increase of the ash and crude protein contents as compared to fresh sample. Their aim was to obtain a dehydrated seaweed to reduce losses of material, degradation of nutritional values and poor quality during conventional solar drying. Cox et al. (2012) analyzed the effect of various methods (drying pretreatment, boiling, steaming and microwave) on the phytochemicals of *Himantalia elongate* seaweeds. Lee et al. (2010) reported TPC and antioxidant activity values of *Hizikia fusiformis* seaweeds dried using vacuum drying at 20 and 30 ºC and hot air drying at 40 and 60 ºC. Cruces et al. (2016) studied the effect of freeze–drying, silica–drying, oven–drying and airdrying on TPC and antioxidant activity (DPPH) of *Lessonia spicata* seaweed. Whereas freeze–drying showed the best extraction yields and silica– and oven–drying reduced around 20% of phlorotannin extraction yields. The effect of drying treatment was more notorious for DPPH test where scavenging activity was reduced from 65% (freeze–dried) to 21 % (airdried).

Wong & Cheung (2001) studied influence of two drying treatments (oven– and freeze–drying) on the proximate composition and physicochemical properties (swelling, water holding and oil holding capacity) of *Sargassum hemiphyllum*, *Sargassum henslowianum*, *Sargassum patens* seaweeds. Authors reported that freeze–dried seaweeds had greater potential to conservation of food ingredients since proximate composition and physicochemical properties of products were less altered. Norra et al. (2016) evaluated TPC and DPPH values of sun–dried, oven–dried and freeze–dried different seaweeds of *Sargassum spp.* TPC value of varied significantly with drying treatments showing oven–dried the higher TPC and DPPH values. It was outstanding that the less aggressive drying method (freeze–drying) produced the lowest TPC and DPPH values. Drying conditions are critical for thermal sensible compounds as phlorotannins, however extraction conditions could be used to compensate the detrimental effects of drying.

However, hot air convective drying might have some undesirable secondary effects, on later operations (i.e., rehydration), color changes (Maillard reaction) and nutritional modifications (Tello–Ireland, 2015) that could suppose worse taste properties and subsequently decreasing the consumer acceptance. Drying of foodstuffs, including seaweeds, must be carried out by finding a solution that deals with nutritional and bioactive properties, energy, time, costs, and appealing features (Moreira et al., 2016 & 2017).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Other relevant aspect in food operation (drying or extraction processes) is the particle size of the sample. Particle size is critical since operation time depend on the diffusion of compounds in the bulk of the materials. As particle size decreases the outer cells constitute a large proportion and specific surface increases, improving mass transfer flows (for drying and extraction operations). Nevertheless, size reduction is limited by several reasons such as generation of airborne particles, high pressure drop in the extractor, low drainage rates, presence of fine particles in the extracts, flow instability, and entrainments (Oreopoulou, 2003; Moreira et al., 2016).

6.1. ASSAYED SYSTEMS

The objective of Section 6 was to prepare the fresh alga *Ascophyllum nodosum* to ensure its availability throughout the Thesis. The fresh seaweed was dried and grounded (Section 4.2). Finally, with seaweed flour (AF) its average particle size and its water retention capacity (Section 4.3) were determined. The scheme of the research carried out could be seen in Figure 6.1.

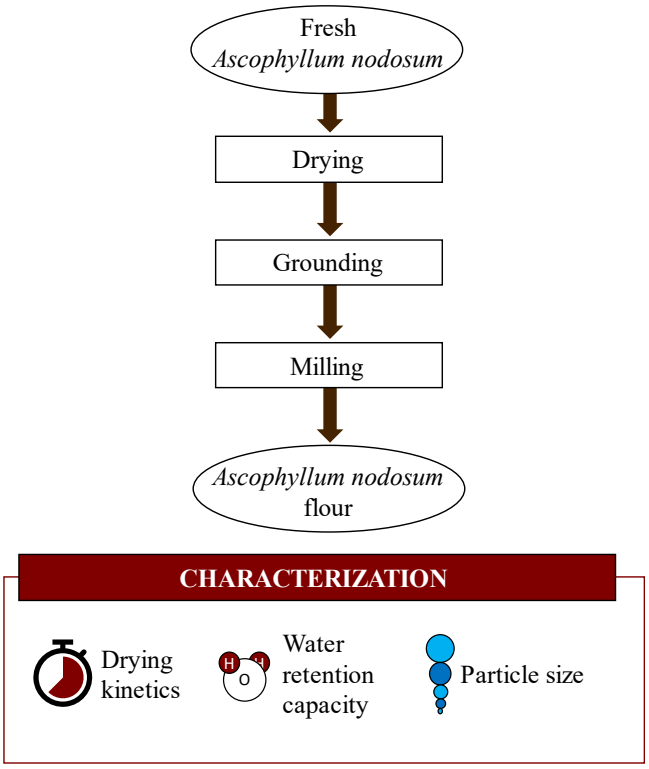


Figure 6.1. *A nodosum* flour obtention, drying, grounding, milling pretreatments.

6.2. DRYING KINETICS

Experimental drying kinetics of *Ascophyllum nodosum* (*A. nodosum*), for deep–bed configuration (load density of 2.0 ± 0.1 kg/m^2) at 50 ºC is shown in Figure 6.2. Drying at 50 ºC needed at least 450 ± 5 min to achieve the equilibrium moisture content (X_{eq}) of 0.02 kg water/kg dried solid ($kg_W/kg_{d.b}$). Similar drying time (480 min) were reported by Chenlo et al., (2018) at the same conditions and seaweed. Moisture content of fresh seaweed (73.7 ± 3.8% d.b), air–dried (4.3 ± 0.02% d.b) and milled (2.9 ± 0.01% d.b) were determined drying samples in a vacuum dryer to determine the moisture content evolution during the flour production (Eq. (4.1)).

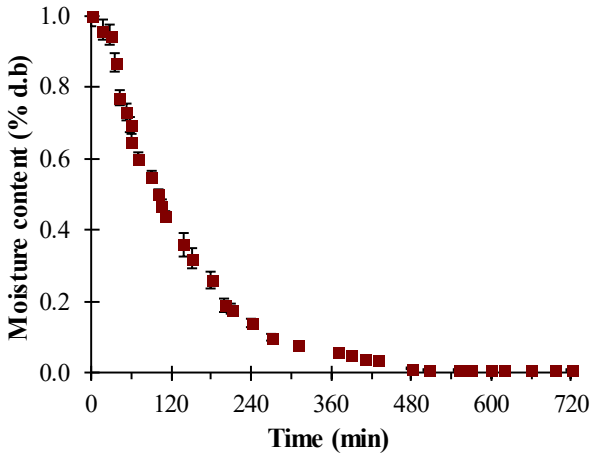


Figure 6.2. Experimental kinetics of *Ascophyllum nodosum* seaweed drying at dry temperature of air at 50 ºC, 30% relative humidity, 2 m/s of air velocity and 2 kg/m^2 of load density.

6.3. PARTICLE SIZE DISTRIBUTION

Particle size distribution of sieved *A. nodosum* flour are shown in Figure 6.3. The largest mass fraction corresponded to 250 to 500 μm range with the 50% of the total mass particles. Particle size fractions with the second highest mass fraction percentage was found in the size ranges 200–250 and 125–200 μm with 19.0 and 16.9% of the total mass particles, respectively. The minimum mass fraction was obtained for particles with a lower size than 40 μm with just 0.1% of the total mass particles. Average particle size, Eq. (4.2), was 276 ± 8 μm.

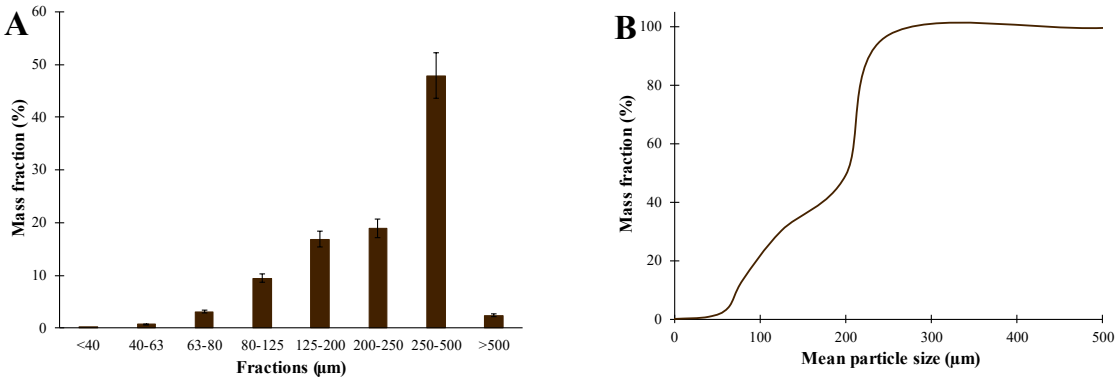


Figure 6.3. Differential (A) and accumulated particle size (B) distribution curve from *A. nodosum* flour after drying and milling.

The obtained flour was used as raw material for all the extractions, characterization, and foods additives studies. Average particle size was in the range of average commercial food additives (Le Bleis et al., 2015) and slightly higher than size (201 µm) reported by Moreira et al. (2016) working with the same seaweeds.

6.4. WATER RETENTION CAPACITY

Water retention capacities (WRC, g_W/g_{DS}) of *A. nodosum* flour was calculated using Eq. (4.3) and corresponded to 6.0 ± 0.9. The obtained seaweeds powders showed WRC value in range with those reported by Gómez–Ordóñez et al. (2010) for *Bifurcaria bifurcata* (4.9 g_W/g_{DS}) and Rupérez & Saura–Calixto (2001) for *Fucus vesiculosus* (5.5 g_W/g_{DS}).

HIGHLIGHTS

After the drying, milling and flour characterization of fresh seaweed the main source of phlorotannins, carbohydrates, and alginates of the Thesis was prepared.

Seaweed treatments ensured the use of seaweed flour and their conditions along the whole Thesis time lapse .

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

7. EXTRACTION SOLVENT AND METHOD ASSESSMENT

Current Section 7 was adapted from:

Gisbert M, Sineiro J, Moreira R (2022) Polyphenols extraction kinetics from *Ascophyllum nodosum* seaweed employing sweet water and saltwater: Effect of ultrasound sonication. *Algal Research–Biomass Biofuels and Bioproducts* 66, 10773. DOI: 10.1016/j.algal.2022.102773 (See Section 17)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

7.1. GENERALITIES

Seaweeds have increasing interest by their high content of bioactive compounds, polyphenols and polysaccharides such as fucoidan, laminarin, ascophyllan, alginates and others (Charoensiddhi et al., 2016; Tavakoli et al., 2021; Cebrián–Lloret et al., 2022). These compounds have demonstrated to have several bioactive characteristics in Sections 1.4, 1.5 and 1.6.

Ascophyllum nodosum (*A. nodosum*) brown seaweed is one of the most widely studied seaweeds by their high content of these bioactive and antioxidant compounds (Kadam et al., 2015c; Catarino et al., 2019). *A. nodosum* polyphenolic compounds (phlorotannins) are secondary metabolites, produced by polyketide pathway reaction of phloroglucinol (Koivikko et al., 2007). Phlorotannins have been reported to act as shelters against seaweeds distresses such as changes of salinity, temperature and desiccation (Tierney et al., 2013a).

Researchers are focused on studying best processing, manufacture, and preservation conditions that maximize bioactive compounds extraction from algae (Naveen et al., 2021; Kadam et al., 2015a). Conventional solid–liquid extraction (SLE) with intermediate polarity solvent mixtures, supercritical fluid extraction, pressurized liquid extraction and centrifugal partition extraction have been proposed; but these methods have some drawbacks previously commented in Section 3.1.

Ultrasound–assisted extraction (UAE) is considered an eco–friendly and highly efficient extraction technique (EPA 2012). It offers high operational reproducibility with short extraction times, simplified and low–cost equipment, reduce solvent consumption, low energy usages, is highly effective to produce enriched antioxidant extracts and can be operated at low temperature that will be discussed in Section 8. In fact, temperature control during phlorotannins extraction is critical due to their thermal sensitive characteristics (Tello–Ireland et al., 2011). Adequate extraction conditions reduce processing and environmental costs and specific bioactive compounds extraction can be enhanced (Chemat & Vian, 2014). Selection of extraction method and operation conditions facilitate high yields and reducing product purities depending on the targeted compounds nature. Solvent is one of the key aspects on seaweed phlorotannins yield and "eco–friendly" extraction.

USC DE SANTIAGO DE COMPOSTELA

Water is the safest solvent usually employed in industrial extractions according to green chemistry principles, however, has low extraction efficiency compared with organo–solvents (EPA 2012; Naveen et al., 2021). Aqueous mixtures of methanol, ethanol, acetone, or acids have shown to be more effective extractants of seaweed phlorotannins based on their polarity affinity (Catarino et al., 2019). Nevertheless, these solvents are volatile, inflammable, and toxic (Chemat & Vian, 2014; Tavakoli et al., 2021;). Water use reduces subsequent purification stages and waste residue generation. The use of seawater as extraction solvent may be an interesting alternative to reduce costs associated to water treatments and is the natural environment of seaweeds implying that solvent and seaweeds could be collected jointly (Charoensiddhi et al., 2016). The effect of ionic strength of solvent on phytochemical extracts characteristics must be determined because phlorotannins and bioactive compounds change their solubility (Noubigh et al., 2007; Tyagi et al., 2021;).

7.2. ASSAYED SYSTEMS

Extracts were chemically characterized by means of polyphenols (Section 4.6.1), carbohydrates (Section 4.6.2) and uronic acids (Section 4.6.3) content and antioxidant capacities by DPPH (Section 4.6.4), ABTS (Section 4.6.5) and FRAP (Section 4.6.6) methods. Extracts carried out with saline solvent (S–extracts) showed lower TPC than those obtained with distilled water (D) using two different extraction methods, conventional solid–liquid extraction (Section 4.4.1) and ultrasound–assisted extraction (Section 4.4.2). SLE kinetics employing both solvents (D and S) were successfully modelled by Peleg's model (Section 4.4.1.1). Long–time UAE (15 min) increased phytochemical content in both D– and S–extracts with respect to SLE. Extracts showed three main (A, B, and C) regions from RP–HPLC gradient mode (Section 4.9) with different peak areas and shapes. At these conditions, saltwater can be employed as eco–friendly solvent according to the green chemistry principles. The aim of Section 7 was to determine and model polyphenol extraction kinetics from *A. nodosum* seaweed by means of different procedures (SLE and UAE) to establish extraction times and, on the other hand, to measure the ionic strength effect using two distilled aqueous based solvents for the extraction, distilled water (D) and salty water (S) on aqueous extracts features as it is summarized in Figure 7.1.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

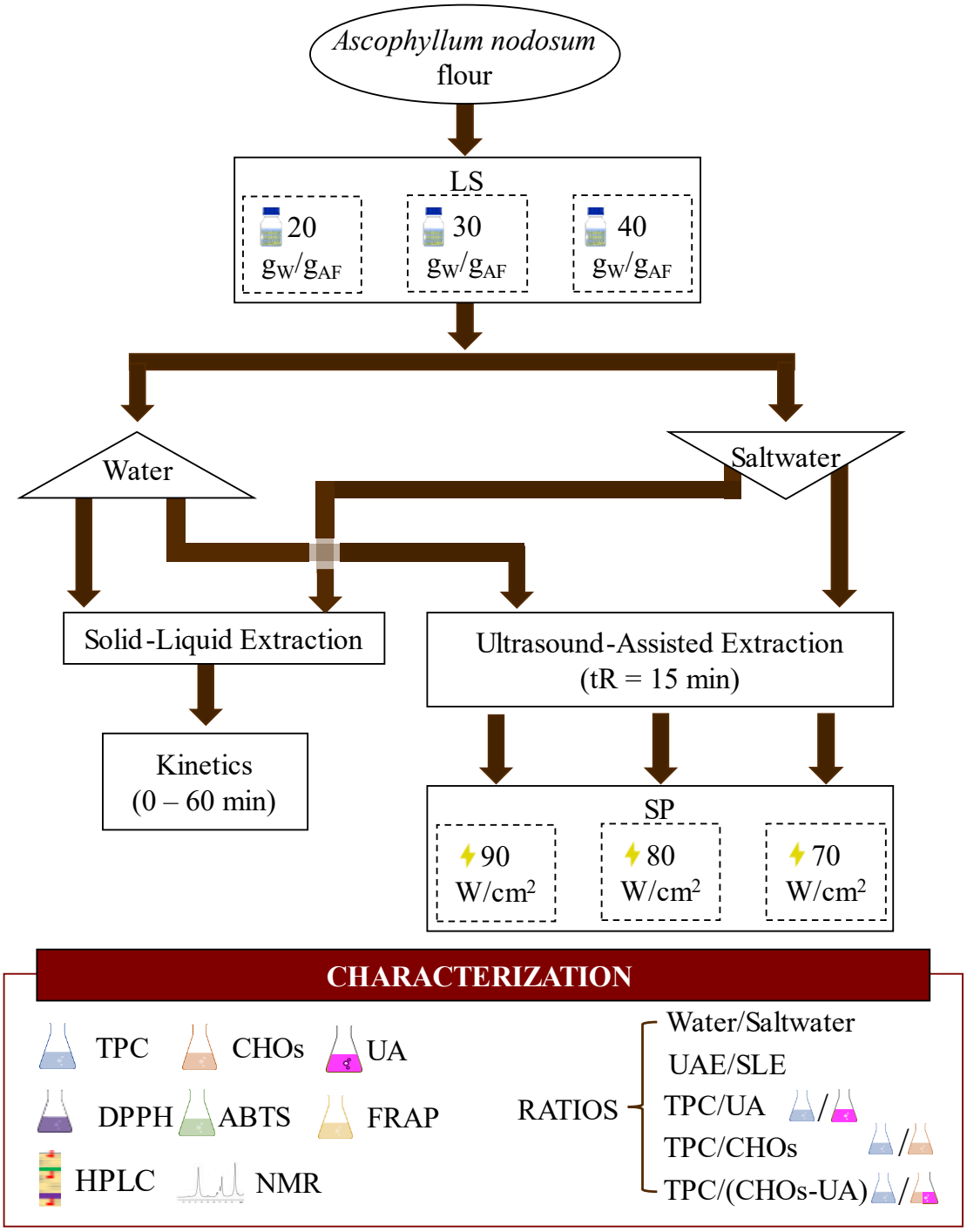


Figure 7.1. *A. nodosum* extracts characterization to evaluate extraction method, solid–liquid extraction, and ultrasound–assisted extraction.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

7.3. SOLID–LIQUID EXTRACTION (SLE)

The main results (TPC, CHOs, UA, and antioxidant activities (DPPH, ABTS and FRAP)) from the kinetic studies of solid–liquid extraction (SLE) of are shown in Figure 7.2. These studies were performed at 20, 30 and 40 g_W/g_{AF} solid–liquid ratio for 60 min of contact at rt., between dried *A. nodosum* seaweed flour (AF) and double–distilled (D) or saltwater (S) solvent, maintaining a stirring speed of 170 rpm. The selected assayed determinations were chosen as rapid, easy, highly reproducible, low–cost and very extensively used for plant and seaweeds extracts characterisation (Shodehinde & Oboh, 2014; Kumar et al., 2020; Naveen et al., 2021;).

TPC of D–extracts (Figure 7.2A) initial value (2 min) was 1.91 ± 0.04 until reached steady state (10 – 15 min) with a value of 2.1 ± 0.04 g_{PE}/L. However, S–solvent gave initial TPC values of 1.6 ± 0.02 that slightly ($p > 0.05$) increased up to 1.7 ± 0.1 g_{PE}/L at the stationary state (achieved about 15 min). TPC initial value of D30 and D40 (Figure 7.2A) were 1.1 ± 0.04 and 1.0 ± 0.04 gPE/L, respectively. Similarly, to LS20 extracts, both reached steady state (10–15 min) with values of 1.6 ± 0.2 and 1.3 ± 0.2, respectively. Conversely, S–samples initial TPC values were 0.9 ± 0.1 and 0.5 ± 0.03 for S30 and S40, respectively, that increased up to 1.2 ± 0.04 and 0.7 ± 0.03, respectively, at the stationary state (achieved about 15 min). CHOs (g_{GE}/L) values of D–extracts (Figure 7.2B) values started with 1.6 ± 0.1 (D20), 0.8 ± 0.1 (D30) and 0.8 ± 0.1 (D40) at short time and increased until 2.2 ± 0.03, 1.5 ± 0.1 and 1.1 ± 0.1, respectively, at the steady state, CHOs of S–extracts values started at 0.8 ± 0.1 (D20), 0.4 ± 0.03 (D30) and 0.2 ± 0.07 (D40) and reached higher steady values of 1.2 ± 0.03, 0.9 ± 0.1 and 0.6 ± 0.06, respectively. Steady CHOs content at 15–30 min, with both solvents and all LS ratios, was reached. Both initial UA (Figure 7.2C) values for LS20 samples were 0.5 ± 0.02 and 0.5 ± 0.04 until achieving 0.9 ± 0.04 and 0.6 ± 0.04 g_{GE}/L after 15–30 min for D– and S–solvent, respectively. D30 started with 0.3 ± 0.1 (D) and 0.3 ± 0.04 (S) that increased up to 0.6 ± 0.03 and 0.4 ± 0.04 at the steady state, respectively. Finally, LS40 samples started with 0.2 ± 0.02 and 0.2 ± 0.04 until achieving 0.5 ± 0.04 and 0.2 ± 0.05 after 15 min for D– and S–solvent, respectively.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Antioxidant activities of the extracts employing saltwater were systematically (slightly) lower with respect to distilled water. DPPH (Figure 7.2D) scavenging activity kinetics begun with slightly similar value of 2.8 ± 0.1 (LS20), 2.4 ± 0.1 (LS30) and 1.8 ± 0.04 (LS40), for both solvents. Then, reached close values of 3.6 ± 0.3, 2.6 ± 0.1 and 2.1 ± 0.1 mM_{TE}, respectively, at the steady state (10–15 min). Similar trend was observed from ABTS (Figure 7.2E) results, starting both D– and S–extracts, at 0.3 ± 0.02, 0.2 ± 0.01 and 0.1 ± 0.01 achieving similar steady state (10–15 min) values of 0.4 ± 0.02, 0.3 ± 0.04 and 0.2 ± 0.03 mM_{TE} for samples at 20, 30 and 40 g_W/g_{AF} of LS ratios, respectively. Finally, FRAP (Figure 7.2F) average values started at 36.9 ± 1.8 (LS20), 25.1 ± 1.9 (LS30) and 16.8 ± 2.0 (LS40) until both, D– and S–samples, reached the steady state (10–15 min) value of 46.9 ± 2.3, 30.7 ± 1.5 and 24.8 ± 0.5 μM_{TE}, for 20, 30 and 40 of LS ratio, respectively.

From extraction kinetics, it can be observed that steady state was achieved after 15 min of extraction time for tested chemical and phytochemical characteristics, independently of extraction solvent and the LS ratios employed. Both, solvent and LS ratio had a notorious effect on all studied compounds content (TPC, CHOs and UA) being significant ($p < 0.05$) higher for D– than S–extracts. However, no significant ($p < 0.05$) differences of antioxidant activities of these extracts were found. It was concluded that S–solvent was worse phytochemicals extractant than D–solvent, but S–extracts bioactivity was slightly lower.

Saline effect on extraction kinetics can be explained by several reasons. Ionic strength is a crucial factor that could affect to quality, properties and extraction yields of some proteins, since saline environments increase the proteins' solubility and prevent undesired protein unfolds (Koysuren et al., 2021). UA values reduction could be explained by a partial precipitation of alginic acid into sodium alginate (Pamies et al., 2010). These facts could contribute to obtain higher antioxidant activities than expected in saline extracts with low TPC content (SLE samples). Other authors found that low ionic strength solvents (D–solvent) promote the disruption of the structural integrity of the cell wall that caused a notorious bioactive compound releasing (Charoensiddhi et al., 2016). These authors demonstrated that saline buffers (saline, phosphate, and acetate) reduced the alga osmotic initial shock preventing cell–wall disruption and compounds releasing as behaviour observed in Figure 7.2.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

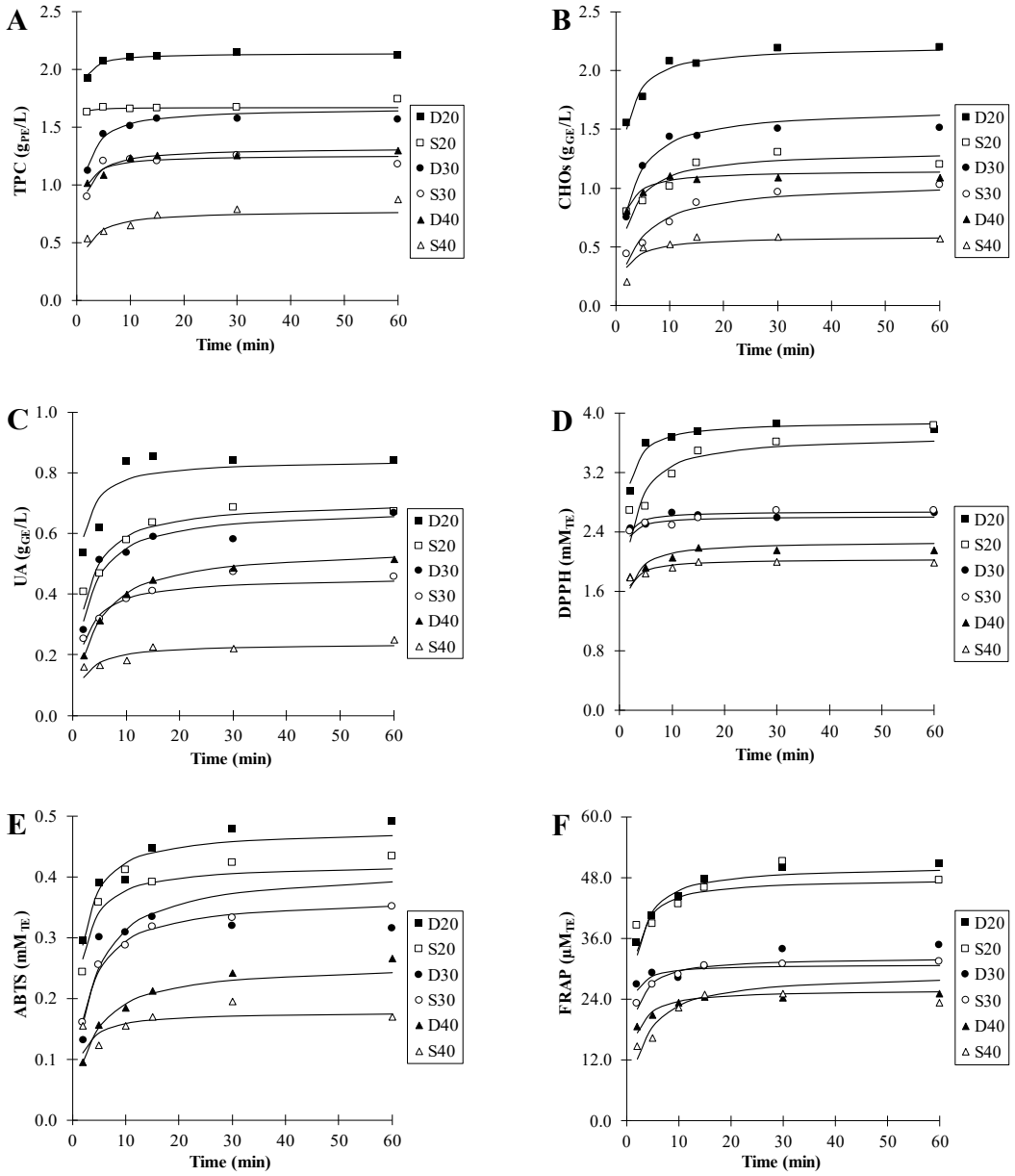


Figure 7.2. Experimental data (dots) and Peleg's model values (lines) for TPC (A), CHOs (B) UA (C), DPPH (D), ABTS (E) and FRAP (F) of SLE kinetics using water (D) and saltwater (S) for extractions at 20, 30 and 40 g_W/g_{AF} of liquid–solid ratio.

Obtained extracts showed higher content than those previously reported. Tierney et al. (2014) obtained lower TPC (75 to 150 $\mu g_{PE}/mg_{extract}$) and FRAP values (50 to 150 $\mu g_{TE}/mg_{extract}$) using different extraction conditions (LS = 10 g_W/g_{AF}, 170 rpm, rt, unspecified time). Kadam et al. (2015b) using the same LS value (20 g_W/g_{AF}) at 70 ºC for 2.5 h obtained lower TPC values (0.2 mg_{PE}/g_{AF}). However, other processing conditions (particle size, pre– and posttreatments) can notoriously affect the extraction rate and (pseudo–) equilibrium.

7.3.1. SOLID–LIQUID EXTRACTION KINETICS MODELLING

SLE kinetics of D– and S–samples (Figure 7.2) showed high initial extraction rates followed by a slow–down (around 15 min) by the decrease of driving force for mass transfer, until pseudo–equilibrium was reached. Results indicated that times longer than 15 min have not a relevant influence on the content in the extracts. Specifically, D–samples reached equilibrium before 10–15 min, but S–samples needed at least 15 min to achieve it.

Peleg's model (Eq. (4.4)) was satisfactorily ($R^2 > 0.81$ and RMSE < 2.97) employed for extractions kinetics modelling and the agreement between experimental data and predicted values can be observed in Figure 7.2. Higher K_1 and K_2 values mainly observed from S–samples, evidenced the slightly lower extraction rates and extraction capacities for TPC, CHOs and UA content with respect to D–samples (Table 7.1). The equilibrium values (X_{eq}) of bioactive compounds were higher for D–samples for all phytochemical characterizations. Nevertheless, these differences were not directly observed for antioxidant activity values, making necessary further analyses.

7.4. ULTRASOUND–ASSISTED EXTRACTION (UAE)

Experimental bioactive compounds (TPC, CHOs and UA) content and antioxidant activities (DPPH ABTS and FRAP) of SLE extracts at equilibrium (15 min) with D– and S–solvent previously shown (Section 7.3.1) were compared with UAE carried out at 20, 30 and 40 g_W/g_{AF} of LS ratio, named as UAE20–90, UAE30–90 and UAE40–90, respectively for 15 min, using D– and S–solvents and 90 W/cm^2 of sonication power (Figure 7.3). Additionally, UAE20 samples were sonicated at 70 (UAE20–70) and 80 (UAE20–80) W/cm^2 to determine the effect of sonication power on compounds extracted.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 7.1. Peleg's model parameters (K_1 and K_2) and $X_{eq} = 1/K_2$ for extracts bioactive compounds content (TPC, CHOs and UA) and antioxidant activities (DPPH, ABTS and FRAP) for SLE kinetics using water (D) and saltwater (S) at 20, 30 and 40 g_W/g_{AF}.

Param.	LS	Sol.	TPC (Y = g_{PE}/L)	CHOs (Y = g_{GE}/ L)	UA (Y = g_{GE}/L)	DPPH (Y = mM_{TE})	ABTS (Y = mM_{TE})	FRAP (Y = μM_{TE})
K_1 (min/Y)	*20*	*D*	0.1 ± 0.01^{a}	0.4 ± 0.04^{a}	1.0 ± 0.10^{a}	0.1 ± 0.01^{a,b}	2.8 ± 0.3^{a}	0.02 ± 0.00^{a}
		S	0.02 ± 0.00^{a}	1.5 ± 0.2^{c}	2.9 ± 0.3^{b}	0.3 ± 0.03^{c}	2.8 ± 0.3^{a}	0.02 ± 0.00^{a}
	30	*D*	0.5 ± 0.03^{b}	1.3 ± 0.1^{b,c}	3.5 ± 0.4^{b}	0.08 ± 0.01^{a}	7.9 ± 0.8^{b}	0.01 ± 0.00^{a}
		S	0.4 ± 0.04^{b}	3.7 ± 0.4^{e}	4.2 ± 0.4^{b}	0.09 ± 0.01^{a}	6.8 ± 0.7^{b}	0.03 ± 0.00^{a,b}
	40	*D*	0.6 ± 0.04^{b}	0.7 ± 0.1^{a,b}	6.8 ± 0.7^{c}	0.3 ± 0.03^{c}	13.4 ± 1.3^{c}	0.04 ± 0.00^{b}
		S	1.7 ± 0.2^{c}	2.7 ± 0.3^{d}	7.7 ± 0.7^{c}	0.2 ± 0.02^{b}	6.9 ± 0.7^{b}	0.10 ± 0.01^{c}
K_2 (Y^{-1})	*20*	*D*	0.5 ± 0.04^{a}	0.5 ± 0.04^{a}	1.2 ± 0.1^{a}	0.3 ± 0.03^{a}	2.1 ± 0.2^{a}	0.02 ± 0.00^{a}
		S	0.6 ± 0.1^{a,b}	0.8 ± 0.1^{a,b,c}	1.4 ± 0.1^{a,b}	0.3 ± 0.03^{a}	2.4 ± 0.2^{a}	0.02 ± 0.00^{a,b}
	30	*D*	0.6 ± 0.1^{a,b}	0.6 ± 0.04^{a,b}	1.5 ± 0.2^{a,b}	0.4 ± 0.04^{a,b}	2.4 ± 0.2^{a}	0.03 ± 0.00^{c}
		S	0.8 ± 0.1^{b}	1.0 ± 0.1^{c}	2.2 ± 0.2^{b}	0.4 ± 0.04^{a,b}	2.7 ± 0.2^{a,b}	0.03 ± 0.00^{b,c}
	40	*D*	0.8 ± 0.1^{a,b}	0.9 ± 0.1^{b,c}	1.8 ± 0.2^{a,b}	0.4 ± 0.04^{b}	3.9 ± 0.3^{b}	0.04 ± 0.00^{c}
		S	1.3 ± 0.1^{c}	1.7 ± 0.2^{d}	4.2 ± 0.4^{c}	0.5 ± 0.05^{b}	5.6 ± 0.4^{c}	0.03 ± 0.00^{c}
X_{eq} (Y)	*20*	*D*	2.1 ± 0.2^{c}	2.2 ± 0.2^{d}	0.8 ± 0.1^{d}	3.9 ± 0.4^{b}	0.5 ± 0.05^{c}	50.4 ± 5.0^{b}
		S	1.7 ± 0.2^{b,c}	1.3 ± 0.1^{b,c}	0.7 ± 0.1^{c,d}	3.7 ± 0.4^{b}	0.4 ± 0.04^{c}	47.8 ± 4.8^{b}
	30	*D*	1.7 ± 0.2^{b,c}	1.7 ± 0.1^{c}	0.7 ± 0.1^{c,d}	2.7 ± 0.3^{a}	0.4 ± 0.04^{c}	30.9 ± 3.1^{a}
		S	1.3 ± 0.1^{a,b}	1.1 ± 0.1^{a,b}	0.5 ± 0.04^{b}	2.6 ± 0.3^{a}	0.4 ± 0.04^{b,c}	32.3 ± 3.2^{a}
	40	*D*	1.3 ± 0.1^{a,b}	1.2 ± 0.1^{b}	0.6 ± 0.04^{b,c}	2.3 ± 0.2^{a}	0.3 ± 0.03^{a,b}	25.9 ± 2.6^{a}
		S	0.8 ± 0.1^{a}	0.6 ± 0.04^{a}	0.2 ± 0.02^{a}	2.0 ± 0.2^{a}	0.2 ± 0.02^{a}	29.1 ± 2.9^{a}
R^2	*20*	*D*	1.0 ± 0.1	1.0 ± 0.10	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
		S	0.9 ± 0.1	0.9 ± 0.09	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
	30	*D*	1.0 ± 0.1	1.0 ± 0.10	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
		S	1.0 ± 0.1	1.0 ± 0.10	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
	40	*D*	1.0 ± 0.1	1.0 ± 0.10	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
		S	0.9 ± 0.1	1.0 ± 0.10	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
RMSE	*20*	*D*	0.02 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.02 ± 0.00	1.5 ± 0.2
		S	0.03 ± 0.00	0.08 ± 0.01	0.03 ± 0.00	0.2 ± 0.02	0.02 ± 0.00	3.0 ± 0.3
	30	*D*	0.04 ± 0.00	0.06 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	2.4 ± 0.2
		S	0.06 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.06 ± 0.01	2.3 ± 0.2	0.6 ± 0.1
	40	*D*	0.04 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.08 ± 0.01	0.01 ± 0.00	0.7 ± 0.1
		S	0.06 ± 0.01	0.06 ± 0.01	0.02 ± 0.00	0.05 ± 0.01	0.02 ± 0.00	2.4 ± 0.2

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

TPC (g_{PE}/L) values (Figure 7.3A) notoriously increased with sonication for both solvents for D (from 2.1 ± 0.04 to 3.1 ± 0.08) and S (from 1.7 ± 0.1 to 2.8 ± 0.1) in samples with LS = 20 g_W/g_{AF}. Meanwhile LS = 30 g_W/g_{AF} extracts with D–solvent increased from 1.6 ± 0.2 up to 1.9 ± 0.2 and S–solvent from 1.2 ± 0.1 up to 1.8 ± 0.2. And samples with LS = 40 g_W/g_{AF} ranged, from 1.3 ± 0.1 up to 1.7 ± 0.2 in D– samples and from 0.7 ± 0.1 up to 1.4 ± 0.1 in S–samples. In all cases, TPC increased notoriously with SP and inversely with LS values

Figure 7.3B showed that the lowest CHOs (g_{GE}/L) values (1.08 ± 0.11 and 0.58 ± 0.05, for D– and S–solvents, respectively) were obtained with SLE at the highest LS value (40 g_W/g_{AF}). Meanwhile, the highest CHOs values (2.6 ± 0.3 and 2.1 ± 0.2) were obtained at the lowest LS value (20 g_W/g_{AF}) and highest SP (90 W/cm^2). Similar trend was observed from UA values (Figure 7.3C) where the lowest UA (g_{GE}/L) content corresponded to SLE40 (D = 0.5 ± 0.04 and S = 0.2 ± 0.03) and the highest UA content were obtained with UAE treatment and the lowest LS (1.0 ± 0.1 and 0.9 ± 0.1 for D– and S–samples, respectively). The use of sonication had notorious effect on CHOs but UA increased in a narrow range. Similar results could be seen in Section 8. where sonication increased the CHOs and UA content

DPPH (Figure 7.3D), ABTS (Figure 7.3E) and FRAP (Figure 7.3F) showed similar trends with solvent type, LS ratio and SP value. These results indicate that extraction method had effects on bioactive compounds (TPC, CHOS and UA) extraction and antioxidant activities (DPPH, ABTS and FRAP) values. Sonication improved extraction due to seaweed tissue disruption (Kadam et al., 2015a; Tavakoli et al., 2021).

As an example of the notorious effect that UAE method it can be indicated that DPPH (mM_{TE}) increased from 3.8 ± 0.4 until 8.1 ± 0.8 in D–extracts and from 3.5 ± 0.3 up to 6.5 ± 0.6 in S–samples, in both cases from UAE40 up to UAE20 both at 90 W/cm^2. It was also evaluated the effect of sonication power (SP = 70, 80 and 90 W/cm^2) and not significant ($p > 0.05$) effect on phytochemical compounds as well as antioxidant activities of extracts, showing at the highest SP (90 W/cm^2) extracts the highest activities, mainly with D–solvent. Lower SP studied conditions (70 and 80 W/cm^2) did no show significant ($p < 0.05$) differences. This could be due to more amount of disrupted (deeper) tissue or the apparition of locally heated zones promoted by sonication.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

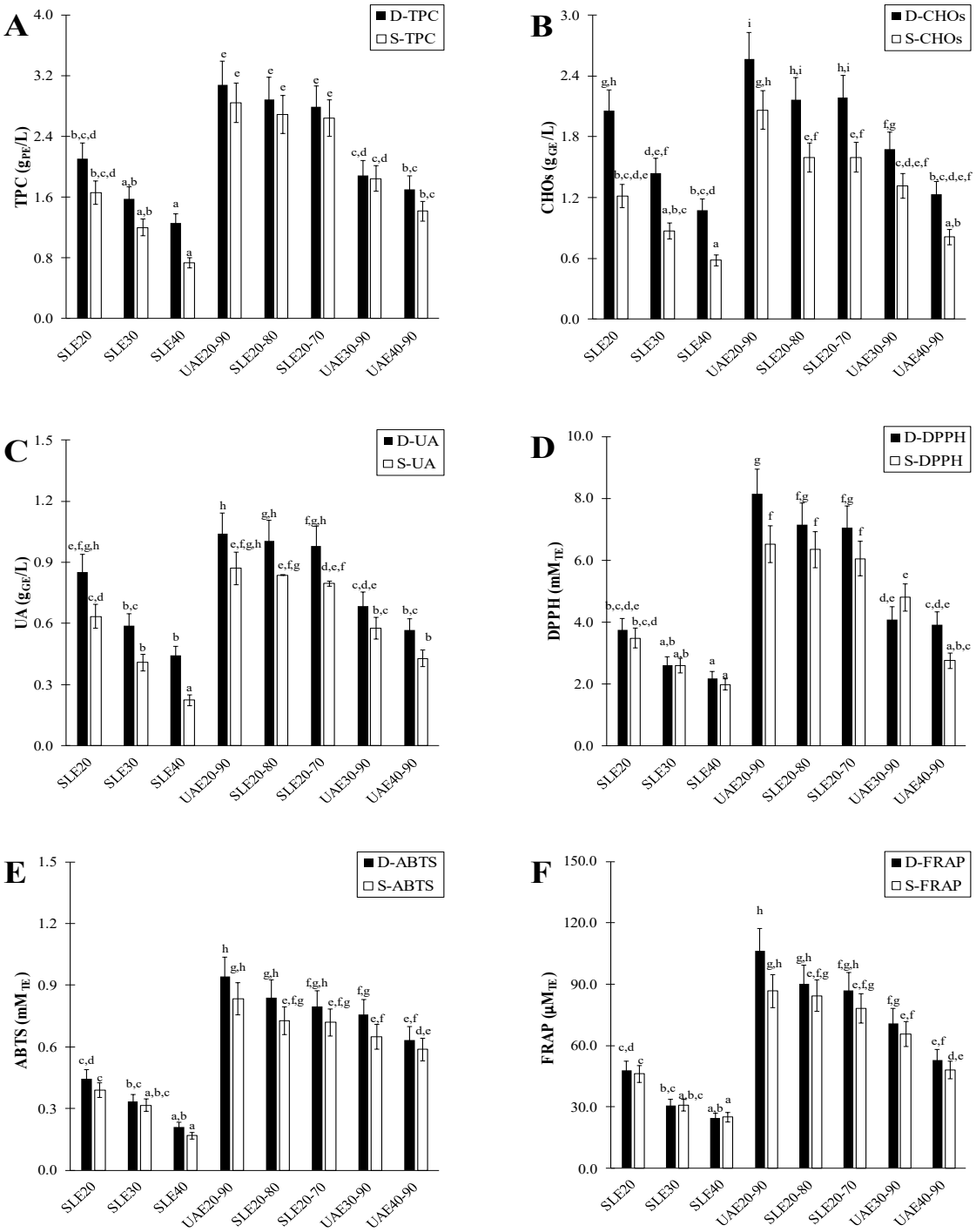


Figure 7.3. TPC (A), CHOs (B), UA (C), DPPH (D), ABTS (E) and FRAP (F) values of UAE (sonication power of 90, 80 and 70 W/cm^2) and SLE using D– and S–solvents at 15 min of extraction with 20, 30 and 40 g_W/g_{AF} of LS ratios. Different superscript letters above bars mean significant ($p < 0.05$) differences between values.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

With regards to previous research on UAE extraction, it was reported that 15 min of UAE guarantee efficient TPC values (Kadam et al., 2015a,b and c; Tavakoli et al., 2021). Nevertheless, long–term UAE could increase the thermal degradability of polyphenols (Tello–Ireland et al., 2011) and to avoid it, additional refrigeration costs are involved. Furthermore, long–term UAE prone the coextraction of other seaweed compounds such as alginates, fucoidans or proteins (Moreira et al., 2016; Catarino et al., 2019). Short sonication times are enough to obtain high polyphenols content in the extracts from *A. nodosum* seaweed, further seen in Section 8. By this reason, seaweeds at LS = 20 g_W/g_{AF} using D–solvent were subjected firstly to a SLE (15 min, rt and 170 rpm) and secondly to a UAE (90 W/cm^2, below 40 ºC) for only 2 min. Aqueous extracts showed TPC values of 2.8 ± 0.01 g_{PE}/L. This value was just 1.11–fold lower than the reported in this study for D–extracts sonicated at 90 W/cm^2 employing sonication 7.5–fold times longer. Further, antioxidant activities measured by DPPH, ABTS and FRAP methods increased just up to 1.1–, 1.1– and 1.7–fold times, respectively. Secondary compounds such as CHOs (1.5 ± 0.02 g_{GE}/L) and UA (0.8 ± 0.04 g_{GE}/L) notoriously decreased 1.68– and 1.8–fold, respectively. So, short–term UAE post–extraction (2 min) after 15 min of SLE produced extracts with TPC and antioxidant activities in the range of those obtained after continuous UAE with lower energy consumption and carbohydrates (CHOs and UA) content, increasing extraction selectivity of polyphenols.

By comparing UAE results in this research work with literature, the yields were lower than those obtained by Kadam et al. (2015a) for dried *A. nodosum* (12 h at 40 °C) under severe UAE conditions (25 min of extraction time, LS = 10 g_W/g_{AF} and SP = 75.8 W/cm^2) where authors showed TPC value of 14.3 g_{GE}/L expressed in gallic acid equivalents, CHOs of 8.7 g_{GE}/L and UA of 11.7 g_{GE}/L. However, UAE data were slightly higher than those reported by Moreira et al. (2016) for dried *Fucus vesiculosus* (8 h at 50 °C) using moderate UAE conditions (4 min, LS = 30 g_W/g_{AF} and SP = 80 W/cm^2) obtained extracts with 0.5 g_{PE}/L, 1.7 g_{GE}/L and 0.7 g_{GE}/L of TPC, CHOs and UA values, respectively.

Linear correlations between antioxidant activities (DPPH, ABTS, and FRAP) and TPC of SLE and UAE extracts at different SP (90, 80 and 70 W/cm^2) and LS (20, 30 and 40 g_W/g_{AF}) were found (Figure 7.4). Three methods showed suitable correlations ($R^2 > 0.93$) when data were treated without distinction between solvents (D and S). These correlations made evident that solvents had not significant ($p < 0.05$) effects on extracted phlorotannins profile.

These correlations showed that S–solvent was an effective extractant of seaweed polyphenols. Interestingly, obtained correlations evidenced that polyphenols present in the extracts, regardless of extraction conditions (SLE or UAE), remained chemically similar (Connan et al., 2004) and TPC gave the main antioxidant responses of the extracts. Other researchers have found these linear correlations in aqueous extracts from marine seaweeds (Moreira et al., 2016).

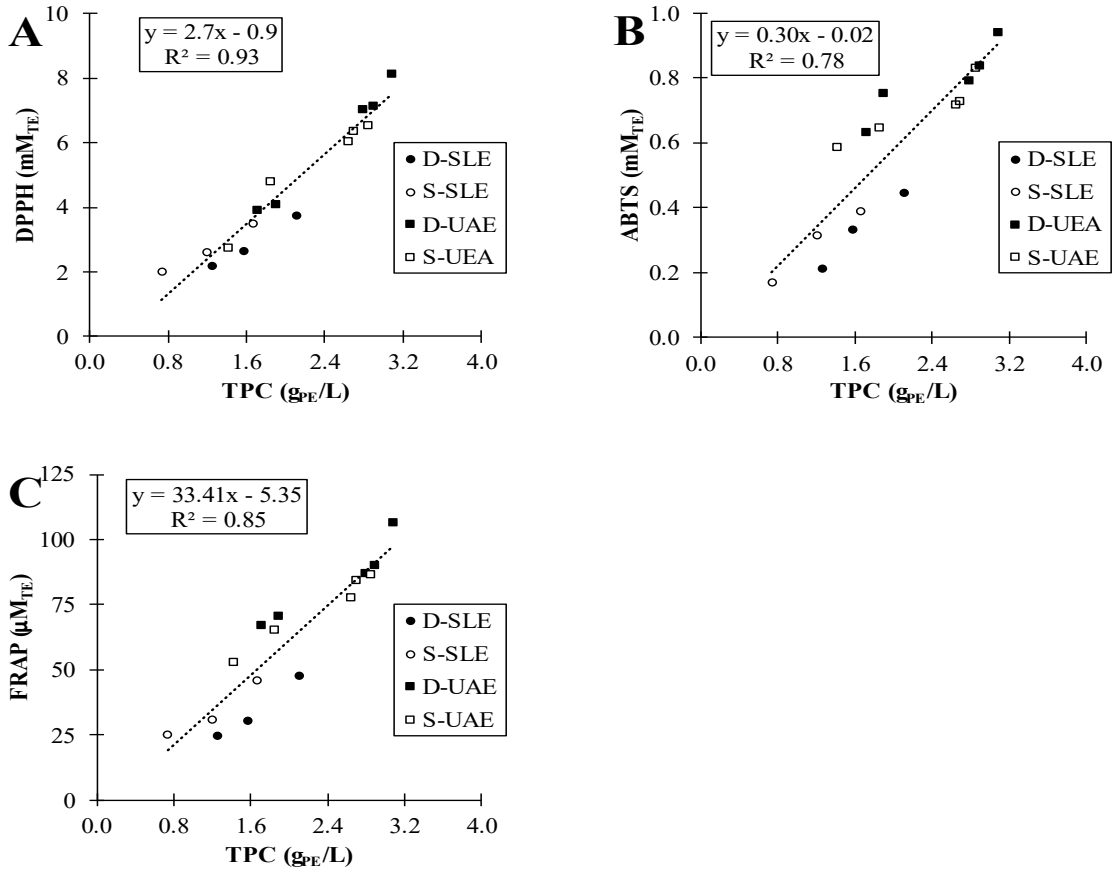


Figure 7.4. Linear relationships between TPC and antioxidant activities, DPPH (A), ABTS (B) and FRAP (C), from SLE and UAE (70, 80 and 90 W/cm^2) extracts using D– and S–solvents at 15 min of extraction with 20, 30 and 40 g_W/g_{AF} LS ratios.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

7.4.1. ANALYSIS OF COMPOSITION AND ACTIVITY RATIOS

Each extract, from SLE and UAE, using D– or S–solvent and from different LS ratio (20, 30 or 40 g_W/g_{AF}) for SP = 90 W/cm^2 gave different phytochemical compositions. It was proposed the deep study of the extracts by ratios of their different features determined, setting maximum TPC as main criterion. Studied ratios were extraction yield (Table 7.2), TPC ratios (Table 7.3), assayed method ratio (Table 7.4) and solvent ratio (Table 7.5). Ratio UAE/SLE was proposed to evaluate the effect of extraction method. UAE/SLE ratio (Table 7.4) showed that all phytochemical compounds, polyphenols, carbohydrates, uronic acids as well as antioxidant activities, presented values higher than 1, evidencing superior UAE extractive capacities had against SLE for any LS ratio (20, 30 and 40 g_W/g_{AF}) and solvent type used (D and S). UAE/SLE ratio showed greater values for S–samples due to the low extraction values they had in SLE that were compensated in UAE treatment Table 5.5 (solvent type ratio).

Table 7.2. Extraction yields (Eq. (4.5)) in SLE and UAE (SP = 90 W/cm^2) samples of phytochemical compounds (TPC, CHOs, UA and antioxidant activities) from *A. nodosum* seaweed with 20, 30 and 40 g_W/g_{AF} of LS ratios using D– and S–solvents. Different superscript letters within a column indicate significant ($p < 0.05$) differences between values.

Method	LS	Sol.	TPC (mg_{PE}/g_{AF})	CHOs (mg_{GE}/g_{AF})	UA (mg_{GE}/g_{AF})	DPPH (mg_{TE}/g_{AF})	ABTS (mg_{TE}/g_{AF})	FRAP (mg_{TE}/g_{AF})
SLE	20	D	42.7 ± 0.8^{b}	$44.1 \pm 1.2^{c,d}$	$16.9 \pm 0.7^{c,d}$	19.5 ± 2.0^{a}	$2.4 \pm 0.2^{a,b}$	0.3 ± 0.03^{a}
		S	33.3 ± 1.5^{a}	26.3 ± 0.6^{a}	$14.1 \pm 0.6^{b,c}$	18.5 ± 1.9^{a}	$2.1 \pm 0.2^{a,b}$	0.2 ± 0.02^{a}
	30	D	$49.9 \pm 5.2^{b,c}$	$50.5 \pm 1.7^{c,d}$	20.5 ± 0.3^{e}	20.1 ± 2.0^{a}	3.1 ± 0.3^{c}	0.2 ± 0.02^{a}
		S	37.7 ± 1.2^{a}	$31.4 \pm 4.5^{a,b}$	13.8 ± 1.5^{b}	19.6 ± 2.0^{a}	$2.8 \pm 0.3^{b,c}$	0.2 ± 0.02^{a}
	40	D	$52.9 \pm 6.0^{b,c,d}$	$45.9 \pm 0.3^{c,d}$	22.2 ± 2.0^{e}	$22.7 \pm 2.3^{a,b}$	$2.6 \pm 0.3^{b,c}$	0.3 ± 0.03^{a}
		S	31.1 ± 1.1^{a}	23.8 ± 2.2^{a}	9.5 ± 2.0^{a}	20.4 ± 2.0^{a}	1.8 ± 0.2^{a}	0.3 ± 0.03^{a}
UAE	20	D	$61.6 \pm 4.7^{c,d,e}$	51.4 ± 9.2^{d}	20.8 ± 0.3^{e}	40.8 ± 12.6^{c}	$4.7 \pm 0.04^{d,e}$	0.5 ± 0.01^{c}
		S	$56.9 \pm 4.3^{c,d,e}$	$41.3 \pm 0.1^{b,c,}$	17.4 ± 0.1^{d}	$32.7 \pm 0.8^{a,b,c}$	4.2 ± 0.04^{d}	0.4 ± 0.02^{b}
	30	D	$63.8 \pm 2.1^{d,e}$	$50.3 \pm 1.9^{c,d}$	20.6 ± 0.1^{e}	$30.7 \pm 2.7^{a,b,c}$	5.7 ± 0.1^{f}	0.5 ± 0.03^{c}
		S	$58.7 \pm 3.8^{c,d,e}$	$39.4 \pm 3.9^{b,c}$	$17.3 \pm 0.2^{c,}$	$36.6 \pm 1.6^{a,b,c}$	4.9 ± 0.2^{e}	$0.5 \pm 0.01^{b,c}$
	40	D	68.2 ± 0.4^{e}	$49.4 \pm 1.0^{c,d}$	22.7 ± 0.2^{e}	$39.4 \pm 3.2^{b,c}$	6.4 ± 0.1^{g}	0.7 ± 0.05^{d}
		S	$60.0 \pm 5.5^{c,d,e}$	32.4 ± 2.0^{b}	$17.2 \pm 0.1^{c,}$	$27.6 \pm 10.3^{a,b,c}$	$5.9 \pm 0.3^{f,g}$	0.5 ± 0.01^{c}

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Extraction yield (%) of phytochemicals from AF varied significantly ($p < 0.05$) among studied compounds. TPC yield (g_{PE}/g_{AF}) ranged from 31.1 ± 1.1 (S–SLE40) up to 68.2 ± 0.4 (D–UAE40–90). Interestingly, SLE showed significant ($p < 0.05$) differences between extracts obtained at the same conditions varying the solvent type; however, these differences did not remain constant after the UAE treatment. UAE showed a double positive effect increasing polyphenol extraction related to all SLE samples and supplied the low extraction capacities of S–solvent. These results evidenced the prominent extractive capacities of UAE technology, widespread reported by literature (Mekinic et al., 2019).

