TESIS DOCTORAL

AÑO 2023

TÍTULO DE LA TESIS

COMPUESTOS BIOACTIVOS EN ALGAS Y EN ALIMENTOS ENRIQUECIDOS CON ALGAS: CARACTERIZACIÓN ANALÍTICA Y ENSAYOS DE ACTIVIDAD BIOLÓGICA

AUTOR

CARLOS GERÓNIMO TERRIENTE PALACIOS

PROGRAMA DE DOCTORADO EN CIENCIAS

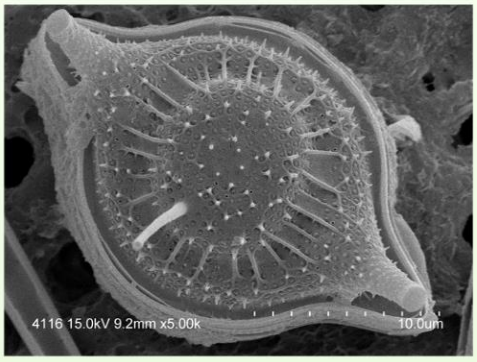
DIRECTOR

MASSIMO CASTELLARI

Compuestos bioactivos en algas y en alimentos enriquecidos con algas: Caracterización analítica y ensayos de actividad biológica

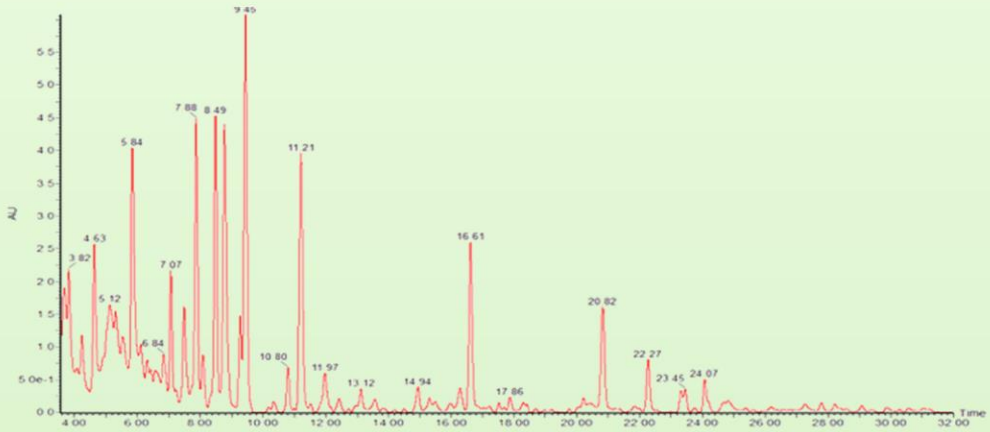
Carlos Gerónimo Terriente Palacios

Agosto 2023



Odontella aurita
(scanning electron micrograph)

Bifurcaria bifurcata



Compuestos bioactivos en algas y en alimentos enriquecidos con algas: caracterización analítica y ensayos de actividad biológica

POR

CARLOS GERÓNIMO TERRIENTE PALACIOS

Memoria presentada para optar al grado de Doctor en Ciencias por la Universidad Nacional de Educación a Distancia.

Fdo. Carlos G. Terriente Palacios

Gerona, a 15 de agosto de 2023

El Dr. D. MASSIMO CASTELLARI, Investigador principal del Programa de Funcionalidad y seguridad alimentarias del Instituto de Investigación y Tecnología Agroalimentarias - IRTA

CERTIFICA

Que el trabajo que se presenta en esta tesis doctoral bajo el título:

"Compuestos bioactivos en algas y en alimentos enriquecidos con algas: caracterización analítica y ensayos de actividad biológica", ha sido realizado bajo mi dirección en los laboratorios del Instituto de Investigación y Tecnología Agroalimentarias - IRTA y que reúne todos los requisitos legales, académicos y científicos para hacer que el doctorando D. Carlos Gerónimo Terriente Palacios pueda optar al grado de Doctor en Ciencias por la UNED.

Y para que conste, expido y firmo el presente certificado en

Fdo. Dr. Massimo Castellari

Gerona, a 15 de agosto de 2023

TABLA DE CONTENIDOS

RESUMEN

La sociedad actual se caracteriza por tener, cada vez más, un mayor conocimiento de la relación que existe entre la dieta y la salud, por lo que suele demandar una alimentación basada en productos naturales, seguros y que sean también funcionales. Es por ello que diferentes ramas de la ciencia se han ocupado en el desarrollo de estos alimentos funcionales, que además de proporcionar nutrientes suficientes para satisfacer los requerimientos metabólicos de un individuo, producen una serie de efectos fisiológicos beneficiosos, más allá de los beneficios nutricionales aceptados.

Los alimentos funcionales se caracterizan por tener en su composición una serie de compuestos que son responsables de tales funciones, a los que se denominan compuestos bioactivos.

Las algas, aunque consumidas en Oriente desde tiempos inmemoriales, están empezando a abrirse camino en los mercados occidentales por su fama, cada vez más creciente, de ser "superalimentos" con múltiples propiedades beneficiosas para la salud humana.

Esta memoria recoge los resultados obtenidos durante la realización de la tesis doctoral titulada "**Compuestos bioactivos en algas y en alimentos enriquecidos con algas: caracterización analítica y ensayos de actividad biológica**".