Maximum TPC yield was in range with reported by Kadam et al. (2015b) (82.7 mg_{GAE}/g_{DB}, expressed in gallic acid equivalents per g of dried algae) working with *A. nodosum*, but using 10 min (0.66–fold lower than in the current study) of UAE (75.8 W/cm^2 of sonication power) and subsequently 22 h (88–fold higher than in the current study) of SLE of extraction time using acidic water (0.03 M HCl) as extractant. Yields increased with LS ratio, however, LS20 samples were selected to be characterized, since higher dilution ratios seem to diminish the effect UAE treatment. This fact can be caused by the local increase of solution temperature by the soundwaves instead of the cell–wall disruption that promotes bioactive compound releasing (Mekinic et al., 2019).

TPC/CHOs (g_{PE}/g_{GE}) ratios varied in a wide range from 1.0 ± 0.03 (D–SLE20) up to 1.7 ± 0.1 (S–UAE40–90). Specifically, the highest TPC/UA ratios was obtained with a sonicated extract with S–solvent because CHOs content of these extracts was less than 0.8 g_{GE}/L. TPC/UA (g_{PE}/g_{GE}) ratios ranged into a narrow interval from 2.4 ± 0.1 up to 3.3 ± 0.1, but the maximum value was again found in the extracts S–SLE20, S–UAE20–90 and S–UAE40–90. SLE using S–solvent was the least favourable for both polyphenols (0.7 ± 0.1 g_{PE}/L) and uronic acids (0.5 ± 0.04 g_{GE}/L) extractions, since it was obtained with the highest liquid–to–solid ratio (40 g_W/g_{AF}), slightly lower efficient extractant (S–Solvent) and low extractive method (SLE). However, both UAE samples with the highest TPC/UA ratio (S–UAE20–90 and S–UAE40–90) were owed by the high extractive capacities of UAE of polyphenols combined with the low efficiencies that S–solvent had to extract uronic acids.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Other ratio, TPC/(CHOs – UA) (g_{PE}/g_{GE}), was the relationship between polyphenols and all those sugars that are not alginic acid isoforms. This ratio was used to discern between the fraction of easily separated alginic acid/alginates and those sugars whose separation from polyphenols would require alternative methods (Audibert et al., 2009). This ratio varied from 1.6 ± 0.1 to 3.7 ± 0.3, and the maximum value was obtained again from S–UAE40 extract. This result agreed with previous studies, in which it was observed that extreme extraction conditions decreased the TPC ratio values, because carbohydrates and uronic acids extraction was more favoured in these conditions in comparison to polyphenols (Section 8).

Table 7.3. Polyphenols–carbohydrates and polyphenols–energy ratios from *A. nodosum* seaweed with 20, 30 and 40 g_W/g_{AF} of LS ratios using D– and S–solvents using SLE and UAE (SP = 90 W/cm^2) extraction methods. Different superscript letters within a column indicate significant ($p < 0.05$) differences between values.

Method	LS	Sol.	$\frac{\mathbf{TPC}}{\mathbf{CHOs}}$ (g_{PE}/g_{GE})	$\frac{\mathbf{TPC}}{\mathbf{UA}}$ (g_{PE}/g_{GE})	$\frac{\mathbf{TPC}}{\mathbf{CHOs - UA}}$ (g_{PE}/g_{GE})	$\frac{\mathbf{Energy}}{\mathbf{TPC}}$ (kJ L/g_{PE})
SLE	20	D	1.0 ± 0.03^{a}	2.5 ± 0.04^{a,b}	1.6 ± 0.1^{a}	19.7 ± 0.79^{a}
		S	1.3 ± 0.1^{a,b}	2.4 ± 0.1^{a}	2.7 ± 0.2^{c}	24.9 ± 1.0^{a}
	30	D	1.0 ± 0.1^{a}	2.4 ± 0.1^{a,b}	1.7 ± 0.1^{a}	26.3 ± 1.1^{a}
		S	1.2 ± 0.2^{a,b}	2.7 ± 0.1^{b,c}	2.1 ± 0.2^{a,b,c}	34.5 ± 1.4^{a}
	40	D	1.2 ± 0.1^{a,b}	2.4 ± 0.1^{a}	2.2 ± 0.2^{a,b,c}	33.0 ± 1.3^{a}
		S	1.3 ± 0.1^{a,b}	3.3 ± 0.2^{d}	2.2 ± 0.2^{abc}	56.3 ± 2.3^{b}
UAE	20	D	1.2 ± 0.2^{a,b}	3.0 ± 0.1^{c,d}	2.0 ± 0.2^{a,b}	105.2 ± 4.2^{c}
		S	1.4 ± 0.1^{b}	3.3 ± 0.1^{d}	2.4 ± 0.2^{b,c}	113.9 ± 4.5^{c}
	30	D	1.1 ± 0.1^{a,b}	2.8 ± 0.03^{b,c}	1.9 ± 0.2^{a,b}	171.5 ± 6.9^{d}
		S	1.4 ± 0.1^{b}	3.2 ± 0.1^{d}	2.5 ± 0.2^{b,c}	175.7 ± 7.0^{d,e}
	40	D	1.4 ± 0.02^{b}	3.0 ± 0.01^{c,d}	2.6 ± 0.2^{b,c}	190.0 ± 7.6^{e}
		S	1.7 ± 0.1^{c}	3.3 ± 0.1^{d}	3.7 ± 0.3^{d}	229.0 ± 9.2^{f}

The energy consumption (kJ) was evaluated considering 15 min of extraction time for both SLE and UAE treatments. The energy/TPC ratio (kJ L/g_{PE}) ranged from 19.7 ± 0.8 (D–SLE20) to 229.0 ± 9.2 (S–UAE40–90). Extraction method had a notorious effect on energy consumption since SLE consumption was lower than 56.3 ± 2.3 (S–SLE40), meanwhile UAE samples at least needed 2–fold times this value (D–UAE20–90, 105.2 ± 2.3). Energy/TPC ratio was suitable to determine those extracts with the highest energy efficiency for polyphenols extraction, obtained with LS20 samples for both extractions (SLE and UAE) with both extractants.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The use of UAE/SLE ratio was proposed to evaluate the effect of extraction method. It can be seen in Table 7.4 that all phytochemical compounds, polyphenols, carbohydrates, uronic acids as well as antioxidant activities, presented greater values than one evidencing superior UAE extractive capacities against SLE for any LS ratio (20, 30 and 40 gW/gAF) and solvent type used (D and S). UAE/SLE ratio showed greater values for S–samples due to the low extraction values they had in SLE that were compensated in UAE treatment.

Table 7.4. Ratio UAE/SLE of compound extraction (TPC, CHOs and UA) and antioxidant capacities of the extracts (DPPH, ABTS and FRAP) from *A. nodosum* seaweed with 20, 30 and 40 g_W/g_{AF} of LS ratios using D– and S–solvents using SLE and UAE (SP = 90 W/cm^2) extraction methods. Different superscript letters within a column indicate significant ($p < 0.05$) differences between values.

LS	Sol.	TPC	CHOs	UA	DPPH	ABTS	FRAP
20	D	$1.5 \pm 0.1^{a,b,c}$	$1.3 \pm 0.1^{a,b}$	1.2 ± 0.1^{a}	2.2 ± 0.1^{a}	2.1 ± 0.1^{a}	2.2 ± 0.1^{a}
	S	$1.7 \pm 0.1^{b,c}$	1.7 ± 0.1^{b}	1.4 ± 0.1^{a}	1.9 ± 0.1^{a}	2.1 ± 0.1^{a}	1.9 ± 0.1^{a}
30	D	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.6 ± 0.1^{a}	2.3 ± 0.1^{a}	$2.3 \pm 0.1^{a,b}$
	S	$1.5 \pm 0.1^{a,b,c}$	$1.5 \pm 0.1^{a,b}$	1.4 ± 0.1^{a}	1.9 ± 0.1^{a}	2.1 ± 0.1^{a}	2.1 ± 0.1^{a}
40	D	$1.4 \pm 0.1^{a,b}$	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}	1.8 ± 0.1^{b}	3.0 ± 0.1^{c}	2.8 ± 0.1^{b}
	S	1.9 ± 0.1^{c}	$1.4 \pm 0.1^{a,b}$	1.9 ± 0.2^{b}	1.4 ± 0.1^{c}	3.5 ± 0.1^{b}	2.1 ± 0.1^{a}

D/S ratio evaluated solvent type effect on bioactive compound extractions. Table 7.5 evidenced that D–solvent was better extractant that S–solvent. Obtained ratios for TPC, CHOs and UA showed at least 1.27–, 1.66– and 1.34–fold more polyphenols, carbohydrates, and uronic acids than saline solvent, respectively, when D–solvent was used. Meanwhile antioxidant activities (DPPH, ABTS and FRAP) showed similar results, obtaining D/S ratio values between 0.98 and 1.25, evidencing that D–solvent was slightly better extractant than S–solvent.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 7.5. Ratio D/S on compound extraction (TPC, CHOs and UA) and antioxidant capacities of the extracts (DPPH, ABTS and FRAP) from *A. nodosum* seaweed with 20, 30 and 40 g_W/g_{AF} of LS ratios using D– and S–solvents using SLE and UAE (SP = 90 W/cm^2) extraction methods. Different superscript letters within a column indicate significant ($p < 0.05$) differences between values.

LS	Method	TPC	CHOs	UA	DPPH	ABTS	FRAP
20	SLE	$1.3 \pm 0.1^{a,b}$	$1.7 \pm 0.1^{a,b}$	1.3 ± 0.1^{a}	$1.1 \pm 0.1^{a,b}$	1.1 ± 0.1^{a}	1.0 ± 0.1^{a}
	UAE	1.1 ± 0.1^{a}	1.25 ± 0.1^{a}	1.2 ± 0.1^{a}	$1.3 \pm 0.1^{a,b}$	1.1 ± 0.1^{a}	1.2 ± 0.1^{a}
30	SLE	$1.3 \pm 0.1^{a,b}$	$1.66 \pm 0.1^{a,b}$	1.4 ± 0.1^{a}	$1.0 \pm 0.1^{a,b}$	1.1 ± 0.2^{a}	1.0 ± 0.1^{a}
	UAE	1.0 ± 0.1^{a}	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}	0.9 ± 0.1^{a}	1.2 ± 0.2^{a}	1.1 ± 0.1^{a}
40	SLE	1.7 ± 0.1^{b}	1.8 ± 0.1^{b}	2.0 ± 0.1^{b}	$1.1 \pm 0.1^{a,b}$	1.3 ± 0.1^{a}	1.0 ± 0.1^{a}
	UAE	1.2 ± 0.1^{a}	$1.5 \pm 0.1^{a,b}$	1.3 ± 0.2^{a}	1.4 ± 0.1^{b}	1.1 ± 0.1^{a}	1.3 ± 0.1^{a}

TPC–antioxidant activities correlation, extraction yields, TPC ratios, D/S and UAE/SLE ratio were interesting tools or indices to evaluate the extraction characteristics to increase the bioactivities of the *A. nodosum* extracts. Thus, extractions carried out with LS = 20 g_W/g_{AF}, using D– and S–solvents for both extraction methods (SLE and UAE) were chosen to be chromatographically analysed.

7.5. CHROMATOGRAPHY PROFILING: SLE *VS* UAE

Several authors have previously used RP–HPLC method to separate and identify phlorotannins from *A. nodosum* seaweed (Koivikko et al 2007; Gonçalves–Fernández et al., 2019). UV wavelength recording at 267 nm is widely reported as the optimal for phloroglucinol molecules (Catarino et al., 2019) and so as for phlorotannins. RP–HPLC gradient mode (Section 4.14) of extracts from both extractions SLE (5 and 15 min), and UAE (90 W/cm^2, 15 min) using both D– and S–solvents and LS = 20 g_W/g_{AF} were shown in Figure 7.5.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

All extracts (non–sonicated and sonicated, SLE and UAE) profiles were characterized by reasonably well–separated peaks, corresponding to different size phlorotannin oligomers with a region where high–polymeric phlorotannin structures were eluted together in a hump. Three different regions could be differenced in the profiles: 10–20 min (A), 28–40 (B), and 45–70 (C). Areas under time set regions were evaluated (using PowerChrom 2.5 software). These three regions suggested that similar compounds were found in extracts, however the different signal intensities and peak shapes evidenced that both extraction method and solvent type had effects on the phlorotannin profiles.

Injections of 20 µL at 0.5 mg/mL phlorotannin standard (i.e., phloroglucinol) was performed with retention times around 32 min. Molecular weight of polyphenols directly affected to retention time of the peaks (Koivikko et al., 2007). A–region corresponded with the aqueous predominance of mobile–phase gradient and peaks in this region can be associated to low molecular weight polyphenols with some interferences of carbohydrate compounds as alginates (simple sugars show very low but not totally "zero" signal at 266 nm). Some proteins may be extracted, promoted by the S–solvent use because high ionic force can stabilize charged species (Koysuren et al., 2021; Tavakoli et al., 2021;) and this effect can be enhanced by UAE process.

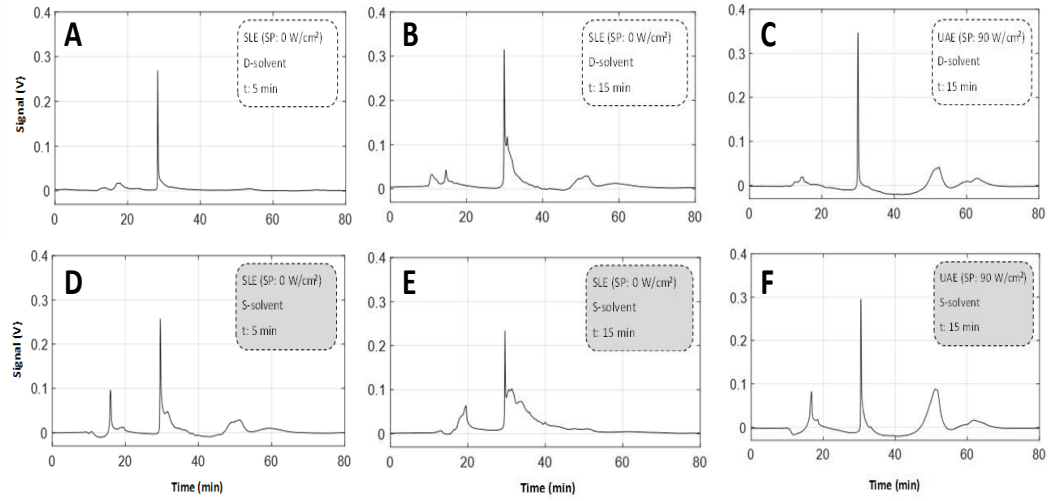


Figure 7.5. RP–HPLC profiles (LS = 20 g_W/g_{AF}) of SLE (5 and 15 min) and UAE (15 min) at 90 W/cm^2, extracts using D– (A, B and C), and S–solvents (D, E and F).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Our results showed more intense signals in A region for S– extracts, corroborating these hypotheses. A–region did not show relevant changes during D–SLE kinetics; nevertheless, S–SLE showed at short time a high signal that decreased with extraction time. A–region of sonicated saline extracts is like that determined at short time (5 min) S–SLE profile.

B–region was associated to main polyphenolic family formed by phlorotannin isomers with relative similar molecular size and chemical features with similar retention times. B–region coincided with phloroglucinol standard injections and with methanolic increasing gradient of mobile–phase, that prone their elution thorough C18 column (Gonçalves–Fernández et al., 2019). Low definition into B–region was observed with the presence of sodium chloride (S–solvent) regarding D–solvent. Proteins extracted by S–solvent could form complexes with some B–region phlorotannins affecting the profile sharpness, intensity, and retention time of peaks. B–region changed with extraction time (SLE), D–and S– samples increased peak tails with time. This B–region tail evolution could be due to phlorotannins deterioration, oxidation, or complexation along time; effects promoted by ionic strength. It was noted that UAE profiles had a higher B–region peak signal with lower trace of peak–tail, consequently polyphenols from B region in UAE extracts were more homogenous molecules compared with SLE samples (Tierney et al., 2014; Kadam et al 2015c,) and sonication prevented the formation of intermolecular complexes (Kumar et al., 2020).

C–region placed at higher retention times was associated with polyphenols with high molecular sizes. Peaks in C–region were more evident in UAE extracts (independently of solvent). This result seemed to indicate the sonication facilitated the leaching of polyphenols with high molecular size that during conventional extraction remained in the seaweeds tissue. Catarino et al. (2019) also observed that polyphenols with longer retention time came out of the column in a hump hindering their identification and quantification. Areas under A–, B and C regions (from 2 to 60 min) were normalized by the maximum measured total area (corresponding to 60 min) from SLE samples for both solvents (Figure 7.6). At any time, normalized B–region was the highest (around 0.8 at long times) and normalized C–region was the lowest (around 0.06 at long times) independently of solvent employed. S– solvent achieved the highest area due to B–peak tails (Figure 7.5).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

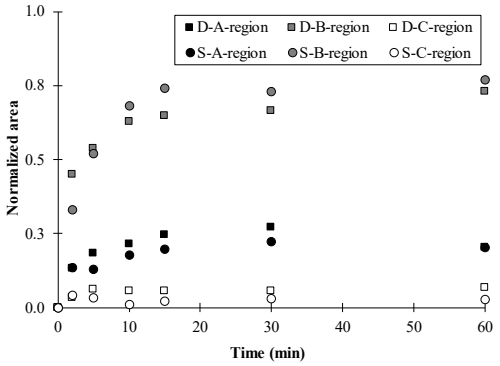


Figure 7.6. Evolution of normalized area values during SLE (LS = 20 g_W/g_{AF}) from RP–HPLC profiles using D– and S–solvent, of A–, B– and C–regions.

All normalized areas were practically invariant after 15 min of extraction indicating that pseudo–equilibrium is simultaneously achieved in all fractions. In fact, normalized area corresponding to C–region (heavy fraction) was practically constant after 5 min meaning that these compounds were already leached at short times, probably, because they were located at the seaweed surface (previously exudated during drying). Diffusion transport of these compounds through seaweed tissue was limited by their large size and no additional contribution of this fraction at longer times was observed. Seaweed extracts are formed by a complex matrix of compounds and the lack of commercial standards make difficult their chemical characterizations, however RP–HPLC technique gave a reproducible and deep–insight of nature, kinetics and proximate structural features of these bioactive molecules obtained from SLE and UAE carried out using D– and S–solvents.

7.5.1. RELATIONSHIP HPLC AREAS–ANTIOXIDANT CAPACITIES

Normalized areas of A–, B– and C–regions were employed to establish relationships with antioxidant activities determined from the SLE extracts at different extraction times from both solvents (D and S). Figure 7.7 shows DPPH, ABTS and FRAP trends with normalized RP–HPLC regions and total area (at constant extraction time). It can be observed high correlation and acceptable linear regressions coefficients ($R^2 > 0.8$) between normalized total area and DPPH, ABTS and FRAP values independently of employed solvent.

Furthermore, additional linear regressions could be obtained with normalized B–region areas and antioxidant activities. The slope the linear regression equations with total area and B–region was similar (for D– and S– solvents). These results confirmed that the main responsible fraction of antioxidant activity of extracts is the B–region previously associated to the polyphenolic fraction formed by phlorotannin isomers.

Several authors have reported that RP–HPLC method is not very effective in separating complex mixtures of phlorotannins (Tierney et al., 2014), and needs to be complemented with MS and/or NMR techniques (Koivikko et al., 2007). Nevertheless, acceptable peaks separation, reproducible and insight of nature, kinetics, proximate structural features and interesting relationships between chromatographic peaks and antioxidant activities were obtained.

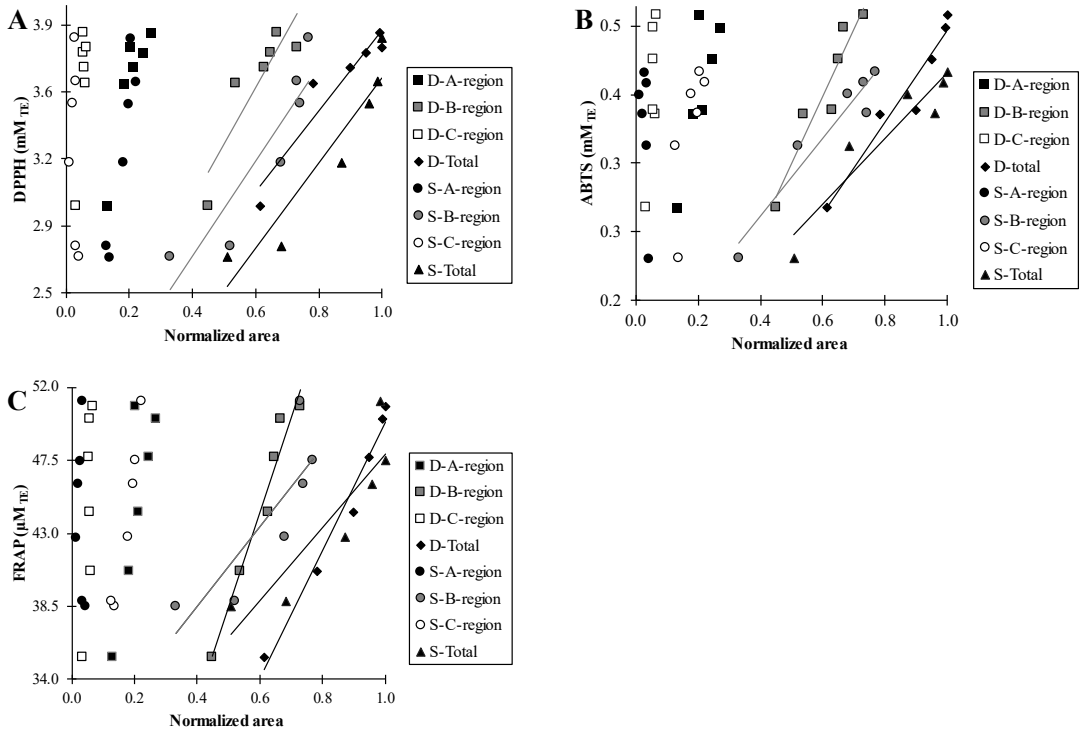


Figure 7.7. Normalized area values from the SLE (LS = 20 g_W/g_{AF}) extracts (2 to 60 min) from D– and S– solvents of A–, B–, C–regions and total correlated with measured antioxidant activities (DPPH, ABTS and FRAP).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

HIGHLIGHTS

Saltwater (S–solvent) was a suitable to be employed in both solid–liquid (SLE) and ultrasound–assisted (UAE) extractions to obtain bioactive extracts.

TPC (up to 1.7–fold), CHOs (up to 1.9–fold) and UA (up to 2.0–fold) values of D–samples were higher than S–extracts.

Antioxidant activities measured by DPPH (up to 1.4–fold), ABTS (up to 1.2–fold) and FRAP (up to 1.3–fold) were only slightly higher values in D– than S –samples.

Extraction kinetics were successfully modelled by Peleg's model and pseudo–equilibrium was achieved after 15 min.

Sonication (15 min) significantly increased all phytochemical parameters of extracts, with values of 1.9–, 1.7–, 1.9–, 2.2–, 2.1– and 2.75–fold higher for TPC, CHOs, UA, DPPH, ABTS and FRAP, respectively, regarding conventional liquid–solid extracts.

Extracts obtained with low liquid–solid ratios (20 g_W/g_{AF}) provided higher TPC, CHOs and UA values and antioxidant activities than higher LS samples (> 30 g_W/g_{AF}).

Chromatographic analysis showed the presence of three regions whereas B–region was the phlorotannins that successfully correlated with antioxidant activities.

Saline solvent favours the extraction of non-phenolic compounds that formed intermolecular complexes.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

8. ULTRASOUND-ASSISTED EXTRACTION OPTIMIZATION

Current Section 8 adapted from:

Gisbert M, Barcala M, Rosell CM, Sineiro J, Moreira R (2021) Aqueous extracts characteristics obtained by ultrasound-assisted extraction from *Ascophyllum nodosum* seaweeds: effect of operation conditions. *Journal of Applied Phycology* 33, 3297–3308. DOI: 10.1007/s10811–021–02546–5 (See Section 17)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

8.1. GENERALITIES

The high content of polysaccharides, polyunsaturated fatty acids, minerals, fiber, vitamins, and bioactive molecules in seaweeds (Pereira, 2011) has put them in the spotlight to be used in several applications, as it was commented in Sections 1.5 and 1.6. The global demand for seaweed–derived products is producing relevant socio–economic benefits. Nowadays, the algae–based refinery concept is promoted and being extensively focus of research activity. The aim is to develop biorefineries to produce valuable compounds with seaweeds as unique raw material (Herrero & Ibáñez, 2015). The extraction of bioactive compounds can be carried out with water as unique solvent; the manufacture does not require high temperatures and the possibility of successively extract multiple compounds minimizes the amounts of residues generated, concepts that follow green chemistry principles (EPA, 2012).

Brown seaweeds are resource for several industries since they present high contents of polyphenols, fucoidans and alginate (Holdt & Kraan, 2011). These nutraceuticals have recently gained relevance for their beneficial health applications since they have shown notorious bioactive features deeply seen in Sections 1.4, 1.5 and 1.6. Seaweed biochemical composition is variable and depends on species, season, geographic origin, contaminants, and their natural individual variability (Tabassum et al., 2016). Polyphenols extracted from brown seaweeds are potential food additives that act as competitive inhibitors of digestive α–amylase and α–glucosidase enzymes, inhibiting carbohydrates hydrolysis that cause a reduction of the sugar intake levels (Rodrigues et al., 2015) previously seen in Sections 1.7.2, 1.7.3 and 3.3.

Ultrasound–assisted extraction (UAE) is one of the most widespread polyphenols extraction methods from different natural raw materials (Cikos et al., 2018; Bordoloi & Goosen, 2020) largely explored in Section 1.7.1. This method has recently attracted attention since it is a low–cost equipment and procedure (Kadam et al., 2015a), and it is even considered an eco–friendly technology according with the Environmental Protection Agency of United States of America. (Talmaciu et al., 2015). In comparison to other green techniques, ultrasound technology is simpler and faster than microwave–assisted extraction (Zhu et al., 2017) and less dependent on the algal biochemical composition compared with enzyme–assisted extraction (Hahn et al., 2012).

USC
DE SANTIAGO
DE COMPOSTELA

Several authors have applied UAE to nutraceuticals extraction from marine macroalgae, discussed in Sections 1.7.1 and 3.1.2, since it has three major extraction benefits: promotes the solvents penetration into cellular materials, improves mass transfer and disrupts plant cell walls facilitating cell content release (Povey & Mason, 1998). UAE is more effective to produce enriched antioxidant extracts (Dang et al., 2017b). Moreira et al., (2016) studied the effect of fresh seaweed drying temperature on the polyphenols–enriched extracts obtained by UAE and observed total polyphenol content (TPC), antioxidant activity decreased, and uronic acids increased with drying temperature. Additionally, a previous study about air drying effect on three brown seaweeds (*Fucus vesiculosus*, *Bifurcaria bifurcata* and *A. nodosum*) concluded that drying at 50 °C and 30% of relative humidity were the optimal drying conditions to improve antioxidant properties of polyphenols extracts by UAE (Moreira et al., 2017).

Kadam et al. (2015a) compared the efficiency of aqueous and acidic solutions during UAE of laminarin and polyphenols from *A. nodosum* and *Laminarina hyperborea*. Rodrigues et al. (2015) compared enzymatic and UAE methods on the properties of extracts from three algae species (*Osmundea pinnatifida*, *Sargassum muticum* and *Codium tomentosum*) obtaining the highest TPC from brown seaweed and the highest carbohydrate content from red seaweed. This study obtained the extracts carrying out extractions after seaweed drying at 50 °C and sonication at 400 W for 10 min at 50 °C in a batch process.

A. nodosum brown edible seaweed was selected for this study for its high polyphenols content (14% dry basis) (Holdt & Kraan, 2011). The characterization of seaweed extracts usually includes TPC values and the evaluation of their antioxidant activity by means of several methods (Mesa–Vanegas et al., 2015; Dang et al. 2017a;) that are quick and easy methods with high reproducibility for antioxidant capacities characterization of aqueous ultrasound–assisted extracted polyphenols from seaweed, explored in Section 3.2.

UAE habitually transfers polysaccharides and uronic acids to liquid phase together with polyphenols. This fact demands additional characterization techniques to evaluate the total carbohydrate content (CHOs) and the uronic acid content (UA) of the extracts (Leyton et al., 2016; Moreira et al., 2016). Carbohydrates and uronic acids could be considered contaminants of extracted polyphenols because they can interact forming complexes that could modify chemical and structural polyphenols features (Wang et al., 2016).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Water is an efficient solvent for polyphenols extraction (Leyton et al., 2016). Additionally, the use of water reduces subsequent purification stages required when binary solutions as solvents are used. However, aqueous blends of acetone (Koivikko et al., 2005), acids (Kadam et al., 2015a) or ethanol (Audibert et al., 2010) improve the extraction of polyphenols since they extract soluble and insoluble fractions of polyphenols. Parameters such as cost, efficiency and feasibility of the process must be considered. Operation temperature must be maintained under 40 °C to minimize the loss of heat–sensitive compound (Li et al., 2017; Catarino et al., 2019). The aim of Section 8 was to determine the best conditions of aqueous UAE of polyphenols by the evaluation of the effects, following a Box–Behnken (BB) experimental design, the sonication power (SP), sonication time (tR) and the mass solvent/solid ratio (LS) using *A. nodosum* seaweed. Extracts were characterized by total polyphenol (TPC,), total carbohydrates (CHOs) and total uronic acids (UA) content and antioxidant capacities (DPPH, ABTS and FRAP methods) of extracts were also measured.

8.2. ASSAYED SYSTEMS

Aqueous extracts from *Ascophyllum nodosum* brown edible seaweeds were obtained after continuous ultrasound–assisted extraction (Section 4.4.2). The effect of sonication time (from 2 to 6 min), sonication power (from 70 to 90 W/cm^2) and solvent–to–solid ratio (from 20 to 40 g_W/g_{AF}) on the extracts features was studied employing a Box–Behnken experimental design (Section 4.4.2.1). Extracts were characterized by polyphenols (Section 4.6.1), carbohydrates (Section 4.6.2), and uronic acid (Section 4.6.3) content. Response surface methodology (by means of a quadratic model) was employed to relate chemical composition of extracts and operation conditions. Optimal experimental conditions were established to obtain maximal polyphenols content and several polyphenols–carbohydrates ratios together with the conditions that minimize energy consumption per extracted polyphenols amount relationship were proposed to find out optimal extraction conditions. Antioxidant capacities of extracts were determined employing different DPPH (Section 4.6.4), ABTS (Section 4.6.5) and FRAP (Section 4.6.6) methods. Positive linear correlations between antioxidant activity and total polyphenols content were established. The proposed research scheme is showed in Figure 8.1.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

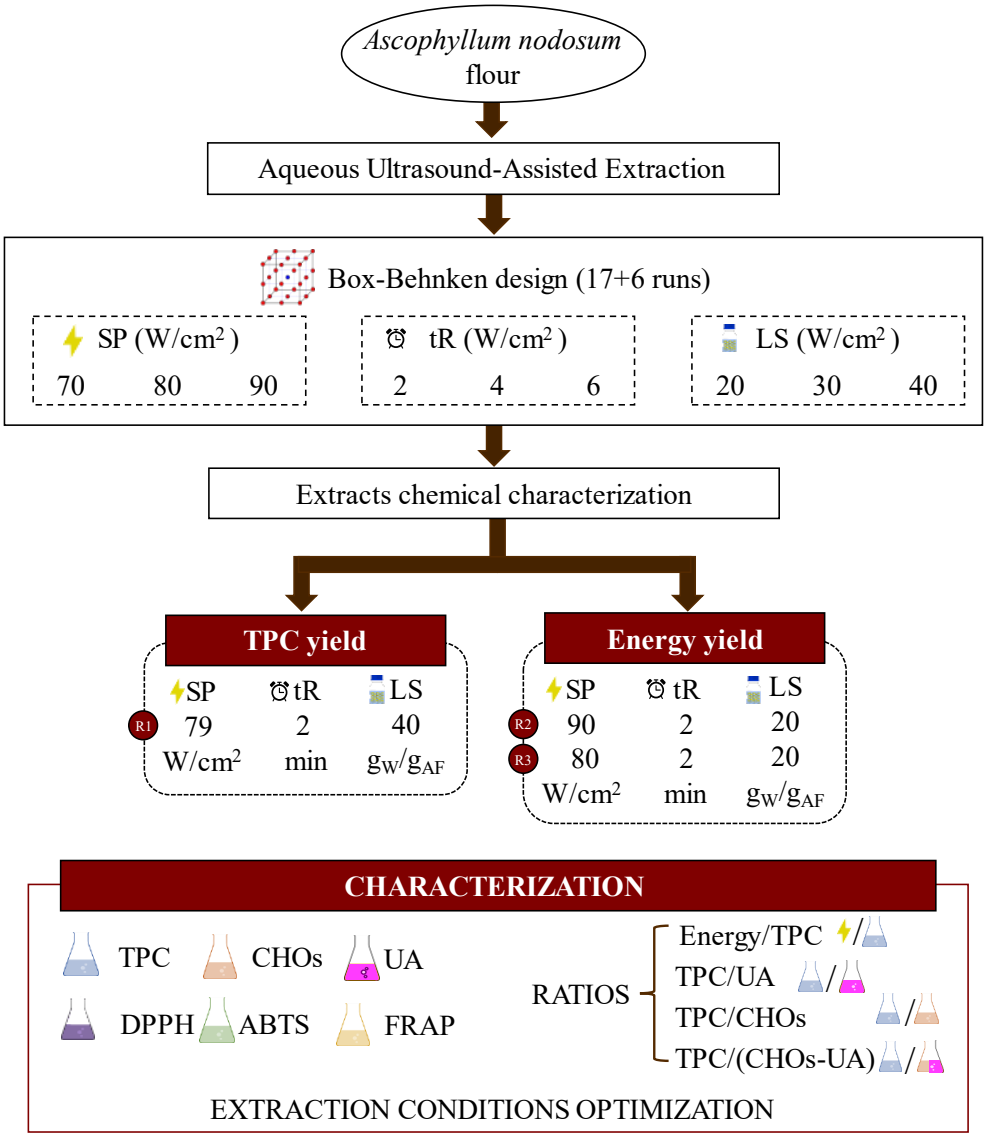


Figure 8.1. Box–Behnken experimental designing in order to optimize and set up ultrasound–assisted extraction of *A. nodosum* bioactive compounds.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

8.3. EXTRACTS CHEMICAL CHARACTERIZATION

The experimental values obtained for chemical parameters from the 17 runs corresponding to Box – Behnken (BB) design are shown in Table 8.1. Experimental values for total polyphenol content (TPC) ranged from 1.3 ± 0.02 (runs 6, 11, 12, 13 and 17) to 2.8 ± 0.01 g_{PE}/L (run 14). Total carbohydrate content (CHOs) was in the range 0.5 ± 0.06 (run 6) to 1.5 ± 0.02 g_{GE}/L (run 14). Finally, experimental total uronic acids content (UA) ranged from 0.04 ± 0.02 (run 3) to 0.8 ± 0.05 g_{GE}/L (run 14). Maximal TPC, CHOs and UA extracts content were obtained in the run 14 at conditions SP = 90 W/cm^2, tR = 2 min and LS = 20 g_W/g_{AF}. Globally, it can be observed that the extracts obtained, when low LS ratios were employed, provided high TPC, CHOs and UA content. The variation of the residence time, between 2 and 6 min, seemed to have slight influence on the TPC and UA content of extracts; however, CHOs increased with increasing residence times. Finally, the SP values increased the extraction of polyphenol and carbohydrates. Kadam et al. (2015c) working with dried *A. nodosum* (40 °C for 12 h) and employing longer extraction times (from 5 to 25 min) than those employed in this Thesis, HCl solvent concentration (up to 0.06 M) and ultrasound amplitude from 22.8 to 114 μm, determined a maximum TPC of 14.3 g_{GAE}/L expressed as gallic acid equivalents and was accompanied with maximal CHOs extraction of 8.7 g_{GE}/L and UA of 11.7 g_{GE}/L. operating at the highest sonication power and acidic concentration and the longest times. Moreira et al. (2016) obtained aqueous extracts with values of 0.5 g_{PE}/L, 1.7 g_{GE}/L and 0.7 g_{GE}/L of TPC, CHOs and UA, respectively, from UAE extraction carried out for tR = 4 min, LS = 30 g_W/g_{AF} and SP = 90 W/cm^2 employing *F. vesiculosus* dried at 50 °C for 8 h and determined the high impact of the drying temperature (above 50 °C) of seaweeds on TPC and UA content of the extracts.

These results and those obtained in the current Thesis can be compared with conventional solvent extraction of polyphenols from brown seaweeds. In example, Zhang et al. (2007) obtained high TPC of 5.6 g_{PE}/L with fresh *A. nodosum*, employing low liquid–solid ratios (12.5 g_W/g_{AF}) and ethanol (50% v/v) as solvent during very long extraction times (90 min) and at high temperature (80 ºC). Ummat et al. (2020) obtained extracts with the highest TPC values of 4.8 g_{PE}/L using hydroalcoholic solutions as solvent (ethanol 50% v/v), with a very low solvent–solid ratio (10 g_W/g_{AF}) and very long extraction times (30 min) of UAE from *F. vesiculosus* seaweed previously dried at 50 °C for 9 days.

USC DE SANTIAGO DE COMPOSTELA

Table 8.1. Experimental (Exp) and modeled (Mod) TPC (Eq. (8.1)), CHOs (Eq. (8.2)) and UA (Eq. (8.3)) values from aqueous extraction of *A. nodosum* seaweeds using UAE at different SP, tR and LS conditions following a BB design.

Run	Variables			TPC (g_{PE}/L)		CHOs (g_{GE}/L)		UA (g_{GE}/L)	
	SP (W/cm^2)	tR (min)	LS (g_W/g_{AF})	Exp	Mod	Exp	Mod	Exp	Mod
1	70	2	30	1.6 ± 0.04^{e}	1.6	$0.7\pm0.01^{b,c,d,e}$	0.7	$0.1\pm0.05^{a,b,c}$	0.1
2	70	4	20	2.4 ± 0.02^{f}	2.4	$1.2\pm0.06^{h,i}$	1.1	$0.1\pm0.04^{b,c,d}$	0.2
3	70	4	40	$1.4\pm0.10^{b,c}$	1.4	$0.7\pm0.06^{b,c,d}$	0.6	$0.04\pm0.02^{a,b}$	0.1
4	70	6	30	$1.5\pm0.04^{c,d,e}$	1.7	0.9 ± 0.01^{g}	0.9	$0.2\pm0.06^{c,d}$	0.1
5	80	2	20	$2.5\pm0.04^{f,g}$	2.6	1.3 ± 0.01^{j}	1.3	0.6 ± 0.03^{i}	0.5
6	80	2	40	1.3 ± 0.10^{a}	1.3	0.5 ± 0.06^{a}	0.4	$0.06\pm0.04^{a,b,c}$	0.04
7	80	4	30	$1.5\pm0.04^{c,d,e}$	1.6	$0.7\pm0.06^{b,c,d,e}$	0.8	$0.3\pm0.02^{e,f,g}$	0.3
8	80	4	30	$1.6\pm0.05^{d,e}$	1.6	$0.8\pm0.06^{d,e,f,g}$	0.8	0.3 ± 0.01^{g}	0.3
9	80	4	30	$1.6\pm0.01^{d,e}$	1.6	$0.8\pm0.02^{c,d,e,f,g}$	0.8	$0.3\pm0.01^{e,f,g}$	0.3
10	80	4	30	$1.6\pm0.03^{c,d,e}$	1.6	$0.8\pm0.06^{e,f,g}$	0.8	$0.3\pm0.05^{f,g}$	0.3
11	80	6	40	1.3 ± 0.02^{a}	1.3	$0.7\pm0.03^{b,c,d,e}$	0.7	$0.2\pm0.02^{d,e}$	0.2
12	80	6	40	1.3 ± 0.06^{a}	1.3	0.6 ± 0.02^{b}	0.7	$0.2\pm0.02^{a,b,c,d}$	0.2
13	80	6	40	$1.3\pm0.06^{a,b}$	1.3	$0.7\pm0.03^{b,c,d}$	0.7	$0.2\pm0.03^{d,e,f}$	0.2
14	90	2	20	2.8 ± 0.01^{h}	2.7	1.5 ± 0.02^{k}	1.4	0.8 ± 0.05^{j}	0.8
15	90	4	30	$1.6\pm0.06^{c,d,e}$	1.6	0.9 ± 0.04^{g}	0.8	0.5 ± 0.02^{h}	0.4
16	90	4	30	$1.5\pm0.03^{c,d,e}$	1.6	0.9 ± 0.02^{a}	0.8	0.4 ± 0.03^{h}	0.4
17	90	6	40	$1.4\pm0.02^{a,b}$	1.2	$0.7\pm0.02^{f,g}$	0.6	$0.1\pm0.02^{b,c,d}$	0.2
A	70	4	30	1.6 ± 0.00^{e}	1.6	$0.7\pm0.01^{b,c,d,e}$	0.8	$0.04\pm0.03^{a,b}$	0.1
B	70	2	40	$1.5\pm0.08^{c,d}$	1.4	$0.3\pm0.05^{b,c}$	0.3	0.02 ± 0.02^{a}	0.03
C	80	4	20	2.5 ± 0.06^{g}	2.6	1.3 ± 0.02^{j}	1.1	0.6 ± 0.08^{i}	0.5
D	80	6	20	$2.5\pm0.01^{f,g}$	2.6	1.1 ± 0.07^{h}	1.0	0.5 ± 0.06^{h}	0.4
E	90	6	20	2.7 ± 0.04^{h}	2.7	$1.2\pm0.07^{i,j}$	0.8	0.8 ± 0.02^{j}	0.7
F	90	2	40	1.3 ± 0.01^{a}	1.2	$0.7\pm0.04^{c,d,e,f}$	0.5	0.4 ± 0.01^{h}	0.6

where EXP, was experimental value; MOD, was value obtained from the models, TPC, CHOs and UA values were expressed as g of equivalent molecule per liter. Reference molecules used were, phloroglucinol (PE) for TPC and glucose (GE) for CHOs and UA determinations.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

It was concluded that working at room temperature (below 30 °C) with 90 W/cm^2 sonication power, LS ratio of 20 g_W/g_{AF} and very short time, around 2 min (run 14), it was obtained a promising TPC value of 2.8 ± 0.01 g_{PE}/L. It must be considered that data collected from literature about seaweed extraction corresponds to batch experiments, working in some cases with different (alcoholic, organic and/or acidic) solvents, higher temperatures, longer operation times and lower liquid–solid ratios resulting in a wide TPC range. Despite that UAE technology is in the spotlight of seaweed industry and in the research of new antioxidant resources, to carry out a comparison covering all the bibliographic results is difficult since it is necessary to consider that there are several relevant extraction conditions and pre–treatments (mainly drying methods, conditions and procedures) like grinding (mean particle size and particle size distribution), or use of different compounds as quantification standards, could hardly affect to the extracts features.

8.4. RESPONSE SURFACE MODELLING

ANOVA was conducted to evaluate the significance of the models for 95% ($p < 0.05$) confidence interval (Table 8.2). A crossed reduced quadratic model was selected (model $p < 0.0001$) for TPC, CHOs and UA in the aqueous extracts after backward elimination step to eliminate the non–significant terms. The non–significant lack of fit test of 0.06, 0.09 and 0.05 observed for TPC, CHOs and UA content, respectively, indicated that well–fitting model and models were adequate to describe experimental data. In addition, the values of coefficient of determination, R^2, and adjusted–R^2 were higher than 0.91 and 0.88, respectively, for TPC, CHOs and UA confirming the fit goodness of the respective models. The significant ($p < 0.05$) effect on the TPC was contributed by the linear and quadratic terms of liquid–solid ratio (LS), and the interaction term between sonication power and liquid–solid ratio (SPLS). It is worth mentioning the absence of significant ($p < 0.05$) effects of the sonication time on the TPC of extracts.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 8.2. ANOVA for quadratic models of TPC, CHOs and UA content in the UAE aqueous extracts from *A. nodosum* seaweed

	TPC	CHOs	UA
cst	1.5***	0.8***	0.3***
SP			0.2***
LS	–0.6***	–0.3***	–0.2***
SPtR		–0.1**	
SPLS	–0.1**		–0.1***
tRLS		0.2*	0.1*
LS^2	0.4***		
R^2	0.99	0.91	0.96
Adjusted R^2	0.98	0.88	0.94
Lack of fit	0.06	0.091	0.048
F_{exp}	0.99	0.44	1.54
Model *p–value*	0.0001	0.0001	0.0001

where, *, **, ***, represented statistical significance with $p < 0.05$, < 0.01 y < 0.001, respectively; cst, constant coefficient; SP, sonication power; tR, time residence; LS, liquid–solid ratio are the codified variables.

The significant effect on the CHOs was contributed by the linear term LS, and the interaction terms, sonication power and time (SP·tR) and time and liquid–solid ratio (tR·LS). Finally, the significant ($p < 0.05$) effect on the UA was contributed by the linear terms SP and LS and interaction terms SP·LS and tR·LS. These results evidenced that liquid–solid ratio (LS), as well as their interactions with sonication power (SP) and time (tR), played a prominent role in the UAE of polyphenols, carbohydrates, and uronic acids, while sonication power and time residence played secondary roles.The final response equations for TPC, CHOs, and UA content, for coded variables, are given in the Eqs. (8.1), (8.2) and (8.3), respectively:

$$\mathrm{RSM_{TPC}} = 1.5 - \mathrm{LS}(0.6 - 0.1\mathrm{SP} + 0.4\mathrm{LS}) \quad (8.1)$$

$$\mathrm{RSM_{CHOs}} = 0.8 - 0.3\mathrm{LS} - \mathrm{tR}\,(0.1\mathrm{SP} + 0.17\mathrm{LS}) \quad (8.2)$$

$$\mathrm{RSM_{UA}} = 0.3 + 0.2\mathrm{SP} + \mathrm{LS}\,(0.07\,t - 0.1\mathrm{SP} - 0.2) \quad (8.3)$$

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

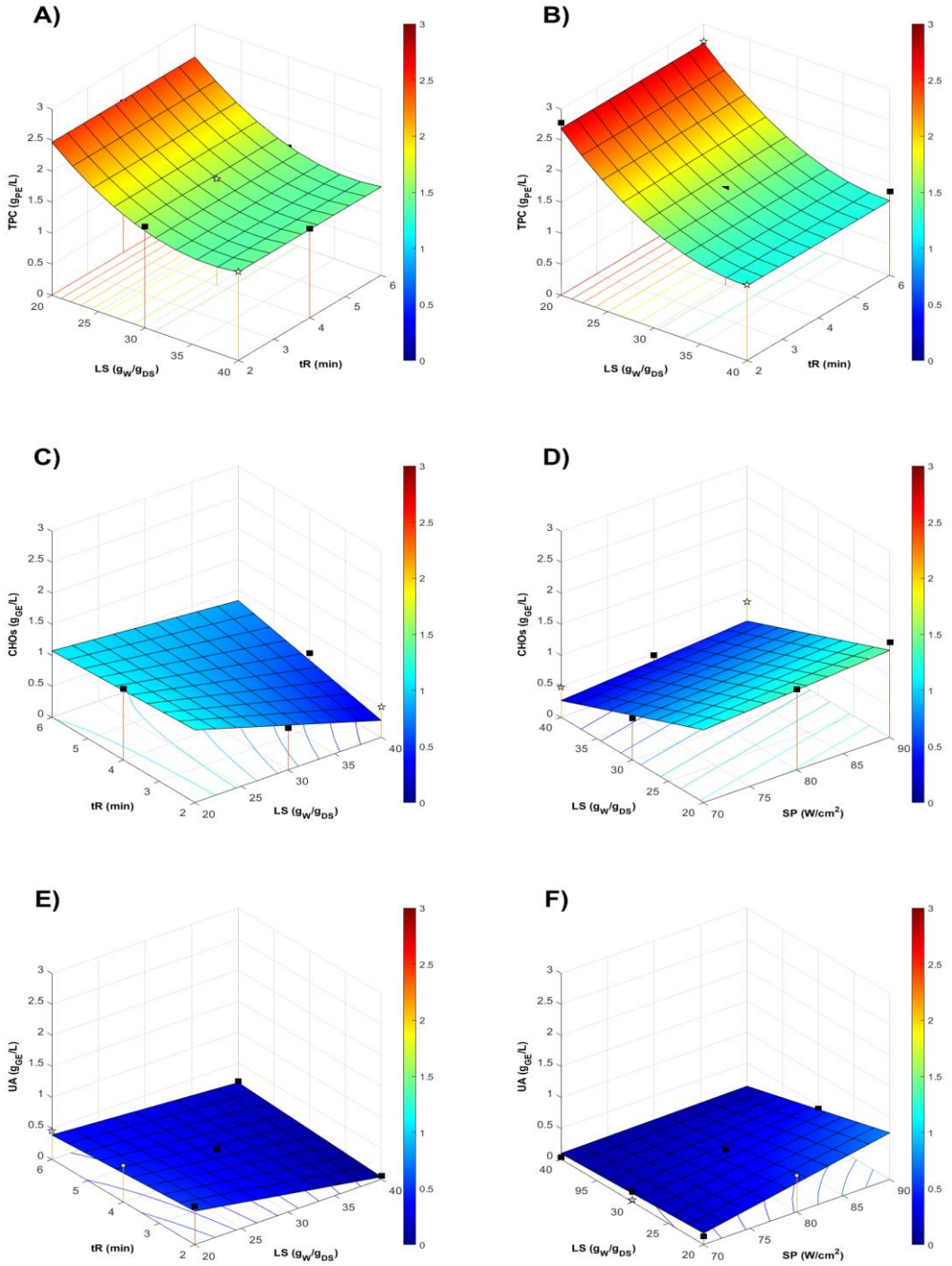


Figure 8.2. Response surface plots for A, B polyphenols (TPC, g_{PE}/L); C, D carbohydrates (CHOs, g_{GE}/L) and E, F uronic acids (UA, g_{GE}/L) content vs studied variables at constant sonication power (SP, W/cm^2) or time residence (tR, min): A, C SP = 70 W/cm^2, E SP = 80 W/cm^2, B SP = 90 W/cm^2, D tR = 2 min and F tR = 4 min. Box–Behnken (black box) and additional points (white star).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Eq. (8.1) estimated the maximum TPC value of 2.8 g_{PE}/L at the same operation conditions than those given by run 14. The minimum CHOs content (0.31 g_{GE}/L) was found (by means of Eq. (8.2) at the highest S, the shortest t and an intermediate P value (79.2 W/cm^2), that is, experimental conditions closer to run 6. Finally, a minimum UA content 0.01 g_{GE}/L), Eq. (8.3) was found at intermediate conditions of P (75.6%) and t (2.5 min), and close to the highest S (38 g_W/g_{AF}).

To observe the different TPC, CHOs and UA trends, several representative plots are collected in Figure 8.2. TPC plot (Figure 8.2A and B) was shown as function of LS and tR with SP set at 90 w/cm^2. CHOS plot (Figure 8.2C and D) as function of SP and LS with tR set at 2 min. UA plot (Figure 8.2E and F) as function of SP and LS at constant tR (4 min). These plots are also evidenced the noticeable agreement between experimental data given by the BB design and estimated value for the respective models. Furthermore, several dots corresponding to the additional extracts performed are also included. It can be observed that the response surfaces are very close to these experimental data, verifying again the suitability of the proposed models.

8.5. ANALYSIS OF TPC RATIOS

Each experimental run gave aqueous extracts with different compositions from *A. nodosum*. Maximum TPC was chosen as criterion to select the optimal extracts conditions (run 14, in this Thesis). Nevertheless, this implies necessarily subsequent operations for polyphenols isolation and purification. It is also important to consider costs, efficiencies and feasibility of the extraction, and the optimal condition must be related to additional aspects such as minimize energy consumption and/or operation time (increase production). Studied TPC ratios are summarized in Table 8.3.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 8.3. TPC ratios in relation to CHOs, UA and consumed energy during for UAE of *A. nodosum* seaweeds at different conditions of SP, tR and LS.