La INTRODUCCIÓN incluye una extensa revisión bibliográfica acerca de los beneficios para la salud humana de diversos compuestos bioactivos presentes en las matrices de alga objeto de estudio, y los beneficios del uso de algas marinas como ingrediente funcional en productos alimentarios. También se incluye una descripción de las técnicas analíticas utilizadas en la caracterización, aislamiento y purificación de compuestos bioactivos, así como de los ensayos de bioactividad

de compuestos y extractos obtenidos a partir de algas y de alimentos enriquecidos con algas.

La PARTE EXPERIMENTAL, expone los resultados obtenidos en el desarrollo de la presente tesis doctoral, y se ha dividido en tres bloques:

El **bloque 1** se ha dedicado a la caracterización de compuestos bioactivos y se ha subdividido en 2 capítulos. En el **capítulo 1** de este bloque se describe la optimización y validación de un nuevo método analítico para la cuantificación simultánea de 3 derivados del ácido sulfónico (Taurina, Homotaurina, Hipotaurina) y 19 aminoácidos en muestras de algas comerciales de uso alimentario, aplicando la cromatografía líquida de alta resolución en fase reversa acoplada a espectrometría de masas de triple cuadrupolo (RP- HPLC -ESI- QQQ/ MS2). En el **capítulo 2**, se demuestra el potencial de esta metodología analítica para evaluar el perfil aminoacídico y de Taurina, Homotaurina y Hipotaurina en un amplio rango de especies de algas y en alimentos enriquecidos con algas, todas ellas disponibles en el mercado para su uso en alimentación humana.

El **bloque 2** se ha centrado en la medición de actividades biológicas, antioxidantes y de modulación de la Sirtuina 1, en muestras de algas. En el **capítulo 3**, se detallan los valores de actividad antioxidante y de activación o inhibición de la Sirtuina 1 por parte de hidrolizados proteicos procedentes de una gran diversidad de especies de algas, tanto comerciales como salvajes. En este trabajo, asimismo, se investigan nuevas correlaciones entre el contenido de ciertos aminoácidos en los hidrolizados proteicos, y las capacidades antioxidantes y de modulación de la Sirtuina 1.

OBJETIVOS

El objetivo general de la presente Tesis Doctoral ha sido la caracterización analítica y la evaluación de la bioactividad de compuestos bioactivos procedentes de algas comestibles y de alimentos enriquecidos con algas. Este amplio objetivo, se ha subdividido en tres objetivos parciales:

1. El primero objetivo ha sido el desarrollo y validación de un método analítico novedoso para la determinación simultánea de 3 aminoácidos derivados del ácido sulfónico (TAD), - taurina, homotaurina, hipotaurina- y 19 aminoácidos en muestras de algas comerciales, tanto macroalgas como microalgas. Para ello, se usó una plataforma de separación cromatográfica con detección por espectrometría de masas RP- HPLC -ESI- Triple Cuadrupolo /MS.

2. el segundo objetivo ha sido la evaluación del contenido de - taurina, homotaurina, hipotaurina- y 19 aminoácidos en un amplio rango de especies de algas y alimentos enriquecidos con algas disponibles en el mercado para consumo humano. Para esto, se ha utilizado el método analítico previamente desarrollado y validado en el punto anterior. Asimismo, para llevar a cabo la cuantificación de los componentes de interés en los alimentos enriquecidos con algas, se ha validado el método analítico antes mencionado, en estas matrices.

3. el tercer objetivo parcial ha sido la evaluación de la bioactividad de hidrolizados proteicos obtenidos de 36 especies diferentes de macroalgas y microalgas comestibles. Para tal fin, se llevó a cabo un aislamiento de la fracción proteica y posterior hidrólisis enzimática de cada especie de alga. Estos estudios de bioactividad incluyen diversos ensayos de la actividad

antioxidante y de la capacidad de modulación de una importante proteína deacetilasa dependiente de NAD, la Sirtuina 1 (SIRT1). Esta proteína está actualmente siendo usada como diana terapéutica de un amplio rango de enfermedades asociadas al envejecimiento, tales como cáncer, enfermedades neurodegenerativas, osteoporosis, enfermedades cardiovasculares, diabetes tipo 2, etc.

.

INTRODUCCIÓN

1. ALGAS COMO FUENTE DE COMPUESTOS BIOACTIVOS

1.1. Macroalgas y microalgas

Las algas forman parte de un grupo muy heterogéneo de organismos fotosintéticos. La división incluye organismos multicelulares, macroalgas, y organismos unicelulares, también conocidos como microalgas (que miden desde 1 mm hasta varios cm) (**Figura 1**) [1].

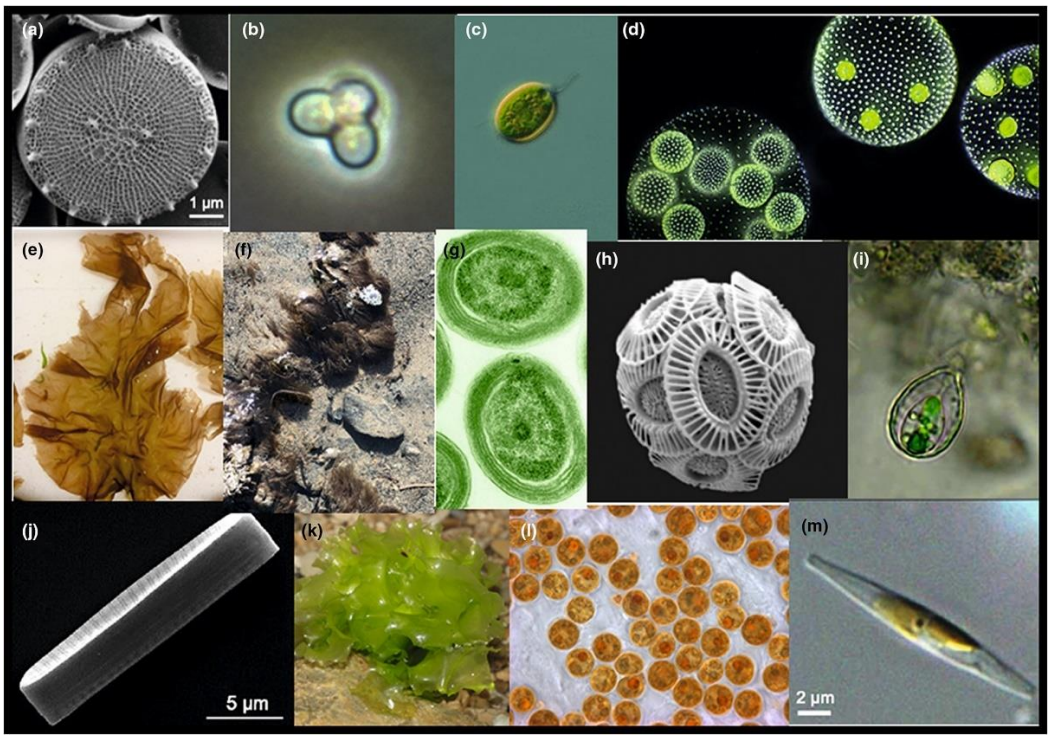


Figura 1. Biodiversidad de las Algas. [2]. (a) *Thalassiosira pseudonana* (Bacillariophyta); (b) *Cyanidioschyzon merolae* (Rhodophyta); (c) *Chlamydomonas reinhardtii* (Chlorophyta); (d) *Volvox* (Chlorophyta); (e) *Porphyra umbilicalis* (Rhodophyta); (f) *Ectocarpus siliculosus* (Phaeophyta); (g) *Prochlorococcusmarinus* (Cyanophyta); (h) *Emiliania huxleyi* (Haptophyta); (i) *Paulinella chromatophora* (Cercozoa); (j) *Fragilariopsis cylindrus* (Bacillariophyta); (k) *Ulva Lactuca* (Chlorophyta); (l) *Symbiodinium microadriaticum* (Dinophyta); (m) *Phaeodactylum tricornutum* (Bacillariophyta).

Las **macroalgas** son organismos marinos macroscópicos que pueden alcanzar varios metros de longitud (algunos tallos de las mismas pueden llegar a alcanzar los 65 metros). Dado que se engloban dentro de los productores primarios, estas se encuentran en la base de la cadena alimentaria marina, sirviendo de alimento a varias comunidades de animales herbívoros, tanto invertebrados como vertebrados. Viven principalmente en agua de mar y, dado que son organismos fotosintéticos, necesitan suficiente luz. Asimismo, la mayoría de las especies requieren de un punto de fijación, y es por esto que las algas marinas habitan más frecuentemente zonas costeras, concretamente en frentes rocosos. Sin embargo, hay unos pocos géneros (*Sargassum*) que no viven adheridos a sustratos rocosos, sino flotando libremente [3].

Las macroalgas son, pues, organismos fotosintéticos acuáticos principalmente marinos y pertenecen al dominio Eukarya y los reinos Plantae (algas verdes y rojas) y Chromista (algas marrones). Aunque los sistemas de clasificación han evolucionado mucho con el tiempo, generalmente se acepta que las macroalgas verdes están incluidas en el filum Chlorophyta, y su pigmentación es idéntica a la de las plantas vasculares (clorofilas a y b y carotenoides); las macroalgas rojas pertenecen al filum Rhodophyta y tienen clorofila a, ficobilinas, y algunos carotenoides como pigmentos fotosintéticos; las macroalgas pardas pertenecen al filum Ochrophyta, y todas ellas se agrupan en la clase Phaeophyceae y su pigmentación es debida, fundamentalmente, a clorofilas a y c y carotenoides (generalmente la fucoxantina, responsable de su color marrón) [3] **Figura 2.**