Run	Energy (KJ)	SP (W/cm^2)	tR (min)	LS (g_W/g_{AF})	$\frac{TPC}{UA}$ (g_{PE}/g_{GE})	$\frac{TPC}{CHOs}$ (g_{PE}/g_{GE})	$\frac{TPC}{(CHOs-UA)}$ (g_{PE}/g_{GE})	$\frac{Energy}{TPC}$ ($KJ/(g_{PE}/L)$)
1	29.3	70	2	30	17.2 ± 0.5^{g}	2.4 ± 0.03^{h}	2.8 ± 0.1^{d}	17.9 ± 0.03^{b}
2	58.6	70	4	20	17.1 ± 0.2^{g}	$2.1\pm0.05^{e,f,g}$	$2.4\pm0.03^{b,c}$	24.2 ± 0.01^{c}
3	58.6	70	4	40	41.2 ± 0.6^{i}	$2.2\pm0.1^{f,g,h}$	2.3 ± 0.1^{b}	40.7 ± 0.04^{e}
4	87.9	70	6	30	9.3 ± 0.4^{f}	1.7 ± 0.03^{a}	2.1 ± 0.05^{a}	57.5 ± 0.03^{k}
5	36.6	80	2	20	$4.1\pm0.1^{a,b}$	$2.0\pm0.01^{b,c,d,e}$	3.7 ± 0.01^{j}	14.5 ± 0.03^{a}
6	36.6	80	2	40	21.9 ± 0.7^{h}	2.7 ± 0.2^{i}	$3.0\pm0.1^{e,f}$	28.9 ± 0.07^{d}
7	73.2	80	4	30	$5.1\pm0.1^{c,d}$	$2.3\pm0.09^{g,h}$	4.0 ± 0.03^{k}	$47.7\pm0.03^{h,i}$
8	73.2	80	4	30	$5.0\pm0.1^{b,c}$	$2.0\pm0.09^{c,d,e,f}$	$3.4\pm0.02^{h,i}$	45.9 ± 0.03^{f}
9	73.2	80	4	30	$5.3\pm0.1^{c,d,e}$	$2.0\pm0.03^{d,e,f,g}$	$3.3\pm0.01^{g,h}$	$46.7\pm0.01^{f,g}$
10	73.2	80	4	30	$5.0\pm0.2^{b,c}$	$2.0\pm0.1^{b,c,d,e}$	$3.2\pm0.04^{f,g}$	$46.9\pm0.02^{g,h}$
11	109.7	80	6	40	6.2 ± 0.1^{e}	$1.8\pm0.04^{a,b}$	2.5 ± 0.02^{c}	86.9 ± 0.02^{m}
12	122.3	80	6	40	$6.1\pm0.1^{d,e}$	$2.1\pm0.1^{e,f,g}$	$3.1\pm0.03^{e,f}$	96.3 ± 0.04^{o}
13	122.3	80	6	40	6.2 ± 0.1^{e}	$2.0\pm0.1^{c,d,e,f}$	$2.9\pm0.03^{d,e}$	93.3 ± 0.04^{n}
14	40.8	90	2	20	3.7 ± 0.1^{a}	$1.8\pm0.02^{a,b,c}$	$3.6\pm0.01^{i,j}$	14.7 ± 0.02^{a}
15	81.6	90	4	30	3.4 ± 0.1^{a}	$1.7\pm0.06^{a,b}$	3.5 ± 0.01^{i}	52.5 ± 0.04^{j}
16	73.2	90	4	30	3.5 ± 0.1^{a}	$1.8\pm0.03^{a,b,c}$	$3.6\pm0.02^{i,j}$	48.1 ± 0.02^{i}
17	109.7	90	6	40	10.0 ± 0.1^{f}	$1.9\pm0.03^{a,b,c,d}$	$2.4\pm0.03^{b,c}$	81.4 ± 0.01^{l}

TPC/UA (g_{PE}/g_{GE}) ratios varied in a wide range from 3.4 ± 0.1 up to 41.2 ± 0.6 and high values of the corresponded to runs with the highest LS (40) ratio. Specifically, the highest TPC/UA ratios were obtained in run 3 (SP = 70 W/cm^2 and tR = 4 min) and run 6 (SP = 80 W/cm^2 and tR = 2 min) with ratios of 41.2 ± 0.6 and 21.9 ± 0.7, because UA content of these extracts were lower than 0.06 g_{GE}/L. TPC/CHOs (g_{PE}/g_{GE}) ratios ranged into a narrow interval from 1.7 ± 0.03 up to 2.7 ± 0.2, and the maximum value was also found in the run 6 where experimental conditions were the least favorable for both polyphenols (1.3 ± 0.1 g_{PE}/L) and sugars (0.5 ± 0.1 g_{GE}/L) extractions, corresponding to medium SP (80 W/cm^2), the lowest tR (2 min) and the highest LS (40 g_W/g_{AF}).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The third analyzed ratio, TPC/(CHOs–UA) (g_{PE}/g_{GE}), was the relationship between polyphenols and all those sugars that were not isoforms of alginic acid. With this ratio, it was discerned between the fraction of easily separable alginic extraction procedures, and those sugars whose separation from polyphenols would require alternative methods (Gonçalves–Fernández et al., 2018). This ratio varied from 2.1 ± 0.1 to 4.0 ± 0.1, and the maximum value was determined in run 7 with intermediate conditions of the BB design (SP = 80 W/cm^2, tR = 4 min and LS = 30 g_W/g_{DS}). This result evidenced that any extreme conditions selected for sonication power, residence time or solvent–solid ratio (within the experimental plan) decreased the ratio value because carbohydrates (not alginic) were more promoted at these conditions to be extracted than polyphenols.

Response surface fittings were used to obtain the corresponding estimated optimal ratios. Maximum TPC/CHOs values (3.2 g_{PE}/g_{GE}) and TPC/(CHOs–UA) ratios were determined at the conditions of the shortest tR, the highest LS and at intermediate SP (79.2 W/cm^2), close to run 6. Maximum TPC/UA ratio (41.2 g_{PE}/g_{GE}) was obtained at the lowest SP, the highest LS and intermediate tR (4.3 min), close to run 3. These results indicated that some experimental points were close enough to optimal values and consequently no additional experiments were necessary to corroborate these theoretical values. Energy consumption (KJ) was evaluated, considering sonication area (6.2 cm^2), time and applied power. The energy/TPC ratio (KJ/(g_{PE}/L)) ranged from 14.6 ± 0.1 up to 96.3 ± 0.1, showing that extracts with the highest energy efficiency for polyphenols extraction were runs 5 and 14.

Both extracts achieved relatively high TPC values employing the lowest sonication energy (mainly related to short sonication times). Runs 5 and 14 gave the lowest energy/TPC ratio, and no significant ($p < 0.05$) differences were found between their values of TPC/UA, TPC/CHOs and TPC/(CHOs–UA) ratios. Particularly, the last ratio gave a promising high value (3.6 ± 0.1 g_{PE}/g_{GE}) indicating favorable conditions for the extraction of polyphenols and uronic acids against other carbohydrates. Globally, it was be concluded that conditions that maximized polyphenol extraction with low energy consumption corresponded to low LS (20 g_W/g_{AF}), short tR (2 min) and SP above 80 W/cm^2. Polyphenols, fucoidans and alginates are the objective of this PhD Thesis and correspond to the most bioactive compounds. Consequently, the extract obtained under the conditions of run 14, was the main working extract in the subsequent Sections 9 and 11 of this Thesis.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

8.6. ANTIOXIDANT ACTIVITY OF EXTRACTS

Antioxidant activities of ultrasound–assisted extracts were studied by DPPH, ABTS and FRAP methods. The DPPH scavenging activity assays showed radical scavenging capacities from 413 ± 73 (runs 6 and 11) up to 718 ± 55 μM_{TE} (run 14). ABTS scavenging activity values ranged from 232 ± 4 (run 6) to 491 ± 1 μM_{TE} (run 14) and FRAP activities between 10.2 ± 0.4 (run 6) and 16.5 ± 0.1 μM_{TE} (run 14). The antioxidant activity of the extracts showed similar trends for the three assayed methods. Indeed, independently of the employed method, run 14 (SP = 90 W/cm^2, tR = 2 min and LS = 20 g_W/g_{AF}) showed the highest antioxidant capacity. Prominent antioxidant activity of run 14 was used to select them as further working extract in this Thesis. Meanwhile the lowest antioxidant capacity slightly varied between methods and was always obtained in run 6 (SP = 80 W/cm^2, tR = 2 min and LS = 40 g_W/g_{AF}). Linear correlations ($R^2 > 0.9$) between antioxidant responses (DPPH, ABTS and FRAP) and TPC of extracts were found (Figure 8.3A, B and C).

Radical scavenging activities of seaweed extracts have been studied by several authors such as Goswami et al. (2016), who employing phloroglucinol standard molecule and reported values around 46 $mg_{PE}/mmol_{DPPH}$. Wang et al. (2009) studied extracts from *A. nodosum* seaweed by a liquid–solid extraction (20 g_W/g_{DS}) using two different solvents, water and acetone 70% (v/v), during 24 h at room temperature, and the highest IC_{50} (43.5 $mg_{PE}/mmol_{DPPH}$) was obtained with acetone blends. Kirke et al. (2017) reported IC_{50} values between 20 and 40 mg_{PE}/L at room temperature and 70 and 80 mg_{PE}/L at 50 °C measured from purified fractions extracts obtained from *F. vesiculosus* carrying out extractions with a liquid–solid ratio of 10 g_W/g_{DS} using water–methanol 80% (v/v) solution for 24 h. Audibert et al. (2010) presented IC_{50} values around 19 $mg_{PE}/mmol_{DPPH}$ from purified polyphenols fractions obtained from *A. nodosum* after liquid–solid extraction with methanol at 4 g_W/g_{DS}, 3 h at 40 °C.

Matanjun et al. (2008) studied several seaweed species and obtained ABTS values from 375 to 475 $mmol_{TE}/mg_{DE}$ and FRAP capacities of 63 and 92 mg_{TE}/mg_{DE} for extracts obtained from *Dictyota dichotoma*, *Sargassum polycystum* and *Padina* species, employing methanol or diethyl ether as solvents at ratios of 10 g_W/g_{DS} for 72 h. Audibert et al. (2010) reported ABTS values around 235–540 μg_{TE}/L. FRAP values obtained by Ummat et al. (2020) were around 50 μg_{TE}/L.

USC DE SANTIAGO DE COMPOSTELA

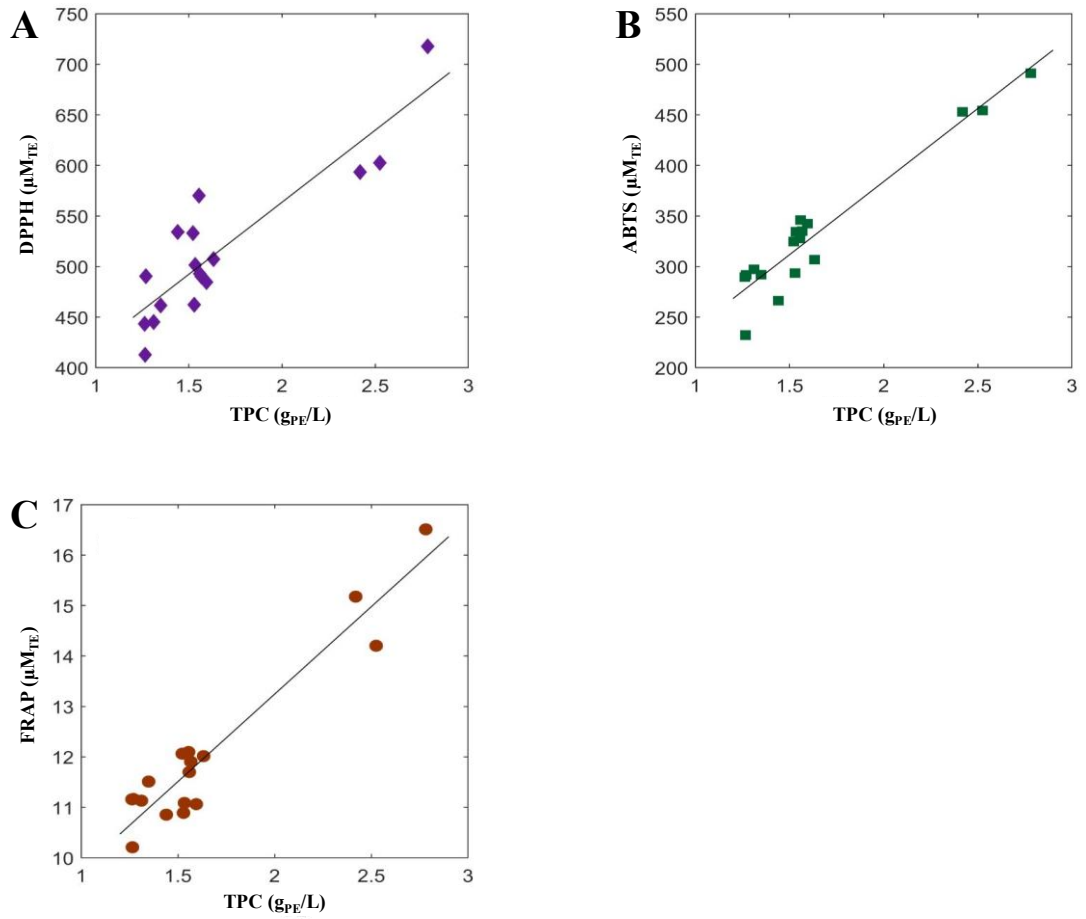


Figure 8.3. Linear correlations of antioxidant activities determined by DPPH (A), ABTS (B) and FRAP (C) methods, against TPC for UAE from *A. nodosum*.

Briefly, in comparison to above mentioned studies, obtained IC_{50} values from DPPH assays in this study showed lower scavenging activities than phloroglucinol, *A. nodosum* extracts or purified fractions from *F. vesiculosus*. Furthermore, reported that ABTS values were higher than those obtained here and, finally, FRAP values were in the same range. It is worthy to note that despite lower antioxidant responses of extracts were determined in this study, they were obtained after very short times of extraction with low energy consumption without further purification or fractionizing processes of the extracts compared with consulted literature.With regards to the linear correlations of DPPH, ABTS and FRAP with TPC, other authors also found these trends with other seaweeds.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Moreira et al. (2016) obtained these linear relationships with *F. vesiculosus* extracts and Matanjun et al. (2008) for red (*Eucheuma cottonii*, *Eucheuma spinosum* and *Halymenia durvillaei*), green (*Caulerpa lentillifera* and *Caulerpa racemosa*) and brown (*Dictyota dichotoma*, *Sargassum polycystum* and *Padina spp.*) algae. This linear trend suggested that polyphenols present in the extracts, regardless of extraction conditions, remained chemically similar (Connan et al., 2004) and TPC gave the main antioxidant responses of the extracts.

HIGHLIGHTS

Continuous UAE extraction modelling of *A. nodosum* at room temperature indicated that TPC of extracts depended on sonication power (SP) and liquid–solid ratio (LS) and minor dependence of time residence (tR).

Maximal TPC value (2.8 ± 0.01 g_{PE}/L) was obtained at SP= 90 W/cm^2, LS = 20 g_W/g_{DS} and tR = 2 min.

Carbohydrate and uronic acid contents depended on sonicated time, amplitude and solvent–solid ratio following a quadratic model.

Minimal energy consumption per gram of extracted phlorotannin (around 14.7 KJ L/g_{PE}) corresponded to: LS = 20 g_W/g_{AF}, tR = 2 min and SP > 80 W/cm^2

Antioxidant activities (DPPH, ABTS and FRAP) of extracts using short sonication times were comparable to those extracts obtained after long extraction (> 5 h) and organic solvents.

Antioxidant capacities were linearly correlated with the total polyphenol contents of the extracts confirming that main antioxidant activity of seaweed derived products is owed to their phlorotannins content.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

9. INFLUENCE OF OXIDATION AND DIALYSIS ON BIOACTIVITY

Current Section 9 adapted from:

Gisbert M, Sineiro J, Moreira R (2022) Influence of oxidation and dialysis of phlorotannins on bioactivity & composition of ultrasound–assisted extracts from *Ascophyllum nodosum*. *Marine drugs.* DOI: 10.3390/md20110706 (See Section 17)

USC DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

9.1. GENERALITIES

Isolation and chemical characterization of phlorotannins has gained special attention in recent years due to their bioactivities among the other specific characteristics mentioned along the Thesis. Nevertheless, their composition still remains scarcely studied and very few commercial standards are available in the market (Tierney et al., 2013). Several isolation methods have been reported in literature, but these components are in nature mainly high molecular weight polymers and their depolymerization process may be necessary. Phlorotannins oxidation can be developed in seaweed tissues, hence, this fact is suggested as one of the main steps in the complexation with carbohydrates and/or proteins during the formation of the cell membranes and walls, see Figure 1.4 (Steevensz et al., 2012; Breton et al., 2011). Additionally, seaweeds processing (milling, drying, extraction, and preservation) can promote these reactions. Xu et al. (2018) suggested the degree of oxidation can be used as variable to control the bioactivity of phlorotannins itself, or to modify color or taste, as it is done in production of tea, cacao, or wine (Lund, 2021). Quinones are one of the oxidized form of phlorotannins that maintain only partially the bioactivity of original phloroglucinol–derivatives, but this fact is still unclear (Zhang et al., 2018).

Phlorotannins extraction could be carried out with water, the safest solvent according to green chemistry principles, as unique solvent (Kadam et al., 2015a). Nonetheless, water shows lower extraction yields compared with organo–solvents, such as acetone or alcohols (Koivikko et al., 2007; Kadam et al., 2015a; Wang et al., 2016; Catarino et al., 2019; Allwood et al., 2020). Moreover, water simultaneously solubilizes polysaccharides and proteins, producing extracts formed by a complex mixture of compounds (Wang et al., 2016). In fact, it is not clear whether the extraction with organic solvents can promote the extraction of un–bound phlorotannins (Catarino et al., 2019) or alternatively, organo–solvents reduce the formation of phlorotannins complexes when they are extracted (Koivikko et al., 2007; Tierney et al., 2014). Thus, the selection of solvent type is critical and must be carried out with the objective of obtaining higher polyphenol content, higher antioxidant activity, lower economic– time–consuming together with the final use of phlorotannins as was aforementioned in Section 3.1.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The wide range of molecular sizes of phlorotannins (and/or complexation state) makes their separation, purification, and characterization difficult due to the oligomeric structure and similar polarity of those biopolymers (Parys et al., 2007). It was reported that antioxidant capacities of phlorotannins are related to their structure and molecular size (degree of polymerization), in fact, oligomeric phlorotannins are generally more active than highly polymerized compounds (Breton et al., 2011).

9.2. ASSAYED SYSTEMS

The aim of the current section was to generate phlorotannin–enriched extracts from *A. nodosum* UAE extracts using methods based on both size (using different cut–off membrane dialysis) and oxidation state. Ultrasound–assisted extraction (90 W/cm^2, 2 min and 20 $g_{solvent}/g_{AF}$; Sections 4.4.1 and 8) was carried using water (EW) and 70% (v/v) acetone:water mixture (EA) as extraction solvents. Phlorotannins–enriched extracts were separated through dialysis membranes with different cut–off values and the corresponding fractions were chemically analyzed. Besides, PW extract was dialyzed (Section 4.8) in cut–off cassettes (20, 10, 3.5 and 2 kDa), whereas it was obtained the compositional distribution by size. Additionally, the oxidation of phlorotannin–enriched fraction was studied to assess the resistance to oxidation of phlorotannins. Oxidation of PW extract was evaluated under natural oxidation (PON), forced aeration (POA) and hydrogen peroxide (POP) for 120h at room temperature (Section 4.7). Extracts were characterized by means of total polyphenols content (Section 4.6.1), carbohydrates (Section 4.6.2) and antioxidant activities (Section 4.6.4). Several spectrometry techniques such as MALDI–TOF–MS (Section 4.10), FT–IR (Section 4.11) and ^{1}H–NMR (Section 4.13) were carried out. And chromatography (RP–HPLC in on-off mode from Section 4.9) analysis of the extracts were employed to support the identification of some phlorotannins in the different extracts and fractions. The scheme of the research carried out could be seen in Figure 9.1

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

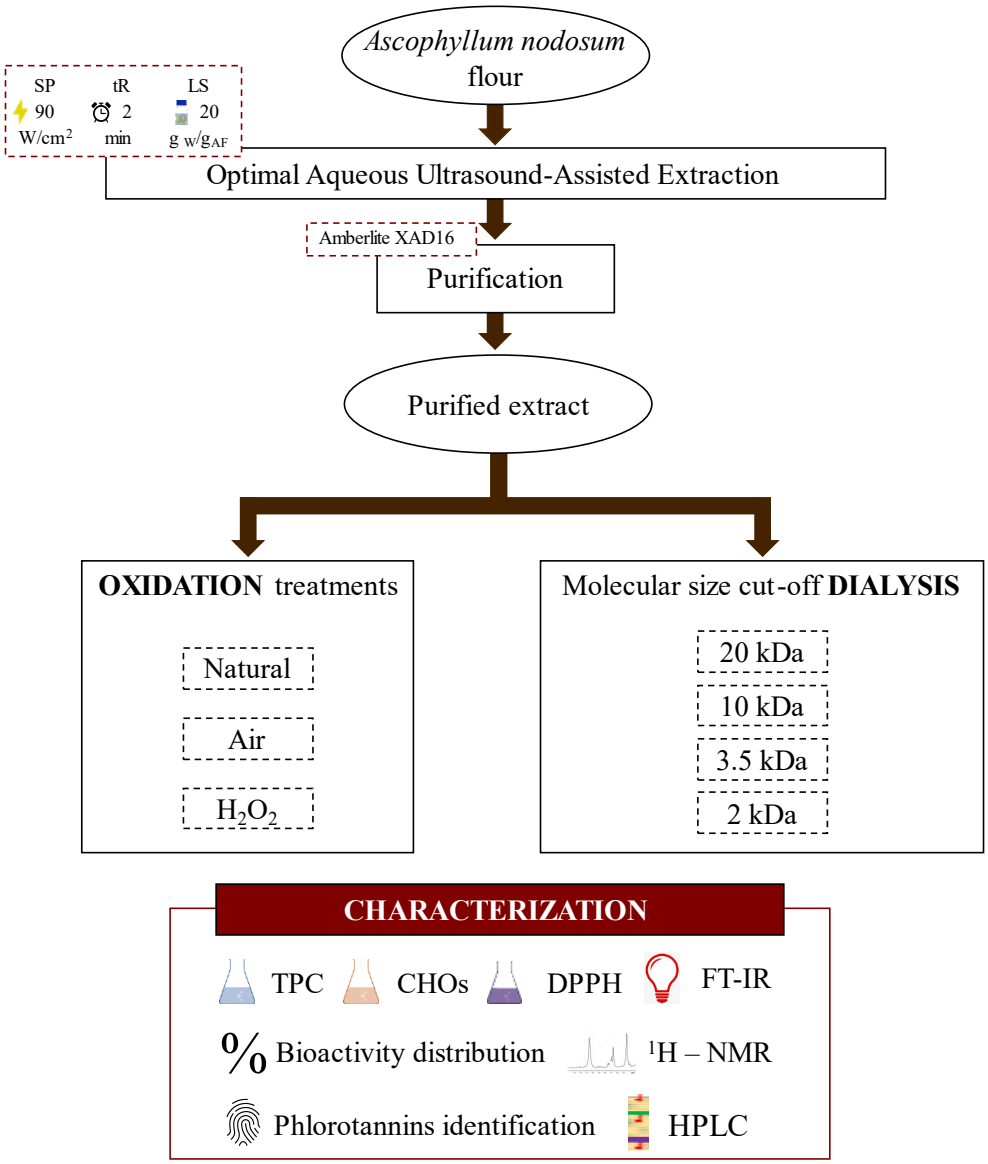


Figure 9.1. Systems experimentally obtained to evaluate the influence of oxidation and dialysis of phlorotannins on bioactivity and composition of ultrasound–assisted extracts from *Ascophyllum nodosum*.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

9.3. BIOACTIVITY CHARACTERIZATION

Several phytochemical fractions were obtained from *A. nodosum* Firstly, crude extracts were obtained from water (EW) and acetone:water (AE) extraction; thereafter, the EW was purified using Amberlite XAD16 column ro reduce the carbohydrate content (i.e., alginate isoforms) and the purified EW fraction (PW) was obtained (Section 11). PWs were subjected to several oxidation treatments employing air (natural (PON) and forced aeration (POA)) and hydrogen peroxide (POP). Finally, PWs were dialyzed using 2–20 kDa cut–off cassettes given additional extracts (PD20, PD10, PD3, PD2). Experimental values of total polyphenol content (TPC), total carbohydrate content (CHOs) and antioxidant activity by DPPH scavenging method (RSA) of obtained extracts are shown in Figure 9.2.

TPC values (Figure 9.2A) of crude extracts (EA and EW) varied in a wide range between 1.54 ± 0.14 and 5.67 ± 0.08 g_{PE}/L; CHOs values were ranged from 0.61 ± 0.05 up to 0.88 ± 0.02 g_{GE}/L, and DPPH values were between 1.38 ± 0.08 and 1.88 ± 0.05 g_{TE}/L. The highest content was obtained for acetone:water extract (EA), evidencing the notorious effect of the extracting capacity of this solvent type. Despite EA extract showed higher TPC value, its antioxidant activity was not so notorious as could be expected (Figure 9.2C) when compared with EW. Hence, EA extract also showed higher carbohydrate content than EW**,** evidencing that acetone:water 70% v/v was not so selective for polyphenols extraction as has been reported in several studies (Koivikko et al., 2007; Catarino et al., 2019). These results indicate that further separation of phlorotannins from carbohydrates (i.e., purification) is necessary to remove carbohydrates, mainly uronic acids in *A. nodosum*, when ultrasound–assisted extraction is performed, even when acetone:water mixture is used as solvent.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

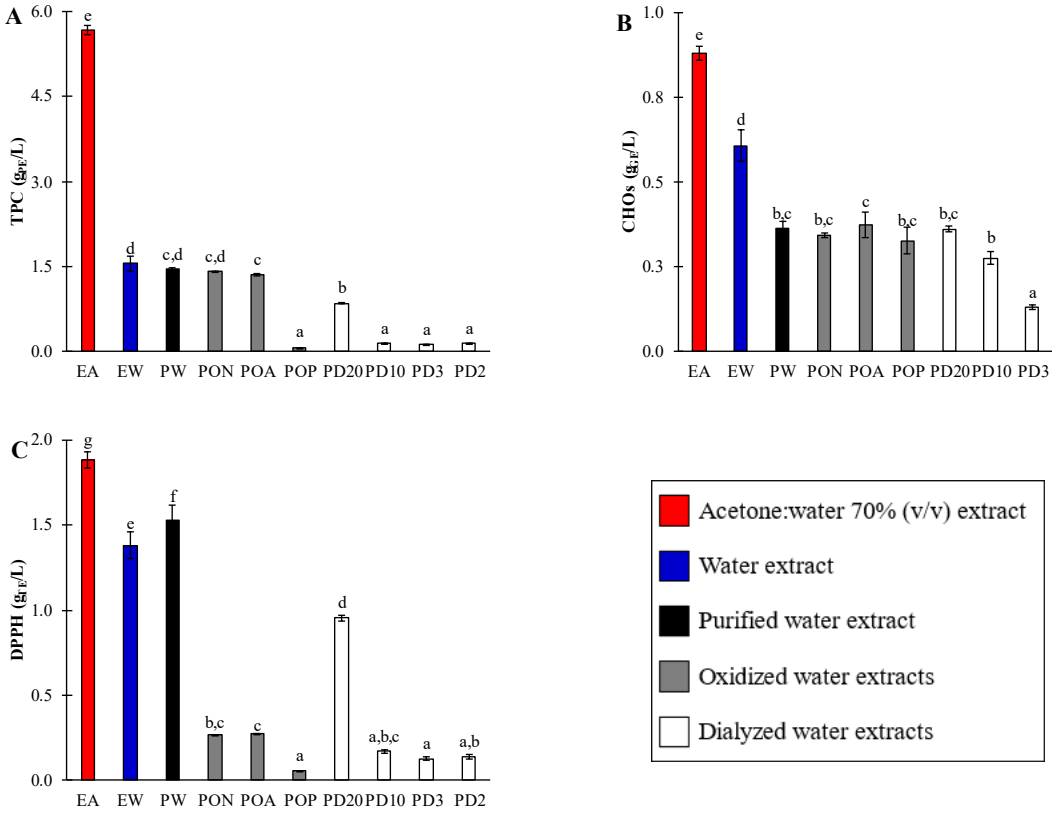


Figure 9.2. Phytochemical and antioxidant activity (TPC, A; CHOs, B and DPPH, C) of crude (EA and EW), purified (PW), oxidized (PON, POA and POP) and dialyzed, (PD20, PD10, PD3 and PD2) extracts. Different superscript letters indicate significant ($p < 0.05$) differences among values.

From these results, water was selected to be purified and to carry out the further oxidation assays and the dialysis fractionation, because of its character of reliable, cheap and food–friendlier solvent, as compared with acetone. PW showed similar TPC (TPC = 1.44 ± 0.02 g_{PE}/L, PW/EW = 1.1–fold higher) and DPPH values (1.53 ± 0.09 g_{TE}/L, 1.1–fold higher) to EW, however, with a significantly lower content of CHOs (0.36 ± 0.02 g $_{GE}$/L, 1.8–fold lower). Hence, when PW was compared with AE by means of ratios (± 0.1) corresponding to TPC (TPC_{AE}/TPC_{PW}) and CHOs ($CHOs_{AE}/CHOs_{PW}$) were 3.9–fold and 2.4–fold lower, respectively, but RSA (RSA_{AE}/RSA_{PW}) was just 1.2–fold lower. These results evidenced that water permitted the extraction of phlorotannins with affinities for carbohydrates that significantly reduced the antioxidant activity of EW that will be deeply seen in Section 11 that some authors name as phlorotannin–carbohydrate complexes (Wang et al., 2016). Thus, purification of EW is deeply recommended to achieve extracts comparable to EA. Indeed, in previous studies, it was reported that Amberlite XAD16 purification increased both antioxidant and amylase and glucosidase

enzyme–inhibiting activities of extracted polyphenols (Section 11). Thus, PW extract as an eco–friendlier option was selected for further oxidation assays and dialysis to determine how bioactivity depends on oxidation state and/or molecular size.

Catarino et al. (2019) obtained *Fucus vesiculosus* extracts and Wang et al. (2009) *A. nodosum* extracts, with TPC values of 0.86 and 0.28 g_{PE}/L, respectively. Both authors studied phlorotannins extraction with 70% (v/v) acetone:water solution with conventional solid–liquid extraction, at liquid–solid ratio of 20 $g_{solvent}/g_{algae}$ for 24 at rt. TPC values were significantly lower despite of very long conventional extraction (24h) employed in comparison to values achieved in this study (5.67 ± 0.08 g_{PE}/L) with UAE method. Zhang et al. (2007) obtained similar TPC values of 5.6 g_{PE}/L, applying conventional solid–liquid extraction, but employing 12.5 $g_{solvent}/g_{algae}$ of liquid–solid ratio using water–ethanol (50% v/v) as solvent and *A. nodosum* seaweed during long extraction times (90 min) and high temperature (80 ºC). Ummat et al. (2020) obtained extracts with TPC values of 4.8 g_{PE}/L using water–ethanol (50% v/v) as solvent, 10 $g_{solvent}/g_{algae}$ of liquid–solid ratio and long UAE times (30 min) from *Fucus vesiculosus* seaweed. Consequently, both authors reported close values to EA extract (5.67 ± 0.08 g_{PE}/L) but employing higher temperature, longer time, and lower liquid–solid ratio.

In the current Thesis, limited oxidation of PW extract was performed under oxidation conditions varying from soft (natural, PON and aerated, POA) to severe (hydrogen peroxide, POP), with the aim of evaluating the extract remaining antioxidant activity, to evaluate sensitivity of the phlorotannins enriched extracts. Oxidation of PW showed outstanding results. TPC values (Figure 9.2A) of oxidized extracts varied in a wide range from a minimum value for POP (0.06 ± 0.001 g_{PE}/L) to both PON and POA (1.4 ± 0.04). Regarding PW sample, natural and air–driven oxidation conditions caused minimal TPC reduction in both samples (1.63–fold lower), whereas POP showed a sharp TPC change (25–fold lower). Conversely, CHOs results (Figure 9.2B) showed no significant ($p < 0.05$) differences among all oxidation assays (PON, POA and POP) with minor changes concerning with PW extract (0.36 ± 0.02 g_{GE}/L) with only 1.05–fold higher values, because carbohydrates were not oxidable by hydrogen peroxide or oxygen in the assayed conditions here applied. Nevertheless, DPPH values (Figure 9.2C) showed that all oxidation procedures had a notorious effect on antioxidant activity, which values varied from 0.05 ± 0.01 (POP) up to 0.27 ± 0.04 g_{TE}/L (PON and POA), in contrast with an original value of PW (1.53 g_{TE}/L).

Oxidation had a dramatic effect on antioxidant activity, reducing DPPH values around 5.7–fold for PON and POA and around 29.8–fold for POP. Oxidations conditions evidenced that polyphenols maintained their structure and content (TPC values) but their antioxidant capacities (DPPH values) were reduced. Indeed, these results could also be used to assess the adequacy of the determination methods for screening antioxidants, whereas TPC method could not show different values due to the existence of partially oxidized phlorotannins.

Dialysis of PW extract was carried out throughout 20 (PD20), 10 (PD10), 3.5 (PD3) and 2 kDa (PD2) cut–off dialysis cassettes. TPC values (Figure 9.2A) varied between 0.22 ± 0.01 g_{PE}/L (PD10, PD3 and PD2) and 0.84 ± 0.01 (PD20). Similarly, DPPH scavenging activity (Figure 9.2C) showed the highest values for PD20 (0.96 ± 0.02 g_{GE}/L) and the other dialysates (0.14 ± 0.01). These results indicated that polyphenol molecules with sizes ranged from 2 up to 10 kDa were practically negligible (together with obviously the corresponding antioxidant activity). Finally, CHOs (Figure 9.2B) values increased with dialysis cut–off size from 0.07 ± 0.01 g_{GE}/L (PD2) up to 0.36 ± 0.01 (PD20). These results agreed with those of reported by Hermund et al. (2018), that proposed that the relationship between bioactivity and molecular size is related with the enclosed structure of each phlorotannin and the amount unavailable OH–groups, that are both, dependent on the branching of the phlorotannins.

To compare the phytochemical composition data with other studies the percentage distribution of TPC, CHOs and DPPH among the dialyzed fractions was calculated (Figure 9.3) assuming as total the content determined from PW (TPC = 1.44 ± 0.020 g_{PE}/L; DPPH = 1.53 ± 0.09 and CHOs = 0.36 ± 0.02 g_{GE}/L). CHOs were distributed between 0 and 20 kDa the following mode: < 2 kDa fraction accounted for 19.4 ± 2.0 % of CHOs, 2–3.5 kDa fraction rendered 16.7 ± 1.2 %, 3.5–10 kDa fraction was the major fraction, accounting for 38.9 ± 1.5 %, the 10–20 kDa fraction accounted for 25 ± 1.1 % and negligible CHOs percentage was detected in the fraction with size higher than 20 kDa (0.2 ± 0.07) (Figure 9.3). In Section 12, it was determined that the size of polymeric alginates extracted from *A. nodosum* after phlorotannins UAE was ranged from 133 and 428 kDa. However, no signals were obtained for sizes higher than 20 kDa, evidencing that purification and molecular size cut–off dialysis removed high molecular size alginic forms, then, CHOs signals corresponded to their smaller size oligomers that were found in all cut–off ranges.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

TPC and DPPH analysis considering polyphenols molecular sizes distribution determined the existence of three main regions: lower than 2 kDa (8.5 ± 1.0 and 9.2 ± 1.7), between 10 and 20 kDa (49.7 ± 0.2 and 53.6 ± 0.6) and larger than 20 kDa (41.8 ± 1.7 and 37.3 ± 2.8). These results showed that the largest population (around 50% of total TPC of the purified extract) and the most bioactive fraction (> 53% of total DPPH of the purified extract) was found at 10–20 kDa range. These observations agreed with several authors reporting that the larger phlorotannins were the main bioactive compounds in the seaweeds, such as Audibert et al. (2010), who found that size polyphenol fractions > 50 kDa showed the highest antioxidant activity, and Connan et al. (2004) that reported that higher bioactivities were obtained when dialyzed extracts were around 100 kDa.

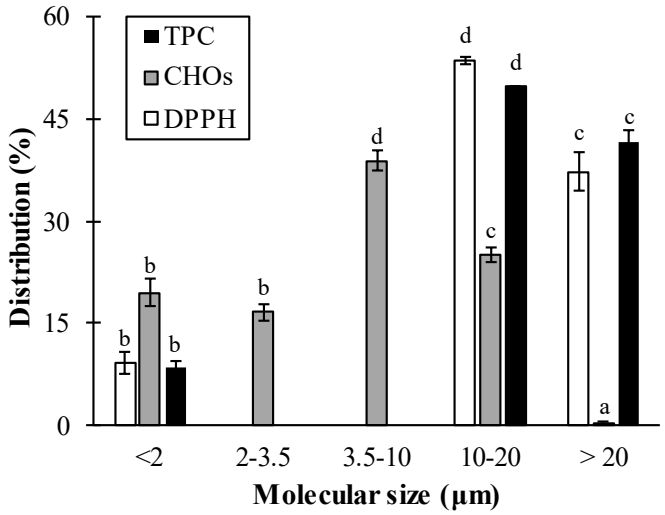


Figure 9.3. Distribution percentage of TPC, DPPH and CHOs values among dialyzed extracts.

Nevertheless, both Tierney et al. (2013) and Kubanek et al. (2004) reported that phlorotannins from *A. nodosum* and *Fucus vesiculosus* were around 3.5–100 kDa (40–60 % of polyphenols) and at least 32% of molecules were higher than 100 kDa. Paiva et al. (2017) working with *Fucus spiralis* also reported that highest bioactive fraction was obtained with 3 kDa ultra–filtrated extract. Bogolitsyn et al. (2019b) studied molecular size of phlorotannins from *Fucus vesiculosus* seaweed ranged from 8.7 to 49.3 kDa. Authors determined that highest TPC value (0.98 $g_{PE}/ g_{extract}$) was achieved with fraction around 30 kDa, whereas the highest antioxidant activity (0.91 $g_{AAE}/g_{extract}$, expressed as mg of ascorbic acid equivalents per $g_{extract}$) was obtained from 14.5 kDa fraction, demonstrating that the fraction ranging from 8 to 18 kDa was the most active.

Then, the obtained results in this Thesis agreed with all those authors, that explained the lower bioactivity of high molecular phlorotannins were owed by conformational changes caused by the formation of intra– and inter–molecular bonds (i.e., bioaccessibility to phlorotannins is limited with their high molecular fractions). Then, to achieve the isolation and/or identification of the main phlorotannins group that provided the best results, additional characterization techniques were applied.

9.4 CHROMATOGRAPHIC CHARACTERIZATION

There are no commercially available standards for phlorotannin polymers being resorcinol and phloroglucinol the only one available (Steevensz et al., 2012). Commercial and representative of the major compounds found in seaweeds were injected to test the elution pattern and deduce where oligomers of sugars, uronic acids, proteins and phlorotannin could be found. Additionally, extracts profiles were compared with commercial D–(+)–glucose, D–(+)–glucuronic acid, bovine serum albumin (BSA), resorcinol and phloroglucinol to determine sugar/protein presence. Residence times (tR) of assayed standard compounds weere 16.5, 19.1, 52.0, 52.1 and 52.1 min for glucuronic acid, glucose, phloroglucinol, BSA and resorcinol, respectively (Figure 9.4A). Detection was carried out at 266 nm reported as maximal phloroglucinol absorption wavelength (Catarino et al., 2019). Non–phenolic standards produced a much minor signal compared with prominent peaks observed from resorcinol and phloroglucinol (Figure 9.4A) confirming that main signals obtained from RP–HPLC could be assumed as phlorotannins isomers.

A. nodosum crude (EA and EW), purified (PW), oxidized (PON, POA and POP), and dialyzed (PD20, PD10, PD3 and PD2) extracts were analyzed with RP–HPLC technique. Profiles were characterized by widely separated peaks, corresponding to different phlorotannin oligomers (Figure 9.4 B and C) with relative similar chemical characteristics (i.e., affinities). This chromatographic profiling was carried out to with retention times and peak shapes determine the existence and relative abundance of different molecule families.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

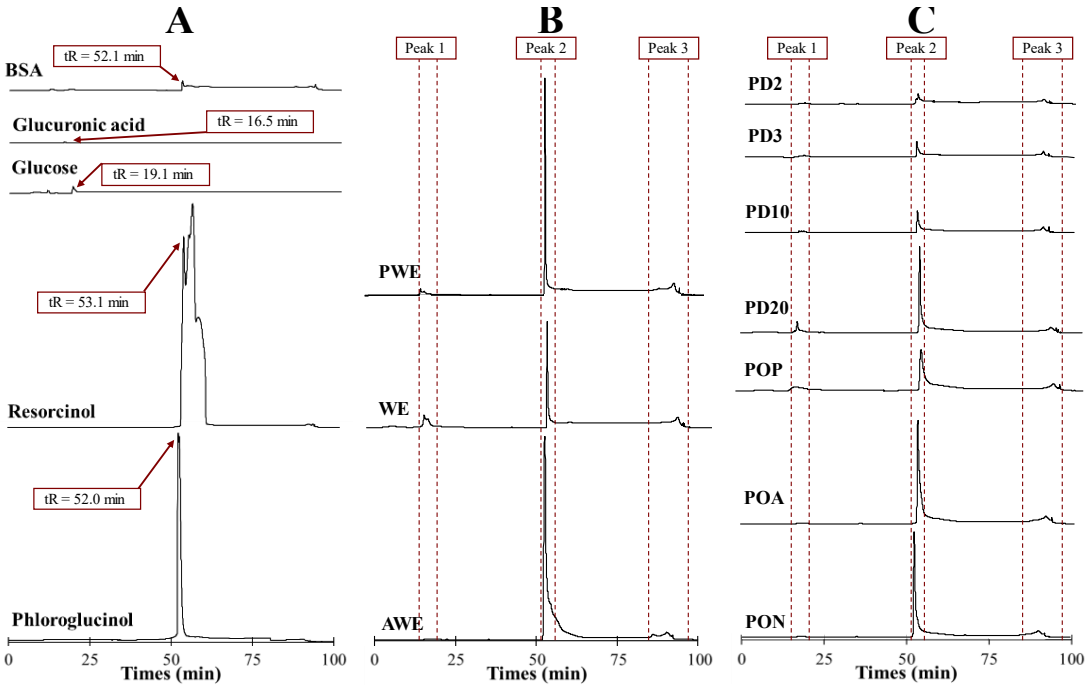


Figure 9.4. RP–HPLC profiles of (A) standard compounds (phloroglucinol, resorcinol, glucose, glucuronic acid and BSA); (B) crude and purified (EA, EW, and PW), (C) oxidized, (PON, POA and POP) and dialyzed (PD20, PD10, PD3 and PD2) extracts.

The chromatograms of the extracts (Figure 9.4B and C) showed three clearly defined regions, common in all extracts, meaning that three main groups of molecules (i.e., same retention time) were present in all extracts with different content values (i.e., different peak intensity). Variation of signal intensities evidenced that the use of different solvents and subsequent post–treatments modified the phlorotannin profiles. Peak 1 (tR = 16 ± 3 min) corresponded to the set of polar components that gave some absorbance at 266 nm and coincided with glucose and glucuronic acid tR values. This peak was associated to the presence of oligosaccharides or proteins with low affinity that elute rapidly. Peak 2 (tR = 53 ± 2 min) corresponded to main phlorotannin isomers with relative similar molecular size that coincided with tR values of phloroglucinol and resorcinol standard injections. Additionally, Peak 2 was in all samples the most notorious peak in comparison to Peaks 1 and 3. Finally, Peak 3 (tR = 90 ± 6 min) was associated with a high affinity compound for C18 column, assumed as high molecular size oligomers that eluted in a hump (Catarino et al., 2019).

EA showed a more intense Peak 2 signal compared with EW, that together with TPC values (Figure 9.3C) corroborated that acetone:water was a more efficient solvent for polyphenols

extraction than water; however, PW showed a prominent peak 2 signal like EA. The differences between EA and EW were related to their different content of phlorotannin–carbohydrate complexes. Tierney et al., (2013) also reported no significant ($p < 0.05$) difference between water and ethanol–water mixture, evidencing that phlorotannins profile was not much different. Extraction with water or acetone:water produced co-extracted carbohydrates (Figure 9.2B), however, Peak 1 (Figure 9.3B) signals were hindered by the prominent signal of Peak 2. CHOs reduction after purification, increased the amount of phlorotannin amount capable to interact with column (Peak 2) provided by PW sample.

All oxidized PWEs (PON, POA and POP) showed a Peak 2 that was present in all samples, but its signal intensity was notoriously reduced due to the partial loss of OH groups (Figure 9.3C). Peak 3 signals did not show significant ($p < 0.05$) changes among oxidized samples. It was Peak 2 the main difference among oxidizes extracts (PON > POA > POP) that reduce signals of each treatment. RP–HPLC results (Figure 9.3C) allowed to differentiate between both soft oxidation conditions, where air–driven oxidation (POA) showed a major reduction in Peak 2 than just natural oxidation. Finally, and according with phytochemical characterizations (Figure 9.2), POP extract showed a lower intensity peak concerning with PON, POA and PW extracts. This effect evidenced that bioactivity together with chemical nature of POP sample were altered after this severe oxidation treatment.

All dialyzed PWs fractions (PD20 > PD10 > PD3 > PD2) showed lower peaks signals than original PW (Figure 9.3C). Lower Peak 2 intensity of PD20 corroborated the proposed distribution of TPC and DPPH values (49.7% and 53.6%) where the main bioactive fraction was found from 10 to 20 kDa range. Peak 1 signals were related to different small alginate oligomers that were still present after both treatments (dialysis or oxidation) of PW, which presence was shown in Figure 9.3.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

9.5. SPECTROSCOPIC CHARACTERIZATION

9.5.1. FOURIER TRANSFORMED INFRARED (FT–IR)

FT–IR technique was used to appreciate notorious differences among assayed extracts, such as number of peaks (due to chemical reactions), peak intensities (differences in compounds content) and peak shapes (presence of similar groups). Then, FT–IR spectra for crude (EA and EW), purified (PW), oxidized (POP) and dialyzed (PD20 and PD2) extracts was showed in Figure 9.5. Oxidized POA and PON did not show significant ($p < 0.05$) differences with PW and dialyzed samples PD10 and PD3 showed similar spectra to PD2.

Three regions were evaluated as the main characteristic signals from polyphenolic compounds, Region 1 located around 2900 cm^{-1}, Region 2 around 1550 cm^{-1} and Region 3 around 1140 cm^{-1}. Region 1 was related to vibrational stretch of inter– and intra– molecular hydroxyl groups. Hence, these peaks are often used as criterion to measure the strength of the hydrogen bonding of polar molecules (Ertani et al., 2018). Region 2 corresponded to main phlorotannins and alginate signals. The 1700 and 1600 cm^{-1} were signals associated to aryl–aryl and ethyl ether bonds, both formed during polymerization of phloroglucinol in the formation of phlorotannins. Thus, FT–IR did not permit to discern among phlorotannin families (Hesse et al., 2005).

FT–IR spectra from two crude extracts (EW and EA) showed several peaks in EA slightly observed in EW sample (Figure 9.5). When Region 1 was analyzed, the spectrum of EA showed three peaks at 2830, 2910 and 3000 cm^{-1}, whereas only two peaks were observed in the spectrum of EW. The peak around 3000 cm^{-1} showed a lower signal in EW compared to AE, this change was associated to hydrogen bonding between phlorotannins and water, forming adducts that could explain the high intensity in EW rather than EA. The prominent peak around 2830 cm^{-1} seen in EA, that was negligible in all the other samples, was associated with traces of acetone. And the signal around 2910 cm^{-1} could be explained as hydrogen bonding between phlorotannins and carbohydrates (and/or alginates).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

EA showed, Figure 9.2B, a high CHOs value (as EW) due to the lack of selectivity of UAE. However, this signal was dramatically reduced in PW because of the carbohydrate and alginate isoforms removal after purification treatment also seen in Section 11. This fact was confirmed in oxidized and dialyzed extracts because this signal was minimally detected (Figure 9.5). Three signals of Region 1 were scarcely observed in dialyzed samples. PD20 maintained the peak corresponding to water content at 3400 cm^{-1}, however PD2 showed a displaced signal that hindered hydrogen bonds signals in Region 1. This result was explained by the high accessibility of small phlorotannins (PD2) that can interact with solvent traces and sugars.

Region 2 was the characteristic zone of stretching vibrations of C=C bonds of aromatic rings, with signals of carboxylic acids, aldehydes and/or ketones (Hesse et al., 2005). Indeed, 1600 cm^{-1} signal was associated with asymmetric stretching vibrations of carboxylate vibration in alginic acid (that will be seen in Section 12). Then, these signals at 1700 and 1600 cm^{-1} were associated to the presence of phlorotannins and alginate oligomers in the extracts. Crude extracts (EA and EW) analysis showed similar intensities in phlorotannin content that differed from TPC results (Figure 9.2A). Signal at 1700 cm^{-1} corresponded to different oxidation states that could be present in the enriched phlorotannins extracts. PW and PD20 samples showed similar intensity for the signal at 1700 cm^{-1} that increased in POP and PD2 samples. Similar FT–IR spectra of POP and PD2 together with results of TPC (Figure 9.2A), DPPH (Figure 9.2A) and Peak 2 of chromatograms (Figure 9.4) suggested that 1700 cm^{-1} signal was due to higher content in oxidized phlorotannin forms.

The low alginates content after purification was confirmed since their characteristic signal (1600 cm^{-1}) was negligible in PW extract and subsequent samples (oxidized and dialyzed). FT–IR confirmed that purification treatment was suitable to remove alginates and other carbohydrates. Consequently, phlorotannins were more accessible and at the same TPC the bioactivity increased (DPPH value). Furthermore, it was previously reported that purified and crude extracts showed different enzymatic inhibition activities (seen in Section 11). FT–IR results were complemented with ^{1}H–NMR and MALDI–TOF techniques to isolate possible chemical structures that explained those bioactivities observed in extracts.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

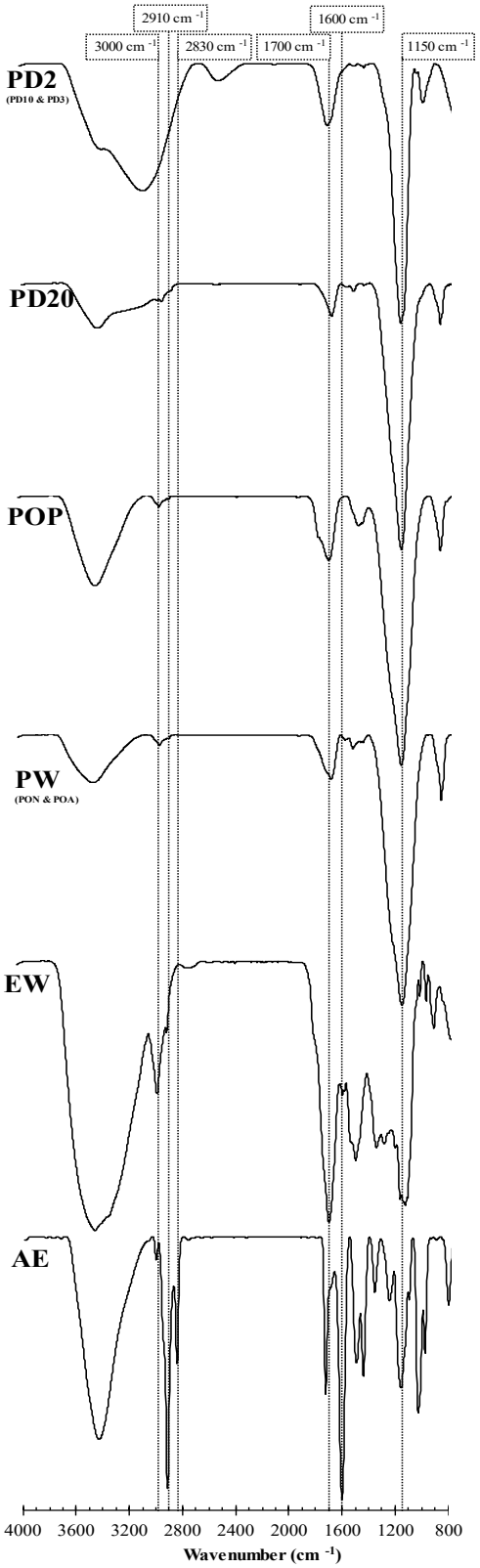


Figure 9.5. FT–IR spectra of crude (EA and EW), purified (PW), oxidized (POP) and dialyzed (PD20 and PD2) extracts.

USC
DE SANTIAGO
DE COMPOSTELA

9.5.2. HYDROGEN NUCLEAR MAGNETIC RESONANCE (^{1}H–NMR)

^{1}H–NMR analysis was carried out to give a proximate insight of chemical structure of the crude (EA and EW), purified (PW), oxidized (POP) and dialyzed (PD20 and PD2) extracts. Similarly, to which was found for FT–IR spectra, no significant ($p < 0.05$) differences were observed between POA and PON with PW and between PD10 and PD3 with PD2. ^{1}H–NMR spectra of these samples showed noticeable signals in the range 4.8–9.6 ppm (Figure 9.6), where the main polyphenolic compounds could be identified together (Breton et al., 2001; Zhang et al., 2004; Parys et al., 2007; Vissers et al., 2017).

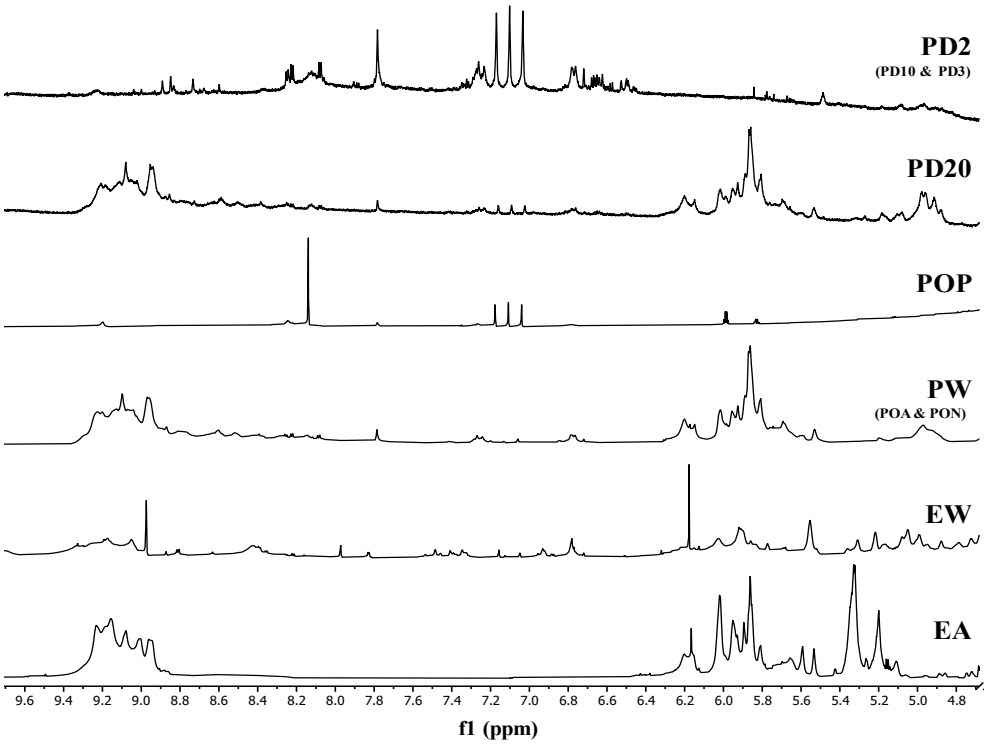


Figure 9.6. ^{1}H–NMR (DMSO–d_6) spectra of crude (EA and EW), purified (PW), oxidized (POP) and dialyzed (PD20 and PD2) extracts.

^{1}H–NMR of crude extracts (EA and EW) showed noticeable differences (Figure 9.6). EA extract showed two clearly differentiated areas where all the signals were aggregated, between 8.8 to 9.4 ppm and 5.0 to 6.4 ppm whereas EW spectrum showed peaks throughout all the spectrum, with two noticeable peaks at 6.15 and 8.95 ppm, matching to those group of signals found in EA spectrum.