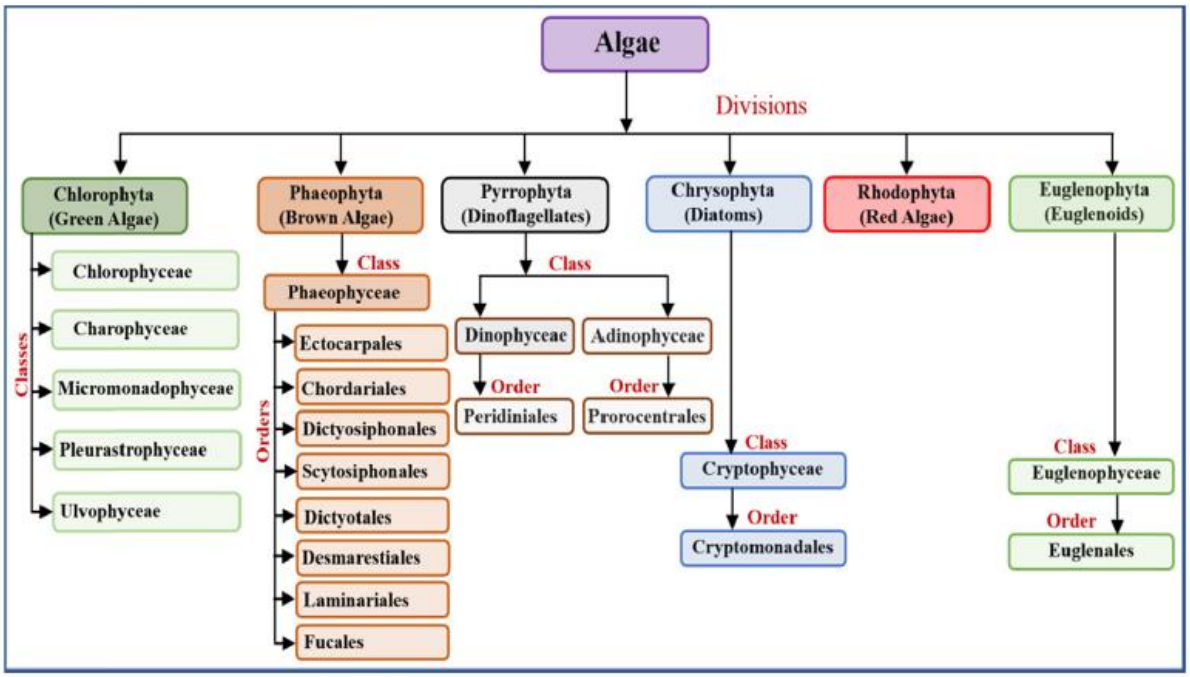


Figure 2. Esquema de clasificación de las macroalgas. [4]

Algunas de las especies de macroalgas que han sido utilizadas en la parte experimental de esta tesis doctoral, se muestran en la **Figura 3**.

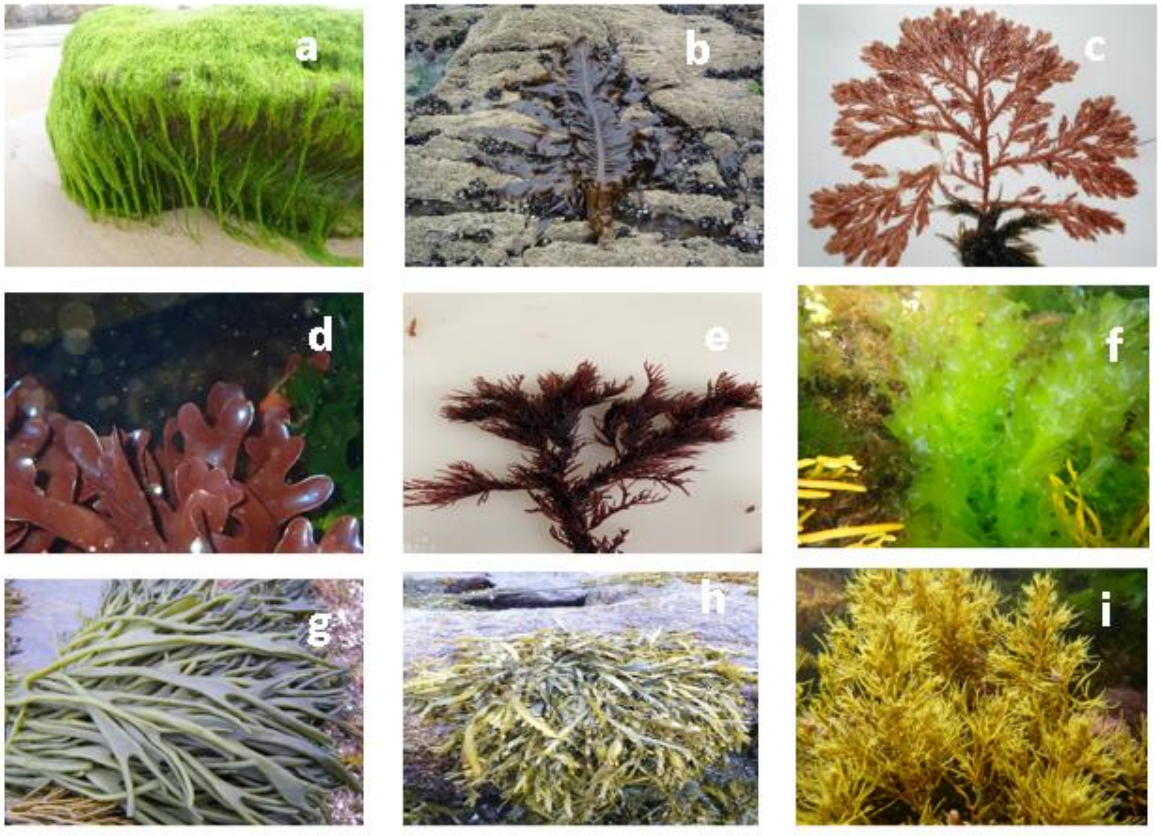


Figura 3. Algunas macroalgas usadas en esta tesis doctoral**. a** *Ulva intestinalis*; **b** *Undaria pinnatifida*; **c** *Plocammium cartilagineum*; **d** *Chondrus crispus*; **e** *Halopitys incurva*; **f** *Ulva sp.*; **g** *Codium sp.*; **h** *Ascophyllum nodosum*; **i** *Gongolaria baccata*. (Fuente: Elaboración propia; fotos cedidas por el Dr. César Peteiro (IEO-CSIC).