The observed differences between crude extracts could be related to the presence of carbohydrates and phlorotannins complexes. It seems that acetone:water extraction minimized the binding of phlorotannins with other compounds, showing two main groups: one composed of almost pure phlorotannins, assigned to the high chemical shift (around 9 ppm) and a second group of partially bound (6 ppm) phlorotannins, closer to "sugars" region (< 5 ppm). However, the use of water solvent water seemed to promote complexation mechanisms, several peaks appearing along spectra evidencing a wide variety of compounds (and/or complexes). This hypothesis was supported when purified extract (PW) was analyzed (Figure 9.6) and it showed more similarities with EA rather than EW.

Interestingly, it was found that oxidized PW extracts showed that ^{1}H–NMR spectra of PON and POA (data not shown) did not show significant ($p < 0.05$) differences with PW samples. Hence, as it was seen in TPC (Figure 9.2A) and FT–IR (Figure 9.5) results, soft oxidation conditions were not enough to alter proton profile shifts. Nevertheless, hydrogen peroxide oxidation dramatically changed the NMR spectrum. Three main group of signals was observed in POP spectrum, a peak around 8.2 ppm, three small peaks at 7.1 ppm, and two smaller peaks close to 5.9 ppm. Hydrogen peroxide oxidation was strong enough to almost remove the bioactive capacities of the extracts, however, the low signals explained their last remaining antioxidant capacities (Figure 9.2C). Indeed, these signals could be further used as partial reference of quinones presence assessment.

Breton et al., (2011) defined the purity of their samples by the apparition of exclusive peaks between 5.5 and 6.5 ppm. Authors did not report signals at chemical shifts higher than 8.8 (due to alcohols proton) because they were only visible with deuterated DMSO solvent. Thus, phlorotannin signals in EA and PW samples that seemed more abundant at 5.0 to 6.4 ppm range were assumed as indicator of purity. When PW dialysates were analyzed (Figure 9.6), it was noted a difference between PD20 and the other dialysates below 10 kDa (PD10, PD3 and PD2). PD20 showed some minor differences at 7.1 ppm, with three small peaks like POP extract that did not appear in PW. PD20 after 120h of dialysis treatment could contain quinones formed as self–oxidation process corresponding to that three peaks signal.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

According to TPC (Figure 9.2A), DPPH (Figure 9.2C) values, dialysates below 10 kDa showed notorious differences as compared with the original PW extract similar as it was seen in FT–IR (Figure 9.5). ^{1}H–NMR of dialysated extracts showed peaks located between 6.4 and 8.4 ppm, in coincidence with three characteristic peaks found in POP spectrum (around 7.0 ppm) corresponding to the oxidized polyphenols (quinones).

^{1}H–NMR spectra similarities between PW and PD20 samples evidenced that the main bioactive fraction was obtained between 10 and 20 kDa, as it was observed previously in the analysis of components distribution (Figure 9.3). Additionally, spectra were able to match the different bioactive capacities observed when comparing <2 kDa dialyzed samples with PD20 and PW extracts. Hence, it was corroborated the low phlorotannins content in the dialysate fractions (< 10 kDa) and that molecules were partially oxidized explaining their low bioactivities together with Peak 2 signals observed in chromatographic profiles.

9.5.3. MALDI–TOF–MS

MALDI–TOF–MS analysis of purified (PW) and dialyzed extracts (PD20 and PD2) as the most pure, clean, and treated samples, was performed to tentatively identify some of the phlorotannins extracted form *A. nodosum* seaweed. Spectra of PW measurable signals was ranged from 969 to 30722 *m/z* (mass–to–charge ratio), showing the high molecular weight and the complexity to characterize the phlorotannins in the extract even after purification, being major signals found at *m/z* values lower than 4000. PD20 ranged from 1001 up to 20506 *m/z* with widely separated peaks (1010–2797 *m/z*) by 162 *m/z* neutral loss. However, PD20 sample still showed several and hampered signals that difficulted a suitable phlorotannin identification. Finally, PD2 extract ranged from 500 up to 1988 *m/z* also showed separated peaks by 162 *m/z* and three peaks (586, 702 and 928 *m/z*) that did not match proposed fragmentation pattern. The mass fragment proposed of [*m/z*] = 162 was observed by several authors indicating that it may be related to the cross–ring cleavages (Catarino et al., 2019). Identifications and degree of polymerization were carried out based on the loss of phloroglucinol (PGU) moieties (126/125/124 *m/z*) and two hydroxyl/water (OH, H_2O) moieties (17/18 *m/z*) that corresponded to 162 *m/z* separation of PD20 and PD2 samples (Figure 9.7).

USC UNIVERSIDADE DE COMPOSTELA

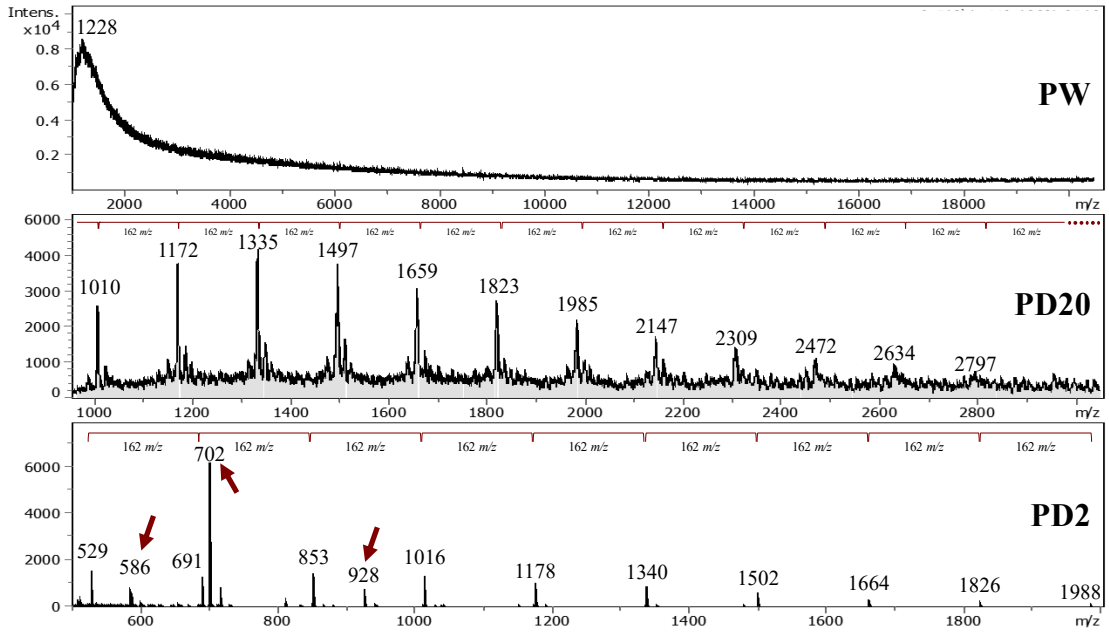


Figure 9.7. MALDI–TOF–MS spectra of purified (PW) and dialyzed extracts (PD20 and PD2).

Phloroglucinol as the unique monomer of phlorotannins and the loss of water molecules (and/or OH groups) and deprotonation are effects generally considered during phlorotannins identification (Stevenz et al., 2012; Visser et al., 2017; Catarino et al., 2019; Allwood et al 2020). Thus, 1 PGU with 2 H_2O molecules, was used to determine the degree of polymerization (DP) of main signals by Equation (9.1):

$$DP = \frac{[m/z]_m + n[H_2O]}{[PHL] + 2[H_2O]} \quad (9.1)$$

where DP was degree of polymerization in phloroglucinol units (PGU); $[m/z]_m$ was the measured [*m/z*] value of each peak; [PHL] was the [*m/z*] value of phloroglucinol, *n* was the number of water molecules lost; and $[H_2O]$ was the [*m/z*] value of water.

DP value of dialyzed extracts showed that phlorotannins ultrasound–assisted extracted from *A. nodosum* seaweed were ranged from 4–9 and 7–17 PGUs for PD2 and PD20, respectively. Several literature reports confirmed that average phlorotannin DP value is ranged around 3 up to 15 PGUs (Table 9.1), that could reach values of 39 PGUs (Steevensz et al., 2012).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 9.1. Phloroglucinol units of dialyzed PD20 and PD2 extracts in comparison with extracts from brown seaweeds reported by bibliography.

Literature reference	Seaweed *spp.*	DP range
This Thesis	*A. nodosum*	4 – 16
Allwood et al., (2020)	*A. nodosum*	11 – 18
Bogolitsyn et al., (2019a)	*Fucus vesiculosus*	3 – 8
Catarino et al., (2019)	*Fucus vesiculosus*	5 – 10
Hefferman et al. (2015)	*Fucus vesiculosus*	3 – 16
Kadam et al., (2015b)	*A. nodosum*	4 – 12
Kellogg et al. (2014)	*Fucus distichus*	3 – 18
Lopes et al. (2018)	*Fucus vesiculosus*	3 – 6
Sardari et al., (2021)	*A. nodosum*	3 – 6
Steevensz et al., (2012)	*A. nodosum*	4 – 39
Tierney et al., (2014)	*A. nodosum*	6 – 11
Vissers et al. (2017)	*Laminaria digitata*	3 – 27
Wei et al. (2003)	*Sargassum kjellmanianum*	9 – 12

Phlorotannins empirical formula was employed (Table 9.2) due to the several possible conformations of phloroglucinol that could be found in seaweed that are still under discussion (Steevensz et al., 2012; Ford et al., 2019). The highest signal detected was around 702 *m/z*, also reported by Allwood et al. (2020) without identification and assumed as a fragmentation residue. Here, it was hypothesized that could correspond to a fragment of dieckol ($C_{30}O_{22}H_{18}$) without two water molecules or isomer of the dimeric form of hydroxytrifuhalol A, reported by Corona et al., (2016), here forming an adduct with OH^-.

The exact structure, bindings and sequence of phloroglucinol units remain uncertain, particularly for those with high molecular weights since linkage positions cannot be easily assigned. For instance, Nwosu et al. (2011) reported that phlorotannins isolated from *A. nodosum* were derived from 7–phloroeckol, however, Allwood et al., (2020) suggested that phlorotannins in *A. nodosum* seaweed were predominantly fucophlorethol–A type due to preponderance of cleavages of ether bonds. This result agrees to previous FT–IR analysis where ethyl–ether signal (1410 cm^{-1}) was prominent, especially for those derived from PW (i.e., dialyzed, and oxidized samples).

USC DE SANTIAGO DE COMPOSTELA

Indeed, obtained families in Table 9.2 were possibly related with the different group of phlorotannins shown in Figure 1.3. The main drawback of phlorotannins is that despite they could have similar DP values or molecular sizes, they produce different fragmentation patterns (Catarino et al., 2019) that do not allow their identification.

Table 9.2. List of phlorotannins identified in (Figure 9.7) and its water loss n[H_2O] comparing data with Steevensz et al., (2012) & Kellogg et al. (2014). Superscript letters were used to identify different phlorotannins families within the extract

Formula	Literature	[H_2O] loss	This study *[m/z]*	DP
$C_{30}H_{22}O_{15}$	621	5	529[a]	4
	621	2	586[b]	4
$C_{36}H_{26}O_{18}$	745	3	691[a]	5
$C_{84}H_{58}O_{42}$	868	1	853[a]	5
$C_{90}H_{62}O_{45}$	930	0	927[c]	6
$C_{150}H_{102}O_{75}$	1033	1	1015[a]	6
$C_{114}H_{78}O_{57}$	1178	0	1177[a]	7
$C_{264}H_{178}O_{132}$	1364	1	1339[a]	8
$C_{294}H_{198}O_{147}$	1519	1	1501[a]	9

Allwood et al. (2020) found that dialysis could also modify the fragmentation patterns. Thus, another explanation to the lack of matching between obtained values in the present research and literature data can be the consideration of the presence of oxidized molecules in PD2. Oxidation state showed their prominent effect in bioactive (Figure 9.2), chromatographic (Figure 9.3) and spectroscopic (FT–IR, Figure 9.5, and ^{1}H–NMR Figure 9.6) characterizations, that could also affected to MALDI–TOF–MS analysis producing a different fragmentation pattern. The existence of an important fraction of formed quinones, which may be more prone to form adducts with water because of its higher its polarity than phenols, could several of the mass fragments detected by MALDI–TOF–MS. Then, obtained results contribute to the current literature for the fragmentation pattern and DP values of phlorotannins from *A. nodosum*. The results derived from the present research may be further used to determine possible presence of oxidized species of phlorotannins, that may lead to understand the nature and behavior of these complex and bioactive compounds.

HIGHLIGHTS

Aqueous mixture of acetone (EA) was better phlorotannins extractant than water (EW) (3.7–fold higher) but renders an extract with significant ($p < 0.05$) amount of carbohydrates and alginates (1.5– and 1.4–fold higher, respectively).

Purification of EW extract (PW) reduced the carbohydrate content (i.e., removing high molecular size alginate oligomers) leaving a similar polyphenol content but with higher antioxidant activities than EW

Purification treatment removed alginate isoforms bonded to some phlorotannins, leaving free–phlorotannins that after purification showed higher antioxidant activities.

Antioxidant activity was partially maintained after oxidation (natural, PON and aeration, POA) and only hydrogen peroxide oxidation (POP) achieves a significant ($p < 0.05$) oxidation of PW extract phlorotannins.

Phlorotannins with a size between 10 and 20 kDa were the main bioactive phlorotannins, with TPC and DPPH retaining 49.7 and 53.6%, respectively of the total antioxidant activity of PW.

Dialyzed extracts with the minimum molecular size < 2 kDa showed low antioxidant activity as compared with highr size phlorotannins oligomers.

MALDI–TOF–MS allowed the identification of degree of polymerization (4–16) and main fragmentation pattern based on the loss of 1 molecule of phloroglucinol and 2 of water. Conversely, some peaks did not follow this pattern evidencing the presence of different groups of phlorotannin.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

10. *ASCOPHYLLUM NODOSUM* & CORN STARCH INTERACTIONS

Current Section 10 adapted from:

Gisbert M, Aleixandre A, Sineiro J, Rosell CM, Moreira R (2022) Interactions between *Ascophyllum nodosum* seaweeds polyphenols and native and gelled corn starches. *Foods* 11, 1165 DOI: 10.3390/foods11081165 (See Section 17)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

10.1. GENERALITIES

The demand for gluten–free products is increasing since celiac disease affects around 1% of world population. However, food industry seems to be more focused on technological than nutritional quality (O'Shea et al., 2014; Mihaylova et al., 2021). Gluten–free diet can include starchy foods that usually show high glycemic index and fat content together with low protein content (Matos & Rosell, 2011). The Food and Agriculture Organization of the United Nations together with the World Health Organization recommends reducing the intake of these high glycemic–index meals, since its continuous consumption increases metabolic disorders such as glucose intolerance, obesity, and type–II diabetes (Dall'Asta et al., 2020). The design of new gluten–free products using natural and safe additives, with health benefits to celiac patients, is a current challenge (Parada et al., 2019).

The main polyphenols from seaweeds, i.e., phlorotannins, are promising nutraceuticals deeply explored in Section 1.4. They are being considered as a valuable source of antioxidants by drugs and food industries (FAO, 2018). Polyphenols extracted from *Ascophyllum nodosum* (*A. nodosum*) brown seaweed are potential food additives that may act as competitive inhibitors of digestive α–amylase and α–glucosidase enzymes, inhibiting the hydrolysis carbohydrates and thus reducing the glucose absorption levels (Parada et al., 2019) largely discussed in Sections 1.6 and 3.3. Ingested phenolic compounds usually have low bioavailability due to its high sensitivity to the gastrointestinal tract environments (Qiu et al., 2016).

In vitro studies are easy, rapid, widespread, and reproducible methods to evaluate inhibitory phlorotannins capacities (Sun et al., 2020; Diez–Sánchez et al., 2021). The inhibiting capacity of polyphenols against starch digestive enzymes (mainly α–amylase and α–glucosidase) is most often studied by mixing polyphenols with the enzyme followed by addition of substrates promoting polyphenols–enzymes interactions. In real food consumption, the inhibition of starch digestive enzymes can be achieved either by taking digestive enzymes inhibitor pills of chemical–handmade (i.e., acarbose, miglitol and voglibose) or increasing the intake of natural polyphenols (Dall'Asta et al., 2020).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

An alternative strategy may be the integration of polyphenols within food matrices; therefore, it is necessary to ensure the polyphenol contact with digestive enzymes at small intestine. The polyphenols–starch interactions might influence their enzymatic inhibitory capacities, stressing the relevance of the selection of the most adequate method for an optimal intestine delivery, bioavailability, and beneficial health of this new generation foods (Wang et al., 2021).

Phlorotannins are easily affected by oxygen, pH, ions, light, and temperature (Amoako & Awika, 2016). As reported by Guo et al. (2021), the design and production of foods with added phlorotannins have synergic beneficial effect, since meals quality is improved and polyphenols deterioration rate is reduced (Guo et al., 2021). Starch matrices have demonstrated to act as shelters of various food bioactive compounds such as fatty acids, phenolic compounds, vitamins, and probiotics (Zhu, 2017). Polyphenol–starch blends have been largely studied because starches are low cost, abundant, edible, non–allergenic, film–forming capacities, emulsification properties (Deladino et al., 2015) and classified as Generally Recognized as Safe (GRAS). Nowadays, starchy–based encapsulation is being applied for diverse biomedical and industrial applications (Qiu et al., 2016). The polyphenols can interact with starch and other macromolecules in reversible (solubilization, emulsification, film–forming, and sorption) or irreversible ways (crosslinking, acetylation, esterification, and oxidation) (Guo et al., 2021).

There is extensive research literature reporting mechanisms of sorption, complexation, interaction, or encapsulation of polyphenols on several starchy materials (wheat, corn, potato, yuca, green pea, beans), using a wide variety of bioactive sources (tea leaf, yerba mate, sorghum) and using different immobilization methodologies (Qiu et al., 2016; Wang et al., 2021). However, studies considering polyphenols from seaweeds into these new bioactive starchy products are scarce, as it was previously commented (Sections 1.7.2, 1.7.3 and 3.3). In the present work it is proposed to add the seaweeds to recipes acting as a two–step process with an initial solid–liquid extraction of polyphenols from *A. nodosum* powder (AF) followed by the sorption/complexation process between polyphenols and starch.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The use of the whole raw material (dried seaweed) as source of bioactive compounds involves a reduction of time, costs, and energy consumption of processing, since extraction and purification processes are omitted (Martínez–Maqueda et al., 2018). As main drawback, seaweeds can contain undesirable components, such can be an eventual excessive iodine content, which oral intake is limited by law in foods, or some heavy metals, depending on their origin. Additionally, the special taste of brown seaweeds are critical aspects for the production and consumer–acceptance of these seaweeds–derived products. A proper selection of the seaweed raw material (species, origin, seasonal harvesting), processing (drying, preservation) and extraction and purification of bioactive compounds, together with their integration in the food matrices, can be used to diminish these problems (Parada et al., 2015). Moreover, seaweed–starch complexations have demonstrated to extend food shelf–life, protect bioactive molecules against gastrointestinal tract conditions, and release them in intestine (Deladino et al., 2015). The characteristics of starch gelatinization process in the presence of seaweeds (or polyphenols from them) is proposed as key aspect on bioactive immobilization on starchy materials in the present research. Increasing the knowledge of this process would suppose a relevant advance in food science and technology since products with tunable sensory, bioactivity and rheological properties could be achieved with simple operational and manufacture control (Igoumenidis et al., 2018).

10.2. ASSAYED SYSTEMS

The effect of several blending procedures between *A nodosum* seaweed flour (AF) and corn starch (CS) on the interactions between polyphenols and starch was studied in the current Section 10. These methods comprised the blending of AF with native starch (NT), with previously gelled starch gel (GL) and promoting the gelling of corn starch in the presence of AF (CGL), as it was explained in Section 4.14. Different AF–CS ratios, from 1:0.5 to 1:25 were studied. The liquid phase was chemically characterized by polyphenols (Section 4.6.1), carbohydrates (Section 4.6.2) content and the antioxidant activity by assayed by three methods: DPPH scavenging activity (Section 4.6.4), ABTS reduction (Section 4.6.5), and iron–reducing capacity by FRAP method (Section 4.6.6) methods. The solid phase was characterized by FT–IR (Section 4.11) and SEM (Section 4.16) techniques. The Halsey model successfully fitted the

USC DE COMPOSTELA

equilibrium TPC in liquid and polyphenols adsorbed/retained (Section 4.14) by the solid phase of tested systems. The obtained results would allow us to determine the enhancement of the polyphenols retention to achieve starchy foods with high bioactivity. The scheme of the research carried out could be seen in Figure 10.1.

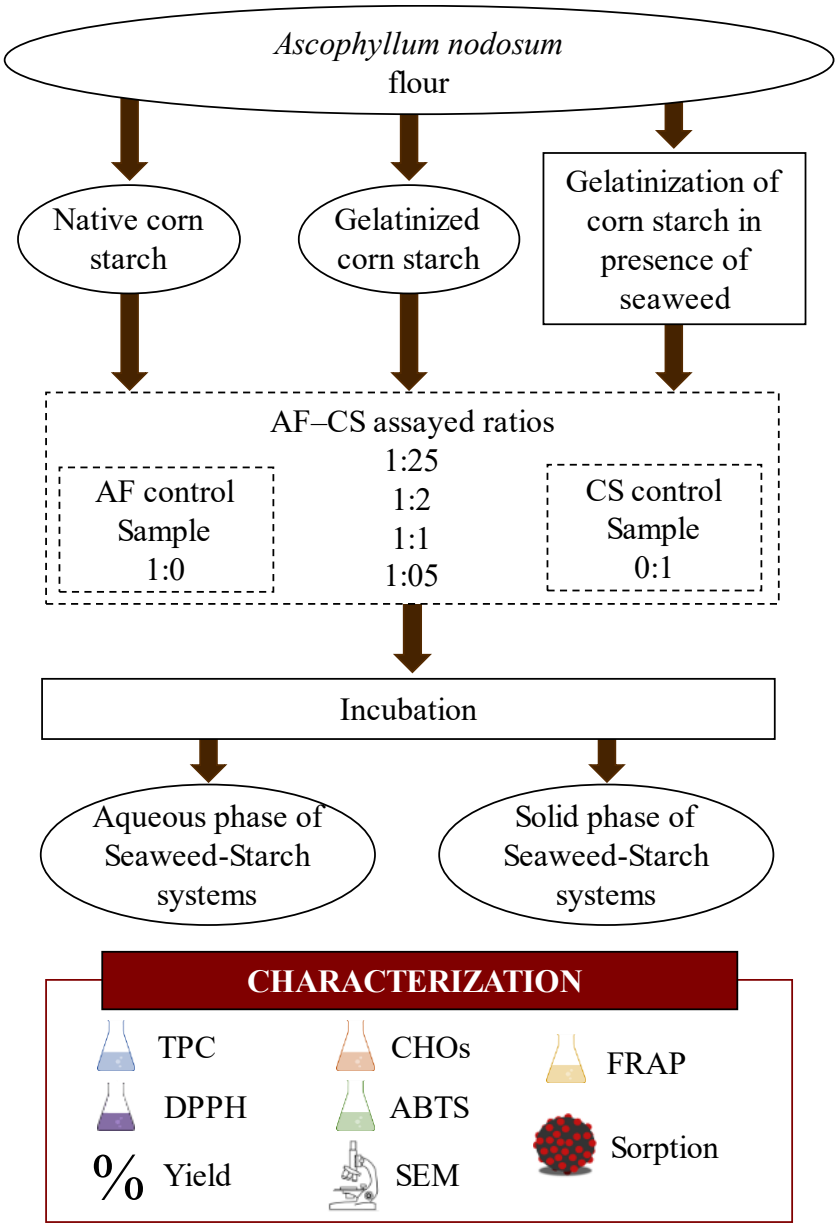


Figure 10.1. Schematic process used to determine *A. nodosum* seaweed interactions with corn starch based on different mixing procedures.

10.3. AQUEOUS PHASE CHEMICAL CHARACTERIZATION

Aqueous phase from *Ascophyllum nodosum* seaweed powder (AF), corn starch (CS) and AF–CS blends were chemically characterized, determining polyphenols content (TPC), carbohydrates content (CHOs) and their antioxidant activities by DPPH, ABTS and FRAP methods. TPC and CHOs values of AF–CS blends and control samples are summarized in Figure 10.2A and B, respectively. For the CS control, it was necessary to evaluate according to CS structures, and native (NT samples) and gelled CS (GL and CGL samples) controls were employed in each case.

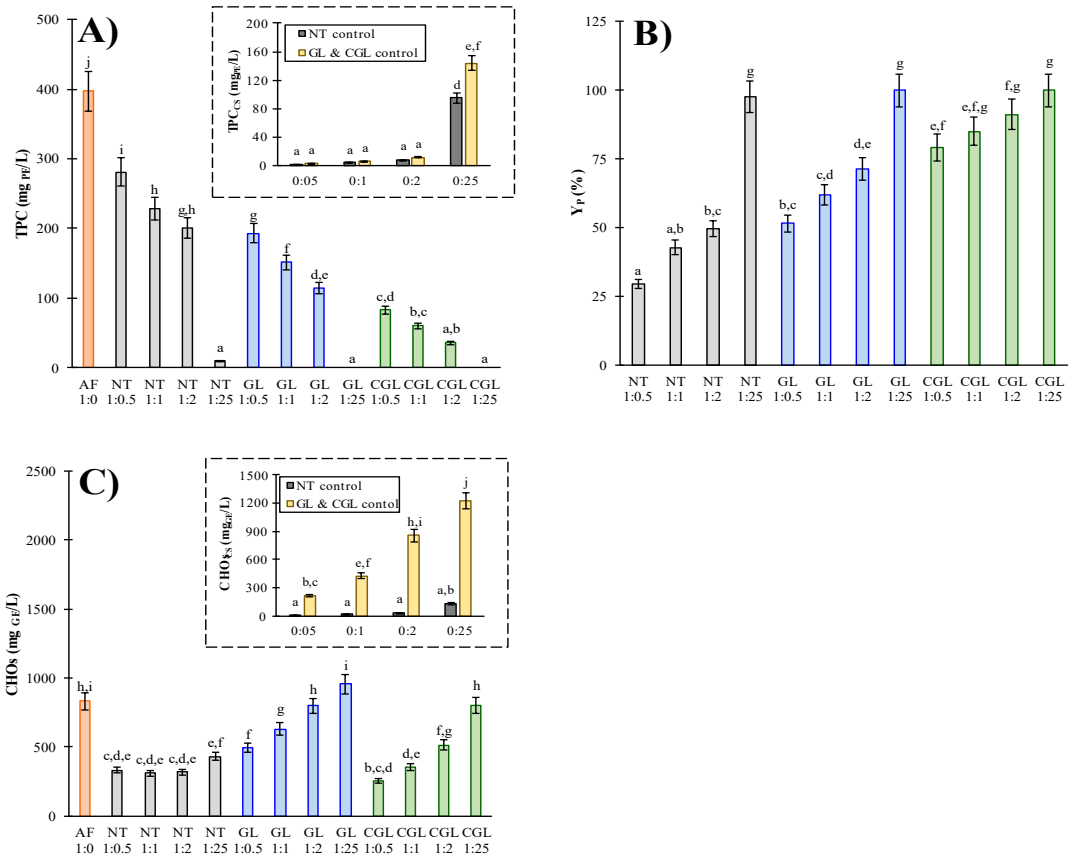


Figure 10.2. TPC values (A), sorption yields, Y_P (B), and CHOs values (C) of AF (), CS (native () and gelled () control samples and AF–CS blending methods of NT (), GL () and CGL () samples. Different letters indicate significant ($p < 0.05$) differences among samples.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

10.3.1. TOTAL POLYPHENOLICS CONTENT (TPC)

The average total polyphenols content of *A. nodosum* control sample in solution (TPC_{AF}) was 396.9 ± 28.6 mg_{PE}/L. The TPC evaluation of corn starch control samples was performed to correct the values of total polyphenols for some reducing power that starch–derived carbohydrates might have on Folin–Ciocalteu reagent. Starch concentration almost did not affect the TPC values obtained (Figure 10.2A, ratios 0:0.5, 0:1 and 0:2), excepting the value at low liquid–solid ratios (blend 0:25), which was significantly ($p < 0.05$) higher. Slight differences were found between TPC from native CS (from 1.9 ± 0.1 to 94.9 ± 6.8 mg_{PE}/L and gelled CS (from 2.9 ± 0.2 to 143.4 ± 10.3). The control samples evaluation showed that starch, either gelled or not, did not significantly contributed to final TPC values, but it was not negligible for 0:25 samples. The obtained TPC values from both control samples separately (AF and CS) rendered higher values than those measured with the corresponding blends (Figure 10.2A). TPC values of the NT blends ranged from 9.4 ± 0.7 to 280.5 ± 20.2 mg_{PE}/L, GL samples showed lower content, from 0.02 ± 0.01 to 192.9 ± 13.9 mg_{PE}/L and CGL showed the lowest values (at constant AF–CS ratio) with a maximum value of 82.8 ± 6.0 for 0:0.5 ratio (Figure 10.2A).

These results indicate that the polyphenols released from seaweed to the liquid phase interacted with CS (native or gel) structures remaining bound to or trapped into the solid phase. For the three assayed methods (NT, GL and CGL), the higher the corn starch ratio, the lower the net TPC values in liquid, thus negligible values were obtained at 1:25 ratio samples with starch gels. GL values corresponding to gelled CS showed lower ($p < 0.05$) polyphenols content in relation to NT. The different available sorption surface area between native starch (granular structure) and gelled starch could explain these differences (Wang et al., 2021).

The blending procedure that involves the gelling of starch in the presence of seaweeds polyphenols (CGL) caused a dramatically decrease of TPC values. The NT and GL methods involved the direct contact between AF and CS (native or gelled) at rt during 15 min meanwhile CGL put them in contact during the gelatinization process carried out at 100 ºC (20 min), a cooling step (20 min), and the final dispersion and homogenization in water.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

During CS gelling with the presence of AF, both the *A. nodosum* flour and extracted polyphenols can be trapped within the gel structure during gels formation and subsequent retrogradation (Igoumenidis et al., 2018). Other factor that could be responsible for the decrease of polyphenols content is the thermal treatment, which could promote the polyphenols partial decomposition (Tello–Ireland et al., 2011; Liu et al., 2021b;) or the formation of new bonds and substances (Barros et al., 2014).

Wang et al. (2021) studied native starch, starch gel, and gelatinized corn starch in the presence of tannic acids, reporting that the formation of bindings between tannins and gelled starch was more abundant than between tannins and native starch. The gelling of starch in the presence of tannic acid, similarly to "CGL blend" here reported, also showed notorious lower TPC values. A notoriously higher retention of polyphenols in gelled starch (GL and CGL), as compared with native starch, was also reported by Barros et al. (2014). In fact, these authors obtained different bioactive compounds content for oven dried (105 ºC) and for freeze–dried starchy–sorghum matrixes. Initial TPC is a critical aspect in equilibrium adsorption experiments; in the current study, this value (396.9 ± 39.7 mg_{PE}/L) was lower than those employed by Wang et al. (2021), (10, 20 and 30 mg of tannic acids per mL) and are comparable to those employed by Barros et al. (2014), with initial 485 mg of gallic acid equivalents per g of sorghum. Nevertheless, both authors obtained similar trends. Even working with higher liquid–solid ratios, the polyphenols extraction was enough to show relevant and measurable interaction with native and gelled corn starch.

TPC from the aqueous phase of AF–CS blends and AF and CS control samples were used to determine the sorption yield (Y_P) of the samples (Figure 10.2B) using Eq. (4.26). Y_P varied between 29.3 ± 1.8 (NT 1:0.5) and 99.8 ± 5.9 (both GL and CGL 1:25). Y_P values showed higher retention capacities of the gelled corn starch (GL and CGL), with similar trends for three methods tested, where the higher the CS content the higher the polyphenols retention yield. NT samples showed lower sorption yield values by the lower active surface area of native starch to interact with polyphenols (Barros et al., 2012). To understand both effects, AF–CS blending methods (NT, GL and CGL) and CS structure (native and gelled), on polyphenol sorption, different ratios of Y_P (GL/NT, CGL/NT and CGL/GL) were calculated and the corresponding values are presented in Table 10.1.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The lowest ratios value was 1 at ratio 1:25 because the adsorption yield was near 100% when the highest starch amount was employed. The highest values were 1.75 ± 0.07, 2.70 ± 0.05 and 1.54 ± 0.08 for GL/NT, CGL/NT, and CGL/GL ratios, respectively, when the lowest adsorbent (1:0.5 ratio) was used.

Table 10.1. Polyphenol sorption yields (Y_P) ratios from NT, GL and CGL assayed methods. Different superscript letters indicate significant ($p < 0.05$) differences between ratio values

	GL/NT	CGL/NT	CGL/GL
1:0.5	1.8 ± 0.1^{d}	2.7 ± 0.04^{f}	1.5 ± 0.1^{c}
1:1	$1.5 \pm 0.1^{b,c}$	2.0 ± 0.04^{e}	$1.4 \pm 0.1^{b,c}$
1:2	$1.3 \pm 0.1^{b,c}$	$1.8 \pm 0.04^{d,e}$	1.3 ± 0.07^{b}
1:25	1.0 ± 0.04^{a}	1.0 ± 0.02^{a}	1.0 ± 0.03^{a}

These values indicated that the use of starch gel increased almost twice the polyphenols retained in comparison to native starch. These values could be related to higher surface area developed in the gel structure in comparison to granular structure. When the formation of starch gel took place in the presence of seaweeds flour increased almost 3– and 1.54–times regarding NT method and GL methods, respectively. This notorious increase could be explained by additional retention of polyphenols beyond adsorption, as entrapment or encapsulation of bioactive molecules in the gel structure. As the amount of starch increased (1:1 and 1:2 ratios) the differences decreased due to the respective increase in sorption yields.

Polyphenols retained by CS, q (m_{gPE}/g_{CS}), determined by means of Eq. (10.1) against TPC values of the liquid phase are plotted in the Figure 10.3. For NT, GL and CGL samples, q values were lower than 22.9 ± 1.6; 42.0 ± 2.9 and 65.0 ± 4.5, respectively, achieved when the lowest starch amount were employed. Furthermore, q values were near zero employing 1:25 ratio because yield were around 100 %. Figure 3 clearly shows the enhancement of the polyphenol–starch affinity in GL and CGL samples regarding NT samples. In example, at q = 15 mg_{PE}/g_{CS}, equilibrium is achieved at TPC of 240, 115 and 35 mg_{PE}/L of TPC for NT, GL and CGL samples, samples.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

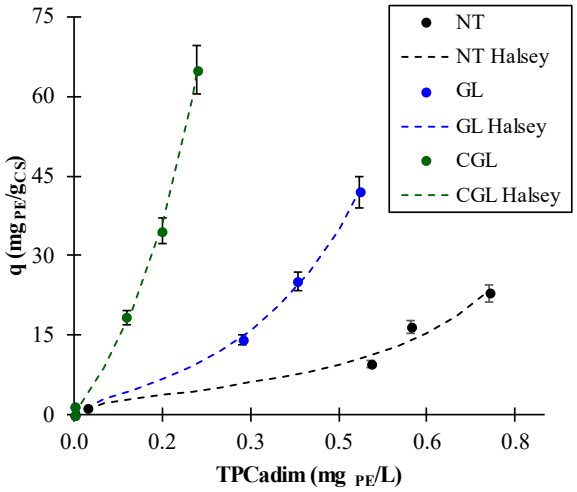


Figure 10.3. Adsorbed/retained polyphenols by CS, q, vs TPC of aqueous phase for AF–CS samples (NT, GL, and CGL) at 20 ºC. Lines are Halsey model (Eq. (10.1)).

These results confirmed that low sorption surface area was available when native corn starch granules were employed in comparison to area developed by starch gel structures in GL and CGL samples. In all cases, the shapes, convex to the abscissa axis over the tested range, of equilibrium adsorption/retention isotherms of polyphenols on starch allowed their classification as III according to the BET classification (Brunauer et al. 1940). An empirical two–parameters model, Halsey model, Eq. (10.1), was employed to fit the experimental data (Halsey, 1948).

$$q = \left(\frac{-A}{\ln\left(\frac{TPC}{TPC_{AF}}\right)} \right)^{\frac{1}{B}} \qquad (10.1)$$

where A and B are the fitting parameters.

Table 10.2 shows the A and B values of Eq. (10.1) for NT, GL and CGL samples. According to the values of coefficient of determination ($R^2 > 0.98$) and root mean square error (RMSE < 3.6) of Eq. (4.30), the goodness of fitting can be considered acceptable; Figure 10.3 show the modelled values. Values of parameter A varied in a narrow range (from 5.2 ± 0.7 to 6.1 ± 0.4) with blending procedure, but exponent, B^{-1}, varied from almost linear (1.1) for NT, quadratic (1.9) for GL, and cubic (3.0) for CGL.

Table 10.2. Parameters of Halsey equation (Eq. (4.30)).

Method	A	B	R^2	RMSE
NT	6.0 ± 0.6	0.9 ± 0.1	0.98	3.57
GL	5.2 ± 0.7	0.5 ± 0.02	0.99	0.43
CGL	6.1 ± 0.4	0.3 ± 0.04	0.99	1.22

10.3.2. TOTAL CARBOHYDRATE CONTENT (CHOS)

Carbohydrate content (mg_{GE}/L) values (Figure 10.2C) were notoriously high for gelled corn starches and provided by both raw materials, *A. nodosum* seaweed flour and corn starch. CHOs increased with CS content, indicating that CS carbohydrates were partially solubilized, mainly because of the thermal treatment, as can be deduced from the results of control gelled samples (GL and CGL controls in Figure 10.2C). CHOs evaluation made mandatory to handle data comparing AF–CS blends with control samples of AF and CS. AF control sample (1:0) showed an initial CHOs value of 830.4 ± 59.8 mg_{GE}/L while NT control values varied between 8.2 ± 0.6 and 129.5 ± 9.3 meanwhile gelled CS methods (GL and CGL) ranged up 213.2 ± 15.4 to 1223 ± 88 for 1:0.5 to 1:25 AF–CS ratios, respectively. CHOs significant ($p < 0.05$) differences stressed out the notorious effect that gelatinization had on CHOs signals.

At constant AF–CS ratios, CHOs values were significantly ($p < 0.05$) higher in GL (495 ± 35 to 955 ± 69) in comparison to CGL (252 ± 18 and 805 ± 58) and samples (333 ± 24 to 432 ± 31) samples. Higher CHOs values of GL samples were associated with the leaching of some polysaccharides (mainly amylose) during starch gelatinization, and, in addition, with the extracted carbohydrates from AF, increased the total carbohydrates content; this effect was clearly evidenced in assays performed at 1:25 ratio. Low CHOs values determined in CGL samples can be again explained by the gel trapping effect. The formation of CS gel in presence of AF limited the release of carbohydrates from algae to liquid phase, that notoriously ($p < 0.05$) reduced CHOs in AF–CS samples.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Besides, thermal deterioration during gelatinization could also partially alter sugars (Barros et al., 2014) making them not detectable. Scarce sugar content evaluation is carried out in literature, however, it is necessary to correctly evaluate the adsorbent amount at equilibrium and is also useful to understand the physical and chemical phenomena involved during the formulation of these new bioactive starchy foods, since many of *A. nodosum* carbohydrates have demonstrated relevant phytochemical features.

10.3.3. ANTIOXIDANT ACTIVITIES (DPPH, ABTS AND FRAP)

Antioxidant activities were determined by DPPH (Figure 10.4A) and ABTS (Figure 10.4B) radical scavenging activities and FRAP as method for electron donor capacity (Figure 10.4C) for aqueous phases of tested samples at equilibrium. As expected, the highest antioxidant activity was obtained for AF control sample (Figure 10.4A, B and C, isolated dots). The lowest antioxidant activity values were obtained for CGL samples. This last result could be related to the thermal deterioration of polyphenols during heating treatment and the entrapment of bioactive compounds into the starch gel.

An acceptable linear correlation ($R^2 > 0.93$) between DPPH and TPC values, without distinction among employed blending procedure, was found indicating that scavenging activities of polyphenols measured by this method depend only on TPC and possible deterioration of bioactive compounds is not detected. Nevertheless, to achieve suitable linear correlations ($R^2 > 0.88$) for ABTS–TPC results, experimental data had to be separated in two sets according to AF thermal–treatment, subjected (CGL) and non–subjected (NT and GL) to heating process, and consequently employing this more sensitive method different antioxidant activities trends with TPC could be successfully established. Finally, a linear correlation for FRAP–TPC data was lower than 0.65 whereas only NT samples showed satisfactory R^2 values around 0.93. Thus, FRAP values corresponding to GL and, particularly, CGL methods were very low.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

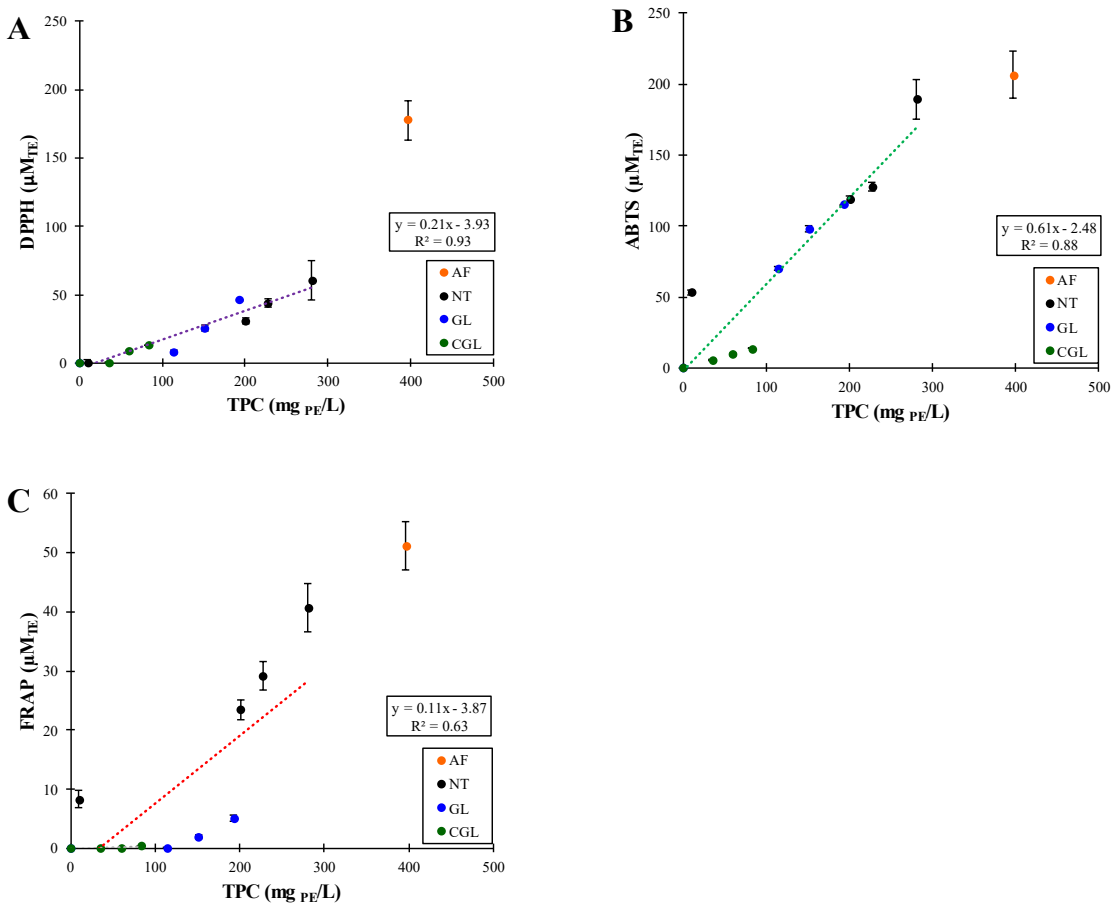


Figure 10.4. Antioxidant activities determined by DPPH (A), ABTS (B) and FRAP (C) against TPC in control (AF) and AF–CS samples (NT, GL, and CGL, methods).

This last result demonstrated that the most bioactive polyphenols became notoriously trapped by CS gel remaining low bioactivity in the liquid phase. Other researchers also reported linear correlations in polyphenol–starch blends (van Hung et al., 2013). In this manner, AF–CS matrices can be considered as promising polyphenols shelters against digestive tract (Guo et al., 2021; Zhu et al., 2017). Moreover, the different CGL–TPC trends (for ABTS and FRAP methods) with very low antioxidant activities could be also related to partial thermal degradation (oxidation, polymerization, etc.) of polyphenols during processing that significantly ($p < 0.05$) reduces the bioactivity of non–adhered phytochemicals on starch surface.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

10.4. FOURIER TRANSFORMED INFRARED (FT–IR)

FT–IR technique was carried out to evaluate the nature of bindings formed between CS and polyphenols by means of the analysis of AF, CS, and AF–CS blends (1:1 ratio) from NT, GL, and CGL methods. FT–IR can give relevant structural information and was previously employed in polyphenols–starch systems (Hu & Du, 2019; Wang et al., 2021). All samples showed a notorious peak at 3400 cm^{-1} that corresponded with the vibrational stretching of inter– and intra–molecular hydroxyl groups (–OH), Figure 10.5.

However, around 2800 cm^{-1}, AF and CGL showed two peaks not observed in other samples corresponding to hydrogen bridge bonds. Indeed, intensity values of this region are often used as criterion to measure the amount (concentration) of hydrogen bridge bonds (Hesse et al., 2005). An explanation about the presence of this signals is that these bonds mainly are between polyphenol molecules forming complexes and the existence in CGL samples corroborated that polyphenols are entrapped in the gel structure and these hydrogen bridge bonds remained intact.

The region around 1600 cm^{-1} corresponds to the characteristic peak of aromatic rings (stretching vibrations of C=C bonds). In this region significant ($p < 0.05$) different quantitative signals among samples with and without AF were found and was straightly related to polyphenols (phlorotannins) content (AF > CGL > GL > NT > CS samples). Particularly, CGL´s high peak intensity indicated the increasing retention by entrapment of polyphenols by CS, even after gelatinization treatment (Liu et al., 2019a). NT and GL samples, according to the literature (Barros et al., 2012; Igoumenidis et al., 2018; Hu & Du, 2019), interactions between AF and CS based on physical bonds.

Physisorption has a relatively low degree of specificity and is therefore regarded as non–specific (Wang et al., 2021; Rouquerol et al., 1999). No new peaks were observed in the FT–IR spectra in AF–CS samples demonstrating that interactions consisted in non–specific physical bonds. These results together with linear correlation found for adsorbed polyphenols at equilibrium (Figure 10.3) revealed that the main kind of interactions between polyphenols and CS could be considered of the same nature and they were invariant with the blending procedure.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

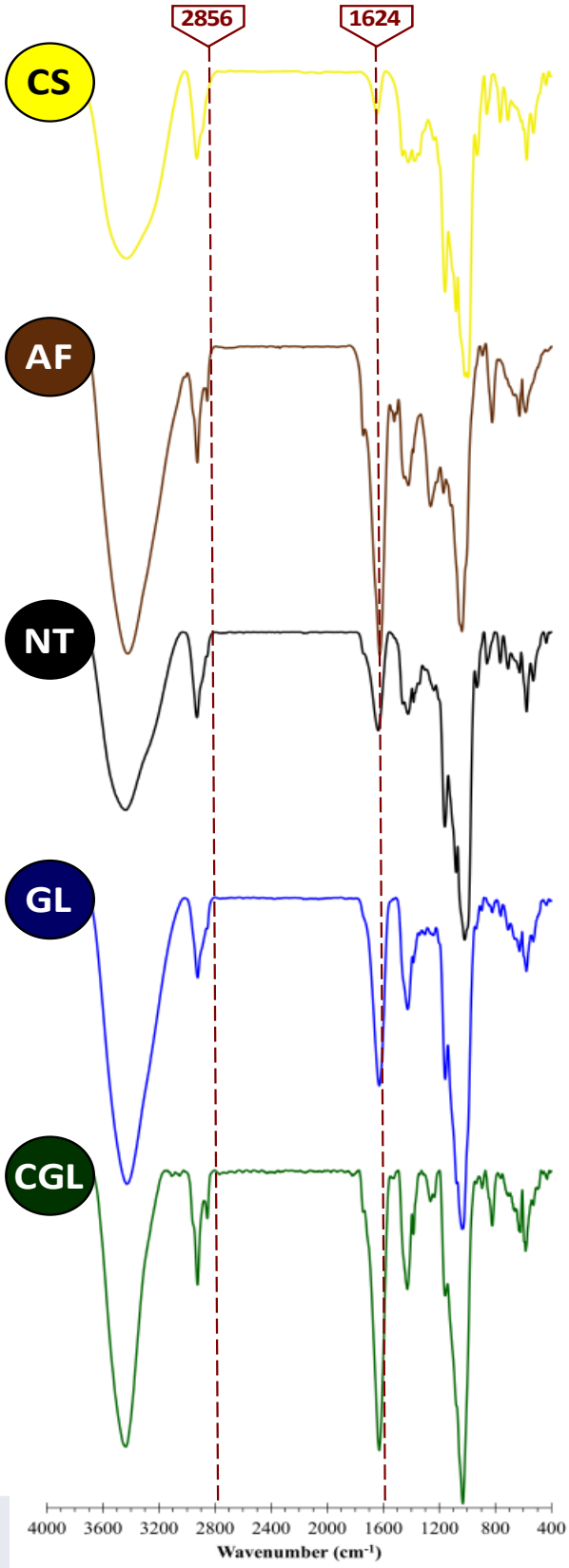


Figure 10.5. FT–IR spectra of control (AF, —, and CS, —) and AF–CS (1:1 ratio) samples (NT, —, GL, —, and CGL, —).

10.5. SCANNING ELECTRON MICROSCOPY (SEM)

SEM micrographs of AF, CS and AF–CS blends (1:1 ratio) were carried out to observe the proposed physical bonding (NT, GL and CGL) and trapping (CGL) interactions between AF and CS (Figure 10.6). AF showed amorphous structures due to drying and milling treatments of the seaweed (Figure 10.6A) defined in Section 6. CS control sample (Figure 10.6B) showed characteristic granular structures of native starch, NT method showed starch granules and a heterogeneous and rough film that partially covered some particles and acted as cohesive structure forming bridges between granules (Figure 10.6C). Both GL (Figure 10.6D) and CGL (Figure 10.6E) showed widespread and characteristic honeycomb gelled starch structure (van Hung et al., 2013; Wang et al., 2021). It was noted gelled micrographs (GL and CGL) did not show any residual starch granules; therefore, full gelatinization was achieved. In fact, GL walls (Figure 10.6D) were thicker than those in CGL (Figure 10.6E) but the size of the holes did not show significant ($p < 0.05$) differences.

Additionally, micrographs of AF–CS blends showed some particles along granule surface and gel walls that were associated with *A. nodosum* particles. NT (Figure 10.6C) and GL (Figure 10.6D) showed these particles on CS surface meanwhile CGL (Figure 10.6E) showed these particles below the walls surface causing notorious prominent and dispersed bulges. These differences confirmed theories proposed where gelling of CS in presence of AF (CGL method) entrapped AF particles and bioactive molecules inside starch gel structure. CGL method seems suitable to improve bioactivity of starchy materials concerning with NT or GL methods where were adsorbed on CS surfaces.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

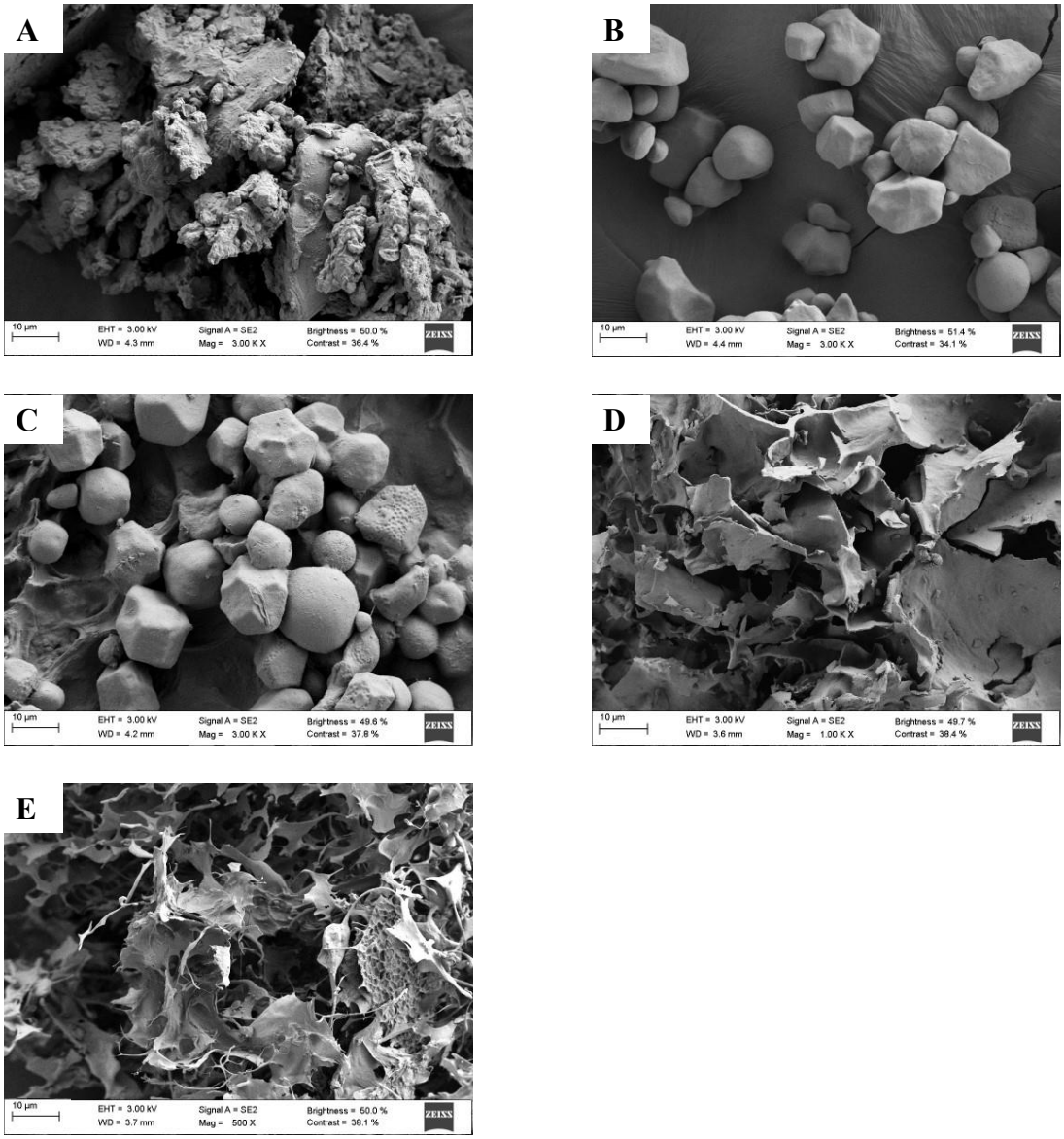


Figure 10.6. Cross–sectional morphologies of solid phase from AF (A), CS (B) and AF–CS (1:1 ratio) samples (NT (C), GL (D) and CGL (E)).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

HIGHLIGHTS

Composition and phase transitions promoted by heating operations modified the phytochemical features, interactions, and cross–sectional morphologies of these meals.

Starch showed different phlorotannins retention yields depending on native or gelled structures.

Physisorption (hydrogen bonds) of phlorotannins on starch was the main mechanism when seaweed was put in contact with native (higher than 25%) and gelled (higher than 50%) starch.

Phlorotannins retention increased when starch gelatinization was carried out in the presence of seaweed flour (higher than 75%) because phlorotannins were physically adsorbed on the surface of starch gel and trapped inside starch gel walls.

The procedures used serve to quantify in a simple way the capacities and nature of retention mechanisms of bioactive molecules by gelled starch foods.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

11. ENZYME-INHIBITING ACTIVITY OF A*SCOPHYLLUM NODOSUM* EXTRACTS

Current Section 11 adapted from:

Aleixandre A, Gisbert M, Sineiro J, Moreira R, Rosell CM (2022) *In vitro* inhibition of starch digestive enzymes by ultrasound–assisted extracted polyphenols from *Ascophyllum nodosum* seaweeds. *Journal of Food Science* 87, 2405–2416 DOI: 10.3390/foods11081165 (See Section 17)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

11.1. GENERALITIES

In the last decade, carbohydrates digestion has attracted much attention due to its direct relationship with postprandial glycemic response, which in turn has been associated with an increased risk of developing metabolic diseases (Dall'Asta et al., 2020). Digestive carbohydrates are mainly present in human diets as starch, sucrose, free glucose fructose, and oligosaccharides. One strategy to manage high blood glucose levels is the inhibition of *α*–amylase and *α*–glucosidase, digestive enzymes involved in the breakdown of starch and disaccharides to glucose (Rios et al., 2015).

Some antidiabetic drugs such as acarbose are both *α*–amylase and *α*–glucosidase enzymes inhibitors and allow the control of postprandial glucose in diabetic patients (Zhao et al., 2021). However, current research is focused on discovering natural *α*–amylase and *α*–glucosidase inhibitors involving plant– and seaweed–derived compounds such as polyphenols, because of their health beneficial features (Kan et al., 2020).

Likewise, *A. nodosum* is being considered as a valuable source of polyphenols to be used in drugs and the food industry (Leandro et al., 2020). The composition and polyphenols features of *A. nodosum* extracts were recently determined employing liquid chromatography–mass spectroscopic techniques evidenced the presence of high structural diversity of phlorotannins (Allwood et al., 2020). Those authors confirmed the presence of dibenzodioxin linked phlorotannins, sulphated phlorotannins, and phenolic acids. Moreover, Apostolidis & Lee (2010) showed the pharmaceutical potentiality of *A. nodosum* against hyperglycaemia seen in Sections 1.6, 1.7.3 and 3.3.

Nevertheless, the extraction procedures and conditions employed to produce polyphenols extracts strongly affect their inhibition capacity against *α*–amylase and *α*–glucosidase (Alu'datt et al., 2017). Considering the efficiency of UAE (Section 3.1), the initial hypothesis is that aqueous polyphenolic extracts obtained *A. nodosum* through UAE might have prominent inhibition capacities against digestive enzymes. As it was shown in Section 8, 2 min of aqueous UAE employing high sonication powers (>70 W/cm^2) were enough for the extraction of polyphenols, but secondary compounds (mainly, carbohydrates and, specifically, uronic acids) were also co–extracted diminishing the selectivity.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Thus, it is convenient to determine the chemical characterization of these raw extracts and to evaluate the effect of additional purification operations on the bioactive activities and inhibition capacities after the secondary compounds removal, resulting enriched extracts (Goncalves–Fernandez et al., 2019). The bioactivity and inhibitory characterization of raw and purified extracts will extend their further food applications.

11.2. ASSAYED SYSTEMS

In Section 11 the inhibitory effects of aqueous *Ascophyllum nodosum* extracts, obtained by ultrasound–assisted extraction (Section 4.4.2) with different sonication powers (70–90 W/cm^2) and purified (Section 4.5), against α–amylase and α–glucosidase enzymes (Section 4.15) were studied. Different inhibition methodologies were carried out, preincubating the extract either with the enzyme or the substrate. Chemical characterization was done in terms of proximate analysis, polyphenols (Section 4.6.1) carbohydrates (Section 4.6.2), antioxidant capacity by both DPPH (Section 4.6.4) and FRAP (Section 4.6.6) methods, proteins, lipids, and ash (Section 4.6.7), RP–HPLC (Section 4.9 in gradient mode) and ^{1}H–NMR (Section 4.13.2) to explain inhibitory activities of extracts. RP–HPLC together with ^{1}H–NMR spectra allowed relating the presence of uronic acid–polyphenols complexes and quinones in the extracts with the different inhibiting capacities of samples. The scheme of the research carried out could be seen in Figure 11.1

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

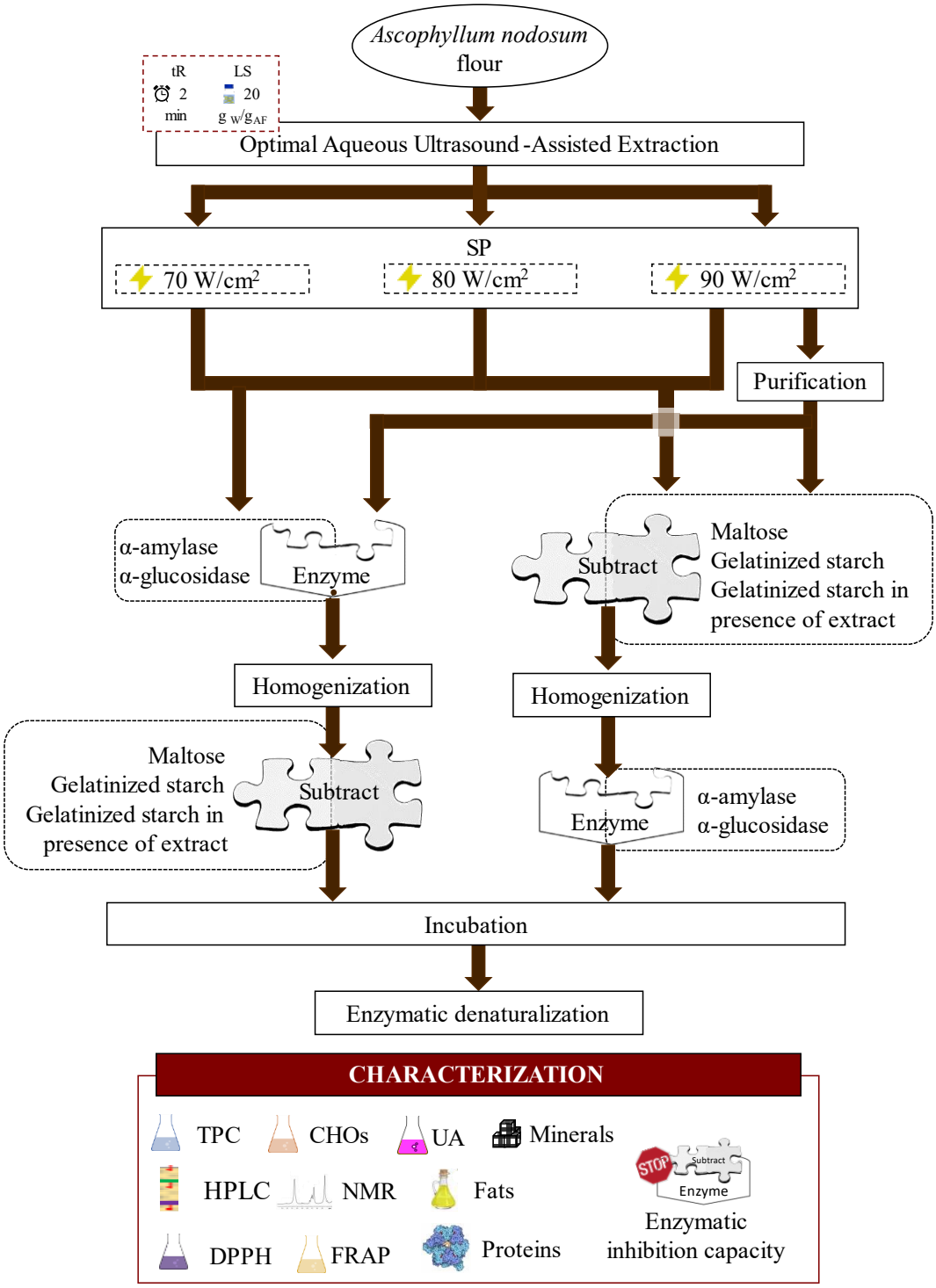


Figure 11.1. Assayed systems for the determination of the digestive enzyme inhibitory capacities of *A. nodosum* extracts and their bioactivities.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

11.3. SEAWEED EXTRACTS CHEMICAL COMPOSITION

Aqueous UAE is largely effective for the extraction of polyphenols, but other compounds like carbohydrates and particularly UA were also co–extracted. Because of that proximate composition, antioxidant activities and UA content were selected to evaluate the impact of UAE and further purification. The moisture, protein, mineral, fat, CHOs, total polyphenol (TPC), UA content, and antioxidant activities (DPPH and FRAP methods) were determined from *A. nodosum* ultrasound–assisted crude (E90, E80 and E70), and purified (P90) extracts (Table 11.1). Sonication power did not significantly($p < 0.05$) modify the proximate composition of the extracts ($p > 0.05$), since protein, mineral, and fat content were around 7.5 ± 0.1, 29.8 ± 0.3, and 4.1 ± 0.1 (%, dry weight, DW), respectively, CHOs (0.1 ± 0.03) and UA (0.2 ± 0.06 mg_{GE}/mg_{DW}) content. TPC (mg_{PE}/mg_{DW}), CHOs (mg_{GE}/mg_{DW}), and UA (mg_{GE}/mg_{DW}) were also invariant with sonication power with values of 0.2 ± 0.04, 0.2 ± 0.02, and 0.2 ± 0.04, respectively. Finally, moisture (%) varied in a narrow range 8.6 ± 0.3 (E80) up to 10.1 ± 0.6 (E70). No significant ($p > 0.05$) differences of antioxidant activities were observed between extracts obtained under different conditions, with mean values of 39.4 ± 1.0% of DPPH scavenging activity and 1.1 ± 0.04 $\mu g_{TE}/g_{DW}$ of Fe^{2+} reducing power. Kadam et al. (2015a, b and c) working with *A. nodosum* aqueous extracts obtained from UAE (35.6 W/cm^2 for 15 min) reported slightly lower TPC values (0.16 mg_{PE}/g_{DW}) with a DPPH decay value of 61.46%.

Nevertheless, purification of the extracts significantly ($p < 0.05$) decreased protein (0.93–fold), mineral (0.28–fold), fat (0.73– fold), and UA (0.27–fold) content and increased CHOS (1.65–fold) and TPC (2.21–fold) content. Simultaneously, an interestingly relevant increase of DPPH decay (1.29–fold) and FRAP (1.61–fold) content was determined in the purified extract.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 11.1. Chemical composition of freeze dried (E90, E80 and E70) and purified (P90) extracts obtained by UAE from *A. nodosum* brown seaweed. Means within a raw followed with different letter are significantly ($p < 0.05$) different.

	P90	**E90**	**E80**	**E70**
Moisture (%, DW)	16.4 ± 0.12^{c}	8.8 ± 0.15^{a}	8.56 ± 0.3^{a}	10.1 ± 0.6^{b}
Proteins (%, DW)	7.0 ± 0.30^{a}	7.5 ± 0.02^{b}	7.37 ± 0.1^{b}	7.57 ± 0.1^{b}
Minerals (%,DW)	8.2 ± 0.2^{a}	29.5 ± 0.2^{b}	29.8 ± 0.1^{b}	30.0 ± 0.4^{b}
Fats (%,DW)	3.0 ± 0.04^{a}	4.2 ± 0.04^{b}	4.0 ± 0.9^{b}	4.2 ± 0.1^{b}
CHOs (g_{GE}/g_{DW})	0.2 ± 0.04^{b}	0.1 ± 0.02^{a}	0.1 ± 0.01^{a}	0.1 ± 0.02^{a}
TPC (g_{PE}/g_{DW})	0.4 ± 0.04^{b}	0.2 ± 0.03^{a}	0.2 ± 0.03^{a}	0.2 ± 0.03^{a}
UA (g_{GE}/g_{DW})	0.05 ± 0.01^{a}	0.2 ± 0.04^{b}	0.2 ± 0.02^{b}	0.2 ± 0.04^{b}
DPPH (mM_{TE}/g_{DW})	128 ± 0.05^{d}	120 ± 0.01^{c}	118 ± 0.02^{b}	107 ± 0.01^{a}
FRAP (mg_{TE}/g_{DW})	1.8 ± 0.05^{a}	1.1 ± 0.01^{b}	1.1 ± 0.1^{b}	1.1 ± 0.04^{b}

According to Wang et al. (2016), polyphenols and UA could be partially forming complexes. The complexation effects on extracts bioactivity are still not elucidated. Regarding this interaction, hydrogen bond or hydrophobic interactions (Koivikko et al., 2005) and covalent bonds of ether, ester, and hemiacetal bonds (Salgado et al., 2009) have been proposed. The bonds of these complexes could affect their structural properties and promote or avoid some chemical interactions. However, the presence of these complexes was not directly discerned by common TPC and UA analyses and antioxidant activities (DPPH and FRAP; Table 11.1). Purification step reduced UA content and increased bioactivity of extracts. UA/TPC ratio was employed as a measure of cleaning of the extracts, since CHOs values varied in a narrow range (from 0.1 to 0.2 mg_{GE}/mg_{DW}) after purification. In fact, UA/TPC ratio decreased significantly ($p < 0.05$) from 0.96 ± 0.08 (E90, E80 and E70) up to 0.1 ± 0.02 (P90) after purification with Amberlite XAD16. These results indicated that this resin was an efficient adsorbent for the removal of UA in the extracts, improving bioactive features (TPC, DPPH, and FRAP) of purified extract. The reduction of UA/TPC ratio could help to explain further enzymatic inhibitory differences of tested extracts.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

11.4. INHIBITION EFFECT OF EXTRACTS AGAINST DIGESTIVE ENZYMES

Comparative study was conducted to determine the capability of the four different *A. nodosum* extracts (E90, E80, and E70 and purified P90) to inhibit α–amylase and α–glucosidase activity following three methodologies: preincubation of extract + enzyme (M1), preincubation of extract + gelatinized starch (M2), and gelatinization of starch + extract (M3).

It is shown in Figure 11.2 the α–amylase and α– glucosidase inhibition induced by the different extracts, in comparison with acarbose, which was taken as standard inhibitor (Table 11.2). In M1 method (Figure 11.2), increasing the UAE sonication power significantly ($p < 0.05$) decreased the IC_{50} meaning that the inhibition capacity of the extracts increased (up to 35% and 15% for α–amylase and α–glucosidase), despite extracts showed similar TPC and DPPH values. The extract purification with Amberlite (P90) largely improved the inhibition efficiency decreasing the IC_{50} of α–amylase and α–glucosidase by 3.0 and 6.1 times, respectively. This result might be explained by its higher TPC (Table 11.1) compared with those measured in the extracts. Comparing the effect of tested extracts, higher extract concentrations were required to inhibit α–amylase compared to α–glucosidase. In relation to acarbose, a specific competitive inhibitor, the results were lower; IC_{50} values (40.0 and 3.2 μg/mL) of P90 were four times higher than that of acarbose on α–amylase and 20 times higher for α–glucosidase (Table 11.2). These results support the findings described by Apostolidis and Lee (2010) when analyzing an aqueous extract from *A. nodosum*, reporting higher inhibition activity against yeast α–glucosidase than against porcine pancreas α–amylase. Conversely, Pantidos et al. (2014) found that a tannin–rich fraction of *A. nodosum* obtained with different solvents was more effective inhibiting porcine pancreas α–amylase than rat intestinal α–glucosidase. Divergences might be ascribed to the different composition of the extracts, which depends on the extraction method. Zhang et al. (2007) determined an IC_{50} = 77 μg/mL against α–glucosidase working with water–ethanol A. nodosum extracts. Although, Liu et al. (2016) determined IC_{50} from 8.9 to 36.3 μg/mL against α–glucosidase from ethanolic extracts of *A. nodosum* in the same range than those found in this work. Nevertheless, discussion of results based on previously published data becomes complicated due to the wide range of experimental conditions used which makes it very difficult to compare IC_{50} values, which are specific for the enzyme type, the substrate used, and the reaction conditions.

USC UNIVERSIDADE DE SANTIAGO

Table 11.2. IC_{50} values of seaweed extracts against α–amylase and α–glucosidase of the different analyzed methodologies: extract + enzyme (M1), extract + gelatinized starch (M2), gelatinization of starch with extract (M3). Means within a column followed with different letter were significantly ($p < 0.05$) different.

	IC_{50} (µg/mL)				
	α–amylase			**α–glucosidase**	
	A1	**A2**	**A3**	**G1**	**G2**
Acarbose	11.5 ± 0.6[a]	56.1 ± 3.0[a]	142.8 ± 12.1[a]	0.2 ± 0.01[a]	0.1 ± 0.01[a]
P90	40.0 ± 1.1[b]	74.0 ± 2.6[b]	810.5 ± 24.3[b]	3.2 ± 0.1[b]	3.6 ± 0.2[b]
E90	119.6 ± 0.7[c]	309.2 ± 4.1[c]	4066.3 ± 50.1[c]	19.5 ± 0.7[c]	26.2 ± 0.8[c]
E80	128.8 ± 2.9[d]	342.6 ± 0.7[d]	4451.0 ± 49.2[d]	20.3 ± 0.3[c]	28.1 ± 0.04[d]
E70	152.9 ± 4.3[e]	439.8 ± 9.8[e]	4777.7 ± 42.5[e]	29.2 ± 0.4[d]	30.4 ± 0.7[e]

Given the influence of the procedure carried out to analyze enzyme inhibition, besides the sonication power during extraction and purification step, two additional methodologies (M2 and M3) were applied to identify the effect of polyphenols interaction with the enzymes on the level of inhibition. The most effective inhibition was obtained when the extracts were previously incubated with the enzyme (M1) (Figure 11.2), where the interaction between polyphenols and enzyme was favored before substrate (starch or maltose) addition for α–amylase (A1) or α–glucosidase (G1), respectively. Conversely, higher IC_{50} values were required with M2 when the extract was previously mixed with gelatinized starch for α–amylase (A2) (IC_{50} = 74.0, 309.2, 342.6, and 439.8 µg/mL for P90, E90, E80, and E70, respectively) or with maltose for α–glucosidase (G2) (IC_{50} = 3.6, 26.2, 28.1, and 30.4 µg/mL for P90, E90, E80, and E70, respectively) (Figure 11.2). These observations suggest that in the M2 method, the substrate hinders the polyphenol accessibility to the enzymes, increasing IC_{50} values. Pantidos et al. (2014) also observed lower effectiveness of a tannin–rich fraction, as porcine pancreas α–amylase inhibitor, when it was preincubated with gelatinized potato starch. Lordan et al. (2013) described porcine pancreas α–amylase inhibition (IC_{50} = 0.05 mg/mL) with *A. nodosum* extracts obtained from a three–stage process of at least 3 h duration, like the one obtained with P90 extract in M2.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Similarly, to the previously commented polyphenol–enzyme binding, phenolic compounds can bind starch by non–covalent interactions, modulating starch digestion kinetics (Giuberti et al., 2020). This lower inhibition effect when the extract was preincubated with the starch highlights the significance of the hydrolysis kinetics during *in vitro* analysis and subsequently to the *in vivo* studies, where pancreatic α–amylase and intestinal α–glucosidase both are secreted into the gut lumen, where they would meet the seaweed extract and the starch mixture. Furthermore, the inhibitory effect against α–amylase dramatically decreased when extracts were added to native starch, and the blend was subjected to high temperatures to gelatinize the starch (A3) (Figure 11.2). IC_{50} values varied between 810.5 (P90) and 4777.7 (E70) μg_{DW}/mL. Nevertheless, in this case, besides the starch impediment previously mentioned for A2, polyphenols and starch interaction during the gelatinization process or polyphenols stability might be considered. Wu et al. (2011) described the existence of hydrogen bonding interaction between tea polyphenols and rice starch during gelatinization.

On the other hand, high temperature can affect the stability of polyphenols and antioxidant activity (Moreira et al., 2016), therefore affecting its ability as enzyme inhibitors. Betoret & Rosell (2020) analyzed the effect of temperature (70, 80, and 90 ºC for 20 min) on phenolic compounds of *Brassica napobrassica* blended with maize and rice starches. These authors reported the protective role of starch with phenolic compounds in which the high apparent viscosity might contribute to protect the bioactive compounds. Authors linked the changes of bioactive compounds after thermal treatments with thermal degradation, matrix un–structuring effect and interaction with other ingredients, protecting them from degradation. Nevertheless, to better understand the interactions between *A. nodosum* bioactive compounds and digestive enzymes additional techniques, such as scanning electron microscopy, Fourier transform infrared spectrophotometry or X–ray diffraction, would provide valuable information.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

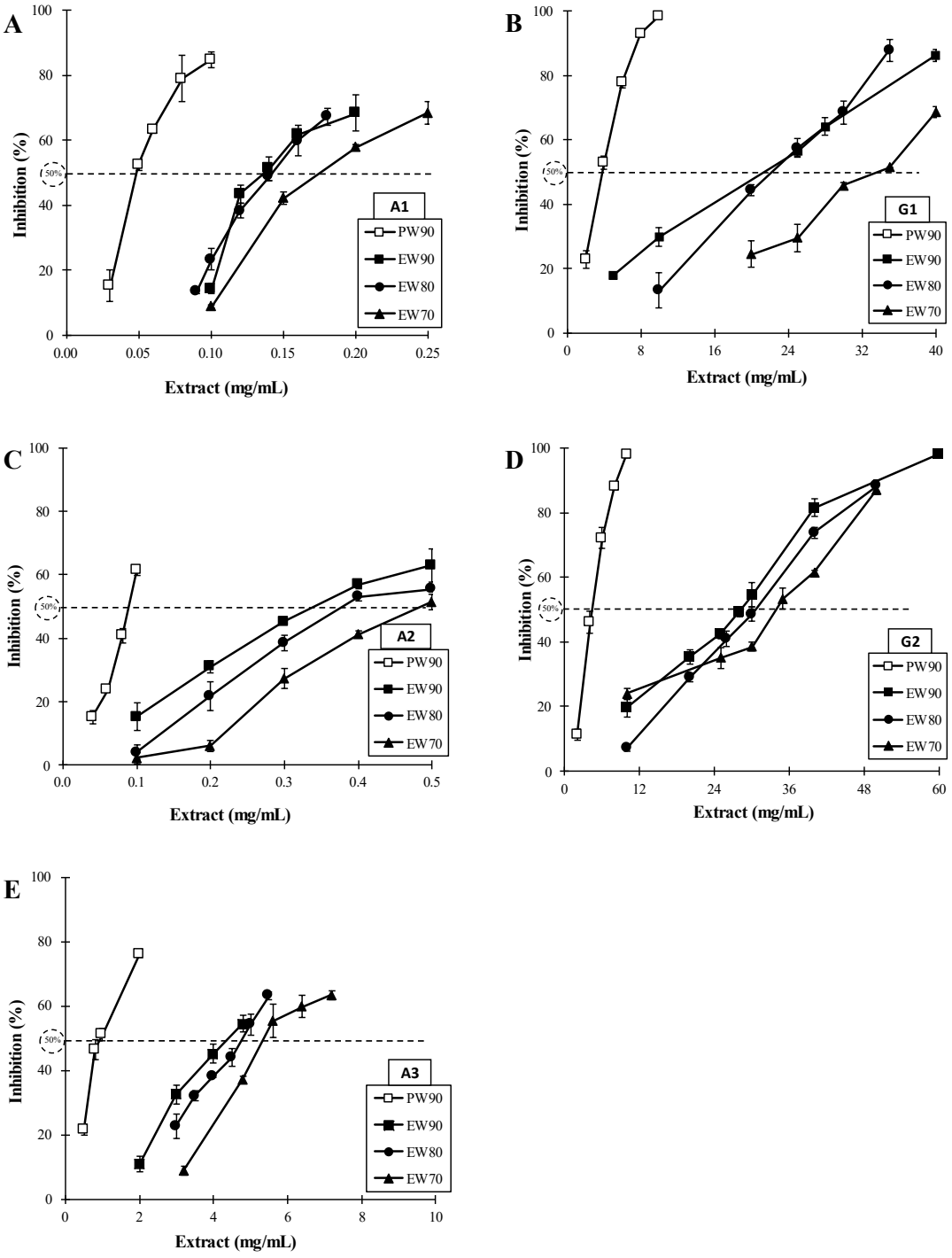


Figure 11.2. Inhibitory capacity of purified (P90) and raw (E90, E80 and E70) seaweed extracts against α–amylase (A = A1; C = A2 and E = A3) and α–glucosidase (B = G1 and D = G2).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

11.5. CHROMATOGRAPHY AND SPECTROSCOPIC ANALYSIS

Crude (E90, E80 and E70) and purified (P90) polyphenolic extracts were chromatographically analyzed using phloroglucinol as standard. Chromatograms showed a unique peak (Figure 11.3) around 60 min of retention time, with no signals around 40 min that was the retention time of the standard used (i.e., phloroglucinol), concluding that the compounds detected had higher molecular mass. This result was expected since phloroglucinol or very small oligomers does not accumulate in the *A. nodosum* tissues, owing to the rapid polymerization reactions (Tierney et al., 2014; Sardari et al., 2021). Standardized phlorotannins chromatographic characterization methodology is limited since commercial standards are scarce. Very small peak signals located up 20 to 40 min of retention time show the presence of polysaccharides, in accordance with additional carbohydrates and UA standards (glucose and D (+)–guluronic acid). No significant ($p < 0.05$) differences on chromatographic spectra of crude extracts (E90, E80 and E70) were observed, indicating phlorotannin structure differences were not detectable with RP–HPLC technique. The increase in TPC value of P90, measured by Folin–Ciocalteu, was also confirmed by HPLC analysis, where 50 µL of 2.5 mg_{FD}/mL of P90 presented a similar peak than 50 µL of 5.0 mg_{DW}/mL from E90 extract.

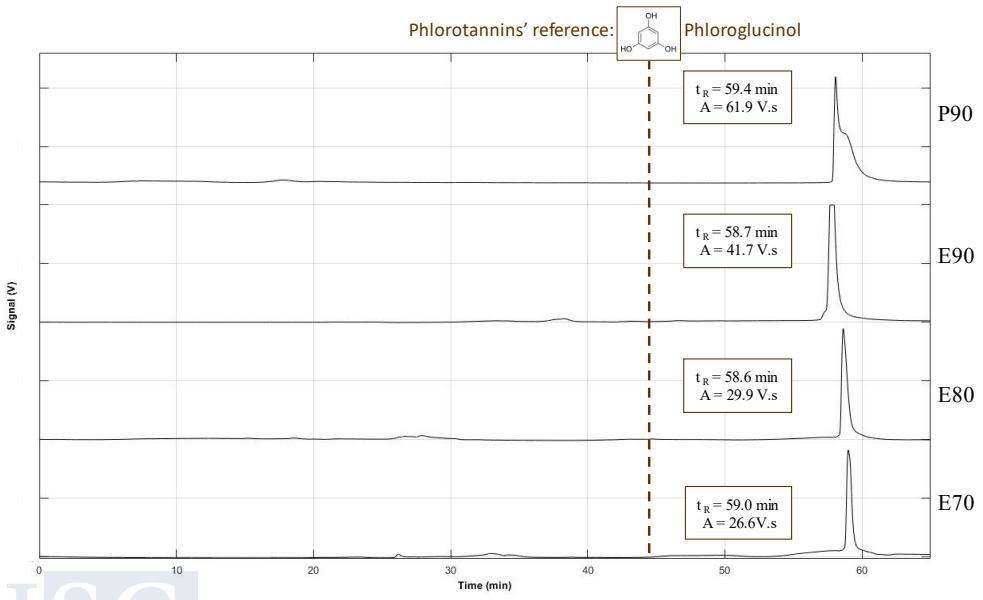


Figure 11.3. RP–HPLC profiles of purified (P90, 2.5 mg_{FD}/mL) and crude (E90, E80, E70, 5.0 mg_{FD}/mL) aqueous extracts obtained by UAE from *A. nodosum* seaweed.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Chromatographic results indicated that all UAE extracts contain polyphenols with high polymer size without significant ($p < 0.05$) presence of oligomers (expected at shorter times). Hence, differences in enzyme inhibition activities among extracts were not explained by HPLC results. Other authors have previously reported that HPLC method was ineffective separating large polymeric phlorotannins from *A. nodosum* (Tierney et al., 2014), and needs to be complemented with mass spectroscopy and/or NMR techniques (Koivikko et al., 2007).

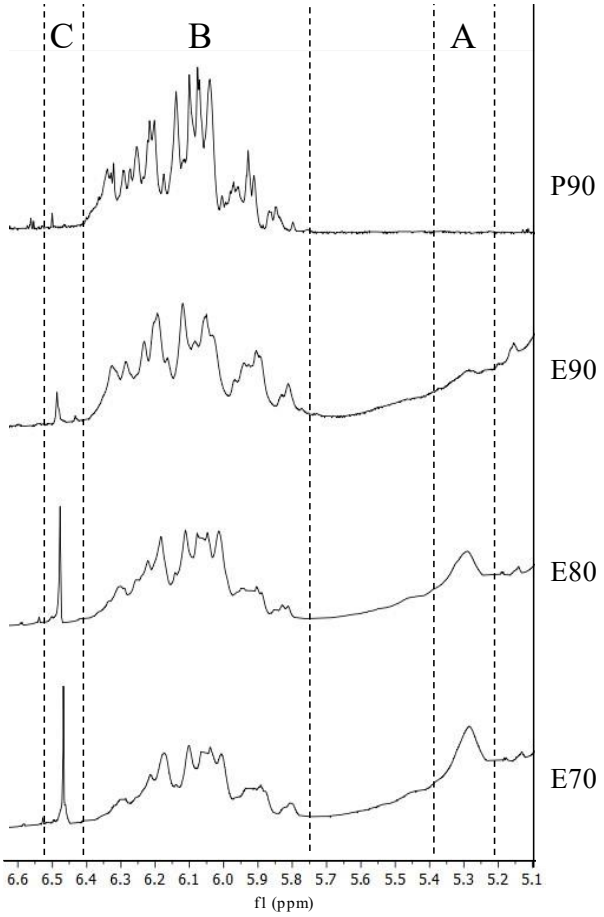


Figure 11.4. ^{1}H–NMR spectra in D_2O of freeze–dried extracts purified (P90) and crude (E90, E80 and E70) obtained by UAE from *A. nodosum* seaweed.

An adequate method for identifying and quantifying purified polyphenols is ^{1}H–NMR, but it is only qualitative in complex mixtures, since isomers appear at approximately the same chemical shifts. Nonetheless, ^{1}H–NMR analysis could give a proximate insight of overall structure of the assayed extracts and understand inhibition results. Figure 11.4 shows ^{1}H–NMR spectra of purified (P90) and crude UAE (E90, E80 and E70) extracts from *A. nodosum* seaweed.

Three different ranges could be identified at 5.20–5.40 (A), 5.75–6.40 (B) and 6.40–6.55 (C) ppm in tested samples, which agree with seaweed's polyphenols signals reported between 5.0 and 6.5 ppm (Audibert et al., 2010; Gager et al., 2020; Susano et al., 2021). In addition, ^{1}H–NMR spectra of crude UAE extracts showed below 5.6 ppm a notorious increasing signal associated with the presence of UA and carbohydrates (Zhang et al., 2004). Polyphenols extracted from brown seaweed are composed of phloroglucinol moieties linked by a mix of aryl–aryl and aryl–ether bonds (Choi et al., 2014). Hydrogens located near to aryl–ether bonds showed values around 6.5 ppm (C–region), meanwhile the aryl–aryl bonds signals around 5.7 to 6.3 ppm (B–region) (Choi et al., 2014; Fukuyama et al., 1990; Kim et al., 2019). This was also corroborated by phloroglucinol standard molecule spectra that showed a peak at 5.85 ppm from aryl–aryl bonds. A–region signal has been related to the presence of quinones derived from partial oxidation of polyphenols (Dobado et al., 2011). In fact, this signal dramatically decreased with sonication power, and disappeared in P90. This trend could be associated to the use of high power increased the lixiviation of less oxidated polyphenols from inner cell–structures (i.e., *physodes*) (Koivikko et al., 2007) and, oppositely, when low power was employed surface polyphenols from cell–wall (more exposed to oxygen) were mainly extracted. All extracts presented B–region signals and sonication power seemed to only change overall shape. Several peaks observed in this region evidenced the presence of different isomers of phlorotannins extracted during UAE treatment.

Prominent signal in C–region for E70 and E80 was observed in comparison to low signal in E90, meaning that C–region signal decreased with increasing sonication power. After Amberlite purification process (P90), this signal practically disappeared. Chemical composition of extracts (Table 11.1) showed a noticeable reduction of UA/TPC ratio for P90 regarding to E90. Based on these results, it is hypothesized that signals of C–region could be indicative of the presence of some polyphenolic complexes, mainly with uronic acids. The gradual signal reduction with increasing sonication power during UAE could be related to the disruption of these complexes, that could increase polyphenol availability for enzymes inhibition. Then, NMR spectra and *in vitro* inhibitory activities suggested that polyphenols–UA complexes are present on the extracts. The observed trend of digestive enzyme inhibitory activities of the extracts (Table 11.1) was associated with the higher presence of "free" polyphenols (not complexed) that interacted more easily with α–amylase and α–glucosidase enzymes during *in vitro* inhibitory assays.

USC DE COMPOSTELA

HIGHLIGHTS

Aqueous extracts obtained by UAE were highly effective inhibitors against digestive enzymes, α–amylase (IC_{50} < 4.7 mg/mL) and particularly for α–glucosidase (IC_{50} < 30.4 μg/mL).

Purification of extracts increased polyphenol (2.1–fold higher) content and reduced uronic acids (4.1–fold lower) content that increased their antioxidant activities and their inhibition capacities against both digestive enzymes.

Mixing of extracts with substrates before enzymatic reaction diminished their inhibitory effect (IC_{50} values of M2 and M3 was higher than M1).

Relevant differences were determined by ^{1}H–NMR technique and were associated to the presence of uronic acids–polyphenols complexes and/or quinones were related to the measured different inhibitory capacities.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

12. ALGINATE OBTAINED FROM PHLOROTANNINS EXTRACTION SOLID RESIDUE

Current Section 12 adapted from the following papers:

Montes L, Gisbert M, Hinojosa I., Sineiro J, Moreira R (2021) Impact of drying on the sodium alginate obtained after polyphenols ultrasound–assisted extraction from *Ascophyllum nodosum* seaweeds. *Carbohydrate Polymers* 272, 118455 DOI: 10.1016/j.carbpol.2021.118455 (See Section 17)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

12.1. GENERALITIES

Sodium alginates (alginates isoform) are linear binary copolymers (Figure 12.1a), linked by 1–4–glycosidic bonds, formed by the epimers, α–L–guluronate (G, Figure 12.1b), and β–D–mannuronate (M, Figure 12.1c). Alginates were deeply studied by Grasdalen et al., (1979, 1981 & 1983) to determine their physicochemical features and the procedures to determine them are summarized in an American Society for Testing and Materials (ASTM, 2012). M/G ratio value measures M– and G–blocks distribution along alginate molecule (Gómez–Ordoñez & Rupérez, 2011), and $N_{G>1}$ value measure average block length (Grasdalen et al., 1981). M/G and $N_{G>1}$ values vary among species, within the different parts of an organism and even during the different life stages of the seaweed. M/G ratio is used as an index for the alginate gels physical properties, in fact, lower values (<1) produce strong gels and higher values (>1) produce soft gels (Rehm 2005). These versatile properties make that the alginates can cover a wide spectrum of industrial applications (Gómez–Ordóñez & Rupérez, 2011).

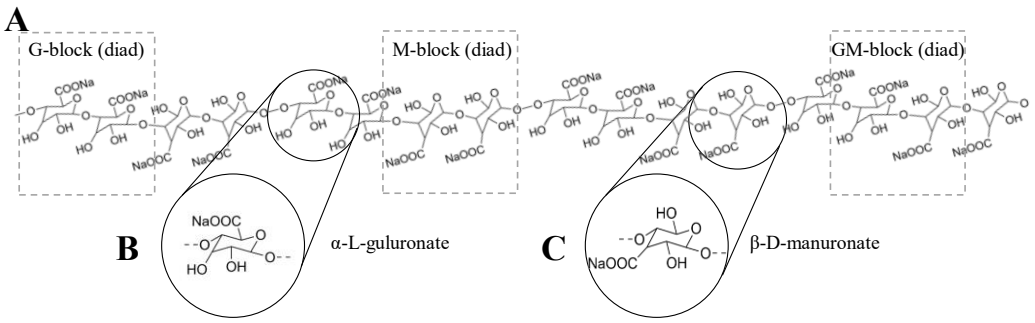


Figure 12.1. Molecular structure of sodium alginate polymer (A), their monomers: α–L–guluronate (G, B) and β–D–mannuronate (M, C) and diad structures (G–, M– and GM–blocks).

Alginate extraction from marine algae is based on the conversion of the alginic acid from the cell wall (Figure 1.4) into alginate salt forms, followed by their precipitation and purification. Firstly, seaweeds are washed and grounded. Formaldehyde is added to remove colored matter. Milled seaweeds are soaked in dilute mineral acid or with $CaCl_2$ (Chee et al., 2011) to remove fucoidans, laminarins, proteins and polyphenols that could modify alginate features. Then, alginic acid is transformed into sodium alginate employing alkaline solutions. Solid residues are removed by centrifugation and filtration.

Then, alginate is precipitated with ethanol to obtain sodium alginate (Donati & Paoletti, 2009). Other alginates are also produced such as calcium alginate, alginic acid, potassium alginate or ammonium alginate using alternatives to alkaline solution (Peteiro, 2018). Then alginate is precipitated with ethanol to produce sodium alginate (Figure 12.2). Finally, alginate fibers were separated, dried and milled (Donati & Paoletti, 2009).

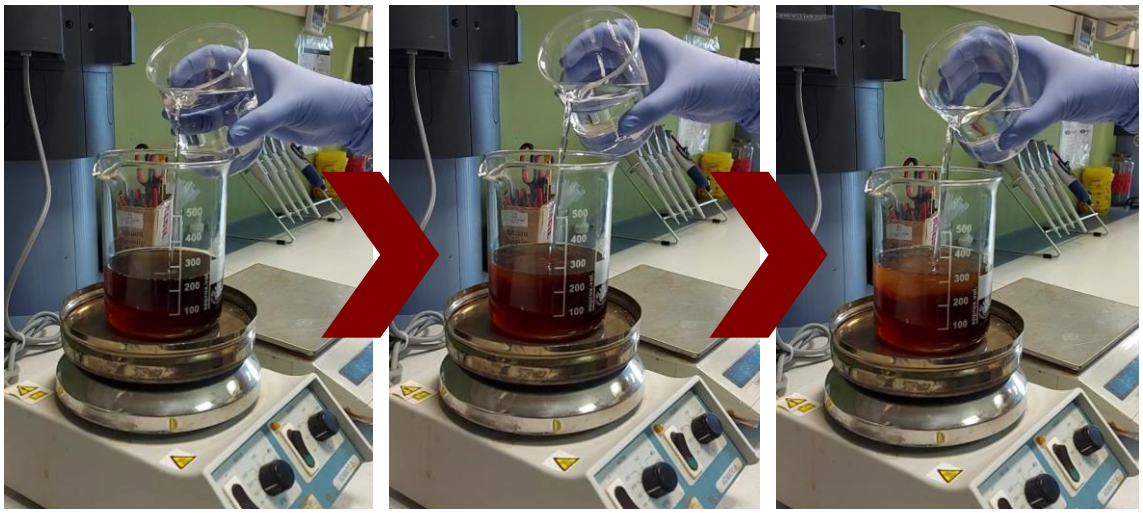


Figure 12.2. Alginate fibers precipitation by addition of ethanol.

Alginates can be characterized by different techniques to achieve deep knowledge of their features to select their optimal application. Some of these widespread techniques to characterize alginates are capillary viscometry (determining the viscosimetric average molecular weight), FTIR and ^{1}H–NMR (physic–chemical composition and structure) and rheometry (gelling and/or thickening capacities) among others. The selection of the optimal alginate according to the uses is critically conditioned by their features such as chemical structure, molecular size or chemical composition, making that alginate characterization a mandatory task after production. Alginate is highly sensitive to temperature, pH, free radicals and contaminants presence (Donati & Paoletti, 2009). Thus, operational conditions must be controlled during alginate production since their depolymerization and oxidation reactions could deteriorate extracted alginates (Smidsrød, et al., 1967). In this Thesis extraction and characterization of alginate was performed for sodium alginate.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Alginates have several industrial applications (foods, dye, biomaterials, medical or pharmaceutical) by their viscosity properties (Uludag et al., 2000). However, polyphenols and other colored compounds (chlorophyl, carotenoids) from algae produce a brown discoloration, during alginate extraction, resulting in an unpleasing dark sodium alginate powder. Bleaching pre–treatments fix these colored compounds to algae tissues reducing brown discoloration, but critically affect the rheological, chemical, and structural features of alginates by modification of molecular weight and M/G ratio (Mohammed et al., 2020). Nowadays, there is an increasing interest in isolating polyphenols for their own unique bioactivity characteristics from algae (Cotas et al., 2020). A suitable strategy for the transformation of current single–product manufacturing process into biorefineries would be the sequential extraction. Polyphenols, promising bioactive molecules above mentioned in Section 1.4 up to 1.6 and, secondly, alginates. To preserve the polyphenols features and to enhance extraction yields, the use of ultrasound–assisted extraction is a promising, efficient, low–cost, and eco–friendly technology (Kadam et al., 2015a, b and c).

The novelty presented in Section 12 was to contribute to the integral valorization of brown seaweeds, by means of the use the solid residue, after the ultrasound–assisted extraction (UAE) of polyphenols from AF seaweed, that still contains other active components, such as alginates. Consequently, our aim was to determine the effect of the process (existence of a drying stage) and operational conditions (drying temperature) on the physicochemical properties of alginates. Alginates were characterized by FT–IR, ^{1}H–NMR, and capillary viscometry.

12.2. ASSAYED SYSTEMS

Ultrasound–assisted extraction (UAE) of polyphenols (Section 4.4) from *A. nodosum* renders a solid phase, which alginates can be extracted from (Section 4.4.3). In this section alginates extracted after the UAE process, with and without an intermediate drying stage at different temperatures (50 and 90 ºC) were characterized in order to valorize UAE residue generated from all the previous Sections 6 up to 11. FT–IR (Section 4.11) and ^{1}H–NMR (Section 4.13.1) analyses showed the high purity of alginates with features in the range of commercial alginates. Drying UAE residues at different temperatures had a notorious effect on average viscosimetric molecular size (Section 4.12) of alginate.

USC UNIVERSIDADE DE COMPOSTELA

Tested process conditions allow the valorization of a residue to produce alginates with different properties together with the possibilities of tune their properties in order to accomplish with different characteristics depending on their future uses. The scheme of the research carried out could be seen in Figure 12.3

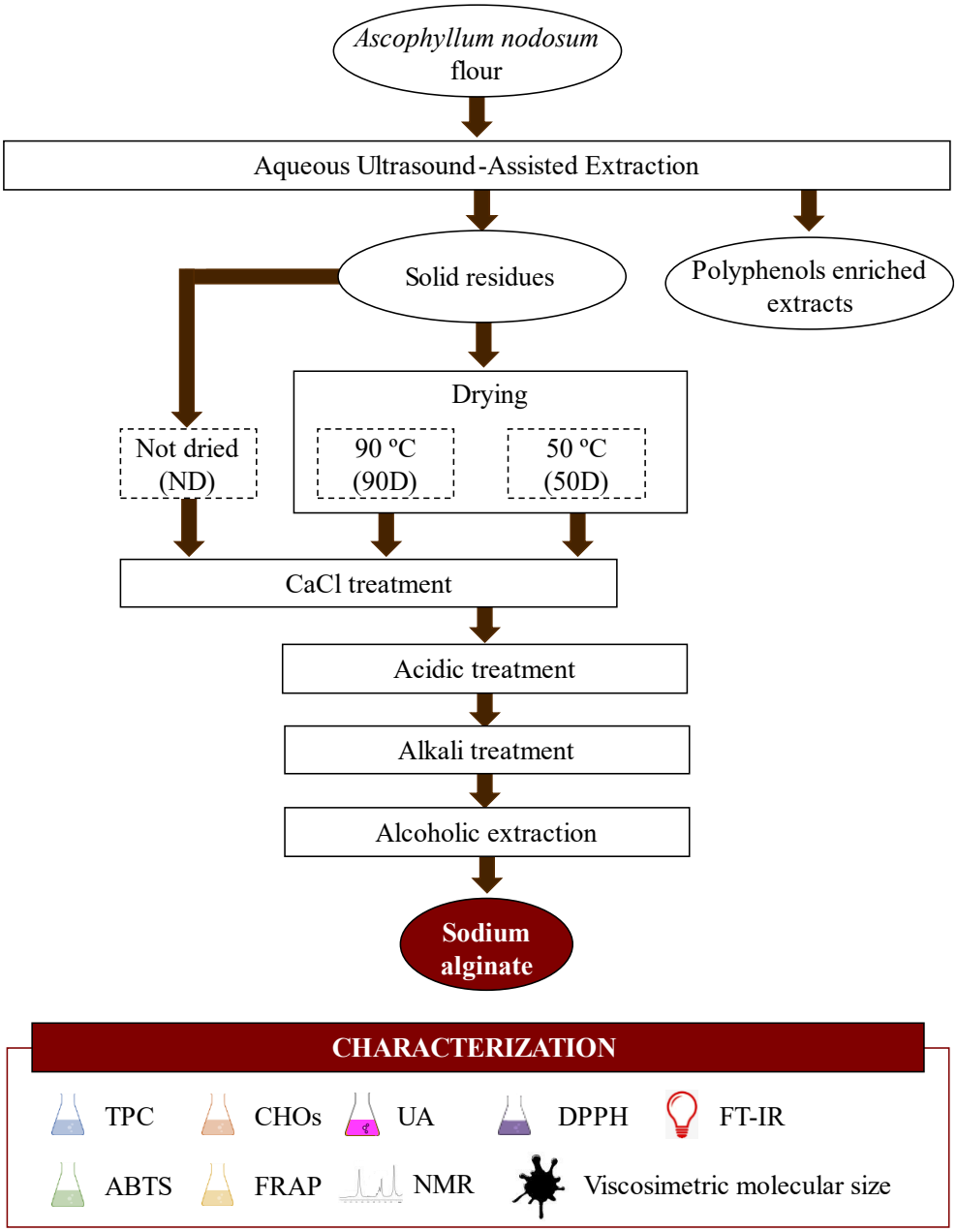


Figure 12.3. Scheme of process used to extract sodium alginate from UAE residues.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

12.3. EXTRACTION YIELD

The alginates extraction yield values, calculated by Eq. (4.6), from UAE undried (ND) and dried solids (50D and 90D) were significantly ($p < 0.05$) different, being their values 20.3 ± 0.9%, 23.7 ± 0.5% and 18.3 ± 0.4%, respectively. Maximum yield was achieved after drying of pellet at 50 ºC and is comparable to that reported (24%) by Rioux et al. (2007) working with *Ascophyllum nodosum* (*A. nodosum*) and is also in the range (from 20 to 41%) reported for other brown seaweeds (Chee et al., 2011). The yield after drying at 90 ºC dramatically decreased due to probably alginates losses from solid (by solubilization) during processing. The low yield at rt may be justified by a dilution effect due to the presence of the water that initially accompanies the solid and that diminishes the effect of the treatments.

12.4. FOURIER TRANSFORMED INFRARED

FT–IR spectra corresponding to extracted alginates from UAE undried (ND) and dried (50D and 90D) solids and CS were shown in Figure 12.4. Sodium alginates presented the characteristic bands up 4000 to 400 cm^{-1} range (Gómez–Ordóñeez & Rupérez, 2011). The peak between 3600 and 3200 cm^{-1} corresponded to stretching vibrations of O–H bonds. The 2930 cm^{-1} peak was stretching vibrations of C–H bonds. These peaks at 4000–2000 range are widespread reported from polysaccharides (El Atouani et al., 2016). The peak range 1680–1620 cm^{-1} was ascribed to asymmetric stretching vibrations of carboxylic groups. The 1415 cm^{-1} peak corresponded to deformation vibrations of C–OH bonds and asymmetric stretching vibrations of –C(=O)–O bonds (Mohammed et al., 2020). Around 1300 cm^{-1} peak appeared in the CA, but not in the *A. nodosum* extracted alginate. Literature refers this peak to S=O bond from sulfated polysaccharides such as fucoidans also extracted from marine brown seaweed (Gómez–Ordóñeez & Rupérez, 2011). The lack of this peak seemed to indicate the absence or low concentration of sulphated carbohydrates in the extracted alginates (Blanco–Pascual et al., 2014). Elemental analysis results (CHNS) confirmed this result, where higher sulfur content (0.09%) in CA than in 50D, 90D and ND samples (0.05, 0.01 and 0.02%, respectively) was determined.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The observed peak in the range 1091–1031 cm^{-1} corresponded to deformation vibrations of C–C–H and O–C–H bonds, stretching vibrations of C–O bonds, and stretching vibrations of C–O and C–C bonds, present in alginate pyranose rings. The 1030 cm^{-1} was promoted by stretching vibrations of C–O bonds (Gómez–Ordóñez & Rupérez, 2011).

Three zones were observed in the range 950–750 cm^{-1}: firstly, 950 to 930 cm^{-1}, corresponding to C–O bonds stretching vibrations from uronic acid residues, secondly, 870 to 883 cm^{-1} corresponding to deformation vibrations of C–H bonds from mannuronic acids, and finally, 815–833 cm^{-1} region to stretching vibration also to C–H bonds (El Atouani et al., 2016).

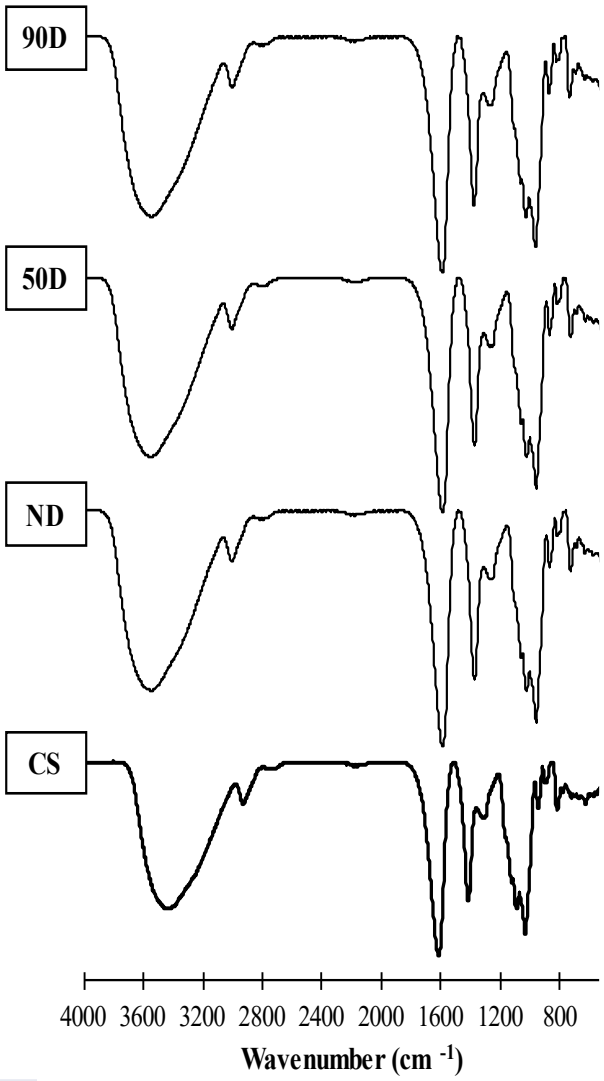


Figure 12.4. FT–IR spectra of commercial alginate from Sigma (CS) and extracted alginate from *Ascophyllum nodosum* sonication residue not dried (ND) and dried at 50 (50D) and 90 ºC (90D).

USC UNIVERSIDADE DE COMPOSTELA

No significant ($p < 0.05$) differences were observed between 50D, 90D and ND samples and minor differences with commercial alginates CS were observed, due to probably different seaweed species origin. Alginates extracted from solid subjected previously to UAE for polyphenols extraction of AF seaweeds showed structural features like commercial alginates. It was also corroborated by alternative seaweeds *F. vesiculosus* and *B. Bifurcata* with no significant ($p < 0.05$) number and intensities within peaks. Further, the absence of additional peaks indicated a low level of impurities. This evidence was also supported by CHNS elemental analysis were pollutants as nitrogen or sulfur were under 1% and 0.1%, respectively.

12.5. NUCLEAR MAGNETIC RESONANCE

Structural features (monads, diads and triads) of commercial (CS) and extracted alginates from *A. nodosum* residue after polyphenols UAE that was undried (ND), dried at 50 ºC (50D) and 90 ºC (90D) evaluated from ^{1}H–NMR spectra (Figure 12.5) were summarized in mannuronic content (F_M) of extracted alginates varied in a very narrow range (0.55 to 0.57) independently of drying conditions. Alginates from *A. nodosum* are characterized by their high mannuronic content in comparison to other seaweeds (Draget et al., 2006). Homopolymer diads blocks of samples presented higher F_{MM} values around 0.30–0.33, than F_{GG} values around 0.17–0.20. Heteropolymer blocks (F_{GM} or F AF) content ranged from 0.23 to 0.26. F_{GGG} triads content ranged from 0.06 (ND) to 0.11 (AF 90D), F_{MGM} and F_{GGM} did not show differences among extracted samples being around 0.15 for extracted alginates. Average block size ($N_{G>1}$) varied from 1.97 (90D) up to 2.15 (ND). Dried samples (50D and 90D) shower lower $N_{G>1}$ values than undried (ND) sample. M/G ratio varied from 1.21 (50D) to 1.33 (90D and ND).