Las algas se distribuyen en ambientes diversos y extremos. Son valiosas debido a su alto contenido en compuestos con diferentes actividades biológicas, incluidos compuestos orgánicos complejos y metabolitos primarios y secundarios. Cabe mencionar, entre ellos, los fitopigmentos (xantofilas y carotenoides), ácidos grasos poliinsaturados (PUFA) como el ácido docosahexaenoico (DHA), compuestos fenólicos, taninos, péptidos, lípidos, enzimas, vitaminas, carbohidratos, terpenoides, y otros. Así, las algas son una fuente de biomasa viable y económica de compuestos valiosos con aplicaciones potenciales en las industrias nutracéutica, farmacéutica, química, alimentaria y cosmética debido a sus propiedades biológicamente activas y regenerativas [5-9].

En los últimos años, las macroalgas han ganado cada vez más interés debido a sus cualidades beneficiosas para la salud humana, sus capacidades para disminuir los riesgos de muchas enfermedades crónicas e incluso ayudar a extender la vida media [10, 11]. Las macroalgas también se pueden utilizar para el tratamiento de aguas residuales o como fertilizante natural en agricultura, mejorando así la calidad de los productos y minimizando la necesidad de fertilizantes químicos [12-14]. El potencial de las macroalgas como fuente de energía renovable también es considerablemente interesante. Estos organismos acuáticos tienen la capacidad de mitigar las emisiones de dióxido de carbono y hoy en día se utilizan como materia prima para producir biocombustibles "limpios" o los denominados "biocombustibles de tercera generación" [15].

Las aplicaciones más importantes de las algas se sintetizan en la **Figura 4**.

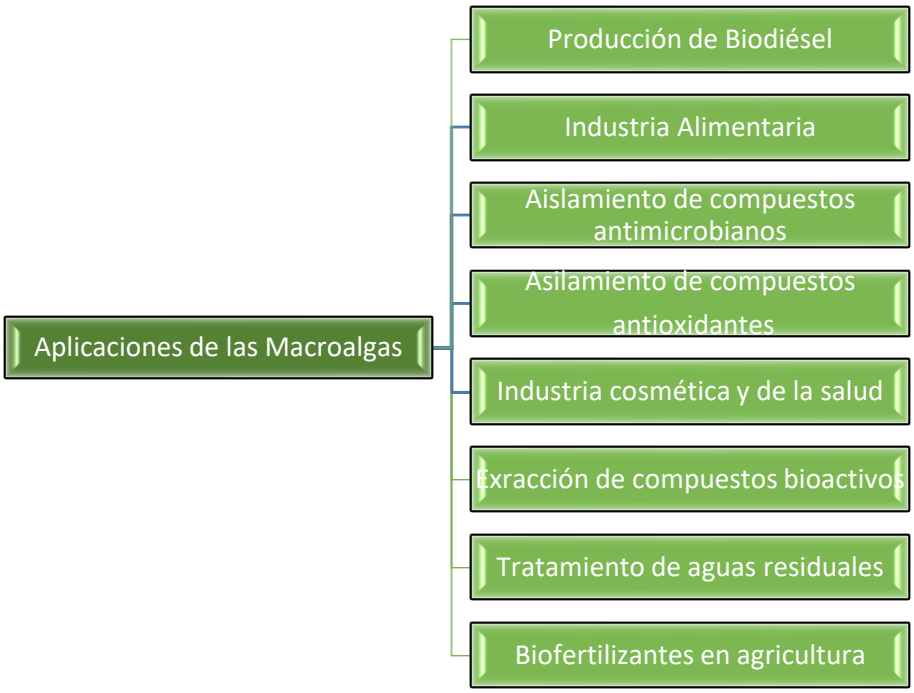


Figura 4. Principales aplicaciones de las Macroalgas.

Las **microalgas** son principalmente organismos fotoautótrofos unicelulares, es decir que utilizan la luz solar como fuente de energía y el CO2 como fuente de carbono para llevar

a cabo la fotosíntesis y producir biomasa orgánica, aunque también se pueden producir cultivos heterótrofos, y utilizar materia orgánica como fuente de carbono y energía. Pueden vivir tanto en agua dulce como en agua de mar, producir biomasa y liberar O_2 a través del consumo de CO_2 y materia inorgánica. Las principales clases de microalgas, sus metabolitos más característicos, y actividades biológicas asociadas, se detallan en la **Figura 5**.