As expected, these results indicated that drying of UAE residue did not modify significantly to monads, diads and triads distribution of the alginates, however $N_{G>1}$ values seemed to decrease by increasing drying temperature. Thus, drying conditions must be carefully controlled to modulate final polymer structural features. Alginates with M/G ratios higher than one are suitable to produce elastic gels for food, cosmetical or pharmaceutical applications (Murillo–Álvarez & Hernández–Carmona, 2007).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

In comparison to commercial alginate (CS), extracted *A. nodosum* alginates showed higher mannuronic content (around 0.56) than CS (0.48). F_{MM} homopolymer content was similar, F_{MM} content of CS (0.35) was notoriously higher, and F_{GM} or F_{MG} (0.18) lower. F_{GGG} content was clearly higher for CS (0.30) than those determined for extracted alginates (0.09) and F_{MGM} and F_{GGM} showed minor differences from 0.12 to 0.15. $N_{G>1}$ value (3.30) of CS was notoriously higher than those of extracted alginates (< 2.15). Finally, M/G ratio of CS was lower than one (0.91) due to glucuronic fraction was predominant above mannuronic molecules.

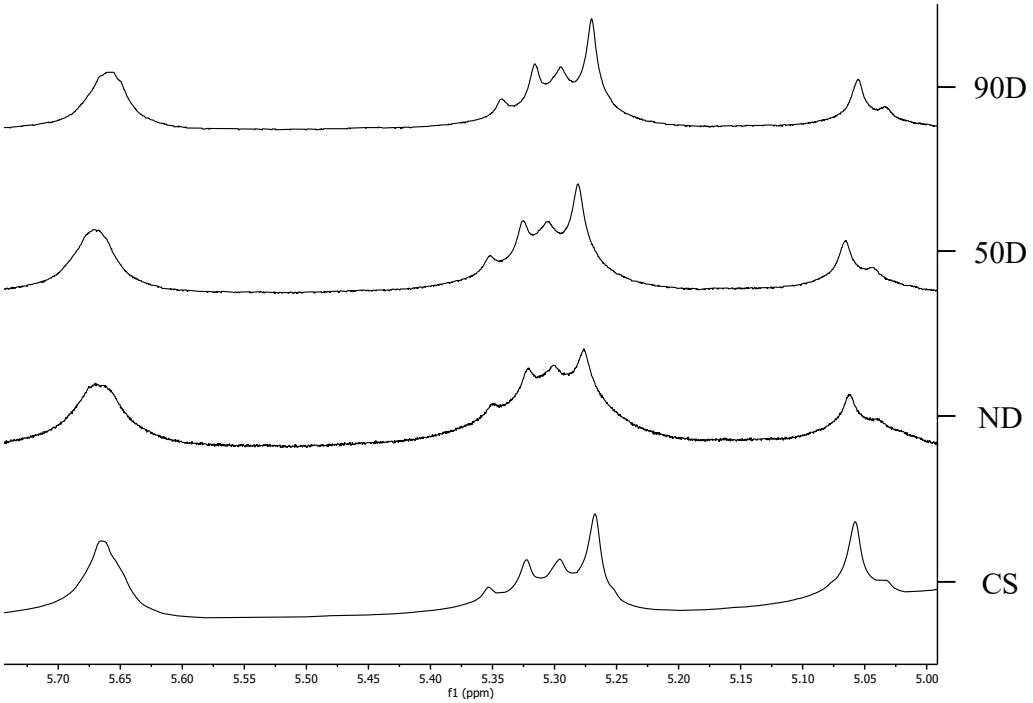


Figure 12.5. NMR spectra in D_2O of commercial alginate from Sigma (CS) and extracted alginate from *A. nodosum* sonication residue not dried (ND) and dried at 50 (50D) and 90 ºC (90D).

The characteristics of the obtained alginates were compared with those previously reported by other authors for alginates from *A. nodosum*. Authors employed different methods and conditions that are necessary to specify to compare results appropriately. In fact, Yuan & Macquarrie (2015) isolated alginates involving several steps at temperatures above 70 ºC during short periods, however, the final alginates showed similar features to extracted alginates in this work. Rioux et al. (2007) characterized dialyzed alginates (>1000 Da) with high average molecular weight. This fact could explain some differences with alginates studied here.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Mannuronic content (F_M) showed similar values and F_{MM} values were slightly lower than those (from 0.38 to 0.39) reported by consulted literature (Donati & Paoletti, 2009; Yuan & Macquarrie, 2015) summarized in Table 12.1 Nevertheless, F_{MM} values were slightly higher than value (0.28) given by Rioux et al. (2007). F_{GG} and F_{GM} values agreed to ranges reported by other authors (Table 12.1). Regarding triads, the values were generally in accordance with ranges previously reported: F_{GGG} and F_{MGM} values agreed and F_{GGM} value was slightly higher than that (0.07) reported by Donati & Paoletti (2009). $N_{G>1}$ values were lower than data (5.0) found by Donati & Paoletti (2009). Higher value of M/G ratio (1.44) was reported than the range (1.22–1.33) determined from extracted alginates in this study (Table 12.1). Average block size differences could be related to different extraction methods and conditions (temperature time) employed (Yuan & Macquarrie, 2015). Also, they can be attributed to seasonal variations and geographical origin of the seaweeds (Tabassum et al., 2016).

Table 12.1. Structural features of commercial alginate (CA) and extracted alginates from and *A. nodosum* undried (ND) and dried at 50 (50D) and 90 ºC and (90D) and comparison with alginates of other authors.

	Thesis				Yuan & Macquarrie (2015)	Donati & Paoletti (2009)	Rioux et al. (2007)
Seaweed	Unknown	*A. nodosum*			*A. nodosum*	*A. nodosum*	*A. nodosum*
Alginate	CS	ND	50D	90D			
F_M	0.48	0.57	0.55	0.57	0.59	0.59	0.46
F_G	0.52	0.43	0.45	0.43	0.41	0.41	0.54
F_{MM}	0.30	0.31	0.30	0.33	0.39	0.38	0.28
F_{GG}	0.35	0.17	0.20	0.20	0.21	0.22	0.36
F_{GM}	0.18	0.26	0.25	0.23	0.21	0.21	0.18
F_{GGG}	0.30	0.06	0.10	0.11		0.13	
F_{MGM}	0.12	0.14	0.15	0.15		0.14	
F_{GGM}	0.12	0.14	0.15	0.15		0.07	
$N_{G>1}$	3.30	2.15	2.07	1.97		5.00	
M/G	0.91	1.33	1.21	1.31	1.44	1.44	1.44

where mannuronate (M) and guluronate (G); $N_{G>1}$: average block length; F_M: M fraction; F_G: G fraction; F_{MM}, and F_{GG}: homopolymer fraction diads; F_{GM}: heteropolymer fraction diads; F_{GGG} and F_{MMM} homopolymer fraction triads; F_{MGM} and F_{GGM}: heteropolymer fraction triads.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

12.6. AVERAGE VISCOSIMETRIC MOLECULAR WEIGHT

Average alginate viscosimetric molecular weight (M_v) decreased significantly ($p < 0.05$) with the existence of a drying step and with increasing drying temperature. M_v values (kDa), obtained for extracted alginates from *A. nodosum*, varied in a wide range from 133.30 ± 1.20 (90D) and 427.80 ± 7.20 (ND). These results indicated clearly that molecular size of extracted alginates can be modulated by the use and conditions employed during drying conditions.

No significant ($p < 0.05$) differences between 90D and CS (156.1 ± 1.5 kDa). Despite different seaweed origin and other aspects, it could be probably related to the industrial conditions employed during extraction process. The molecular weights obtained are within the usual ranges reported for commercial alginates (Dodero et al., 2020a). Similarly, Chen et al. (2021) determined molecular weights of several brown seaweeds (*Sacharina japonica*, *Undaria pinnatiufida*, *Sargassum fusiforme* and *Sargassum hemiphyllum*) ranging from 171.8 to 663.0 kDa.

HIGHLIGHTS

The integral use of seaweeds was achieved with a sequential extraction of phlorotannins by UAE, followed by a controlled drying of solid residue and the subsequent extraction of alginates.

Drying temperature after ultrasound–assisted extraction of phlorotannins affected the extraction yield and rendered alginates with different average block length (from 1.97 up to 2.15) and average viscosimetric molecular weight (from 133.3 up to 257.3 kDa).

Drying could be used to modulate alginate's features producing sodium alginates with different and tuneable properties.

.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

13. CONCLUSIONS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Because of the proposed objectives and the experimental work carried out along this Thesis, as well as the analysis of the results achieved, the conclusions established were:

1 The current literature of phlorotannins extraction from *Ascophyllum nodosum* seaweed by conventional solid–liquid and ultrasound–assisted extractions was reviewed and summarized. It was also analyzed the available resources about the use of phlorotannins (and polyphenols) as food additives and as digestive enzyme inhibitors and their potential to be used glycemic index regulators. Finally, the background for the extraction, characterization and identification of the extracted biopolymers was provided.

2 Along this Thesis it was used different laboratory and pilot plant scale equipments for operations such as drying, freeze–drying, grinding, sieving, extraction, chromatography and dialysis, among others, as well as various experimental techniques for compositional analysis, bioactivity, inhibitions of enzymatic reactions, spectroscopic (FT-IR, NMR, MALDI-TOF-MS) and imaging (SEM) analysis viewed in the Results & Discussion sections.

3 *Ascophyllum nodosum* seaweed was dried, milled and characterized that allowed its adequate preservation and use along this Thesis as unique source of phlorotannins and sodium alginate. The drying, grounding, milling, packaging and preservation (4 ºC) ensured that the seaweed flour maintained the right conditions for use throughout this Thesis.

4 Continuous ultrasound–assisted extraction significantly ($p < 0.05$) increased all phytochemical parameters specially polyphenol content (around 1.4–fold higher) and antioxidant activities (e.g., DPPH increased 2.1–fold) of extracts regarding non-sonicated extracts. Hence, sonication time range studied (from 2 up to 15 min) did not show significant ($p < 0.05$) changes evidencing the notorious capacities of UAE technology to extract bioactive compounds from seaweeds using short operation times.

5 Extraction kinetics were successfully modelled by means of Peleg equation and the pseudo-equilibrium was achieved after 15 min independently of the solvent used. Saltwater (seawater) was a suitable solvent in both SLE and UAE methods to obtain bioactive extracts from *A. nodosum* flour.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

6 Continuous UAE extraction modelling of *Ascophyllum nodosum* carried out at room temperature indicated that phlorotannins content in the extracts depended on sonication power and solvent-solid ratio with minor dependence of sonication time. Maximal phlorotannin extraction was achieved at 90 W/cm^2 of sonication power, 20 g_W/g_{AF} of solvent-solid ratio and 2 min of sonication time. Hence, experimental data modelling allowed to set the optimal extraction conditions, which corresponded with a combination of SLE (15 min) followed by a short time UAE (2 min) to minimize the energy–consumption and to maximize phlorotannins extraction.

7 The phlorotannin-enriched extracts could be satisfactorily purified with a gel filtration chromatography using Amberlite XAD16 whereas carbohydrate content of the extract was reduced (1.7–fold lower) without significant ($p < 0.05$) modification of phlorotannin content. As results, higher antioxidant and enzymatic inhibition capacities increased at least up to 1.2–fold higher for purified extracts in relation to crude extract. These differences between crude and purified extracts were related with the existence of phlorotannin-carbohydrate complexes, that after purification partially disappeared, leaving free phlorotannins in the extracts that were prone to have better bioactivities and enzymatic inhibition capacities.

8 Oxidation of purified phlorotannin-enriched extracts was studied by different treatments, but only severe hydrogen peroxide oxidation was able to achieve a significant oxidation of phlorotannins in extracts. These results evidenced the high antioxidant capacities of the extracts and their resistance against severe oxidative conditions and/or species. The analysis of biological activities as function of molecular size showed that the main bioactive phlorotannins ranged between 10 and 20 kDa, higher than 50% of the total bioactivity of non–dialyzed extract. Hence, extracts with the lowest phlorotannins size showed low bioactivity similar to severe oxidized extract that was suitable seen by NMR, FT–IR and MALDI–TOF–MS techniques.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

9 Regarding starch-seaweeds interactions, the composition of liquid phase and phase transitions of starch promoted by heating operations modified the phytochemical features, and cross-sectional morphologies of these bioactive starchy-based meals. Starch showed different polyphenols retention yields depending on native or gelled structures. Interactions between phlorotannins and starch were owed to physisorption as the main mechanism for native and gelled starches (adsorbent), consequently, adsorbed amount depended on the available surface area. Retention of polyphenols notoriously increased when starch gelatinization was carried out in the presence of seaweeds because phlorotannins were physically adsorbed on the surface of the starch gel and trapped inside starch gel walls. The procedures used served to quantify the retention mode of bioactive molecules in gelled starch foods.

10 Aqueous seaweed extracts showed to be highly effective enzymatic inhibitors against α-amylase and, particularly, α-glucosidase. It was found that tested extracts were more effective inhibitors when they were added directly to the enzyme before to the substrate. The mixing of extracts with substrates (gelatinized or ungelatinized starch, or maltose) prior to the enzymatic reaction reduced the inhibitory effect of the extracts, being especially dramatic for the α-amylase inhibition. The relevant differences observed in inhibition capacities of the extracts could be explained by the presence of alginate–polyphenols complexes and phlorotannins oxidated states (i.e., quinones).

11 The sequential procedure of extraction constituted by, in first step, phlorotannins by UAE technology followed by the extraction of alginates allowed to obtain two valuable products, phlorotannins enriched extracts and sodium alginates. The integral use of seaweeds implies clear economic and environmental advantages.

12 The controlled drying conditions (specifically drying temperature) before extraction allowed to obtain alginates with different properties (average block length ($N_{G>1}$ from 1.97 up to 2.15) and viscosity average molecular weight, M_v, from 133.3 up to 257.3 kDa) that implied that this biopolymer can be produced according to different demands.

USC UNIVERSIDADE DE COMPOSTELA

The challenges of extraction, characterization, identification, and integration of biopolymers from seaweed as antioxidants and enzyme-inhibiting compounds were treated by a multidisciplinary approach (i.e., engineering, chemistry, and food technology) in this Thesis. The knowledge and results achieved satisfactorily accomplished the main aim proposed, to obtain bioactive seaweed derived compounds that may be active controllers for the management of glycemic control, suitable to alleviate and prevent diabetes associated with the gluten–free food diet. As future perspectives, it is proposed the implementation of phlorotannins (as bioactive additives) and alginates (as gluten substitutes) to real bakery matrices and study their effect on textural behavior and glycemic indexes. Another proposed study would be the *in vivo* analysis of phlorotannins to determine effective doses and even long-term effects.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

14. LIST OF FIGURES

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Figure 1.1. Seaweed color classification, main characteristics and representative species.

Figure 1.2. Phlorotannins bioactive capacities.

Figure 1.3. Polyphenol's classification.

Figure 1.4. Proposed matrix of brown seaweed cell–walls.

Figure 1.5. Phlorotannin main groups, all derived from phloroglucinol polyketide pathway reaction

Figure 1.6. *Ascophyllum nodosum* (*Linnaeus*) *Le Jolis* seaweed (A), fronds (B) and flotation vessels (C) from Algaebase database.

Figure 1.7. Geographical distribution of *A. nodosum* seaweed from GBIF database, (2022).

Figure 1.8. Average composition of *A. nodosum* brown seaweed.

Figure 1.9. Industrial potential of seaweeds.

Figure 1.10. Classification of antioxidants.

Figure 1.11. Diagram of celiac disease iceberg.

Figure 3.1. Phlorotannins extraction methods.

Figure 3.2. Scheme of the coetaneous processes in SLE of compounds from seaweed cell.

Figure 3.3. Ultrasound–assisted extraction process.

Figure 3.4. Summary of analytical methods often used for characterization of seaweed biopolymers

Figure 3.5. Antioxidant activity determination methods.

Figure 3.6. Stages in the development of functional foods using seaweed as source of phlorotannins, from a multidisciplinary point of view.

Figure 3.7. Starch gelatinization process and their microstructure changes.

Figure 3.8. Global process of starch metabolic fate.

Figure 4.1. A) Angelantoni Challenge 250 Air convective dryer (Italy), B) Laboratory blender Waring HGBTWT (USA), C) Ultra–centrifugal mill Retsch GmbH, ZM200 (Germany), D) Vibratory sieve FTL 0200, Cisa (Spain) and E) Vacuum packer Sammic V201 (Spain).

Figure 4.2. A) Vacuum oven Vacutherm VT650 (Heraeus Hanau, Germany).

Figure 4.3. Experimental set up for the continuous aqueous ultrasound–assisted extraction of biopolymers from *A. nodosum* seaweed.

Figure 4.4. High–speed laboratory centrifuge Retsch GmbH, ZM200 (Germany, A) and Telstar LyoQuest lyophilizer –55, Telstar Technologies (Spain, B).

Figure 4.5. Concentrator plus/Vacufuge plus, Eppendorf (Germany)

USC DE SANTIAGO DE COMPOSTELA

Figure 4.6. Color change occurred during colorimetric assays.
Figure 4.7. TPC calibration curve.
Figure 4.8. Total polyphenol content determination methodology.
Figure 4.9. CHOs calibration curve.
Figure 4.10. Total carbohydrate content determination methodology.
Figure 4.11. UA calibration curve.
Figure 4.12. Uronic acids content determination methodology.
Figure 4.13. DPPH calibration curve.
Figure 4.14. DPPH scavenging activity determination methodology.
Figure 4.15. ABTS calibration curve.
Figure 4.16. ABTS scavenging activity determination methodology.
Figure 4.17. FRAP calibration curve.
Figure 4.18. Electron donor capacity (FRAP) determination methodology.
Figure 4.19. Scheme of *A. nodosum* aqueous extracts dialysis.
Figure 4.20. Elution gradients used during RP–HPLC analysis of *A. nodosum* extracts, based on gradient mode (A) and on–off mode (B).
Figure 4.21. Average ^{1}H–NMR spectrum shape of alginate used for quantitative analysis.
Figure 4.22. Process scheme summarizing methodology. NT method: AF (seaweeds flour) and corn starch (CS) blending; GL method: AF and CS blending, CGL method: CS gelatinized in presence of AF.
Figure 4.23. Methodology scheme of α–amylase inhibition assays (A1, A2 and A3) and α–glucosidase (G1 and G2) digestive enzymes against aqueous polyphenols extracts from *A. nodosum* seaweed.
Figure 5.1. General scheme of the Results & Discussion section in the current Thesis.
Figure 6.1. *A nodosum* flour obtention, drying, grounding, milling pretreatments.
Figure 6.2. Experimental kinetics of *Ascophyllum nodosum* seaweed drying at dry temperature of air at 50 ºC, 30% relative humidity, 2 m/s of air velocity and 2 kg/m2 of load density.
Figure 6.3. Differential (A) and accumulated particle size (B) distribution curve from *A. nodosum* flour after drying and milling.
Figure 7.1. *A. nodosum* extracts characterization to evaluate extraction method, solid–liquid extraction, and ultrasound–assisted extraction.

USC DE SANTIAGO DE COMPOSTELA

Figure 7.2. Experimental data (dots) and Peleg's model values (lines) for TPC (A), CHOs (B) UA (C), DPPH (D), ABTS (E) and FRAP (F) of SLE kinetics using water (D) and saltwater (S) for extractions at 20, 30 and 40 gW/gAF of liquid–solid ratio.

Figure 7.3. TPC (A), CHOs (B), UA (C), DPPH (D), ABTS (E) and FRAP (F) values of UAE (sonication power of 90, 80 and 70 W/cm2) and SLE using D– and S–solvents at 15 min of extraction with 20, 30 and 40 gW/gAF of LS ratios. Different superscript letters above bars mean significant ($p < 0.05$) differences between values.

Figure 7.4. Linear relationships between TPC and antioxidant activities, DPPH (A), ABTS (B) and FRAP (C), from SLE and UAE (70, 80 and 90 W/cm2) extracts using D– and S–solvents at 15 min of extraction with 20, 30 and 40 gW/gAF LS ratios.

Figure 7.5. RP–HPLC profiles (LS = 20 gW/gAF) of SLE (5 and 15 min) and UAE (15 min) at 90 W/cm2, extracts using D– (A, B and C), and S–solvents (D, E and F).

Figure 7.6. Evolution of normalized area values during SLE (LS = 20 gW/gAF) from RP–HPLC profiles using D– and S–solvent, of A–, B– and C–regions.

Figure 7.7. Normalized area values from the SLE (LS = 20 gW/gAF) extracts (2 to 60 min) from D– and S–solvents of A–, B–, C–regions and total correlated with measured antioxidant activities (DPPH, ABTS and FRAP).

Figure 8.1. Box–Behnken experimental designing in order to optimize and set up ultrasound–assisted extraction of *A. nodosum* bioactive compounds.

Figure 8.2. Response surface plots for A, B polyphenols (TPC, gPE/L); C, D carbohydrates (CHOs, gGE/L) and E, F uronic acids (UA, gGE/L) content vs studied variables at constant sonication power (SP, W/cm2) or time residence (tR, min): A, C SP = 70 W/cm2, E SP = 80 W/cm2, B SP = 90 W/cm2, D tR = 2 min and F tR = 4 min. Box–Behnken (black box) and additional points (white star).

Figure 8.3. Linear correlations of antioxidant activities determined by DPPH (A), ABTS (B) and FRAP (C) methods, against TPC for UAE from *A. nodosum*.

Figure 9.1. Systems experimentally obtained to evaluate the influence of oxidation and dialysis of phlorotannins on bioactivity and composition of ultrasound–assisted extracts from *Ascophyllum nodosum*.

Figure 9.2. Phytochemical and antioxidant activity (TPC, A; CHOs, B and DPPH, C) of crude (EA and EW), purified (PW), oxidized (PON, POA and POP) and dialyzed, (PD20, PD10,

USC DE SANTIAGO DE COMPOSTELA

PD3 and PD2) extracts. Different superscript letters indicate significant ($p < 0.05$) differences among values.

Figure 9.3. Distribution percentage of TPC, DPPH and CHOs values among dialyzed extracts.

Figure 9.4. RP–HPLC profiles of (A) standard compounds (phloroglucinol, resorcinol, glucose, glucuronic acid and BSA); (B) crude and purified (EA, EW, and PW), (C) oxidized, (PON, POA and POP) and dialyzed (PD20, PD10, PD3 and PD2) extracts.

Figure 9.5. FT–IR spectra of crude (EA and EW), purified (PW), oxidized (POP) and dialyzed (PD20 and PD2) extracts.

Figure 9.6. ^{1H}–NMR (DMSO–d6) spectra of crude (EA and EW), purified (PW), oxidized (POP) and dialyzed (PD20 and PD2) extracts.

Figure 9.7. MALDI–TOF–MS spectra of purified (PW) and dialyzed extracts (PD20 and PD2).

Figure 10.1. Schematic process used to determine *A. nodosum* seaweed interactions with corn starch based on different mixing procedures.

Figure 10.2. TPC values (A), sorption yields, YP (B), and CHOs values (C) of AF (▭), CS (native (■) and gelled (▭) control samples and AF–CS blending methods of NT (▭), GL (▭) and CGL (▭) samples. Different letters indicate significant ($p < 0.05$) differences among samples.

Figure 10.3. Adsorbed/retained polyphenols by CS, q, vs TPC of aqueous phase for AF–CS samples (NT, GL, and CGL) at 20 ºC. Lines are Halsey model (Eq. (10.1)).

Figure 10.4. Antioxidant activities determined by DPPH (A), ABTS (B) and FRAP (C) against TPC in control (AF) and AF–CS samples (NT, GL, and CGL, methods).

Figure 10.5. FT–IR spectra of control (AF, ─, and CS, ─) and AF–CS (1:1 ratio) samples (NT, ─, GL, ─, and CGL, ─).

Figure 10.6. Cross–sectional morphologies of solid phase from AF (A), CS (B) and AF–CS (1:1 ratio) samples (NT (C), GL (D) and CGL (E)).

Figure 11.1. Assayed systems for the determination of the digestive enzyme inhibitory capacities of *A. nodosum* extracts

Figure 11.2. Inhibitory capacity of purified (P90) and raw (E90, E80 and E70) seaweed extracts against α–amylase (A = A1; C = A2 and E = A3) and α–glucosidase (B = G1 and D = G2).

Figure 11.3. RP–HPLC profiles of purified (P90, 2.5 mgFD/mL) and crude (E90, E80, E70, 5.0 mgFD/mL) aqueous extracts obtained by UAE from *A. nodosum* seaweed.

USC DE SANTIAGO DE COMPOSTELA

Figure 11.4. ^{1}H–NMR spectra in D_2O of freeze–dried extracts purified (P90) and crude (E90, E80 and E70) obtained by UAE from *A. nodosum* seaweed.

Figure 12.1. Molecular structure of sodium alginate polymer (A), their monomers: α–L–guluronate (G, B) and β–D–mannuronate (M, C) and diad structures (G–, M– and GM–blocks).

Figure 12.2. Alginate fibers precipitation by addition of ethanol.

Figure 12.3. Scheme of process used to extract sodium alginate from UAE residues.

Figure 12.4. FT–IR spectra of commercial alginate from Sigma (CS) and extracted alginate from *Ascophyllum nodosum* sonication residue not dried (ND) and dried at 50 (50D) and 90 ºC (90D).

Figure 12.5. NMR spectra in D2O of commercial alginate from Sigma (CS) and extracted alginate from *A. nodosum* sonication residue not dried (ND) and dried at 50 (50D) and 90 ºC (90D).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

15. LIST OF TABLES

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 1.1. Reactive species.

Table 1.2. Current recognized as safe and edible brown edible seaweeds (FAO, 2018).

Table 1.3. Overview extraction conditions (method, extractant, liquid–solid ratio, time, and temperature) to obtain phlorotannins enriched extracts from *Ascophyllum nodosum*.

Table 1.4. State of art of current phlorotannins isolated from brown seaweeds.

Table 1.5. State of art of inhibitory capacities against α–amylase and α–glucosidase digestive enzymes of polyphenols from *A. nodosum* seaweed.

Table 1.6. State of the art of inhibitory capacities against α–amylase and α–glucosidase digestive enzymes of polyphenols from alternative seaweeds.

Table 1.7 State of art of inhibitory capacities against α–amylase and α–glucosidase digestive enzymes of polyphenols from terrestrial plants.

Table 4.1. BB experimental design of UAE carried at different sonication power (SP), residence times (t) and liquid–solid ratios (LS).

Table 7.1. Peleg's model parameters (K1 and K2) and Xeq = 1/K2 for extracts bioactive compounds content (TPC, CHOs and UA) and antioxidant activities (DPPH, ABTS and FRAP) for SLE kinetics using water (D) and saltwater (S) at 20, 30 and 40 gW/gAF.

Table 7.2. Extraction yields (Eq. (4.5)) in SLE and UAE (SP = 90 W/cm2) samples of phytochemical compounds (TPC, CHOs, UA and antioxidant activities) from *A. nodosum* seaweed with 20, 30 and 40 gW/gAF of LS ratios using D– and S–solvents.

Table 7.3. Polyphenols–carbohydrates and polyphenols–energy ratios from *A. nodosum* seaweed with 20, 30 and 40 gW/gAF of LS ratios using D– and S–solvents using SLE and UAE (SP = 90 W/cm2) extraction methods.

Table 7.4. Ratio UAE/SLE of compound extraction (TPC, CHOs and UA) and antioxidant capacities of the extracts (DPPH, ABTS and FRAP) from *A. nodosum* seaweed with 20, 30 and 40 gW/gAF of LS ratios using D– and S–solvents using SLE and UAE (SP = 90 W/cm2) extraction methods.

Table 7.5. Ratio D/S on compound extraction (TPC, CHOs and UA) and antioxidant capacities of the extracts (DPPH, ABTS and FRAP) from *A. nodosum* seaweed with 20, 30 and 40 gW/gAF of LS ratios using D– and S–solvents using SLE and UAE (SP = 90 W/cm2) extraction methods.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Table 8.1. Experimental (Exp) and modeled (Mod) TPC (Eq. (8.1)), CHOs (Eq. (8.2)) and UA (Eq. (8.3)) values from aqueous extraction of *A. nodosum* seaweeds using UAE at different SP, tR and LS conditions following a BB design.

Table 8.2. ANOVA for quadratic models of TPC, CHOs and UA content in the UAE aqueous extracts from *A. nodosum* seaweed.

Table 8.3. TPC ratios in relation to CHOs, UA and consumed energy during for UAE of *A. nodosum* seaweeds at different conditions of SP, tR and LS.

Table 9.1. Phloroglucinol units of dialyzed PD20 and PD2 extracts in comparison with extracts from brown seaweeds reported by bibliography.

Table 9.2. List of phlorotannins identified in (Figure 9.7) and its water loss n[H2O] comparing data with Steevensz et al., (2012) & Kellogg et al. (2014). Superscript letters were used to identify different phlorotannins families within the extract

Table 10.1. Polyphenol sorption yields (YP) ratios from NT, GL and CGL assayed methods.

Table 10.2. Parameters of Halsey equation (Eq. (4.30)).

Table 11.1. Chemical composition of freeze dried (E90, E80 and E70) and purified (P90) extracts obtained by UAE from *A. nodosum* brown seaweed.

Table 11.2. IC50 values of seaweed extracts against α–amylase and α–glucosidase of the different analyzed methodologies: extract + enzyme (M1), extract + gelatinized starch (M2), gelatinization of starch with extract (M3).

Table 12.1. Structural features of commercial alginate (CA) and extracted alginates from and *A. nodosum* undried (ND) and dried at 50 (50D) and 90 ºC and (90D) and comparison with alginates of other authors.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

16. BIBLIOGRAPHY

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A

Adefegha SA, Oboh G, Ejakpovi II, Oyeleye SI (2015) Antioxidant and antidiabetic effects of gallic and protocatechuic acids: a structure–function perspective. *Comparative Clinical Pathology* 24(6), 1579–1585. DOI: 10.1007/s00580–015–2119–7

Ademiluyi A, Oboh G (2012) Phenolic–rich extracts from selected tropical underutilized legumes inhibit α–amylase, α–glucosidase, and angiotensin I converting enzyme *in vitro*. *Journal of Basic Clinic Physiology and Pharmacology* 23(1), 17. DOI: 10.1515/jbcpp–2011–0005

Agregán R, Munekata PES, Franco D, Carballo J, Barba FJ, Lorenzo JM (2018) Antioxidant potential of extracts obtained from macro– (*Ascophyllum nodosum*, *Fucus vesiculosus* and *Bifurcaria bifurcata*) and micro–algae (*Chlorella vulgaris* and *Spirulina platensis*) assisted by ultrasound. *Medicines* 5, 33. DOI: 10.3390/medicines5020033.

Agregán R, Munekata PES, Franco D, Dominguez R, Carballo J, Lorenzo JM (2017) Phenolic compounds from three brown seaweed species using LCDAD-ESI-MS/MS. *Food Research international* 99(3), 979-985. DOI: 10.1016/j.foodres.2017.03.043

Aguilera JM (2003) Solid–liquid Extraction. In extraction optimization in food engineering, pp 35–39 Marcel Dekker, Inc, Boca Raton, USA.

Ahn GN, Kim KN, Cha SH, Song CB, Lee J, Heo MS, Yeo IK, Lee NH, Jee YH, Kim JS, Heu MS, Jeon YJ (2007) Antioxidant activities of phlorotannins purified from *Ecklonia cava* on free radical scavenging using ESR and H_2O_2–mediated DNA damage. *European Food Research and Technology* 226, 71–79. DOI: 10.1007/s00217–006–0510–y

Ainsworth EA, Gillespie KM (2007) Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature Protocols* 2(4), 875-877. DOI: 10.1038/nprot.2007.102

Akkarachiyasit S, Charoenlertkul P, Yibchok–anun S, Adisakwattana S (2010) Inhibitory activities of cyanidin and its glycosides and synergistic effect with acarbose against intestinal α–glucosidase and pancreatic α–amylase. *International Journal of Molecular Sciences* 11(9). DOI: 10.3390/ijms11093387

Aklakur M (2018) Natural antioxidants from sea: A potential industrial perspective in aquafeed formulation. *Reviews in Aquaculture* 10(2), 385–399. DOI: 10.1111/raq.12167

USC DE SANTIAGO DE COMPOSTELA

Algaebase. *Ascophyllum nodosum* (Linnaeus) Le Jolis. https://www.algaebase.org/search/images/detail/?img_id=3890. Searched on 4 April 2022.

Allsopp P, Crowe W, Bahar B, Harnedy PA, Brown ES, Taylor SS, Smyth TJ, Soler–Vila A, Magee PJ, Gill CIR, Strain CR, Hegan V, Devaney M, Wallace JMW, Cherry, P, FitzGerald, RJ, Strain, JJ, O'Doherty, JV, McSorley EM (2016) The effect of consuming *Palmaria palmata* enriched bread on inflammatory markers, antioxidant status, lipid profile and thyroid function in a randomised placebo–controlled intervention trial in healthy adults. *European Journal of Nutrition* 55, 1951–1962. DOI: 10.1007/s00394–015–1011–1

Alvarez–Rivera G, Ballesteros–Vivas, D, Parada–Alfonso, F, Ibañez E, Cifuentes A (2019) Recent applications of high resolution mass spectrometry for the characterization of plant natural products. *TrAC Trends in Analytical Chemistry* 112, 87–101. DOI: 10.1016/j.trac.2019.01.002

Allwood, J.W.; Evans, H.; Austin, C.; McDougall, G.J. Extraction, enrichment, and LC–MSn–based characterization of phlorotannins and related phenolics from the brown seaweed, *Ascophyllum nodosum*. *Mar. Drugs* **2020**, *18*, 448. DOI: 10.3390/md18090448

Amoako DB, Awika JM (2016) Polymeric tannins significantly alter properties and *in vitro* digestibility of partially gelatinized intact starch granule. *Food Chemistry* 208, 10–17. DOI: 10.1016/j.foodchem.2016.03.096

An EK, Hwang J, Kim SJ, Park HB, Zhang W, Ryu JH, You SG, Jun OJ (2022) Comparison of the immune activation capacities of fucoidan and laminarin extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules* 208, 230–242. DOI: 10.1016/j.ijbiomac.2022.03.122

André R, Guedes L, Melo R, Ascensão L, Pacheco R, Vaz PD, Serralheiro ML (2020) Effect of food preparations on *in vitro* bioactivities and chemical components of *Fucus vesiculosus*. *Foods* 9, 955–975. DOI: 10.3390/foods9070955

Aneiros A, Garateix A (2004) Bioactive peptides from marine sources: Pharmacological properties and isolation procedures. *Journal of Chromatography B* 803, 41–53. DOI: 10.1016/j.jchromb.2003.11.005

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Angell AR, Mata L, de Nys R, Paul NA (2016) The protein content of seaweeds: a universal nitrogen–to–protein conversion factor of five. *Journal of Applied Phycology* 28: 511–524. DOI: 10.1007/s10811–015–0650–1

Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K (2002) Methods for testing antioxidant activity. *Analyst* 127, 183–198. DOI: 10.1039/B009171P

AOAC (2000) Official Methods of Analysis of the AOAC International: Association of Analytical Communities.

Apak R, Çapanoglu E, Shahidi F. (2018) Measurement of antioxidant activity and capacity–In recent trends and applications; John Wiley & Sons Ltd.: Hoboken, USA pp. 278–283.

Apostolidis E, Lee CM (2010) *In vitro* potential of *Ascophyllum nodosum* phenolic antioxidant–mediated α–glucosidase and α–amylase inhibition. *Journal of Food Science* 75, 97–102. DOI: 10.1111/j.1750–3841.2010.01544.x

Artan M, Li Y, Karadeniz F, Lee SH, Kim MM, Kim SK (2008) Anti–HIV–1 activity of phloroglucinol derivative, 6,6–bieckol, from *Ecklonia cava*. *Bioorganic & Medicinal Chemistry Letters* 16, 7921–7926. DOI: 10.1016/j.bmc.2008.07.078

Arufe S, Chenlo F, Sineiro J, Moreira R (2019a) Effect of brown seaweed addition and starch gelatinization on gluten-free chestnut flour doughs and cookies. *Journal of Food Measurement and Characterization* 13, 2571–2580. DOI: 10.1007/s11694–019–00177–6

Arufe S, Della Valle G, Chiron H, Chenlo F, Sineiro J, Moreira R. (2018a) Effect of brown seaweed powder on physical and textural properties of wheat bread. *European Food Research and Technology* 244, 1–10 DOI: 10.1007/s00217–017–2929–8

Arufe S, Sineiro J, Moreira R (2019b) Determination of thermal transitions of gluten–free chestnut flour doughs enriched with brown seaweed powders and antioxidant properties of baked cookies. *Heliyon* 5, e01805. DOI: 10.1016/j.heliyon.2019.e01805

Arufe S, Torres MD, Chenlo F, Moreira R (2018b) Air drying modelling of *Mastocarpus stellatus* seaweed a source of hybrid carrageenan. *Journal of Heat and Mass Transfer* 54, 177–184. DOI: 10.1007/s00231–017–2117–1

ASTM Standard F2259–10 (2012) Standard test method for determining the chemical composition and sequence in alginate by proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy. Westconshohocken, PA: ASTM International.

USC DE SANTIAGO DE COMPOSTELA

Atkinson MJ, Bingman C (2010) Elemental composition of commercial seasalts. *ournal of Aquariculture and Aquatic Sciences* 8, 39–43.

Audibert L, Fauchon M, Blanc N, Hauchard D, Ar Gall E (2010) Phenolic compounds in the brown seaweed *Ascophyllum nodosum*: distribution and radical–scavenging activities. *Phytochemical Analysis* 21, 399–405. DOI: 10.1002/pca.1210

B

Balboa EM, Conde E, Moure A, Falqué E, Domínguez H (2013) *In vitro* antioxidant properties of crude extracts and compounds from brown algae. *Food Chemistry* 138, 1764–1785. DOI: 10.1016/j.foodchem.2012.11.026

Barde SR, Sakhare RS, Kanthale SB, Chandak PG, Jamkhande PG (2015) Marine bioactive agents: A short review on new marine antidiabetic compounds. *Asian Pacific Journal of Tropical Disease* 5, 209–213. DOI: 10.1016/S2222–1808(15)60891–X

Barros F, Awika JM, Rooney LW (2012) Interaction of tannins and other sorghum phenolic compounds with starch and effects on *in vitro* starch digestibility. *Journal of Agricultural and Food Chemistry* 60, 11609–11617. DOI: 10.1021/jf3034539

Barros F, Awika JM, Rooney LW (2014) Effect of molecular weight profile of sorghum proanthocyanidins on resistant starch formation. *Journal of Agricultural and Food Chemistry* 94, 1212–1217. DOI: 10.1002/jsfa.6400

Barrow C, Shahidi F (2007) Marine nutraceuticals and functional foods. (Eds.) Boca Raton (FL): Taylor and Francis, CRC Press, 517.

Bartosz G (2010) Non–enzymatic antioxidant capacity assays: Limitations of use in biomedicine. *Free Radical Research* 44, 711–720. DOI: 10.3109/10715761003758114

Becker EM, Nissen LR, Skibsted LH (**2004)** Antioxidant evaluation protocols: Food quality or health effects. *European Food Research and Technology* 219, 561–571. DOI: 10.1007/s00217–004–1012–4

Behall K, Hallfrisch J (2002) Plasma glucose and insulin reduction after consumption of breads varying in amylose content. *European Journal of Clinical Nutrition* 56, 913–920 DOI: 10.1038/sj.ejcn.1601411

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Belanche A, Jones E, Parveen I, Newbold CJ (2016) A metagenomics approach to evaluate the impact of dietary supplementation with *Ascophyllum nodosum* or *Laminaria digitata* on rumen function in Rusitec fermenters. *Frontiers in Microbiology* 7, 1-14. DOI: 10.3389/fmicb.2016.00299

Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry 239*, 70–76. DOI: 10.1006/abio.1996.0292

Bertness M, Bruno J, Silliman B, Stachowicz J (2014) Marine community ecology and conservation. Sunderland, USA: Sinauer Associates Inc., pp 315–336.

Bhandari MR, Jong–Anurakkun N, Hong G, Kawabata J (2008) α–Glucosidase and α–amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata, Haw*.). *Food Chemistry* 106(1), 247–252. DOI: 10.1016/j.foodchem.2007.05.077

Björck I, Granfeldt Y, Liljeberg H, Tovar J, Asp, NG (1994) Food properties affecting the digestion and absorption of carbohydrates. *American Journal of Clinical Nutrition* 59, 699–705. DOI: 10.1093/ajcn/59.3.699S

Blanco–Pascual N, Montero MP, Gómez–Guillén MC (2014) Antioxidant film development from unrefined extracts of brown seaweeds *Laminaria digitata* and *Ascophyllum nodosum*. *Food Hydrocolloids* 37, 100–110. DOI: j.foodhyd.2013.10.021

Blasco AJ, Rogerio MC, Gonzalez MC, Escarpa A (2005) Electrochemical index as a screening method to determine total polyphenolics in foods: A proposal. *Analytica Chimica Acta* 539, 237–244. DOI:10.1016/J.ACA.2005.02.056

Blumenkrantz N, Asboe–Hansen G (1973) New method for quantitative determination of uronic acids. *Analytical Biochemistry* 54, 484–489. DOI: 10.1016/0003–2697(73)90377–1

Bogolitsyn K, Dobrodeeva L, Druzhinina A, Ovchinnikov D, Parshina A, Shulgina E (2019a) Biological activity of a phenolic complex of Artic brown algae. *Journal of Applied Phycology* 32, 4277–4287. DOI: 10.1007/s10811–019–01840–7

Bogolitsyn K, Druzhinina A, Kaplitsin P, Ovchinnikov D, Parshina A, Kuznetsova M (2019b**)** Relationship between radical scavenging activity and polymolecular properties of brown algae polyphenols. *Chemical Papers* 73, 2377–2385. DOI: 10.1007/s11696–019–00760–7

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Bordoloi A Goosen N (2019) Green and integrated processing approaches for the recovery of high–value compounds from brown seaweeds. *Advances in Botanical Research* DOI: 10.1016/bs.abr.2019.11.011

Bors W, Heller W, Michel C, Saran M (1990) Flavonoids as antioxidants: Determination of radical scavenging efficiencies. *Methods in Enzymology* 186, 343–355. DOI: 10.1016/0076–6879(90)86128–i

Box GEP, Behnken DW (1960) Some new three level designs for the study of quantitative variables. Technometrics 2, 455–475. DOI: 10.2307/1266454

Boyd CE, McNevin AA, Clay J, Johnson HM (2005) Certification issues for some common aquaculture species. *Reviews in Fisheries Science* 13, 231–279. DOI: 10.1080/10641260500326867

Braccini I, Grasso RP, Pérez S (1999) Conformational and configurational features of acidic polysaccharides and their interactions with calcium ions: a molecular modeling investigation. *Carbohydrate Research* 317, 119–130. DOI: 10.1016/S0008–6215(99)00062–2

Brand–Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT–Food Science and Technology* 28, 25–30. DOI: 10.1016/S0023–6438(95)80008–5

Breton F, Cérantola S, Ar Gall E (2011) Distribution and radical scavenging activity of phenols in *Ascophyllum nodosum* (Phaeophyceae). *Journal of Experimental Marine Biology and Ecology* 399, 167–172. DOI: 10.1016/j.jembe.2011.01.002

Brooker BE (1995) Chapter 2. Imaging food systems by confocal laser scanning microscopy. In New physico–chemical techniques for the characterization of complex food syStems. BlackIE Academic & Professional. Glasgow, UK. pp 53

Brunauer S, Deming L, Deming W, Teller E (1940) On a theory of the van der Waals adsorption of gases. *Journal of the American Chemical Society* 62, 1723–1732. DOI: 10.1021/ja01864a025

C

Cano A, Acosta M, Arnao M (2000) A method to measure antioxidant activity in organic media: Application to lipophilic vitamins. *Redox Report* 5, 365–370. DOI: 10.1179/135100000101535933

Catarino MD, Silva AMS, Mateus N, Cardoso SM (2019) Optimization of phlorotannins extraction from *Fucus vesiculosus* and evaluation of their potential to prevent metabolic disorders. *Marine Drugs* 17, 162–185. DOI: 10.3390/md17030162

Carstensen B, Jørgensen ME, Friis S (2014) The epidemiology of diabetes and cancer. *Current Diabetes Reports*. 14: 535. DOI: 10.1007/s11892–014–0535–8.

Castro-Alves VC, Cordenunsi BR (2015) Total soluble phenolic compounds quantification is not as simple as it seems. *Food Analytical Methods* 8(4), 873-884. DOI**:** 10.1007/s12161–014–9961–0

Cebrián–Lloret V, Metz M, Martínez–Abad A, Knutsen SH, Ballance S, López–Rubio A, Martínez–Sanz M (2022) Valorization of alginate–extracted seaweed biomass for the development of cellulose–based packaging films. *Algal Research* 61, 102576. DOI: 10.1016/j.algal.2021.102576.

Charoensiddhi S, Lorbeer AJ, Lahnstein J, Bulone V, Franco CMM, Zhang W (2016) Enzyme–assisted extraction of carbohydrates from the brown alga *Ecklonia radiata*: Effect of enzyme type, pH and buffer on sugar yield and molecular weight profiles. *Process Biochemistry* 51, 1503–1510. DOI: 10.3390/app8101866

Chee SY, Wong PK, Wong CL (2011) Extraction and characterisation of alginate from brown seaweeds (Fucales, *Phaeophyceae*) collected from Port Dickson, Peninsular Malaysia. *Journal of Applied Phycology* 23, 191–196. DOI: 10.1007/s10811–010–9533–7

Chemat F, Rombaut N, Sicaire AG Meullemiestre A, Fabiano–Tixier AS, Abert–Vian M (2017) Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics Sonochemistry* 34, 540–560. DOI: 10.1016/j.ultsonch.2016.06.035.

Chemat F, Vian MA, *Alternative Solvents for Natural Products Extraction*, Springer, 2014.

Chen S, Sathuvan M, Zhang X, Zhang W, Tang S, Liu Y, Cheong KL (2021) Characterization of polysaccharides from different species of brown seaweed using saccharide mapping and chromatographic analysis. *BMC Chemistry* 15, 1. DOI: 10.1186/s13065–2–00727–w

Chen X, Zheng Y, Shen Y (2006) Voglibose (Basen®, AO–128), one of the most important α–glucosidase inhibitors. *Current Medicinal Chemistry* 13, 109–116. DOI: 10.2174/092986706789803035

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Chenlo F, Arufe S, Díaz D, Torres MD, Sineiro J, Moreira R (2018) Air–drying and rehydration characteristics of the brown seaweeds, *Ascophyllum nodosum* and *Undaria pinnatifida*. *Journal of Applied Phycology* 30, 1259–1270. DOI: 10.1007/s10811–017–1300–6

Cho HM, Doan TD, Ha TKQ, Kim HW, Lee BW, Pham HTT, Cho TO, Oh WK (2019) Dereplication by high–performance liquid chromatography (HPLC) with quadrupole–time–of–flight mass spectroscopy (qTOF–MS) and Antiviral activities of phlorotannins from *Ecklonia cava*. *Marine Drugs* 17, 149. DOI: 10.3390/md17030149

Choi JS, Lee K, Lee BB, Kim YC, Kim YD, Hong YK, Cho KK, Choi IS (2014) Antibacterial activity of the phlorotannins dieckol and phlorofucofuroeckol–A from *Ecklonia cava* against *Propionibacterium acnes*. *Botanical Sciences* 92, 425. DOI: 10.17129/botsci.102

Chung HJ, Lim HS, Lim ST (2006) Effect of partial gelatinization and retrogradation on the enzymatic digestion of waxy rice starch. *Journal of Cereal Science* 43, 353–359. DOI:10.1016/j.jcs.2005.12.001

Cikoš AM, Jokic S, Šubaric D, Jerkovic I (2018) Overview on the application of modern methods for the extraction of bioactive compounds from marine macroalgae. *Marine Drugs* 16, 348. DOI: 10.3390/md16100348.

Cirillo G, Kraemer K, Fuessel S, Puoci F, Curcio M, Spizzirri UG, Altimari I, Iemma F (2010) Biological activity of a gallic acid–gelatin conjugate. *Biomacromolecules*, 11(12), 3309–3315. DOI: 10.1021/bm100760x.

Cíž M, Cížová H, Denev P, Kratchanova M, Slavov A, Lojek A (2010) Different methods for control and comparison of the antioxidant properties of vegetables. *Food Control* 21, 518–523.DOI: 10.1016/j.foodcont.2009.07.017

Cladiere M, Delaporte G, Le Roux E, Camel V (2018) Multi–class analysis for simultaneous determination of pesticides, mycotoxins, process–induced toxicants and packaging contaminants in tea. *Food Chemistry* 242, 113–121. DOI: 10.1016/j.foodchem.2017.08.108

Codepen. Willem Karel Dicke. https://codepen.io/misanthrope_murphy/pen/GOZJXR. Searched on 15 July 2022.

Connan S, Goulard F, Stiger V, Deslandes E, Ar Gall E (2004) Interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae. *Botanica Marina* 47, 410–416. DOI: 10.1515/BOT.2004.057

USC DE SANTIAGO DE COMPOSTELA

Corona G, Coman MM, Guo Y, Hotchkiss S, Gill C, Yaqoob P, Spencer JPE, Rowland I (2017) Effect of simulated gastrointestinal digestion and fermentation on polyphenolic content and bioactivity of brown seaweed phlorotannin–rich extracts. *Molecular Nutrition and Food Research* 61, 11–21 DOI: 10.1002/mnfr.201700223

Corona G, Ji Y, Anegboonlap P, Hotchkiss S, Gill C, Yaqoob P, Spencer JPE, Rowland I (2016) Gastrointestinal modifications and bioavailability of brown seaweed phlorotannins and effects on inflammatory markers. *British Journal of Nutrition* 115, 1240–1253. DOI: 10.1017/S0007114516000210

Cotas J, Leandro A, Monteiro P, Pacheco D, Figueirinha A, Gonçalves AMM, Pereira L (2020) Seaweed phenolics: From extraction to applications. *Marine Drugs* 18, 384. DOI: 10.3390/md18080384

Cox E, Abu–Ghannam N, Gupta S (2012) Effect of processing conditions on phytochemical constituents of edible seaweed *Himanthalia elongata*. *Journal of Food Processing and Preservation* 36, 348–363. DOI: 10.1111/j.1745–4549.2011.00563.x

Cozzi G, Gabbana E, Zanchi C, Giudici F, De Leo, L, Ziberna, F, Bramuzzo M, Di Leo G, Not T (2022) 20–Year follow–up study of celiac patients identified in a mass school screening: compliance to gluten–free diet and autoimmunity. *Journal of Pediatric Gastroenterology and Nutrition* 74 91–95. DOI: 10.1097/MPG.0000000000003295

Craigie JS (2011) Seaweed extract stimuli in plant science and agriculture. *Journal of Applied Phycology* 23, 371–393. DOI: 10.1007/s10811–010–9560–4

Cruces E, Rojas–Lillo Y, Ramirez–Kushel E, Atala E, Lopez–Alarcon C, Lissi E, Gomez I (2016) Comparison of different techniques for the preservation and extraction of phlorotannins in the kelp *Lessonia spicata* (Phaeophyceae): assays of DPPH, ORAC–PGR, and ORAC–FL as testing methods. *Journal of Applied Phycology* 28, 573–580. DOI: 10.1007/s10811–015–0602–9

Cumashi A, Ushakova NA, Preobrazhenskaya ME, D'Incecco A, Piccoli A, Totani L, Tinari N, Morozevich GE, Berman AE, Bilan MI, Usov AI, Ustyuzhanina NE, Grachev AA, Sanderson CJ, Kelly M, Rabinovich GA, Iacobelli S, Nifantiev NE (2007) A comparative study of the anti–inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology* 17, 541–552. DOI: 10.1093/glycob/cwm014

USC DE COMPOSTELA

Cummings JH, Englyst HN (1995) Gastrointestinal effects of food carbohydrate. *American Journal of Clinical Nutrition* 61, 938–945. DOI: 10.1093/ajcn/61.4.938S

D

Dall'Asta M, Del Rio D, Tappy L, Potì F, Agostoni C, Brighenti F (2020) Critical and emerging topics in dietary carbohydrates and health. *International Journal of Food Sciences and Nutrition* 71, 286–295. DOI: 10.1080/09637486.2019.1661979

Damonte E, Matulewicz M, Cerezo A (2012) Sulphated seaweed polysaccharides as antiviral agents. *Current Medicinal Chemistry* 11, 2399–2419. DOI: 10.2174/0929867043364504

Dang TT, Bowyer MC, Van Altena IA, Scarlett CJ (2017a) Optimum conditions of microwave–assisted extraction for phenolic compounds and antioxidant capacity of the brown alga *Sargassum vestitum*. Sep. Sci. Technol. 53, 1711–1723. DOI: 10.1080/01496395.2017.1414845.

Dang TT, Vuong QV, Schreider MJ, Bowyer MC, Altena IAV, Scarlet CJ (2017b) Optimization of ultrasound–assisted extraction conditions for phenolic content and antioxidant activities of the alga *Hormosira banksii* using response surface methodology. *Journal of Applied Phycology* 29, 3161–3173. DOI: 10.1007/s10811–017–1162–y

Dao TMA, Waget A, Klopp P, Serino M, Vachoux C, Pechere L, Drucker DJ, Champion S, Barthélemy S, Barra Y, Burcelin R, Sérée E (2011) Resveratrol increases glucose induced GLP–1 secretion in mice: A mechanism which contributes to the glycemic control. *PLoS One* 6, e20700. DOI: 10.1371/journal.pone.0020700

de Boer A, Urlings MJE, Bast A (2016) Active ingredients leading in health claims on functional foods. *Journal of Functional Foods* 20, 587–593. DOI: 10.1016/j.jff.2015.11.025

Deladino L, Teixeira AS, Navarro AS, Álvarez I, Molina–García AD, Martino M (2015) Corn starch systems as carriers for yerba mate (*Ilex paraguariensis*) antioxidants. *Food and Bioproducts Processing* 94, 463–472. DOI: 10.1016/j.fbp.2014.07.001

de la Hera E, Talegon M, Caballero P, Gomez M (2015) Influence of maize flour particle size on gluten–free breadmaking. *Journal of the Science of Food and Agriculture* 93, 924. DOI: 10.1002/jsfa.5826

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Dernbach E Urbich C, Brandes RP, Hofmann WK, Zeiher AM, Dimmeler S (2004) Antioxidative stress–associated genes in circulating progenitor cells: evidence for enhanced resistance against oxidative stress. *Blood* 104(12), 3591–3597. DOI: 10.1182/blood–2003–12–4103

De Souza A, Lajolo FM, Genovese MI (2010) Chemical composition and antioxidant/antidiabetic potential of Brazilian native fruits and commercial frozen pulps. *Journal of Agricultural and Food Chemistry* 58(8), 4666–4674. DOI: 10.1021/jf903875u

Devi KP, Suganthy N, Kesika P, Pandian SK. (2008) Bioprotective properties of seaweeds: *In vitro* evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. *BMC Complementary Medicine and Therapies* 8, 38. DOI: 10.1186/1472–6882–8–38

Diez–Sánchez E, Quiles A, Hernando I (2021) Interactions between blackcurrant polyphenols and food macronutrients in model systems: *In vitro* digestion studies. Foods 10, 847. DOI: 10.3390/foods10040847

Di Matteo V, Esposito E (2003) Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimers disease, Parkinsons disease, and amyotrophic lateral sclerosis. *Current Drug Targets–CNS & Neurological Disorders* 2(2), 95 – 107. DOI**:** 10.2174/1568007033482959

Dobado J, Gómez–Tamayo JG, Calvo–Flores F, Martinez H, Cardona W, Weiss–López B, Ramírez–Rodríguez O, Pessoa–Mahana H, Araya–Maturana R (2011) NMR assignment in regioisomeric hydroquinones. *Magnetic Resonance in Chemistry: MRC*, 49, 358–365. DOI: 10.1002/mrc.2745

Dodero A, Vicini S, Castellano M (2020a) Depolymerization of sodium alginate in saline solutions via ultrasonic treatments: A rheological characterization. *Food Hydrocolloids*, 109, 106–120. DOI: 10.1016/j.foodhyd.2020.106128

Dodero A, Vicini S, Alloisio M, Castellano M (2020b) Rheological properties of sodium alginate solutions in the presence of added salt: an application of Kulicke equation. *Rheological Acta*, 59, 365–374. DOI: 10.1007/s00397–020–01206–8

Dodgson KS (1961) Determination of inorganic sulphate in studies on the enzymic and non–enzymic hydrolysis of carbohydrate and other sulphate esters. *Biochemical Journal* 78, 312–317. DOI: 10.1042/bj0780312

USC DE SANTIAGO DE COMPOSTELA

Donati I, Paoletti S (2009) Material properties of alginates. In B. Rehm (Eds.), Alginates: Biology and Applications. Berlín, Alemania: Springer.

Dong D, Jiao L, Li C, Zhao C (2019) Rapid and real–time analysis of volatile compounds released from food using infrared and laser spectroscopy. *TrAC Trends in Analytical Chemistry* 110, 410–416. DOI: 10.1016/j.trac.2018.11.039

Doyon M, Labrecque J (2008) Functional foods: A conceptual definition. *British Food Journal* 110(11), 1133–1149. DOI: 10.1108/00070700810918036

Draget K, Moe S, Gudmund S, Olav S (2006) Alginates. In A. Stephen G, Phillips P Williams (Eds.), *Food Polysaccharides and Their Applications* (2nd ed.) pp. 289–334) London, United Kingdom: Taylor & Francis.

Duarte K, Justino CIL, Gomes AM, Rocha–Santos TAP, Duarte AC (2014) Green analytical methodologies for preparation of extracts and analysis of bioactive compounds. Comprehensive Analytical Chemistry 65, 59–78. DOI: 10.1016/B978–0–444–63359–0.00004–5

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28 350–356. DOI: 10.1021/ac60111a017

E

Eaggers R, Jaeger PT, Extraction systems. In Tzia C, Liadakis G (2003) Extraction optimization in food engineering, pp 95–97. Marcel Dekker, Inc, Boca Raton, USA.

El Atouani S, Bentiss F, Reani A, Zrid R, Belattmania Z, Pereira L, Mortadi A, Cherkaoui O, Sabour B (2016) The invasive brown seaweed *Sargassum muticum* as new resource for alginate in Morocco: spectroscopic and rheological characterization. *Phycological Research* 64, 185–193. DOI: 10.1111/pre.12135

Eom SH, Lee SH, Yoon NY, Jung WK, Jeon YJ, Kim SK, Leeh MS, Kima YM (2012) α–Glucosidase– and α–amylase–inhibitory activities of phlorotannins from *Eisenia bicyclis*. *Journal of the Science of Food and Agriculture* 92, 2084–2090. DOI: 10.1002/jsfa.5585

Ertani A, Francioso O, Tinti A, Schiavon M, Pizzeghello D, Nardi S (2018) Evaluation of seaweed extracts from *Laminaria* and *Ascophyllum nodosum spp.* As biostimulants in *Zea mays* L. using a combination of chemical, biochemical and morphological approaches. *Frontiers in Plant Science* 9, 428–441. DOI: 10.3389/fpls.2018.00428

Ertesvåg H, Høidal HK, Skjåk–Bræk G, Valla S (1998) The *Azotobacter vinelandii* mannuronan C–5–epimerase AlgE1 consists of two separate catalytic domains. *Journal of Biological Chemistry* 273, 30927–30932. DOI: 10.1074/jbc.273.47.30927

Evans FD, Critchley AT (2014) Seaweeds for animal production use. *Journal of Applied* Phycology 26(2), 891-899. DOI: 10.1007/s10811–013–0162–9

Everette JD, Bryant QM, Green AM, Abbey YA, Wangila GW, Walker RB (2010) Thorough study of reactivity of various compound classes toward the Folin–Ciocalteu reagent. *Journal of Agricultural and Food Chemistry* 58(14), 8139-8144. DOI: 10.1021/jf1005935

F

Fang Z, Bhandari B (2010) Encapsulation of polyphenols – a review. *Trends in Food Science & Technology* 21(10), 510–523. DOI: 10.1016/j.tifs.2010.08.003

FAO (2018) The global status of seaweed production, trade, and utilization. Globefish Research Programme 124, 120–132.

Ferdouse F, Holdt SL, Smith R, Murúa P, Yang Z (2018) The global status of seaweed production, trade and utilization. Globefish Research Programme, Vol. 124, FAO, Rome

Ferdous UT, Yusof ZNB (2021) Medicinal prospects of antioxidants from algal sources in cancer therapy. Frontiers in Pharmacology 12, 593116. DOI: 10.3389/fphar.2021.593116

Firdaus M, Prihanto AA (2014) α–Amylase and α–glucosidase inhibition by brown seaweed (*Sargassum sp*) extracts. *Research Journal of Life Science* 1(1), 6–11. DOI: 10.21776/ub.rjls.2014.001.01.2

Flores FP, Singh RK, Kerr WL, Pegg, RB, Kong F (2013) Antioxidant and enzyme inhibitory activities of blueberry anthocyanins prepared using different solvents. *Journal of Agricultural and Food Chemistry* 61, 4441–4447. DOI: 10.1021/jf400429f

Flores–Fernandez N, Gonzalez Munoz MJ (2017) Ultrasound–assisted extraction of bioactive carbohydrates. In Water extraction of Bioactive Compounds; Dominguez–Gonzalez H, Gonzalez–Munoz MJ, Eds. Elsevier: Amsterdam, The Netherlands, pp. 317–331.

Ford L, Theodoridou K, Sheldrake GN, Walsh PJ (2019) A critical review of analytical methods used for the chemical characterisation and quantification of phlorotannin compounds in brown seaweeds. *Phytochemical Analysis* 1–13. DOI: 10.1002/pca.2851

USC UNIVERSIDADE DE SANTIAGO

Foti MC (2015) Use and abuse of the DPPH. Radical. *Journal of Agricultural and Food Chemistry* 63, 8765–8776. DOI: 10.1021/acs.jafc.5b03839

Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Kido M, Mori H, Nakayama Y, Takahashi M (1989) Structure of an antiplasmin inhibitor, eckol, isolated from the brown algae *Ecklonia kurome* OKAMURA and inhibitory activities of its derivatives on plasma–plasmin inhibitor. *Chemical and Pharmaceutical Bulletin* 37, 349–53. DOI: 10.1248/cpb.37.349

Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Mori H, Nakayama Y, Takahashi M (1990) Anti–plasmin inhibitor. VI.: Structure of phlorofucofuroeckol A, a novel phlorotannin with both dibenzo–1, 4–dioxin and dibenzofuran elements, from *Ecklonia kurome* OKAMURA. *Chemical and Pharmaceutical Bulletin* (Tokyo), 38(1), 133–135. DOI: 10.1248/cpb.38.133

G

Gager L, Connan S, Molla M, Couteau C, Arbona JF, Coiffard L, Cérantola S, Stiger–Pouvreau V (2020) Active phlorotannins from seven brown seaweeds commercially harvested in Brittany (France) detected by ^{1}H–NMR and *in vitro* assays: temporal variation and potential valorization in cosmetic applications. *Journal of Applied Phycology* 32, 2375–2386. DOI: 10.1007/s10811–019–02022–1

Gallagher E, Gormley T, Arendt E (2004) Recent advances in the formulation of gluten–free cereal–based products. *Trends in Food Science & Technology* 15, 143–152. DOI: 10.1016/j.tifs.2003.09.012.

Gambus H, Nowotn A, Ziobro R, Gumul D, Sikora M (2001) The effect of use of guar gum with pectin mixture in gluten–free bread. *Electronic Journal of Polish Agricultural Universities* 4, 9.

Garcia–Pichel F, Belnap J (2021) 7–Cyanobacteria and algae. In Gentry TJ, Fuhrmann JJ, Zuberer DA, Principles and Applications of Soil Microbiology (3rd Edition), pp. 171–189. DOI: 10.1016/B978–0–12–820202–9.00007–1.

Garcia–Vaquero M, Rajauria G, Tiwari B, Sweeney T, O'Doherty J (2018) Extraction and yield optimisation of fucose, glucans and associated antioxidant activities from *Laminaria digitata* by applying response surface methodology to high intensity ultrasound–assisted extraction. *Marine Drugs* 16, 257. DOI: 10.3390/md16080257.

Gauer JS, Tumova S, Lippiat JD, Kerimi A, Williamson G (2018) Differential patterns of inhibition of the sugar transporters GLUT2, GLUT5 and GLUT7 by flavonoids. *Biochemical Pharmacology* 152, 11–20. DOI: 10.1016/j.bcp.2018.03.011

GBIF. *Ascophyllum nodosum* (Linnaeus) Le Jolis global distribution. https://www.gbif.org/species/3196524. Searched on 19th September 2022.

Gimpel JA, Henriquez V Mayfield SP (2015a) In metabolic engineering of eukaryotic microalgae: potential and challenges come with great diversity. *Frontiers in Microbiology* 6, 1376. DOI: 10.3389/fmicb.2015.01376

Gimpel JA, Hyun JS, Schoep NG, Mayfield SP (2015b) Production of recombinant proteins in microalgae at pilot greenhouse scale. *Biotechnology and Bioengineering* 112(2), 339–345. DOI: 10.1002/bit.25357

Giuberti G, Rocchetti G, Lucini L (2020) Interactions between phenolic compounds, amylolytic enzymes and starch: an updated overview. *Current Opinion in Food Science* 31, 102–113. DOI: 10.1016/j.cofs.2020.04.003

Glombitza KW (1979) Antibiotics from algae. *Marine Algae in Pharmaceutical Science* 28, 303-342. DOI: 10.1515/9783110882049.303

Glombitza KW, Keusgen M, Hauperich S (1997) Fucophlorethols from the brown algae *Sargassum spinuligerum* and *Cystophora torulosa*. *Phytochemistry* 46, 1417–1422. DOI: 10.1016/S0031–9422(97)00499–8

Glombitza, KW, Rösener HU (1974) Bifuhalol: Ein diphenyläther aus *Bifurcaria bifurcata*. *Phytochemistry* 13, 1245–1247. DOI: 10.1016/0031–9422(74)80110–X

Glombitza KW, Rösener HU, Müller D (1975) Bifuhalol and diphlorethol of *Cystoseira tamariscifolia*. *Phytochemistry* 14, 1115–1116. DOI: 10.1016/0031–9422(75)85198–3

Glombitza KW, Rösener HU, Vilter H, Rauwald W (1973) Antibiotics from algae. Phloroglucinol from Phaeophyceae. *Planta Medica* 24, 301–303. DOI: 10.1055/s–0028–1099502

Glombitza KW, Schmidt A (1999a) Trihydroxyphlorethols from the brown alga *Carpophyllum angustifolium*. *Phytochemistry* 51(8), 1095-1100. DOI: 10.1016/S0031–9422(99)120–X

Glombitza KW, Schmidt A (1999b) Nonhalogenated and halogenated phlorotannins from the brown alga *Carpophyllum angustifolium*. *Journal of Natural Products* 62(9), 1238-1240. DOI: 10.1021/np990076c

USC DE SANTIAGO DE COMPOSTELA

Glombitza KW, Vogels HP (1985) Antibiotics from algae. XXXV. Phlorotannins from Ecklonia maxima. *Planta Medica* 51(4), 308-312. DOI: 10.1055/s–2007–969498

Gómez–Mascaraque LG, Martínez–Sanz M, Martínez–López R, Martínez–Abad A, Panikuttira B, López–Rubio A, Tuohy MG, Hogan SA, Brodkorb A (2021) Characterization and gelling properties of a bioactive extract from *Ascophyllum nodosum* obtained using a chemical–free approach. *Current Research in Food Science* 4, 354–364. DOI: 10.1016/j.crfs.2021.05.005

Gómez–Ordóñez E, Rupérez P (2011) FTIR–ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. *Food Hydrocolloids*, 25, 1514–1520. DOI: 10.1016/j.foodhyd.2011.02.009

Gonçalves–Fernández C, Sineiro J, Moreira R, Gualillo O (2019) Extraction and characterization of phlorotannin–enriched fractions from the Atlantic seaweed *Bifurcaria bifurcata* and evaluation of their cytotoxic activity in murine cell line. *Journal of Applied Phycology* 31, 2573–2583. DOI: 10.1007/s10811–018–1729–2

Goswami SK, Gangadarappa SK, Vishwanath M, Razdan R, Jamwal R, Bhadri N, Inamdar MN (2016) Antioxidant potential and ability of phloroglucinol to decrease formation of advanced glycation end products increase efficacy of sildenafil in diabetes–induced sexual dysfunction of rats. *The Journal of Sexual Medicine* 4, 106–114. DOI: 10.1016/j.esxm.2015.12.002

Gotama TL, Husni A, Ustadi (2018) Antidiabetic activity of *Sargassum hystrix* extracts in streptozotocin–induced diabetic rats. *Preventive Nutrition and Food Science* 23, 189–195. DOI: 10.3746/pnf.2018.23.3.189

Govan JR, Fyfe JA, Jarman TR (1981) Isolation of alginate–producing mutants of *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas mendocina*. *Journal of General Microbiology*, 125, 217–220. DOI: 10.1099/00221287–125–1–217

Graham LE, Graham J, Wilcox LW (2009) Introduction to algae. In Algae, 2nd edition. Pearson: San Francisco, USA. pp. 1–5.

Grasdalen H (1983) High–field, 1 H–n.m.r . spectroscopy of alginate: sequential structure and linkage conformations. *Carbohydrate Research* 118, 255–260. DOI: 10.1016/0008–6215(83)88053–7.

Grasdalen H, Larsen B, Smidsrød O (1977) ^{13}C–N.m.r studies of alginate. *Carbohydrate Research* 56, C11–C15. DOI: 10.1016/S0008–6215(00)83369–8

USC DE SANTIAGO DE COMPOSTELA

Grasdalen H, Larsen B, Smidsrod O (1979) A P.M.R. study of the composition and sequence of uronate residues in alginates. *Carbohydrate Research* 68, 23–31. DOI: 10.1016/S0008–6215(00)84051–3

Grasdalen H, Larsen B, Smidsrod O (1981) ^{13}C–N.M.R. studies of monomeric composition and sequence in alginate. *Carbohydrate Research* 89, 179–191. DOI: 10.1016/0008–6215(83)88053–7

Guillarme D, Schappler J, Rudaz S, Veuthey JL (2010) Coupling ultra–high–pressure liquid chromatography with mass spectrometry. *TrAC Trends in Analytical Chemistry*, 29, 15–27. DOI. 10.1016/j.trac.2009.09.008

Gunathilaka TL, Samarakoon K, Ranasinghe P, Peiris LD (2020) Antidiabetic potential of marine brown algae–a mini review. *Journal of Diabetes Research* 1230218. DOI: 10.1155/2020/1230218

Gunathilake T, Akanbi TO, Suleria HAR, Nalder TD, Francis DS, Barrow CJ (2022) Seaweed phenolics as natural antioxidants, aquafeed additives, veterinary treatments and cross–linkers for microencapsulation. *Marine Drugs* 20, 445–489. DOI: 10.3390/md20070445

Guo Y, Qiao D, Zhao S, Zhang B, Xie F (2021**)** Starch–based materials encapsulating food ingredients: Recent advances in fabrication methods and applications. *Carbohydrate Polymers* 270, 118358. DOI: 10.1016/j.carbpol.2021.118358

H

Hahn T, Lang S, Ulber R, Muffler K (2012) Novel procedures for the extraction of fucoidan from brown algae. *Process Biochemistry* 47, 1691–1698. DOI: 10.1016/j.procbio.2012.06.016

Hall AC, Fairclough AC, Mahadevan K, Paxman JR (2012) *Ascophyllum nodosum* enriched bread reduces subsequent energy intake with no effect on post–prandial glucose and cholesterol in healthy, overweight males. A pilot study. *Appetite* 58, 379–386. DOI: 10.1016/j.appet.2011.11.002

Halliwell B (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *The Lancet* 344, 721–724. DOI: 10.1016/S0140–6736(94)92211–X

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine (4th eds). pp 19, 269–283 and 451–452. Oxford University Press Inc., New York, USA.

Halliwell B, Gutteridge JMC, Cross CE (1992) Free radicals, antioxidants, and human disease: where are we now. *Journal of Laboratory and Clinical Medicine* 119(6), 598–620.

Halsey G (1948) Physical adsorption on non–uniform surfaces. *Journal of Chemical Physics* 16, 931–937. DOI: 10.1063/1.1746689

Ham YM, Baik JS, Hyun JW, Lee NH (2007) Isolation of a new phlorotannins, fucodiphlorethol G, from a brown alga *Ecklonia cava*. *Bulletin of the Korean Chemical Society* 28, 1595–1597. DOI: 10.5012/bkcs.2007.28.9.1595

Hancock JT, Desikan R, Neill SJ (2001) Role of reactive oxygen species in cell signaling pathways. *Biochemical and Biomedical Aspects of Oxidative Modification* 29(2), 345–350. DOI: 10.1042/0300–5127:0290345

Harnedy PA, FitzGerald RJ (2013) Extraction of protein from the macroalga *Palmaria palmata*. *LWT–Food Science and Technology* 51, 375–382. DOI: 10.1016/j.lwt.2012.09.023

Heffernan N, Brunton NP, FitzGerald RJ, Smyth TJ (2015) Profiling of the molecular weight and structural isomer abundance of macroalgae– derived phlorotannins. *Marine Drugs* 13, 509–528. DOI:10.3390/md13010509

Heo SJ, Hwang JY, Choi JI, Han JS, Kim HJ, Jeon YJ (2009) Diphlorethohydroxycarmalol isolated from *Ishige okamurae*, a brown algae, a potent α–glucosidase and α–amylase inhibitor, alleviates postprandial hyperglycemia in diabetic mice. *European Journal of Pharmacology* 615, 252–256. DOI: 10.1016/j.ejphar.2009.05.017

Hermund DB, Plaza M, Turner C, Jonsdottir R, Kristinsson HG, Jacobsen C, Nielsen KF (2018) Structure dependent antioxidant capacity of phlorotannins from Icelandic Fucus vesiculosus by UHPLCDAD–ECD–QTOFMS. *Food Chemistry* 240, 904–909. DOI: 10.1016/j.foodchem.2017.08.032

Hernández–Carmona G, McHugh DJ, Arvizu–Higuera DL, Rodríguez–Montesinos YE (2002) Pilot plant scale extraction of alginates from *Macrocystis pyrifera* 4. Conversion of alginic acid to sodium alginate, drying and milling. *Journal of Applied Phycology* 14, 445–451. DOI: 10.1023/A:1022372807813

Herrero M, Ibáñez E (2015) Green processes and sustainability: An overview on the extraction of high added–value products from seaweeds and microalgae. *Journal of Supercritical Fluids* 96, 211–216. DOI: 10.1016/j.supflu.2014.09.006

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Hesse M, Meier H, Zeeh B **(**2005**)** Infrared spectroscopy and Raman. In Spektroskopiche Methoden in der organischen Chemie. Georg Thieme Verlag Stuttgart: New york, USA, 44–52.

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology* 23, 543–597. DOI: 10.1007/s10811–010–9632–5.

Houchi S, Mahdadi R, Khenchouche A, Song J, Zhang, W, Pang X, Zhang L, Sandalli C, Du G (2019) Investigation of common chemical components and inhibitory effect on GES–type β–lactamase (GES22) in methanolic extracts of Algerian seaweeds. *Microbial Pathogenesis* 126, 56–62. DOI: 10.1016/j.micpath.2018.10.034

Huang Q, Liu X, Zhao G, Hu T, Wang Y (2018) Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Animal Nutrition* 4(2), 137-150. DOI: 10.1016/j.aninu.2017.09.004

Hutzler P, Fischbach R, Heller W, Jungblut TP, Reuber S Schmitz R, Veit M, Weissenböck G, Schnitzler JP (1998) Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany* 49(323), 953–965. DOI: 10.1093/jxb/49.323.953

I

Ignat I, Volf I, Popa, VI (2011) A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry* 126, 1821–1835. DOI: 10.1016/j.foodchem.2010.12.026

Igoumenidis PE, Zoumpoulakis P, Karathanos VT **(**2018) Physicochemical interactions between rice starch and caffeic acid during boiling. *International Food Research Journal* 109, 589–595. DOI: 10.1016/j.foodres.2018.04.062

Ingram N, Wright TA, Ingoldby CJH (1998) A prospective randomized study of calcium alginate (Sorbsan) versus standard gauze packing following haemorrhoidectomy. *Journal of the Royal College of Surgeons of Edinburgh*, 43, 308–309.

Iwai K. (2008) Antidiabetic and antioxidant effects of polyphenols in brown alga Ecklonia stolonifera in genetically diabetic KK–Ay mice. *Plant Foods for Human Nutrition* 63, 163–169. DOI: 10.1007/s11130–008–0098–4

USC UNIVERSIDADE SANTIAGO

J

Jacobsen C, Sørensen ADM, Holdt SL, Akoh CC, Hermund DB (2019) Source, extraction, characterization, and applications of novel antioxidants from seaweed. Annual Review of Food Science and Technology 10(26), 1–28. DOI: 10.1146/annurev–food–032818–121401

Jayas DS (2016) Food dehydration. *Reference Module in Food Science*. DOI: 10.1016/B978–0–08–100596–5.02913–9

Jégou C, Culioli G, Kervarec N, Simon G, Stiger-Pouvreau V (2010) LC/ESIMSn and 1H HR-MAS NMR analytical methods as useful taxonomical tools within the genus *Cystoseira C. Agardh* (Fucales; Phaeophyceae) *Talanta* 83(2), 613-622. DOI: 10.1016/j.talanta.2010.10.003

Jégou C, Kervarec N, Cérantola S, Bihannic I, Stiger-Pouvreau V (2015) NMR use to quantify phlorotannins: the case of *Cystoseira tamariscifolia*, a phloroglucinol-producing brown macroalga in Brittany (France). *Talanta* 135, 1-6. DOI: 10.1016/j.talanta.2014.11.059

Jensen A (1993) Present and future needs for algae and algal products. In: Chapman ARO, Brown MT, Lahaye M (eds) Fourteenth International Seaweed Symposium. Developments in Hydrobiology, vol 85. Springer, Dordrecht.

Jeon SY, Oh S, Kim E, Imm JY (2013) α–Glucosidase inhibition and antiglycation activity of laccase–catalyzed catechin polymers. *Journal of Agricultural and Food Chemistry* 61(19), 4577–4584. DOI: 10.1021/jf400791r

Jiménez–Escrig A, Jiménez–Jiménez I, Pulido R, Saura–Calixto F (2001) Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture* 81, 530–534. DOI: 10.1002/jsfa.842

Johnston K, Sharp P, Clifford M, Morgan L (2005) Dietary polyphenols decrease glucose uptake by human intestinal Caco–2 cells. *FEBS Letters* 579, 1653–1657. DOI: 10.1016/j.febslet.2004.12.099

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Johnston KL, Clifford MN, Morgan LM. (2003) Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: Glycemic effects of chlorogenic acid and caffeine. *The American Journal of Clinical Nutrition* 78, 728–733. DOI: 10.1093/ajcn/78.4.728

Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG (1994) Flavonoids as antioxidanbts. *Journal of the American Chemical Society* 116, 4846 – 4851. DOI: 10.1021/ja00090a032

Jung HA, Yoon NY, Woo MH, Choi JS (2008) Inhibitory activities of extract from several kinds of seaweeds and phlorotannins from brown alga *Ecklonia stolonifera* on glucose–mediated protein damage and rat lens aldose reductase. *Fisheries Science* 74, 1363–1365. DOI: 10.1111/j.1444–2906.2008.01670.x

Júnior, SQ, Carneiro VHA, Fontenelle TPC, de Sousa LC, Mesquita JX, de Brito, TV, Prudêncio RS, de Oliveira JS, Rolim Medeiros JV, Aragão KS, Ribeiro RA, Barbosa ALR, Freitas ALP (2015) Antioxidant and anti–inflammatory activities of methanol extract and its fractions from the brown seaweed *Spatoglossum schroederi*. *Journal of Applied Phycology* 27, 2367–2376. DOI: 10.1007/s10811–014–0497–x

K

Kabbani TA, Goldberg A, Kelly CP, Pallav K, Tariq S, Peer A, Hansen J, Dennis M, Leffler DA (2012) Body mass index and the risk of obesity in coeliac disease treated with the gluten–free diet. Alimentary Pharmacology and Therapeutics 35(6), 726–729. DOI: 10.1111/j.1365–2036.2012.05001.x

Kadam SU, Álvarez C, Tiwari BK, O'Donnell CP (2017) Extraction and characterization of protein from Irish brown seaweed *Ascophyllum nodosum*. *Food Research International* 99, 1021–1027. DOI: 10.1016/j.foodres.2016.07.018

Kadam SU, O'Donnell CP, Rai DK, Hossain MB, Burgess CM, Walsh D, Tiwari BK (2015a) Laminarin from Irish brown seaweeds *Ascophyllum nodosum* and *Laminaria hyperborea*: Ultrasound–assisted extraction, characterisation, and bioactivity. *Marine Drugs* 13, 4270–4280. DOI: 10.3390/md13074270

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Kadam SU, Tiwari BK, O'Donnell S, O'Donnell CP (2015b) Effect of ultrasound pretreatment on the extraction kinetics of bioactives from brown seaweed (*Ascophyllum nodosum*). *Separation Science and Technology* 50, 670–675. DOI: 10.1080/01496395.2014.960050

Kadam SU, Tiwari BK, O'Donnell CP (2013) Application of novel extraction technologies for bioactives from marine algae. J*ournal of Agricultural and Food Chemistry* 61, 4667–4675. DOI: 10.1021/jf400819p

Kadam SU, Tiwari BK, Smyth TJ, O'Donnell CP (2015c) Optimization of ultrasound assisted extraction of bioactive components from brown seaweed *Ascophyllum nodosum* using surface methodology. *Ultrasonics Sonochemistry* 23, 308. DOI: 316. 10.1016/j.ultsonch.2014.10.007

Kan L, Capuano E, Fogliano V, Oliviero T, Verkerk R (2020) Tea polyphenols as a strategy to control starch digestion in bread: The effects of polyphenol type and gluten. *Food & Function* 11(7), 5933–5943. DOI: 10.1039/D0FO01145B

Kang KA, Lee KH, Chae SW, Koh YS, Yoo BS, Kim JH, Ham YM, Baik JS, Lee NH, Hyun JW (2005) Triphlorethol–A from Ecklonia cava protects V79–4 lung fibroblast against hydrogen peroxide induced cell damage. *Free Radical Research* 39, 883–892. DOI: 10.1080/10715760500161165

Kang SM, Heo SJ, Kim KN, Lee KN, Jeon YJ (2012) Isolation and identification of new compound, 2,700–phloroglucinol–6,60–bieckol from brown algae, *Ecklonia cava* and its antioxidant effect. *Journal of Functional Foods* 4, 158–166. DOI: 10.1016/j.jff.2011.10.001

Karimi–Lotfabad S, Gray MR (2000) Characterization of contaminated soils using confocal laser scanning microscopy and cryogenic–scanning electron microscopy. *Environmental Science & Technology* 34, 16, 3408–3414. DOI: 10.1021/es991026x

Kawamura–Konishi Y, Watanabe N, Saito M, Nakajima N, Sakaki T, Katayama T, Enomoto T (2012) Isolation of a new phlorotannin, a potent inhibitor of carbohydrate–hydrolyzing enzymes, from the brown alga *Sargassum patens*. *Journal of Agricultural and Food Chemistry* 60, 5565–5570. DOI: 10.1021/jf300165j

Kellogg J, Grace MH, Lila MA (2014) Phlorotannins from Alaskan seaweed inhibit carbolytic enzyme activity. *Marine Drugs* 12, 5277–5294. DOI: 10.3390/md12105277

USC DE SANTIAGO DE COMPOSTELA

Kerrison PD, Stanley MS, Edwards MD, Black KD, Hughes AD (2015) The cultivation of European kelp for bioenergy: site and species selection. *Biomass Bioenergy* 80, 229-242. DOI: 10.1016/j.biombioe.2015.04.035

Ketheesan B, Nirmalakhandan N (2011) Development of a new airlift–driven raceway reacator for algal cultivation. *Applied Energy* 88, 3370–3376. DOI: 10.1016/j.apenergy.2010.12.034

Khan B, Ullah A, Khan MA, Amin A, Iqbal M, Khan S, Ateeq M, Aman K, Aziz A, Khattak MNK, Nadeem T, Munir N, Khan S, Ali Q (2024) Anti–hyperglycemic and anti–hyperlipidemic effects of a methanolic extract of *Debregeasia salicifolia* in Alloxan–induced diabetic albino mice. *Brazilian Journal of Biology* 84, e251046. DOI: 10.1590/1519–6984.251046

Kilinç B, Cirik S, Turan G, Tekogul H, Koru E (2013) Seaweeds for Food and Industrial Applications. In: Muzzalupo, I. editor. Food Industry. London: IntechOpen. DOI: 10.5772/53172

Kim SM, Kang SW, Jeon JS, Jung YJ, Kim WR, Kim CY, Um BH (2013) Determination of major phlorotannins in Eisenia bicyclis using hydrophilic interaction chromatography: Seasonal variation and extraction characteristics. *Food Chemistry* 138, 2399–2406 DOI: 10.1016/j.foodchem.2012.11.057

Kim JH, Lee S, Park S, Park JS, Kim YH, Yang SY (2019) Slow–binding inhibition of tyrosinase by *Ecklonia cava* phlorotannins. *Marine Drugs* 17, 359. DOI: 1660–3397/17/6/359.

Kim KY, Nguyen TH, Kurihara H, Kim SM (2010) Alpha–glucosidase inhibitory activity of bromophenol purified from the red alga *Polyopeslancifolia*. *Journal of Food Science* 75, 145–150. DOI: 10.1111/j.1750–3841.2010.01629.x

Kim KT, Rioux LE, Turgeon SL (2014) Alpha–amylase and alpha–glucosidase inhibition is differentially modulated by fucoidan obtained from *Fucus vesiculosus* and *Ascophyllum nodosum*. *Phytochemistry* 98, 27–33. DOI: 10.1016/j.phytochem.2013.12.003.

Kim SK, Wijesekara I (2010) Development and biological activities of marine–derived bioactive peptides: A review. *Journal of Functional Foods* 2, 1–9. DOI. 10.1016/j.jff.2010.01.003

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Kirke DA, Smyth TJ, Rai DK, Kenny O, Stengel DB (2017) The chemical and antioxidant stability of isolated low molecular weight phlorotannins. *Food Chemistry* 221, 1104–1112. DOI: 10.1016/j.foodchem.2016.11.050.

Kobayashi Y, Suzuki M, Satsu H, Arai S, Hara Y, Suzuki K, Miyamoto Y, Shimizu M (2000) Green tea polyphenols inhibit the sodium–dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. *Journal of Agricultural and Food Chemistry* 48, 5618–5623. DOI: 10.1021/jf0006832

Koh LW, Wong LL, Loo YY, Kasapis S, Huang D (2010) Evaluation of different teas against starch digestibility by mammalian glycosidases. *Journal of Agricultural and Food Chemistry* 58(1), 148–154. DOI: 10.1021/jf903011g

Koivikko R, Eränen JK, Loponen J, Jormalainen V (2008) Variation of phlorotannins among three populations of *Fucus vesiculosus* as revealed by HPLC and colorimetric quantification. *Journal of Chemical Ecology* 35, 57-64. DOI: 10.1007/s10886–007–9410–2

Koivikko R Loponen J, Honkanen T, Jormalainen V (2005) Contents of soluble, cell–wall–bound, and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions, *Journal of Chemical Ecology* 31 195–212. DOI: 10.1007/s10886–005–0984–2.

Koivikko R, Loponen J, Honkanen T, Jormalainen V (2007) High–performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. *Phytochemical Analysis* 18, 326–332. DOI: 10.1002/pca.986.

Konermann L, Ahadi E, Rodriguez AD, Vahidi S (2013) Unraveling the mechanism of electrospray ionization. *Analytical Chemistry* 85, 2–9. DOI: 10.1021/ac302789c

Koysuren B, Oztop MH, Mazi BG (2021) Sesame seed as an alternative plant protein source: a comprehensive physicochemical characterisation study for alkaline, salt and enzyme–assisted extracted samples. *International Journal of Food Science* DOI: 10.1111/ijfs.15229

Krentz AJ, Bailey CJ (2005) Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* 65, 385–411. DOI: 10.2165/00003495–200565030–00005

Kubanek J, Lester SE, Fenical W, Hay ME (2004**)** Ambiguous role of phlorotannins as chemical defenses in the brown alga *Fucus vesiculosus. Marine Ecology Progress Series* 277, 79–93. DOI: 10.3354/meps277079

USC DE SANTIAGO DE COMPOSTELA

Kumar LRG, Paul PT, Anas KK, Tejpal CS, Chatterjee1 NS, Anupama TK, Mathew S, Ravishankar CN (2022) Phlorotannins–bioactivity and extraction perspectives. *Journal of Applied Phycology* 34, 2173–2185. DOI: 10.1007/s10811–022–02749–4

Kumar Y, Singhal S, Tarafdar A, Pharande A, Ganesan M, Badgujar PC (2020) Ultrasound assisted extraction of selected edible macroalgae: Effect on antioxidant activity and quantitative assessment of polyphenols by liquid chromatography with tandem mass spectrometry (LC–MS/MS). *Algal Research* 52, 102114. DOI: 10.1016/j.algal.2020.102114

Kwon YI, Apostolidis E, Shetty K (2008) *In vitro* studies of eggplant (*Solanum melongena*) phenolics as inhibitors of key enzymes relevant for type 2 diabetes and hypertension. *Bioresource Technology* 99, 2981–2988. DOI: 10.1016/j.biortech.2007.06.035

L

Lawton RJ (2017) The industrial ecology of freshwater macroalga for biomass appliactions. *Algal research* 24, 486–491. DOI: 10.1016/j.algal.2016.08.019

Lazaridou A, Duta D, Papageorgiou M, Belc N, Biliaderis C (2007) Effects of hydrocolloids on dough rheology and bread quality parameters in gluten–free formulations. *Journal of Food Engineering* 79, 1033–1047. DOI: 10.1016/j.jfoodeng.2006.03.032

Le Bleis F, Chaunier L, Chiron H, Della Valle G, Saulnier L (2015) Rheological properties of wheat our dough and french bread enriched with wheat bran. *Journal of Cereal Science* 65, 167–174. DOI: 10.1016/j.jcs.2015.06.014

Le Bourvellec C, Renard CMGC (2012) Interactions between Polyphenols and macromolecules: quantification methods and mechanisms. *Critical Reviews in Food Science and Nutrition* 52(3), 213. DOI: 10.1080/10408398.2010.499808

Lebovitz HE (1997) a–Glucosidase inhibitors. *Endocrinology and Metabolism Clinics* 26, 539–551. DOI: 10.1016/s0889–8529(05)70266–8

Le Lann K, Jégou C, Stiger–Pouvreau V (2008) Effect of different conditioning treatments on total phenolic content and antioxidant activities in two *Sargassacean* species: Comparison of the frondose *Sargassum muticum* (yendo) fensholt and the cylindrical *Bifurcaria bifurcata R. Ross*. *Phycological Research* 56, 238–245. DOI: 10.1111/j.1440–1835.2008.00505.x

Leandro A, Pacheco D, Cotas J, Marques JC, Pereira L, Gonçalves AM (2020) Seaweed's bioactive candidate compounds to food industry and global food security. *Life* 10, 140. DOI: 10.3390/life10080140

Lee KT, Farid M, Nguang SK (2006) The mathematical modelling of the rehydration characteristics of fruits. *Journal of Food Engineering* 72, 16–23. DOI: 10.1016/j.jfoodeng.2004.11.014

Lee S, Jeon Y (2013) Anti–diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. *Fitoterapia* 86, 129–136. DOI: 10.1016/j.fitote.2013.02.013

Lee SH, Kang SM, Ko SC, Moon SH, Jeon BT, Lee DH, Jeon YJ (2014) Octaphlorethol A: a potent a–glucosidase inhibitor isolated from *Ishige foliacea* shows an antihyperglycemic effect in mice with streptozotocin–induced diabetes. *Food & Function* 5, 2602–2609. DOI: 10.1039/c4fo00420e

Lee SH, Li Y, Karadeniz F, Kim MM, Kima SK (2009) α–Glucosidase and α–amylase inhibitory activities of phloroglucinol derivatives from edible marine brown alga, *Ecklonia cava*. *Journal of the Science of Food and Agriculture* 89, 1552–1558. DOI: 10.1002/jsfa.3623

Lee SH, Park MH, Heo SJ, Kang SM, Ko SC, Han JS, Jeon YJ (2010) Dieckol isolated from *Ecklonia cava* inhibits α–glucosidase and α–amylase *in vitro* and alleviates postprandial hyperglycemia in streptozotocin–induced diabetic mice. *Food and Chemical Toxicology* 48, 2633–2637. DOI: 10.1016/j.fct.2010.06.032

Lee JM, Yang EC, Graf L, Yang JH, Qiu H, Zelzion U, Chan CX, Stephens TG, Weber APM, Boo GH, Boo SM, Kim KM, Shin Y, Jung M, Lee SJ, Yim HS, Lee JS, Bhattacharya D, Yoon HS (2018) Analysis of the draft genome of the red seaweed G*racilariopsis chorda* provides insights into genome size evolution in rhodophyta. *Molecular Biology and Evolution* 35, 1869–1886, DOI: 10.1093/molbev/msy081

Lemanska K, Szymusiak H, Tyrakowska B, Zielinski R, Soffers AE, Rietjens IM (2001) The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Radical Biology and Medicine* 31, 869–881. DOI: 10.1016/s0891–5849(01)00638–4

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Leyton A, Pezoa–Conte P, Barriga A, Buschmann AH, Maki–Arvela P, Mikkola JP, Lienqueo ME (2016) Identification and efficient extraction method of phlorotannins from the brown seaweed *Macrocystis pyrifera* using an orthogonal experimental design. *Algal Research* 16, 201–208. DOI: 10.1016/j.algal.2016.03.019.

Li Y, Fu X, Duan D, Liu X, Xu J, Gao X (2017) Extraction and identification of phlorotannins from the brown alga, *Sargassum fusiforme* (Harvey) Setchell. *Marine Drugs* 15, 49–64. DOI: 10.3390/md15020049.

Li Y, Qian ZJ, Ryu BM, Lee SH, Kim MM, Kim SK (2009a) Chemical components and its antioxidant properties *in vitro*: An edible marine brown alga, *Ecklonia cava*. *Bioorganic & Medicinal Chemistry* 17, 1963–1973. 10.1016/j.bmc.2009.01.031

Li H, Song F, Xing J, Tsao R, Liu Z, Liu S (2009b) Screening and structural characterization of α–glucosidase inhibitors from hawthorn leaf flavonoids extract by ultrafiltration LC–DAD–MS^n and SORI–CID FTICR–MS. *Journal of the American Society for Mass Spectrometry* 20(8), 1496–1503. DOI: 10.1016/j.jasms.2009.04.003.

Li YX, Wijesekara I, Li Y, Kim SK (2011) Phlorotannins as bioactive agents from brown algae. *Process Biochemistry* 46, 2219–2224. DOI: 10.1016/j.procbio.2011.09.015.

Lim SN, Cheung PCK, Ooi VEC, Ang PO (2002) Evaluation of antioxidative activity of extracts from a brown seaweed, *Sargassum siliquastrum*. *Journal of Agricultural and Food Chemistry* 50, 3862–3866. DOI: 10.1021/jf020096b

Liu Y, Chen L, Xua H, Liang Y, Zhenga B (2019a) Understanding the digestibility of rice starch–gallic acid complexes formed by high pressure homogenization. *International Journal of Biological Macromolecules* 134, 856–863. DOI: 10.1016/j.ijbiomac.2019.05.083

Liu C, Ge S, Yang J, Xu Y, Zhao M, Xiong L, Sun, Q (2016a) Adsorption mechanism of polyphenols onto starch nanoparticles and enhanced antioxidant activity under adverse conditions. *Journal of Functional Foods* 26, 632–644. DOI: 10.1016/j.jff.2016.08.036

Liu B, Kongstad KT, Wiese S, Jäger AK, Staerk D (2016b) Edible seaweed as future functional food: Identification of α–glucosidase inhibitors by combined use of high–resolution a α –glucosidase inhibition profiling and HPLC–HRMS–SPE–NMR. *Food Chemistry* 203, 16–22. DOI: 10.1016/j.foodchem.2016.02.001

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Liu X, Luo G, Wang L, Yuan W (2019b) Optimization of antioxidant extraction from edible brown algae *Ascophyllum nodosum* using response surface methodology. *Food and Bioproducts Processing* 114, 205–215. DOI: 10.1016/j.fbp.2019.01.003.

Liu X, Yuan W, Sharma–Shivappa R, Zantan JV (2017) Antioxidant activity of phlorotannins from brown algae. *International Journal of Agricultural and Biological Engineering* 10, 184–191. DOI: 10.25165/j.ijabe.20171006.2854

Liu X, Yuan W, Zhao R (2021a) Extraction of antioxidants from brown algae *Ascophyllum nodosum* Using a binary solvent extraction system. *ACS Food Science & Technology* 1, 1041–1049. DOI: 10.1021/acsfoodscitech.1c00053

Lobban CS, Harrison PJ (1994) Seaweed ecology and physiology. Press Syndicate of the University of Cambridge. New york, USA.

Lopes G, Andrade PB, Valentão P (2017) Phlorotannins: towards new pharmacological interventions for diabetes mellitus type 2. *Molecules* 22, 56–77. DOI: 10.3390/molecules22010056

Lopes G, Barbosa M, Vallejo F, Gil–Izquierdo Á, Andrade PB, Valentão P, Pereira DM, Ferreres F (2018) Profiling phlorotannins from *Fucus spp.* of the Northern Portuguese coastline: Chemical approach by HPLC–DAD–ESI/MSn and UPLC–ESI–QTOF/MS. *Algal Research* 29, 113–120. DOI: 10.1016/j.algal.2017.11.025

López–Hortas L, Flórez–Fernández N, Torres MD, Ferreira–Anta T, Casas MP, Balboa EM, Falqué E, Domínguez H (2021) Applying Seaweed Compounds in Cosmetics, Cosmeceuticals and Nutricosmetics. *Marine Drugs* 19, 552. DOI: 10.3390/md19100552

Lo Piparo E, Scheib H, Frei N, Williamson G, Grigorov M, Chou CJ (2008) Flavonoids for controlling starch digestion: structural requirements for inhibiting human α–amylase. *Journal of medicinal chemistry* 51(12), 3555–3561. DOI: 10.1021/jm800115x

Lordan S, Smyth TJ, Soler–Vila A, Stanton C, Ross RP (2013) The a–amylase and a–glucosidase inhibitory effects of Irish seaweed extracts. *Food Chemistry* 141, 2170–2176. DOI: 10.1016/j.foodchem.2013.04.123

Lund MN (2021**)** Reactions of plant polyphenols in foods: Impact of molecular structure. *Trends in Food Science and Technology* 121, 241–251. DOI: 10.1016/j.tifs.2021.03.056

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

M

Machu L, Misurcova L, Ambrozova JV, Orsavova J, Mlcek J, Sochor J, Jurikova T (2015) Phenolic content and antioxidant capacity in algal food products. *Molecules* 20, 1118–1133. DOI: 10.3390/molecules20011118

Magalhaes LM, Segundo MA, Reis S, Lima JL (2008) Methodological aspects about *in vitro* evaluation of antioxidant properties. *Analytica Chimica Acta* 613, 1–19. DOI: 10.1016/j.aca.2008.02.047

Maheswari V, Azhagum P, Babu S (2022) Comprehensive reviews on phenolic compounds from Phaeophyceae as potential therapeutic agent. *Journal of Applied Biology & Biotechnology* 10(5), 14–21. DOI: 10.7324/JABB.2022.100502

Maki M, Collin P (1997) Coeliac disease. *Lancet* 349, 1755–1759. DOI: 10.1016/S0140–6736(96)70237–4.

Mamat, H, Matanjun, P, Ibrahim, S, Md. Amin SF, Abdul Hamid M, Rameli AS (2014) The effect of seaweed composite our on the textural properties of dough and bread. *Journal of Applied Phycology* 26, 1057–1062. DOI: 10.1007/s10811–013–0082–8

Mancini F, Montanari L, Peressini D, Fantozzi P (2002) Influence of alginate concentration and molecular weight on functional properties of mayonnaise. *LWT–Food Science Technology*, 35, 517–525. DOI: 10.1006/fstl.2002.0899.

Mandel KG, Daggy BP, Brodie DA, Jacoby HI (2000) Review article: alginate–raft formulations in the treatment of heartburn and acid reflux. *Alimentary Pharmacology & Therapeutics*, 14, 669–690. DOI: 10.1046/j.1365–2036.2000.00759.x

Marathe SJ, Jadhav SB, Bankar SB, Singhal RS (2017) Enzyme–Assisted Extraction of Bioactives. In: Puri, M. (eds) Food Bioactives. Springer, Cham. DOI: 10.1007/978–3–319–51639–4_8

Martínez–Maqueda D, Zapatera B, Gallego–Narbón A, Vaquero MP, Saura–Calixto F, Pérez–Jiménez J (2018) A 6–week supplementation with grape pomace to subjects at cardiometabolic risk ameliorates insulin sensitivity, without affecting other metabolic syndrome markers. *Food & Function* 9(11), 6010–6019. DOI: 10.1039/c8fo01323c

Matanjun P, Mohamed S, Mustapha NM, Muhammad K, Ming CH (2008) Antioxidant activities and phenolics content of eight species of seaweeds from north Borneo. *Journal of Applied Phycology* 20, 367–373. DOI: 10.1007/s10811–007–9264–6.

USC UNIVERSIDADE DE SANTIAGO

Matsui T, Tanaka T, Tamura S, Toshima S, Tamaya K, Miyata Y, Tanaka K, Matsumoto K (2007) a–Glucosidase inhibitory profile of catechins and theaflavins. *Journal of Agricultural and Food Chemistry* 55, 99–105. DOI: 10.1021/jf0627672

McHugh DJ (2003) A guide to the seaweed industry. FAO fisheries technical paper number 441, FAO, Rome

McInnes AG, Ragan MA, Smith DG, Walter JA (1985) The high molecular weight polyphloroglucinols of the marine brown alga *Fucus vesiculosus L*. ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. *Canadian Journal of Chemistry* 63, 304–313. DOI: 10.1139/v85–051

Mekinic IG, Skroza D, Šimat V, Hamed I, Cagalj M, Perkovic ZP (2019) Phenolic content of brown algae (*Pheophyceae*) species: extraction, identification, and quantification. *Biomolecules* 9, 244. DOI: 10.3390/biom9060244

Menezes BS, Coelho MS, Meza SLR, Salas–Mellado M, Souza, MRAZ (2015) Macroalgal biomass as an additional ingredient of bread. *International Food Research Journal* 22, 812–817.

Mesa–Vanegas AM, Zapata–Uribe S, Arana LM, Zapata IC, Monsalve Z, Rojano B (2015) Antioxidant activity of different polarity extracts from *Ageratum conyzoides L. Boletín Latinoamericano y del Caribe de Plantas Medicinales Aromaticas*14, 1–10.

Mihaylova D, Popova A, Goranova Z, Petkova D, Doykina P, Lante A (2021) The perspective of nectarine fruit as a sugar substituent in puddings prepared with corn and rice starch. *Foods* 10, 2563. DOI: 10.3390/foods10112563

Milledge JJ, Harvey PJ (2018) Anaerobic Digestion and gasification of seaweed. In Rampelotto P, Trincone A (eds) Grand challenges in marine biotechnology. Grand challenges in biology and biotechnology. Springer, Cham. DOI: 10.1007/978–3–319–69075–9_7

Milledge JJ, Smith B, Dyer PW, Harvey P (2014) Macroalgae–derived biofuel: a review of methods of energy extraction from seaweed biomass. *Energies* 7, 7194–7222. DOI: 10.3390/en7117194

Miller NJ, Diplock AT, Rice–Evans C, Davies MJ, Gopinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science* 84, 407–412. DOI: 10.1042/cs0840407

USC DE SANTIAGO DE COMPOSTELA

MIRACLES: Multi–product integrated biorefinery of algae: from carbon dioxide and light energy to high – value specialties, FP7, Grant agreement no 613588. Available from: http://miraclesproject.eu

Miranda J, Lasa A, Bustamante MA, Churruca I, Simon E (2014) Nutritional differences between a gluten–free diet and a diet containing equivalent products with gluten. *Plant Foods for Human Nutrition*. 69, 182–187. DOI: 10.1007/s11130–014–0410–4

Mohammed A, River A, Stuckey DC, Ward K (2020) Alginate extraction from *Sargassum* seaweed in the Caribbean region: Optimization using response surface methodology. *Carbohydrate Polymers* 245, 116419. DOI: 10.1016/j.carbpol.2020.116419.

Moharram HA, Youssef MM (2014) Methods for Determining the antioxidant activity: A review. *Alexandria Journal of Food Science and Technology*11, 31–42.

Moon HE, Islam MN, Ahn BR, Chowdhury SS, Sohn HS, Jung HA Cho JS (2011) Protein tyrosine phosphatase 1B and α–glucosidase inhibitory phlorotannins from edible brown algae, *Ecklonia stolonifera* and *Eisenia bicyclis*. *Bioscience, Biotechnology, and Biochemistry* 75, 1472–1480. DOI: 10.1271/bbb.110137

Moreira R, Chenlo F, Chaguri L, Fernandes C (2008) Water absorption texture, and color kinetics of air–dried chestnuts during rehydration. *Journal of Food Engineering* 86, 584–594. DOI: 10.1016/j.jfoodeng.2007.11.012

Moreira R, Chenlo F, Sineiro J, Arufe S, Sexto S (2016) Drying temperature effect on powder physical properties and aqueous extract characteristics of *Fucus vesiculosus*. *Journal of Applied Phycology* 28(4), 2485–2494. DOI: 10.1007/s10811–015–0744–9

Moreira R, Sineiro J, Chenlo F, Arufe S, Díaz–Varela D (2017) Aqueous extracts of *Ascophyllum nodosum* obtained by ultrasound–assisted extraction: effects of drying temperature of seaweed on the properties of extracts. *Journal of Applied Phycology* 29, 3191–3200. DOI: 10.1007/s10811–017–1159–6.

Motilva MJ, Serra A, Macia A (2013) Analysis of food polyphenols by ultra high–performance liquid chromatography coupled to mass spectrometry: An overview. *Journal of Chromatography A* 1292, 66–82. DOI: 10.1016/j.chroma.2013.01.012

Mujumdar AS (2006) Handbook of Industrial Drying. Boca Raton, FL, USA: CRC Press.

Munin A, Edwards–Lévy F (2011) Encapsulation of natural polyphenolic compounds; a review. *Pharmaceutics* 3, 793–829. DOI: 10.3390/pharmaceutics3040793

USC DE SANTIAGO DE COMPOSTELA

Munteanu IG, Apetrei C (2021) Analytical Methods Used in Determining Antioxidant Activity: A Review. *International Journal of Molecular Sciences* 22, 3380–2410. DOI: 10.3390/ijms22073380

Murillo–Álvarez JI, Hernández–Carmona G (2007) Monomer composition and sequence of sodium alginate extracted at pilot plant scale from three commercially important seaweeds from Mexico. *Journal of Applied Phycology* 19, 545–548. DOI: 10.1007/s10811–007–9168–5.

Murugan AC, Karim MR, Yusoff MBM, Tan SH, Asras MFBF, Rashid SS (2015) New insights into seaweed polyphenols on glucose homeostasis. *Pharmaceutical Biology* 53(8), 1087–1097. DOI: 10.3109/13880209.2014.959615

N

Nakamura T, Nagayama K, Uchida K, Tanaka R (1996) Antioxidant activity of phlorotannins isolated from the brown alga *Esinia bicyclis*. *Fisheries Science* 62, 923–926. DOI: 10.2331/fishsci.62.923

Naveen J, Baskaran R, Baskaran V (2021) Profiling of bioactives and *in vitro* evaluation of antioxidant and antidiabetic property of polyphenols of marine algae *Padina tetrastromatica*. *Algal Research* 55 102250. DOI: 10.1016/j.algal.2021.102250

Nenna R, Tiberti C, Petrarca L, Lucantoni F, Mennini M, Luparia RPL, Panimolle F, Mastrogiorgio G, Pietropaoli N, Fabio M, Bonamico M (2013) The celiac iceberg: characterization of the disease in primary schoolchildren. *Journal of Pediatric Gastroenterology and Nutrition* 56, 416–421. 10.1097/MPG.0b013e31827b7f64

Niki E (1996) α–Tocopherol. In: Packer L, Cardebas E (eds.) Handbook of antioxidants. Pp 3–25. Marcel Dekker, New York, USA

Norra I, Aminah A, Suri R (2016) Effects of drying methods, solvent extraction and particle size of Malaysian brown seaweed, *Sargassum* sp. on the total phenolic and free radical scavenging activity. *International Food Research Journal* 23, 1559–1563.