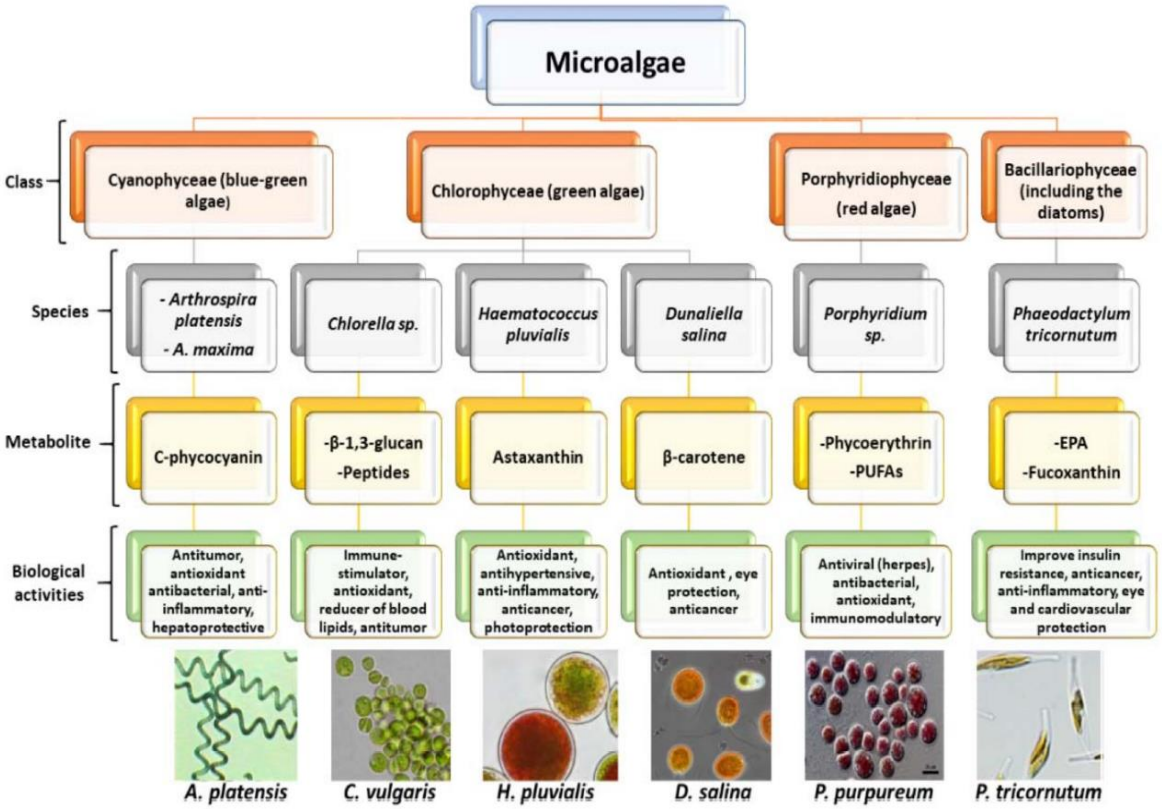


Figura 5. Clases de microalgas, metabolitos característicos y actividades biológicas [16].

Las microalgas sirven, al igual que las macroalgas, como productores primarios en la cadena alimentaria para proporcionar alimento a los organismos y, al mismo tiempo,

afectar el ecosistema microbiano, secretando metabolitos especiales y/o formando relaciones simbióticas con microorganismos [17].

En los últimos años, han sido destacados los efectos beneficiosos de las microalgas en la mejora del medio ambiente. Estas tienen de 10 a 50 veces más capacidad de secuestro de carbono que las plantas terrestres, lo que las convierte en una importante contribución a la reducción de las concentraciones de CO_2 atmosférico [18].

A diferencia de las macroalgas, que tienen una larga historia como alimento y como medicina, las microalgas se han utilizado para la salud humana durante únicamente los últimos 70 años, debido a la dificultad en recolectar grandes cantidades en la naturaleza [19]. Sin embargo, en los últimos años, con la mejora de la tecnología de cultivos, es posible cosechar suficientes cantidades de biomasa de microalgas, lo que promueve aún más el progreso en la investigación de las microalgas en varios aspectos, especialmente en la nutrición y salud humanas [20].

Como fuente de macro y micronutrientes, las microalgas son ricas en compuestos bioactivos, entre los cuales los más importantes son los ácidos grasos poliinsaturados, como el ácido eicosapentaenoico (EPA) y el ácido docosahexaenoico (DHA), polisacáridos, vitaminas (como las vitaminas A, B6, B9, B12, C, D, E y K), y carotenoides (astaxantina, β-caroteno) [21, 22]. Además, las condiciones de cultivo artificial hacen que los compuestos bioactivos producidos por las microalgas sean más seguros. Por ejemplo, la fuente tradicional de ácido omega-3 (DHA) es el aceite de pescado; su uso está limitado por los riesgos para la salud de los antibióticos, metales pesados y otros contaminantes que pueden acumularse en el mismo. Sin embargo, mediante el uso de biorreactores con condiciones controladas de luz, temperatura y dióxido de carbono, se pueden hacen crecer microalgas con altos contenidos de DHA, extraer su biomasa sin uso de disolventes, y obtener aceites ricos en omega-3 y otros compuestos bioactivos, sin estos problemas de acumulación de contaminantes [23].

También es interesante destacar el uso de las microalgas para el desarrollo de nuevas vacunas. Actualmente se han desarrollado prototipos de vacunas fabricadas con microalgas frente a infecciones víricas, bacterianas y parasitarias. En humanos, se han expresado antígenos en microalgas frente a enfermedades causadas por el virus de la inmunodeficiencia humana (VIH), el virus de la hepatitis B, el papilomavirus humano, o el virus Zika, entre otros muchos [24].

1.2. Revisión bibliográfica sobre los beneficios para la salud humana de compuestos bioactivos extraídos de algas

Los compuestos bioactivos se pueden definir como aquellos compuestos tanto esenciales como no esenciales que se encuentran en la naturaleza, que forman parte de la cadena alimentaria, y que pueden tener efectos sobre la salud humana [25]. Estos compuestos se sintetizan durante procesos metabólicos primarios y secundarios en animales, plantas y bacterias [26].