Noubigh A, Mgaidi A, Abderrabba M, Provost E, Fürst W (2007) Effect of salts on the solubility of phenolic compounds: experimental measurements and modelling. *Journal of Agricultural and Food Chemistry* 87, 783–788. DOI: 10.1002/jsfa.2762

Novakova L, Matysova L, Solich P (2006) Advantages of application of UPLC in pharmaceutical analysis. *Talanta* 68(3), 908-918. DOI: 10.1016/j.talanta.2005.06.035

Novotni D, Cukelj N, Smerdel B, Bituh M, Dujmic F, Curic D (2012) Glycemic index and firming kinetics of partially baked frozen gluten–free bread with sourdough *Journal of Cereal Science* 55, 120. DOI: 10.1016/j.jcs.2011.10.008

Nyambe–Silavwe H, Villa–Rodríguez JA, Ifie I, Holmes M, Aydin E, Jensen JM, Williamson G (2015) Inhibition of human α–amylase by dietary polyphenols. *Journal of Functional Foods* 19, 723–732. DOI: 10.1016/j.jff.2015.10.003

Nwosu F, Morris J, Lund VA, Stewart D, Ross HA, McDougall GJ (2011) Anti–proliferative and potential anti–diabetic effects of phenolic–rich extracts from edible marine algae. *Food Chemistry* 126, 1006–1012. DOI: 10.1016/j.foodchem.2010.11.111

O

Oboh G, Ademosun AO, Ayeni PO, Omojokun OS, Bello F (2015a) Comparative effect of quercetin and rutin on α–amylase, α–glucosidase, and some pro–oxidant–induced lipid peroxidation in rat pancreas. *Comparative Clinical Pathology* 24(5), 1103–1110. DOI: 10.1007/s00580–014–2040–5

Oboh G, Agunloye OM, Adefegha SA, Akinyemi, AJ, Ademiluyi AO (2015b) Caffeic and chlorogenic acids inhibit key enzymes linked to type 2 diabetes (*in vitro*): a comparative study. *Journal of Basic and Clinical Physiology and Pharmacology* 26(2), 165–170. DOI: 10.1515/jbcpp–2013–0141

Oboh G, Ogunsuyi O, Ogunbadejo M, Adefegha S (2016) Influence of gallic acid on α–amylase and α–glucosidase inhibitory properties of acarbose. *Journal of Food and Drug Analysis* 24(3), 627–634. DOI: 10.1016/j.jfda.2016.03.003.

Oh SH, Ahn J, Kang DH, Lee HY (2011) The effect of ultrasonificated extracts of *Spirulina maxima* on the anticancer activity. *Marine Biotechnology* 13, 205–214. DOI: 10.1007/s10126–010–9282–2

Okada Y, Ishimaru A, Suzuki R, Okuyama T (2004) A new phloroglucinol derivative from the brown alga Eisenia bicyclis: Potential for the effective treatment of diabetic complications. *Journal of Natural Products* 67, 103–105. DOI: 10.1021/np030323j

Okolie CL, Mason B, Mohan A, Pitts N, Udenigwe CC (2019) The comparative influence of novel extraction technologies on *in vitro* prebiotic–inducing chemical properties of fucoidan extracts from *Ascophyllum nodosum*. *Food Hydrocolloids* 90, 462–471. DOI: 10.1016/j.foodhyd.2018.12.053.

USC UNIVERSIDADE DE SANTIAGO

Okolie CL, Mason B, Mohan A, Pitts N, Udenigwe C.C (2020) Extraction technology impacts on the structure–function relationship between sodium alginate extracts and their *in vitro* prebiotic activity. *Food Bioscience* 37, 100672. DOI: 10.1016/j.fbio.2020.100672

Oreopoulou V, Extraction of Natural Antioxidants. In In Tzia C, Liadakis G (2003) Extraction Optimization in Food Engineering, Marcel Dekker, Inc, Boca Raton, USA pp 329–340.

O'Shea N, Arendt EK, Gallagher E, Shea NO, Arendt EK, Gallagher E (2014) State of the art in gluten–free research. *Journal of Food Science* 79, 1067–1076. DOI: 10.1111/1750–3841.12479

O'Sullivan AM, O'Callaghan YC, O'Grady MN, Queguineur B, Hanni D, Troy DJ, Kerry JP, O'Brien NM (2011) *In vitro* and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland. *Food Chemistry* 126, 1064–1070. DOI: 10.1016/j.foodchem.2010.11.127.

P

Paganga G, Al–Hashim H, Khodr H, Scott BC, Aruoma OI, Hider RC, Halliwell B, Rice–Evans C (1996) Mechanisms of the antioxidant activities of quercetin and catechin. Redox report 2, 359 – 364. DOI: 10.1080/13510002.1996.11747075

Paiva L, Lima, E, Neto AI, Baptista J (2017**)** Angiotensin I–Converting Enzyme (ACE) Inhibitory activity, antioxidant properties, phenolic content and amino acid profiles of *Fucus spiralis* L. protein hydrolysate fractions. *Marine drugs* 15, 311–329. DOI: 10.3390/md15100311

Pamies R, Schmidt RR, Martínez MCL, de la Torre JG (2010) The influence of mono and divalent cations on dilute and non–dilute aqueous solutions of sodium alginates. *Carbohydrate Polymers* 80, 248–253. DOI: 10.1016/j.carbpol.2009.11.020

Pantidos N, Boath A, Lund V, Conner S, McDougall GJ (2014) Phenolic–rich extracts from the edible seaweed, *Ascophyllum nodosum*, inhibit α–amylase and α–glucosidase: Potential anti–hyperglycemic effects. *Journal of Functional Foods* 10, 201–209. DOI 10.1016/j.jff.2014.06.018.

Parada J, Aguilera JM (2011) Review: Starch matrices and the glycemic response. *Food Science and Technology International* 17, 187–204. DOI: 10.1177/1082013210387712

USC UNIVERSIDADE DE SANTIAGO

Parada J, Pérez–Correa J, Pérez–Jiménez J (2019) Design of low glycemic response foods using polyphenols from seaweed. *Journal of Functional Foods* 56, 33–39. DOI: 10.1016/j.jff.2019.03.004

Parada J, Santos, JL (2016) Interactions among starch, lipids, and proteins in foods: Microstructure control for glycemic response modulation. *Critical Reviews in Food Science and Nutrition* 56, 2362–2369. DOI: 10.1080/10408398.2013.840260

Paraskevopoulou D, Boskou D, Paraskevopoulou A (2006) Oxidative stability of olive oil–lemon juice salad dressings stabilized with polysaccharides. *Food chemistry* 101, 1197–1204. DOI: 10.1016/j.foodchem.2006.03.022.

Parys S, Kehraus S, Pete R, Küpper FC, Glombitza KW, König GM (2009) Seasonal variation of polyphenolics in *Ascophyllum nodosum* (Phaeophyceae). E*uropean Journal of Phycology* 44(3), 331-338. DOI: 10.1080/09670260802578542

Parys S, Rosenbaum A, Kehraus S, Reher G, Glombitza KW, König GM (2007) Evaluation of quantitative methods for the determination of polyphenols in algal extracts *Journal of Natural Products (Lloydia)* 70, 12, 1865–1870. DOI: 10.1021/np070302f

Patterson CC, Dahlquist GG, Gyürü E, Green A, Soltész G, EURODIAB Study Group (2009) Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. *Lancet* 373, 2027–2033. DOI: 10.1016/S0140–6736(09)60568–7.

Peleg M (1988) An empirical model for the description of moisture sorption curves. *Journal of Food Science* 53, 1216–1219. DOI: 10.1111/j.1365–2621.1988.tb13565.x

Pereira L (2011) A review of the nutrient composition of selected edible seaweeds. In Pomin V.H. (ed) Seaweed: ecology, nutrient composition and medicinal uses. Nova Science Publishers, New York, USA, 15–50.

Peteiro C (2018) Alginate production from marine macroalgae, with emphasis on kelp farming. In Rehm B, Moradali M. (Eds.), Alginates and their biomedical applications, pp. 27–66. Singapore, Springer.

Picó Y (2013) Ultrasound–assisted extraction for food and environmental samples. *TrAC Trends in Analytical Chemistry* 43, 84–99. DOI: 10.1016/j.trac.2012.12.005

Poole J, Diop A, Rainville LC, Barnabe S (2019) Bioextracting polyphenols from the brown seaweed *Ascophyllum nodosum* from Québec's north shore coastline. *Industrial biotechnology* 15, 212–218. DOI: 10.1089/ind.2019.0008

USC DE SANTIAGO DE COMPOSTELA

Povey MJW, Mason Tj (1998) Ultrasound in food processing, the Way Forward. Blackie Academic & Professional, London.

Prasad PM, Palthur SS, Suresh Kumar C (2010) Nutraceuticals: a conceptual definition. *International Journal of Pharmacy and Pharmaceutical Sciences* 2, 19.

Price ML, Butler LG (1977) Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *Journal of Agricultural and Food Chemistry* 25, 1268–1273.

Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry* 53, 4290–4302. DOI: 10.1021/jf0502698

Proenca C, Freitas M, Ribeiro D, Oliveira EFT, Sousa JLC, Tome SM, Ramos MJ, Silva AMS, Fernandes PA, Fernandes E (2017) α–Glucosidase inhibition by flavonoids: an *in vitro* and in silico structure–activity relationship study. *Journal of Enzyme Inhibition and Medicinal Chemistry* 32(1), 1216–1228. DOI: 10.1080/14756366.2017.1368503.

Pulido R, Bravo L, Calixto FDS (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/ antioxidant power assay. *Journal of Agricultural and Food Chemistry* 48, 3396–3402. DOI: 10.1021/jf9913458

Q

Qiu C, Qin Y, Zhang S, Xiong L, Sun AQ (2013) comparative study of size–controlled worm like amylopectin nanoparticles and spherical amylose nanoparticles: Their characteristics and the adsorption properties of polyphenols. *Food Chemistry* 213, 579–587. DOI: 10.1016/j.foodchem.2016.07.023

R

Ragan MA, Glombitzka KW (1986) Phlorotannins, brown algal polyphenols. *Program Phycology Research* 4, 129-241.

Rajauria G, Jaiswal AK, Abu–Ghannam N, Gupta S (2013) Antimicrobial, antioxidant and free radical–scavenging capacity of brown seaweed *Himanthalia elongata* from western coast of Ireland. *Journal of Food Biochemistry* 37, 322–335. DOI: 10.1111/j.1745–4514.2012.00663.x

Rauch R, Hrbek J, Hofbauer H (2014) Biomass gasification for synthesis gas production and applications of the syngas. *WIREs Energy and Environment* 3, 343–362. DOI: 10.1002/wene.97

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice–Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26, 1231–1237. DOI: 10.1016/S0891–5849(98)00315–3

Regoli F, Winston GW (1999) Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicology and Applied Pharmacology* 156, 96–105. DOI: 10.1006/taap.1999.8637

Rehm BHA (2005) Biosynthesis and applications of alginates. In: Wnek G, Bowlin G (eds) Biosynthesis and applications of alginates. CRC Press, Boca Raton, Encyclopaedia of Biomaterials and Biomedical Engineering 1–9

Rice–Evans C, Miller NJ, Paganga G (1996) Structure–antioxidant activity relationship of flavonoids and phenolic acids. *Free Radical Biology and Medicine* 20, 933–956. DOI: 10.1016/0891–5849(95)02227–9

Ríos JL, Francini F, Schinella GR (2015) Natural products for the treatment of type 2 diabetes mellitus. *Planta Medica* 81. DOI: 10.1055/s–0035–1546131

Rioux LE, Turgeon SL, Beaulieu M (2007) Characterization of polysaccharides extracted from brown seaweeds. *Carbohydrate Polymers* 69, 530–537. DOI: 10.1016/j.carbpol.2007.01.009

Rhoades CJ (2012) Making fuel from algae: Identifying fact amid fiction. In Gordon R, Seckbach J (eds) The science of algal fuels: phycology, geology, biophotonics, genomics and nanotechnology. Springer, Dordrecht, The Netherlands, pp 177–192.

Robertson JA, de Monredon FD, Dysseler P, Guillon F, Amado R, Thibault JF (2000) Hydration properties of dietary fibre and resistant starch: a european collaborative study. *LWT – Food Science and Technology* 33, 72–79. DOI: 10.1006/fstl.1999.0595

Rodrigues D, Sousa S, Silva A, Amorim M, Pereira L, Rocha–Santos TAP, Gomes AMP, Duarte AC, Freitas AC (2015) Impact of enzyme– and ultrasound–assisted extraction methods on biological properties of red, brown, and green seaweeds from the central west coast of Portugal. *Journal of Agricultural and Food Chemistry* 63, 3177–3188. DOI: 10.1021/jf504220e

USC DE SANTIAGO DE COMPOSTELA

Roleda MY, Marfaing H, Desnica N, Jónsdóttir R, Skjermo J, Rebours C, Nitschke U (2019) Variations in polyphenol and heavy metal contents of wild–harvested and cultivated seaweed bulk biomass: Health risk assessment and implication for food applications. *Food Control*, 95, 121–134. DOI: 10.1016/j.foodcont.2018.07.031

Roselló–Soto E, Galanakis CM, Brncic M, Orlien V, Trujillo FJ, Mawson R, Knoerzer K, Tiwari BK, Barba FJ (2015) Clean recovery of antioxidant compounds from plant foods, by–products and algae assisted by ultrasounds processing. Modeling approaches to optimize processing conditions. *Trends in Food Science and Technology* 42, 134–149. DOI: 10.1016/j.tifs.2015.01.002.

Rouquerol F, Rouquerol J, Sing K (1999) Adsorption at the liquid–solid interface: thermodynamics and methodology. In *Adsorption by Powders and Porous Solids. Principles, Methodology and Applications*; Academic Press: London, UK, pp. 129–137.

Roy MC, Anguenot R, Fillion C, Beaulieu M, Bérubé J, Richard D (2011) Effect of a commercially–available algal phlorotannins extract on digestive enzymes and carbohydrate absorption *in vivo*. *Food Research International* 44, 3026–3029. DOI: 10.1016/j.foodres.2011.07.023

Rózylo R, Hassoon WH, Gawlik–Dziki U, Siasta la M, Dziki D (2017) Study on the physical and antioxidant properties of gluten–free bread with brown algae. *CyTA – Journal of Food* 15, 196–203. DOI: 10.1080/19476337.2016.1236839

Rupérez P, Saura–Calixto F (2001). Dietary fibre and physicochemical properties of edible spanish seaweeds. *European Food Research and Technology* 212, 349–354. DOI: 10.1007/s002170000264

S

Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K, Shaw JE, Bright D, Williams R (2019) Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the international diabetes federation diabetes atlas, 9th edition. *Diabetes Research and Clinical Practice* 157, 107843. DOI: 10.1016/j.diabres.2019.107843.

Sahnoun M, Trabelsi S, Bejar S (2017) Citrus flavonoids collectively dominate the α–amylase and α–glucosidase inhibitions. *Biologia* 72(7), 764–773. DOI: 10.1515/biolog–2017–0091.

USC UNIVERSIDADE DE SANTIAGO

Sailler B, Glombitza KW (1999) Phlorethols and fucophlorethols from the brown alga *Cystophora retroflexa*. *Phytochemistry* 50, 869–881. DOI: 10.1002/(sici)1522–7189(199903/04)7:2<57::aid–nt42>3.0.co;2–f

Sajilata MG, Singhal RS, Kulkarni PR (2006) Resistant starch–a review. *Comprehensive Reviews in Food Science and Food Safety* 5, 1–17. DOI: 10.1016/S0031–9422(98)00643–8

Sánchez–Camargo AD, Montero L, Stiger–Pouvreau T, Tanniou A, Cifuentes A, Herrero M, Ibáñez E (2016) Considerations on the use of enzyme–assisted extraction in combination with pressurized liquids to recover bioactive compounds from algae. *Food Chemistry* 192, 67–74. DOI: 10.1016/j.foodchem.2015.06.098.

Santos SAO, Félix R, Pais ACS, Rocha SM, Silvestre AJD (2019) The Quest for Phenolic Compounds from Macroalgae: A Review of Extraction and Identification Methodologies. *Biomolecules* 9(12), 847. DOI: 10.3390/biom9120847

Santos JL, Saus E, Smalley SV, Cataldo LR, Alberti G, Parada J, Gratacòs M, Estivill X (2012) Copy–number polymorphism of the salivary amylase gene: Implications in human nutrition research. *Journal of Nutrigenetics and Nutrigenomics* 5, 117–131. DOI: 10.1159/000339951

Sardari RR, Prothmann J, Gregersen O, Turner C, Karlsson EN (2021) Identification of phlorotannins in the brown algae, *Saccharina latissima* and *Ascophyllum nodosum* by ultra–high–performance liquid chromatography coupled to high–resolution tandem mass spectrometry. *Molecules* 26, 43. DOI: 10.3390/molecules26010043

Sathy R, Kanaga N, Sankar P, Jeeva S (2017) Antioxidant properties of phlorotannins from brown seaweed *Cystoseira trinodis* (Forsskal) C. Agardh. *Arabian Journal of Chemistry* 10, 2608–2614. DOI: 10.1016/j.arabjc.2013.09.039

Scazzocchio B, Varì R, Filesi C, Del Gaudio I, D'Archivio M, Santangelo C, Iacovelli A, GalvanoF , Pluchinotta FR, Giovannini C, Masella R (2015). Protocatechuic acid activates key components of insulin signaling pathway mimicking insulin activity. *Molecular Nutrition & Food Research* 59, 1472–1481. DOI: 10.1002/mnfr.201400816

Schmid M, Guihéneuf F, Stengel DB (2014) Fatty acid contents and profiles of 16 macroalgae collected from the Irish coast at two seasons. *Journal of Applied Phycology* 26(1), 451-463. DOI: 10.1007/s10811–013–0132–2

USC DE SANTIAGO DE COMPOSTELA

Shahidi F, Ambigaipalan P (2015) Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of Functional Foods* 18, 820–897. DOI: 10.1016/j.jff.2015.06.018

Shahidi F, Naczk M, Phenolics in food and nutraceuticals; CRC Press: Boca Raton, FL, USA, 2014. DOI: 10.1201/9780203508732

Shahidi F, Zhong Y (2011) Revisiting the polar paradox theory: A critical overview. J. Agric. *Food Chemistry* 59, 3499–3504. DOI: 10.1021/jf104750m

Sharifuddin Y, Chin YX, Lim PE, Phang SM (2015) Potential bioactive compounds from seaweed for diabetes management. *Marine Drugs* 13, 5447–5491. DOI: 10.3390/md13085447

Shibata T, Ishimaru K, Kawaguchi S, Yoshikawa H, Hama Y (2008) Antioxidant activities of phlorotannins isolated from Japanese *Laminariaceae*. *Journal of Applied Phycology* 20, 705–11. DOI: 10.1007/s10811–007–9254–8

Shimanuki N, Imai M, Nagai K (2020) Effects of counter cations on the water vapor sorption properties of alginic acid and alginates. *Journal of Applied Polymer Science* e49326. DOI: 10.1002/app.49326

Shodehinde SA, Oboh G (2014) Distribution and antioxidant activity of polyphenols in boiled unripe plantain (*Musa paradisiaca*) pulps. E*uropean Journal of Phycology* 38, 293–299. DOI: 10.1016/S2221–1691(13)60095–7

Sibilia JP (1996) Chapter 1 An introduction to materials characterization and chemical analysis. In Sibilia JP, A guide to materials characterization and chemical analysis (2nd edition). Wiley–VCH, Inc. New York. USA.

Sies, H. (2019). Oxidative stress: eustress and distress in redox homeostasis. In Stress: Stress: physiology, biochemistry, and pathology, 3 Pp 153–163. Academic Press. DOI: 10.1016/B978–0–12–813146–6.00013–8

Silva J, Alves C, Freitas R, Martins A, Pinteus S, Ribeiro J, Gaspar H, Alfonso A, Pedrosa R (2019) Antioxidant and Neuroprotective Potential of the Brown Seaweed *Bifurcaria bifurcata* in an *in vitro* Parkinson's Disease Model. *Marine Drugs* 17, 85. DOI: 10.3390/md17020085.

Singleton VL, Orthofer R, Lamuela–Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology* 299, 152–178. DOI: 10.1016/S0076–6879(99)99017–1

USC DE SANTIAGO DE COMPOSTELA

Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16(3), 144–158. DOI: 10.1039/B600518G

Singh I, Bharate S (2006) Phloroglucinol compounds of natural origin. *Natural Product Reports* 23, 558–591. DOI: 10.1039/B600518G

Singh P, Arora A, Strand TA, Leffler DA, Catassi C, Green PH, Kelly CP, Ahuja V, Makharia GK (2018) Global prevalence of celiac disease: systematic review and meta–analysis. *Clinical Gastroenterology and Hepatology* 16, 823–836. DOI: 10.1016/j.cgh.2017.06.037

Singh IP, Sidana J (2013) Phlorotannins. In Woodhead Publishing Series in Food Science, Technology and Nutrition, Functional Ingredients from Algae for Foods and Nutraceuticals; Dominguez, H, Ed.; Woodhead Publishing: Cambridge, MA, USA.

Sivaramakrishnan HP, Senge B, Chattopadhyay P (2004) Rheological properties of rice dough for making rice bread. *Journal of Food Engineering* 62, 37–45. DOI: 10.1016/S0260–8774(03)00169–9.

Skaugrud O, Hagen A, Borgersen B, Dornish (1999) Biomedical and pharmaceutical applications of alginate and chitosan. *Biotechnology and Genetic Engineering Reviews* 16, 23–40. DOI: 10.1080/02648725.1999.10647970.

Smidsrød O, Haug A, Larsen B (1967) Oxidative–reductive depolymerization: a note on the comparison of degradation rates of different polymers by viscosity measurements. *Carbohydrate Research* 5, 482–485. DOI: 10.1016/s0008–6215(00)81123–4.

Soria AC, Villamiel M (2010) Effect of ultrasound on the technological properties and bioactivity of food: A review. *Trends in Food Science and Technology* 21, 323–331. DOI: 10.1016/j.tifs.2010.04.003.

Soto ML, Moure A, Domínguez H, Parajó JC (2011) Recovery, concentration and purification of phenolic compounds by adsorption: A review. *Journal of Food Engineering*105, 1–27. DOI: 10.1016/j.jfoodeng.2011.02.010

Stadler I (1998) Oxygen consumption methods In: Amstrong D., Free radical and antioxidant protocol. Methods in molecular biology 108, p 15 Humana press Buffalo, New York.

Staško A, Brezová V, Biskupic S, Mišík V (2007) The potential pitfalls of using 1,1–diphenyl–2–picrylhydrazyl to characterize antioxidants in mixed water solvents. *Free Radical Research* 41, 379–390. DOI: 10.1080/10715760600930014

USC DE SANTIAGO DE COMPOSTELA

Steinberg PD, Altena IV (1992) Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. *Ecological Monographies* 62, 189–222. DOI: 10.2307/2937093

Stephen MA, Phillips GO, Williams PA (2006) Food Polysaccharides and Their Applications. Boca Raton, FL, USA: CRC Press.

Steevensz AJ, Mackinnon SL, Hankinson R, Craft C, Connan S, Stengel DB, Melanson JE (2011) Profiling phlorotannins in brown macroalgae by liquid chromatography–high resolution mass spectrometry. *Phytochemical Analysis* 23, 547–553. DOI: 10.1002/pca.2354

Stoop JW (1991) The pioneers of pedriatic medicine. *European Journal of Pediatrics* 150, 751–755. DOI: 10.1007/BF00441505

Sugiura Y, Matsuda K, Yamada Y, Nishikawa M, Shioya K, Katsumaki H, Imai K, Amano H (2006) Isolation of a new antiallergic phlorotannins, phlorofucofuroeckol–B, from an edible brown alga, *Eiseenia arborea*. *Bioscience, Biotechnology, and Biochemistry* 70, 2807–2811. DOI: 10.1271/bbb.60417

Sugiura S, Minami Y, Taniguchi R, Tanaka R, Miyake H, Mori T, Ueda M, Shibata T (2017) Evaluation of anti–glycation activities of phlorotannins in human and bovine serum albumin–methylglyoxal models. *Natural Product Communications* 12 1793–1796. DOI: 10.1177/1934578X1701201137

Sui X, Zhang Y, Zhou W (2016) *In vitro* and in silico studies of the inhibition activity of anthocyanins against porcine pancreatic α–amylase. *Journal of Functional Foods* 21, 50–57. DOI: 10.1016/j.jff.2015.11.042.

Sun MF, Jiang CL, Kong YS, Luo JL, Yin P, Guo GY (2022) Recent Advances in Analytical Methods for Determination of Polyphenols in Tea: A Comprehensive Review. *Foods* 11, 1425. DOI: 10.3390/foods11101425

Sun L, Wang Y, Miao M (2020) Inhibition of α–amylase by polyphenolic compounds: Substrate digestion, binding interactions and nutritional intervention. *Trends in Food Science and Technology* 104, 190–207. DOI: 10.1016/j.tifs.2020.08.003

Susano P, Silva J, Alves C, Martins A, Gaspar H, Pinteus S, Mouga T, Goettert MI, Petrovski Ž, Branco LB, Pedrosa R (2021) Unravelling the dermatological potential of the brown seaweed *Carpomitra costata*. *Marine drugs* 19, 135. DOI: 10.3390/md19030135

USC DE SANTIAGO DE COMPOSTELA

T

Tabassum MR, Xia A, Murphy JD (2016) Seasonal variation of chemical composition and biomethane production from the brown seaweed *Ascophyllum nodosum*. *Bioresource Technology* 216, 219–226. DOI: 10.1016/j.biortech.2016.05.071

Talmaciu AI, Volf I, Popa VI (2015) A comparative analysis of the "green" techniques applied for polyphenols extraction from bioresources. *Chemistry & Biodiversity* 12, 1635–1651. DOI: 10.1002/cbdv.201400415

Tadera K, Minami Y, Takamatsu K, Matsuoka T (2006) Inhibition of α–Glucosidase and α–Amylase by flavonoids. *Journal of Nutritional Science and Vitaminology* 52(2), 149–153. DOI: 10.3177/jnsv.52.149

Tan Y, Chang SKC, Zhang Y (2017) Comparison of α–amylase, α–glucosidase and lipase inhibitory activity of the phenolic substances in two black legumes of different genera. *Food Chemistry* 214, 259–268. DOI: 10.1016/j.foodchem.2016.06.100

Tanniou A, Vandanjon L, Incera M, Leon ES, Husa V, Le Grand J, Nicolas JL, Poupart N, Kervarec N, Engelen A, Walsh R, Guerard F, Bourgougnon N, Stiger–Pouvreau V (2014) Assessment of the spatial variability of phenolic contents and associated bioactivities in the invasive alga *Sargassum muticum* sampled along its European range from Norway to Portugal. *Journal of Applied Phycology* 26, 1215-1230. DOI: h10.1007/s10811–013–0198–x

Tavakoli S, Hong H, Wang K, Yang Q, Gahruie HH, Zhuang S, Li Y, Liang Y, Tan Y, Luo Y (2021) Ultrasonic–assisted food–grade solvent extraction of high–value added compounds from microalgae *Spirulina platensis* and evaluation of their antioxidant and antibacterial properties. *Algal Research* 60, 102493. DOI: 10.1016/j.algal.2021.102493

Tello–Ireland C, Lemus–Mondaca R, Vega–Gálvez A, López J, Di Scala K (2011) Influence of hot–air temperature on drying kinetics, functional properties, colour, phycobiliproteins, antioxidant capacity, texture, and agar yield of alga *Gracilaria chilensis*. *LWT–Food Science and Technology* 44, 2112–2118. DOI: 10.1016/j.lwt.2011.06.008

Thomas NV, Kim SK. (2011) Potential pharmacological applications of polyphenolic derivatives from marine brown algae. *Environmental Toxicology and Pharmacology* 32, 325–35. DOI: 10.1016/j.etap.2011.09.004

USC UNIVERSIDADE DE SANTIAGO

Tester RF, Karkalas J, Qi X (2004) Starch structure and digestibility enzyme–substrate relationship. *World's Poultry Science Journal* 60 186–195. DOI: 10.1079/WPS200312

Tierney MS, Smyth TJ, Hayes M, Soler–Vila A, Croft AK, Brunton N (2013a) Influence of pressurized liquid extraction and solid–liquid extraction methods on the phenolic content and antioxidant activities of Irish macroalgae. *International Journal of Food Science & Technology* 48, 860–869. DOI: 10.1111/ijfs.12038

Tierney MS, Smyth TJ, Rai DK, Soler–Vila A, Croft AK, Brunton N (2013b) Enrichment of polyphenol contents and antioxidant activities of Irish brown macroalgae using food–friendly techniques based on polarity and molecular size. *Food Chemistry* 139, 753–761. DOI: 10.1016/j.foodchem.2013.01.019.

Tierney MS, Soler–Vila A, Rai DK, Croft AK, Brunton NP, Smyth TJ (2014) UPLC–MS profiling of low molecular weight phlorotannin polymers in *Ascophyllum nodosum*, *Pelvetia canaliculata* and *Fucus spiralis*. *Metabolomics* 10, 524–535. DOI: 10.1007/s11306–013–0584–z

Tiwari BK (2015) Ultrasound: A clean, green extraction technology. *TrAC Trends in Analytical Chemistry* 71, 100–109. https://doi.org/10.1016/j.trac.2015.04.013.

Topuz OK, Gokoglu N, Yerlikaya P, Ucak I, Gumus B (2016) Optimization of Antioxidant Activity and Phenolic Compound Extraction Conditions from Red Seaweed (*Laurencia obtuse*). *Journal of Aquatic Food Product Technology* 25, 414–422. DOI: 10.1080/10498850.2013.868844

Towler MC, Hardie DG (2007) AMP–activated protein kinase in metabolic control and insulin signaling. *Circulation Research* 100, 328–341. DOI: 10.1161/01.RES.0000256090.42690.05

Tyagi V, Saravanan C, Wang Y, Bhattacharya B (2021) Solvent dependency of sorghum bran phytochemicals acting as potential antioxidants and antibacterial agents. *Food Technology and Biotechnology* 59, 31–43. DOI: 10.17113/ftb.59.01.21.6878

Tzia C, Optimization. In Tzia C, Liadakis G (2003) pp 137–172. Marcel Dekker, Inc, Boca Raton, USA.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

U

Uludag H, De Vos P, Tresco PA (2000) Technology of mammalian cell encapsulation. *Advanced Drug Delivery Reviews* 42, 29–64. DOI: 10.1016/s0169–409x(00)00053–3

Ummat V, Tiwari BK, Jaiswal AK, Condon K, Garcia–Vaquero M, O'Doherty J, O'Donnell C, Rajauria G (2020) Optimization of ultrasound frequency, extraction time and solvent for the recovery of polyphenols, phlorotannins and associated antioxidant activity of brown seaweeds. *Marine Drugs* 18, 250–265. DOI: 10.3390/md18050250

Uribe E, Vega–Gálvez A, Vásquez V, Lemus–Mondaca R, Callejas L, Pastén A (2017) Hot–air drying characteristics and energetic requirement of the edible brown seaweed Durvillaea *antarctica*. *Journal of Food Processing and Preservation* e13313–aE13313. DOI: 10.1111/jfpp.13313

US EPA Green Chemistry (2012) United States Environmental Protection Agency, Washington. https://www.epa.gov/greenchemistry/basics–green–chemistry; Searched on 9 June 2021.

V

Valderrama D (2012) Social and economic dimensions of seaweed farming: a global review. IIFET, Tanzania Proceedings.

van Hung P, Phat NH, Phi NTL (2013) Physicochemical properties and antioxidant capacity of debranched starch–ferulic acid complexes. *Starch/Staerke* 65, 382–389. DOI: 10.1002/star.201200168

Vaz BS, Moreira JB, de Morais MG, Costa J.(2016) Microalgae as a new source of bioactive compounds in food supplements. *Current Opinion In Food Science* 7, 73. DOI: 10.1016/j.cofs.2015.12.006

Venditti A, Maggi F, Vittori S, Papa F, Serrill AM, Di Cecco M, Ciaschetti G, Mandrone M, Poli F, Bianco, A. (2015) Antioxidant and α–glucosidase inhibitory activities of *Achillea tenorii*. *Pharmaceutical biology* 53(10), 1505–1510. DOI: 10.3109/13880209.2014.991833.

Vissers AM, Caligiani A, Sforza S, Vincken JP, Gruppen H (2017) Phlorotannin composition of *Laminaria digitata*. *Phytochemical Analysis* 28(6), 487-495. DOI: 10.1002/pca.2697

Vona R, Pallotta L, Cappelletti M, Severi C, Matarrese P (2021) The impact of oxidative stress in human pathology: focus on gastrointestinal disorders. *Antioxidants* 10, 201–227. DOI: 10.3390/antiox10020201

W

Walker RB, Everette JD (2009**)** Comparative reaction rates of various antioxidants with ABTS radical cation. *Journal of Agricultural and Food Chemistry Journal of Agricultural and Food Chemistry* 57, 1156–1161. DOI: 10.1021/jf8026765

Waltner–Law ME, Wang XL, Law BK, Hall RK, Nawano M, Granner DK (2002) Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. *Journal of Biological Chemistry* 277, 34933–34940. DOI: 10.1074/jbc.M204672200

Wang J, Hu S, Nie S, Yu Q, Xie M (2016) Reviews on mechanisms of *in vitro* antioxidant activity of polysaccharides. *Oxidative Medicine and Cellular Longevity* 5692852. DOI: 10.1155/2016/5692852

Wang T, Jónsdóttir R, Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry* 116, 240–248. DOI: 10.1016/j.foodchem.2009.02.041.

Wang T, Jónsdóttir R, Liu H, Gu L, Kristinsson H, Raghavan S, Olafsdóttir G (2012) Antioxidant capacities of phlorotannins extracted from the brown algae Fucus vesiculosus. *Journal of Agriculture and Food Chemistry* 60, 5874–5883. DOI: 10.1021/jf3003653

Wang Y, Li S, Bai F, Cao J, Sun L (2021) The physical adsorption of gelatinized starch with tannic acid decreases the inhibitory activity of the polyphenol against α–amylase. *Foods* 10, 1233. DOI: 10.3390/foods10061233

Wang W, Okada Y, Shi H, Wang Y, Okuyama T (2005) Structures and aldose reductase inhibitory effects of bromophenols from the red alga Symphyocladialatiuscula. *Journal of Natural Products* 68, 620–622. DOI: 10.1161/CIRCRESAHA.109.213447

Wang X, Wang S, Cai Z (2013) The latest developments and applications of mass spectrometry in food–safety and quality analysis. *TrAC Trends in Analytical Chemistry* 52, 170–185. DOI: 10.1016/j.trac.2013.08.005

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Wei YX, Xu ZH (2003) Studies on antioxidative activity of high molecular weight polyphenols from two kinds of brown algae. *Chinese Traditional and Herbal Drugs* 34, 317–319. DOI: 10.3390/md13010509

Wijesekara I, Yoon NY, Kim SK (2010) Phlorotannins from *Ecklonia cava* (Phaeophyceae): Biological activities and potential health benefits. *Biofactors* 36, 408–414. DOI: 10.1002/biof.114

Wijesinghe WAJP, Jeon YJ (2011) Biological activities and potential cosmeceutical applications of bioactive components from brown seaweeds: a review. *Phytochemistry Reviews* 10(3), 431-443. DOI: 10.1007/s11101–011–9214–4

Williams DE (1966) Structure of 2,2–diphenyl–1–picrylhydrazyl free radical. *Journal of the American Chemical Society* 88, 5665–5666. DOI: 10.1021/ja00975a064

Williamson G (2013) Possible effects of dietary polyphenols on sugar absorption and digestion. *Molecular Nutrition & Food Resarch* 57, 48–57. DOI: 10.1002/mnfr.201200511

Wolter A, Hager AS, Zannini E, Arendt EK (2013) *In vitro* starch digestibility and predicted glycaemic indexes of buckwheat, oat, quinoa, sorghum, teff and commercial gluten–free bread. *Journal of Cereal Science* 58, 431. DOI: 10.1016/j.jcs.2013.09.003

Wong K, Cheung PC (2001) Influence of drying treatment on three Sargassum species. *Journal of Applied Phycology* 13, 43–50. DOI: 10.1023/A:1008149215156

Wood D, Capuzzo E, Kirby D, Mooney-McAuley K, Kerrison P (2017) UK macroalgae aquaculture: what are the key environmental and licensing considerations? *Marine Policy* 83, 29-39. DOI: 10.1016/j.marpol.2017.05.021

Wootton-Beard PC, Moran A, Ryan L (2011) Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after *in vitro* digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Research international* 44(1), 217-224. DOI: 10.1016/j.foodres.2010.10.033

Wright JS, Johnson AER, Di Labio GA (2001) Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *Journal of the American Chemical Society* 123, 1173–1183. DOI: 10.1021/ja002455u

Wu Y, Lin Q, Chen Z, Xiao H (2011) The interaction between tea polyphenols and rice starch during gelatinization. *Food Science and Technology International* 17, 569–577. DOI: 10.1177/1082013211399656.

USC DE SANTIAGO DE COMPOSTELA

X

Xie C, Zhong DF, Yu KT, Chen XY (2012) Recent advances in metabolite identification and quantitative bioanalysis by LC–Q–TOF MS. *Bioanalysis* 4, 937–959. DOI: 10.4155/bio.12.43

Xu L, Li Y, Dai Y, Peng J (2018**)** Natural products for the treatment of type 2 diabetes mellitus: Pharmacology and mechanisms. *Pharmacological Research* 130, 451–465. DOI: doi.org/10.1016/j.phrs.2018.01.015

Y

Yan J, Zhang G, Pan J, Wang Y (2014) α–Glucosidase inhibition by luteolin: Kinetics, interaction and molecular docking. *International Journal of Biological Macromolecules* 64, 213–223. DOI: 10.1016/j.ijbiomac.2013.12.007

Yang HW, Fernando KH, Oh JY, Li X, Jeon YJ, Ryu B (2019) Anti–obesity and anti–diabetic effects of *Ishige okamurae*. *Marine Drugs* 17, 202. DOI: 10.3390/md17040202

Yang Z, Han Y, Gu Z, Fan G, Chen Z (2008) Thermal degradation kinetics of aqueous anthocyanins and visual color of purple corn (*Zea mays L.*) cob. *Innovative Food Science and Emerging Technologies* 9, 341–347. DOI: 10.1016/j.ifset.2007.09.001

Yoon NY, Lee SH, Li Y, Kim SK (2009) Phlorotannins from Ishige okamurae and their acetyl–and butylcholinesterases inhibitory effects. *Journal of Functional Foods* 1, 331–335. DOI: 10.1016/j.bjp.2015.08.005

Yoon JS, Yadunandam AK, Kim SJ, Woo HC, Kim HR, Kim GD (2013) Dieckol, isolated from Ecklonia *stolonifera*, induces apoptosis in human hepatocellular carcinoma Hep3B cells. *Journal of Natural Medicines* 67, 519–527. DOI: 10.1007/s11418–012–0709–0

You HN, Lee HA, Park MH, Lee JH, Han JS (2015) Phlorofucofuroeckol A isolated from *Ecklonia cava* alleviates postprandial hyperglycemia in diabetic mice. *European Journal of Pharmacology* 752, 92–96. DOI: 10.1016/j.ejphar.2015.02.003

Yuan Y, Macquarrie DJ (2015) Microwave assisted step–by–step process for the production of fucoidan, alginate sodium, sugars and biochar from *Ascophyllum nodosum* through a biorefinery concept. *Bioresource Technology* 198 819–827. DOI: 10.1016/j.biortech.2015.09.090

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Yuan Y, Zhang J, Fan J, Clark J, Shen P, Li Y, Zhang C (2018) Microwave assisted extraction of phenolic compounds from four economic brown macroalgae species and evaluation of their antioxidant activities and inhibitory effects on α–amylase, α–glucosidase, pancreatic lipase and tyrosinase. *Food Research International* 113, 288–297. DOI: 10.1016/j.foodres.2018.07.021

Z

Zakaria NA, Ibrahim D, Sulaiman SF, Supardy A (2011) Assessment of antioxidant activity, total phenolic content and in–vitro toxicity of Malaysian red seaweed, *Acanthophora spicifera*. *Journal of Chemical and Pharmaceutical Research* 3(3), 182–191.

Zhang Q, Li N, Liu X, Zhao Z, Li Z, Xu Z (2004) The structure of a sulfated galactan from *Porphyra haitanensis* and its *in vivo* antioxidant activity. *Carbohydrate Research* 339, 105–111. DOI: 10.1016/j.carres.2003.09.015

Zhang BW, Li X, Sun WL, Xing Y, Xiu ZL, Zhuang, CL, Dong YS (2017a) Dietary flavonoids and acarbose synergistically inhibit α–glucosidase and lower postprandial blood glucose. *Journal of Agricultural and Food Chemistry* 65(38), 8319–8330. DOI: 10.1021/acs.jafc.7b02531.

Zhang J, Tiller C, Shen J, Wang C, Girouard GS, Dennis D, Barrow CJ, Miao M, Stewart HS (2007) Antidiabetic properties of polysaccharide– polyphenolic– enriched fractions from the brown seaweed *Ascophyllum nodosum*. *Canadian Journal of Physiology and Pharmacology* 85, 1116–1127. DOI: 10.1139/Y07–105

Zhang D, Wang C, Shen L, Shin HC, Lee KB, Jif B (2018a) Comparative analysis of oxidative mechanisms of phloroglucinol and dieckol by electrochemical, spectroscopic, cellular and computational methods. *RSC Advances* 8, 1963–1972. DOI: 10.1039/C7RA10875C

Zhang R, Yuen AK, Magnusson M, Wright JT, de Nys R, Masters AF, Maschmeyer T (2018b) A comparative assessment of the activity and structure of phlorotannins from the brown seaweed *Carpophyllum flexuosum*. *Algal Research* 29, 130–141. DOI: 10.1016/j.algal.2017.11.027

Zheng Y, Tian J, Yang W, Chen S, Liu D, Fang H, Zhang H, Yea X (2020) Inhibition mechanism of ferulic acid against α–amylase and α–glucosidase. *Food Chemistry* 317, 126346. DOI: 10.1016/j.foodchem.2020.126346

Zhou X, Yi M, Ding L, He S, Yan X (2019) Isolation and purification of a neuroprotective phlorotannin from the marine algae *Ecklonia maxima* by size exclusion and high–speed counter–current chromatography. *Marine Drugs* 17, 212. DOI: 10.3390/md17040212

Zhu F (2017) Encapsulation and delivery of food ingredients using starch based systems. *Food Chemistry* 229, 542–552. DOI: 10.1016/j.foodchem.2017.02.101

Zhu Z, Wu Q, Di X, Li S, Barba FJ, Koubaa M, Roohinejad S, Xiong X, He J (2017) Multistage recovery process of seaweed pigments: investigation of ultrasound assisted extraction and ultra–filtration performances. *Food Bioproducts Process* 104, 40–47. DOI: 10.1016/j.fbp.2017.04.008

Zong G, Lebwohl B, Hu FB, Sampson L, Dougherty LW, Willett WC, Chan AT, Sun Q (2018) Gluten intake and risk of type 2 diabetes in three large prospective cohort studies of US men and women. *Diabetologia* 61, 2164. DOI: 10.1007/s00125–018–4697–9

Zou Y, Qian Z-J, Li Y, Kim MM, Lee S-H, Kim SK (2008) Antioxidant effects of phlorotannins isolated from *Ishige okamurae* in free radical mediated oxidative systems. *Journal of Agricultural and Food Chemistry* 56(16) 7001-7009. DOI: 10.1021/jf801133h

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

17. DIFFUSION OF RESULTS DERIVED FROM THE THESIS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Article (Used in Section 8)

Aqueous extracts characteristics obtained by ultrasound-assisted extraction from *Ascophyllum nodosum* seaweeds: effect of operation conditions.

Journal

Journal of Applied Phycology (2021) 33, 3297–3308; DOI: 10.1007/s10811–021–02546–5; IF: 3.404, Q1: Marine and Freshwater Biology (18/113, 2021); Springer (ISSN: 1573-5176)

Authors

M. Gisbert[1], M. Barcala[1], C.M. Rosell[2,3], J. Sineiro[1], R. Moreira[1]

Affiliation

1. Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain.

2. Institute of Agrochemistry and Food Technology, CSIC, Avenida Agustin Escardino 7, Paterna, 46980 Valencia, Spain

3. Department of Food and Human Nutritional Sciences, University of Manitoba,Winnipeg, Canada

Authorship contribution:

Conceptualization; Data curation; Formal analysis (characterization, response surface modelling, analysis of TPC ratios and antioxidant activity); Investigation; Methodology Software; Roles/Writing-original draft.

Rights & Permissions

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your

intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.



Journal of Applied Phycology (2021) 33:3297–3308
https://doi.org/10.1007/s10811-021-02546-5

Aqueous extracts characteristics obtained by ultrasound-assisted extraction from *Ascophyllum nodosum* seaweeds: effect of operation conditions

Mauro Gisbert[1] · Manuel Barcala[1] · Cristina M. Rosell[2] · Jorge Sineiro[1] · Ramón Moreira[1]

Received: 1 February 2021 / Revised and accepted: 14 June 2021 / Published online: 3 August 2021
© The Author(s) 2021

Article (Used in Section 12)

Impact of drying on the sodium alginate obtained after polyphenols ultrasound–assisted extraction from *Ascophyllum nodosum* seaweeds

Journal

Carbohydrate Polymers (2022) 272, 118455 DOI: 10.1016/j.carbpol.2021.118455

IF: 10.723, Q1: Polymer Science (3/90, 2021). ScienceDirect (ISSN: 0144-8617)

Authors

L. Montes, M. Gisbert, I. Hinojosa, J. Sineiro, R. Moreira

Affiliation

Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain.

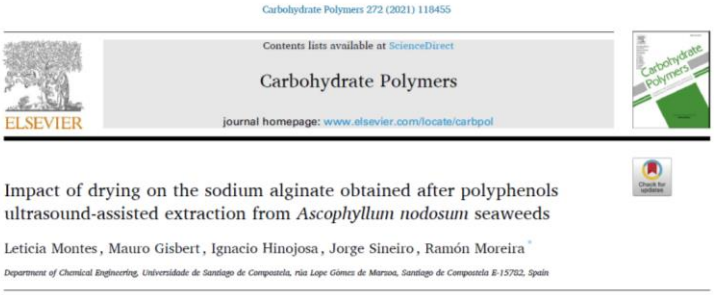
USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Contribution to the Article:

Methodology, Validation, Formal analysis (extraction yields, FT-IR, ^{1}H–NMR and average viscosimetric molecular weight), Investigation, Writing – original draft.

Rights & Permissions

This is an open access article distributed under the terms of the Creative Commons CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Article (Used in Section 10)

Interactions between *Ascophyllum nodosum* seaweeds polyphenols and native and gelled corn starches.

Journal

Foods (2022) 11, 1165; DOI: 10.3390/foods11081165; IF: 5.561, Q1: Food Science & Technology (35/143, 2021). MDPI (ISSN: 2304-8158)

Authors

M. Gisbert[1], A. Aleixandre2, J. Sineiro[1], CM Rosell[2,3], R. Moreira[1]

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Affiliation

1. Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain.

2. Institute of Agrochemistry and Food Technology, CSIC, Avenida Agustin Escardino 7, Paterna, 46980 Valencia, Spain

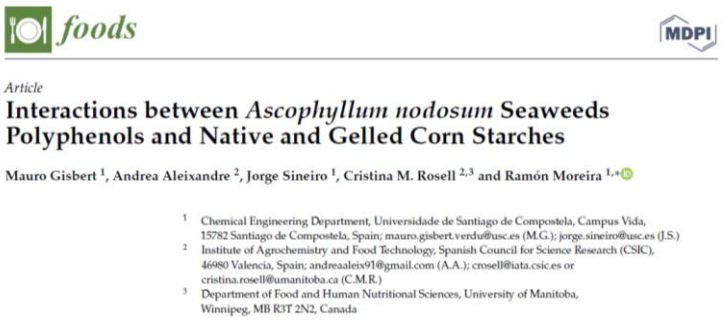
3. Department of Food and Human Nutritional Sciences, University of Manitoba,Winnipeg, Canada

Contribution to the Article:

Conceptualization, Data curation ; Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft.

Rights and permisions

This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



foods

MDPI

Article

Interactions between *Ascophyllum nodosum* Seaweeds Polyphenols and Native and Gelled Corn Starches

Mauro Gisbert [1], Andrea Aleixandre [2], Jorge Sineiro [1], Cristina M. Rosell [2,3] and Ramón Moreira [1,*]

[1] Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, 15782 Santiago de Compostela, Spain; mauro.gisbert.verdu@usc.es (M.G.); jorge.sineiro@usc.es (J.S.)
[2] Institute of Agrochemistry and Food Technology, Spanish Council for Science Research (CSIC), 46980 Valencia, Spain; andreaaleix91@gmail.com (A.A.); crosell@iata.csic.es or cristina.rosell@umanitoba.ca (C.M.R.)
[3] Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

Article (Used in Section 11)

In vitro inhibition of starch digestive enzymes by ultrasound–assisted extracted polyphenols from *Ascophyllum nodosum* seaweeds

Journal

Journal of Food Science (2022) 87, 2405–2416; DOI: 10.1111/1750–3841.16202;

IF: 3.693; Q2: Food Science & Technology (57/143, 2021); Wiley (ISSN: 1750-3841)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Authors

A. Aleixandre[1], M. Gisbert[2], J. Sineiro[2], R. Moreira[2], CM Rosell[1,3]

Affiliation

1. Institute of Agrochemistry and Food Technology, CSIC, Avenida Agustin Escardino 7, Paterna, 46980 Valencia, Spain

2. Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain.

3. Department of Food and Human Nutritional Sciences, University of Manitoba,Winnipeg, Canada

Contribution to the Article:

Data curation, formal analysis, methodology, and writing—original draft.

Rights and permisions

"This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made."

Received: 3 February 2022 | Revised: 14 April 2022 | Accepted: 30 April 2022

DOI: 10.1111/1750-3841.16202

FOOD CHEMISTRY

Journal of Food Science WILEY

In vitro inhibition of starch digestive enzymes by ultrasound-assisted extracted polyphenols from *Ascophyllum nodosum* seaweeds

Andrea Aleixandre[1] | Mauro Gisbert[2] | Jorge Sineiro[2] | Ramón Moreira[2] | Cristina M. Rosell[1,3]

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Article (Used in Section 7)

Polyphenols extraction kinetics from *Ascophyllum nodosum* seaweed employing sweet water and saltwater: Effect of ultrasound sonication.

Journal

Algal Research–Biomass Biofuels and Bioproducts (2022) 66, 10773; DOI: 10.1016/j.algal.2022.102773; IF: 5.276; Q2 Biotechnology & Applied Microbiology (40/158, 2021) ISSN: 2211-9264

Authors

Gisbert M, Sineiro J, Moreira R.

Affiliation

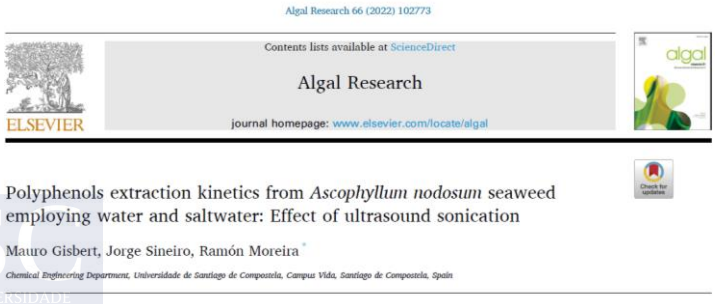
Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain.

Contribution to the Article:

Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Roles/Writing-original draft.

Righsts and permisions

“This is an open access article distributed under the terms of the Creative Commons CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.”



Article (Used in Section 9)

Influence of oxidation and dialysis of phlorotannins on bioactivity and composition of ultrasound–assisted extracts from *Ascophyllum nodosum*.

Journal

Marine drugs (2022). DOI: 10.3390/md20110706 IF: 6.085; Q1 Pharmacology & Pharmacy (48/279, 2021); ISSN: 16603397.

Authors

Gisbert M, Sineiro J, Moreira R.

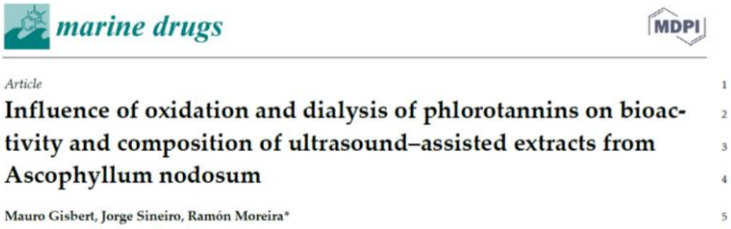
Affiliation

Chemical Engineering Department, Universidade de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain.

Contribution to the Article:

Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Roles/Writing–original draft

Rights & permissions


marine drugs MDPI

Article

Influence of oxidation and dialysis of phlorotannins on bioactivity and composition of ultrasound–assisted extracts from Ascophyllum nodosum

Mauro Gisbert, Jorge Sineiro, Ramón Moreira*

This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

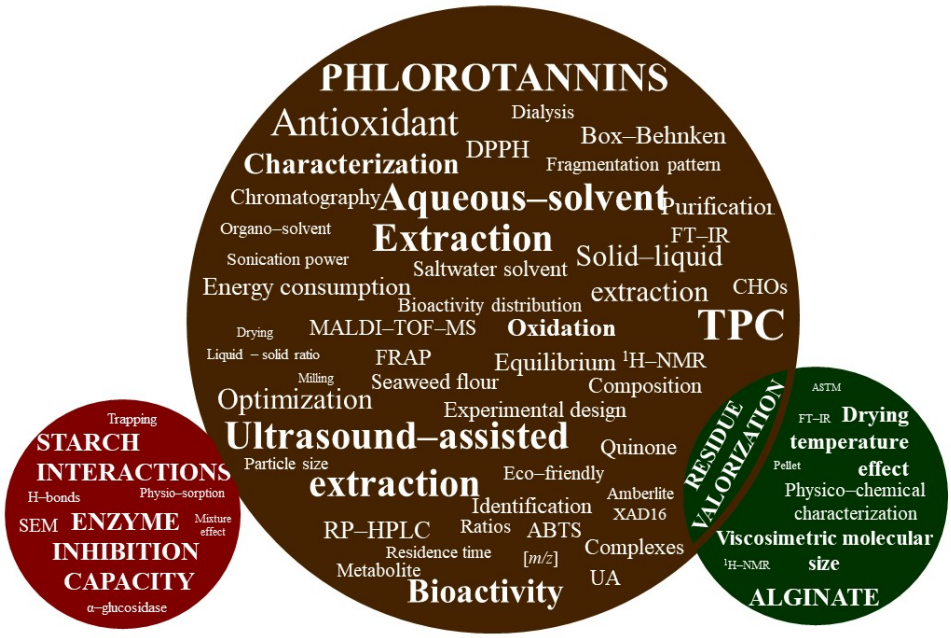
The end

I hope you have enjoyed reading this Thesis as much I did, writing it.

I sincerely expect that you employ all these new knowledge in seaweeds, extractions, biopolymers, phlorotannins, alginates, enzyme inhibition and antioxidants wisely, because:

"With great power comes great responsibilities" (**Amazing Fantasy** #15, 1962)

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



Ascophyllum nodosum seaweeds was used as source of biopolymers. The ultrasound–assisted extraction was used to obtain extracts with high content of bioactive compounds with high antioxidant and enzyme inhibition activities. The physicochemical characterization of these extracts showed a high content of phlorotannins along with other substances (carbohydrates and uronic acids), being necessary subsequent purification steps. By means of gel chromatography and dialysis, extracts with an improved bioactivity were obtained. Extracts with the highest bioactivities were significant inhibitors of α–amylase and α–glucosidase digestive enzymes. Finally, alginates with modulated properties were also obtained improving the integral use of the seaweeds, after the extraction of phlorotannins. Results indicated that extracted biopolymers from *Ascophyllum nodosum* can be employed in gluten–free foods for celiac people and functional formulations.