El **metabolismo primario** representaría todas aquellas rutas o vías metabólicas directamente involucradas en el crecimiento y reproducción celulares. Los principales metabolitos primarios son las proteínas, hidratos de carbono y lípidos [27].

En cuanto al contenido **proteico**, las algas marinas y, en concreto algunas especies de microalgas son muy ricas, llegando a valores cercanos al 70% [28]. Debido al crecimiento de la población mundial, al crecimiento económico, y a otros muchos factores, la demanda global de alimentos crecerá entre un 35% y un 56%, durante el periodo 2010 – 2050, con la consiguiente necesidad de búsqueda de fuentes alternativas de alimentos [29]. En este sentido, las microalgas pueden ser una opción sostenible como fuentes ricas en proteínas. Asimismo, varios autores han aislado diversos péptidos bioactivos de macro y microalgas con diversos usos terapéuticos [30].

Otro estudio [31] se centra en dos especies de clorofitas poco estudiadas, a saber, *Caulerpa racemosa* y *Ulva fasciata*. En este trabajo de investigación se destacó el valor nutricional de ambas algas, que contienen un contenido proteico elevado (8,8–19,9 % para C. racemosa y 8,0–11,1 % para U. fasciata) y una proporción de aminoácidos esenciales (42-45%) comparable a los requisitos de la FAO/OMS.

Los **carbohidratos** pueden presentarse en el alga tanto en sus formas de azúcares simples (glucosa, maltosa, etc.), como en diversas estructuras polisacarídicas con múltiples funciones beneficiosas para la salud humana. Así, algunos de estos polisacáridos han demostrado tener importantes acciones antioxidantes y anticancerígenas [32]. Asimismo, polisacáridos sulfatados de determinadas especies de algas presentan destacadas propiedades antivíricas, y se ha explorado su potencial como agentes antivirales en la prevención del reciente coronavirus (Covid-19) [33]. Otros autores enfatizan la importancia de los polisacáridos de las algas marinas. Ismail y col.[34] exploraron la quimio diversidad de las algas rojas y destacaron sus aplicaciones farmacéuticas. El grupo Rhodophyta son un grupo importante de macroalgas que incluyen aproximadamente 7000 especies. Son una fuente rica de compuestos estructuralmente diversos que incluyen proteínas, polisacáridos sulfatados, pigmentos, ácidos grasos poliinsaturados, vitaminas, minerales y compuestos fenólicos. Los polisacáridos son los componentes principales de la pared celular de las algas rojas, de las cuales representan alrededor del 40-50% del peso seco. Son ampliamente utilizados en las industrias alimentaria y farmacéutica debido a sus propiedades espesantes y gelificantes. Los galactanos, carragenanos y agares son los principales hidrocoloides de la pared celular de las algas rojas y tienen características terapéuticas de amplio espectro.

Los autores también señalan que el contenido químico de las algas es diferente según la especie de alga, la etapa de crecimiento, el medio ambiente y las condiciones externas. Finalmente, las algas rojas podrían ser económicamente relevantes como una fuente sustituta de ingredientes naturales que contribuyen a una amplia gama de bioactividades

como el tratamiento frente al cáncer, agentes antiinflamatorios e inhibidores de la acetilcolinesterasa, etc.

El contenido **lipídico** de las algas puede llegar a ser muy elevado. Cultivos de microalgas de la especie *Botryococcus brauni* pueden llegar a producir hasta un 75% de lípidos [35]. Estos pueden presentarse de diversas formas, desde ácidos grasos poliinsaturados (PUFAs), glucolípidos, fosfolípidos, etc. Dentro de los PUFAs, los más interesantes desde el punto de vista medicinal, son el ácido eicosapentaenoico (EPA), el ácido docosahexaenoico (DHA), el ácido linoleico, por sus acciones beneficiosas frente a enfermedades cardiovasculares, diabetes, cáncer, etc. [36]. Lopes y col. [37], investigaron seis especies de macroalgas comestibles marinas, por su contenido lipídico, especialmente en ácidos grasos poliinsaturados omega-3, y sus efectos sobre varios parámetros relacionados con la salud. Los extractos lipídicos de dos macroalgas verdes, *Ulva rigida* y *Codium tomentosum*, de tres algas rojas *Gracilaria gracilis*, *Palmaria palmata* y *Porphyra dioica*, y el alga parda *Fucus vesiculosus*, se obtuvieron a partir de un sistema de acuicultura multitrófica integrado en tierra, y se analizaron en cuanto a su calidad lipídica, concretamente, en cuanto a sus perfiles de ácidos grasos y bioactividades. Los resultados destacaron la especificidad de especie de los beneficios para la salud de cada alga estudiada. Así, *Ulva rigida* mostró los índices más bajos de aterogenicidad y de trombogenicidad (índices que determinan el potencial de una dieta para producir la obstrucción de las arterias formando placas de grasa o trombos). Los extractos lipídicos de *Palmaria palmata* y *Fucus vesiculosus*, mostraron los valores más elevados en ensayos de actividad antioxidante. *Ulva rigida*, *Codium tomentosum*, *Palmaria palmata* y *Porphyra dioica* inhibieron la actividad de la ciclooxigenasa-2 (Cox-2, enzima sobre expresada en procesos tumorales) hasta en un 80%, mientras que los extractos de P. dioica y P. palmata mostraron la mayor actividad citotóxica potencial en las células de cáncer de mama MDA-MB-231. En conjunto, estos resultados apoyan

el uso de estas macroalgas como alimentos funcionales e ingredientes prometedores para un desarrollo de dietas sostenibles y saludables.

Otro trabajo reciente [38] se centró en estudiar los efectos citotóxicos y apoptóticos de extractos del alga parda *Colpomenia sinuosa*. El extracto de diclorometano: metanol fue el más potente contra la proliferación de líneas celulares de cáncer de colon HCT-116, al aumentar la población de células subG1 e inducir la apoptosis. Este extracto también disminuyó la migración potencial de las células HCT-116 con efectos mínimos sobre las células no tumorigénicas. Estos resultados indican que el alga *Colpomenia sinuosa* es una fuente de compuestos bioactivos que poseen propiedades proapoptóticas y antimigratorias eficaces para ciertos procesos cancerígenos.

El metabolismo **secundario**, incluye todas aquellas rutas celulares que producen compuestos principalmente involucrados en la adaptación de los organismos a su ambiente. Entre estos metabolitos secundarios podemos destacar los compuestos fenólicos, carotenoides, fitoesteroles, terpenos, vitaminas, etc. [39] (**Figura 6**).

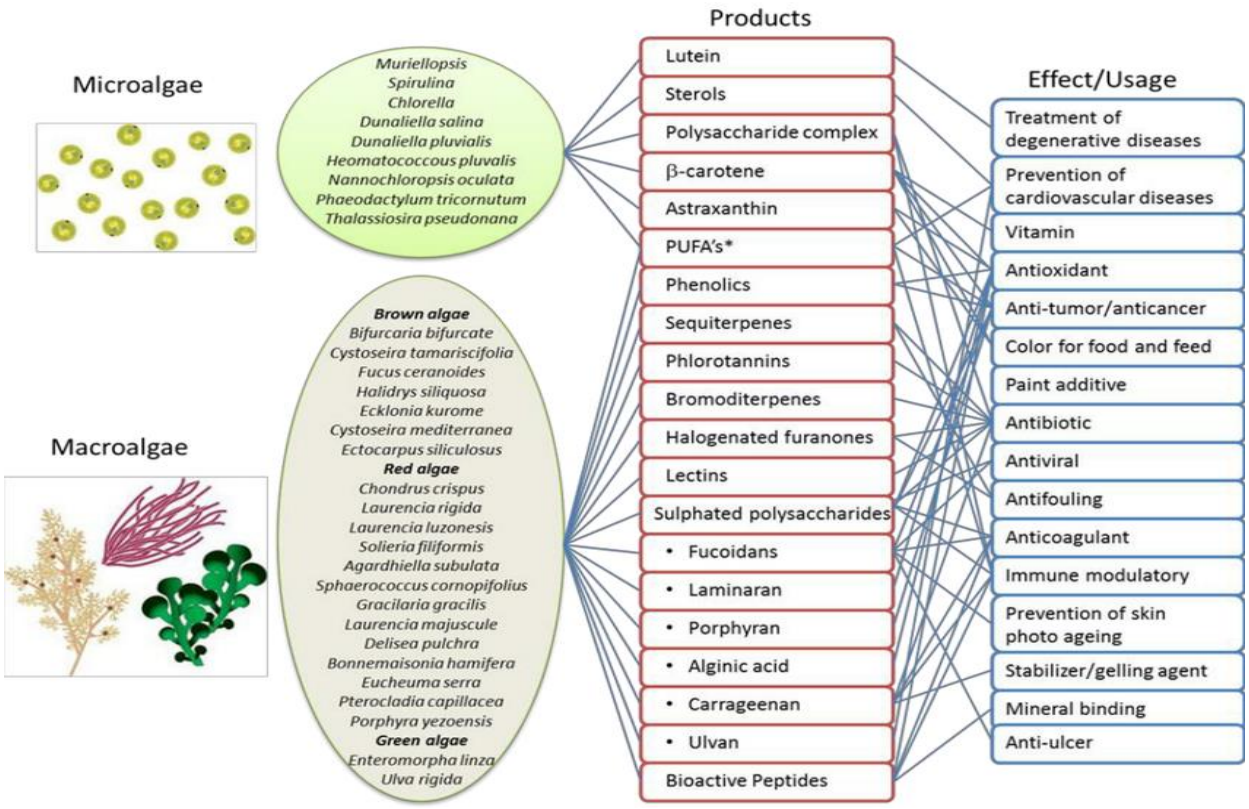


Figura 6. Metabolitos secundarios de algas marinas y sus posibles aplicaciones. [39]

Numerosos metabolitos extraídos de algas marinas poseen actividades biológicas. Estos compuestos bioactivos han sido ampliamente reconocidos por sus posibles beneficios para la salud [40, 41]. Los compuestos bioactivos comerciales procedentes de algas incluyen pigmentos naturales (NP), ácidos grasos poliinsaturados (PUFA), lípidos, proteínas y polisacáridos [42, 43]. Algunos de estos compuestos bioactivos y las fuentes naturales de las que derivan se mencionan en la **Tabla 1**. La variabilidad natural en el contenido de moléculas bioactivas puede atribuirse a relaciones evolutivas, diversificación ecológica y química, etc. [44]. La variación en la concentración de compuestos marinos bioactivos de las poblaciones de algas naturales está influenciada, principalmente, por cambios ambientales como la luz, los nutrientes, los contaminantes, la salinidad, la disponibilidad de CO_2, el pH, la temperatura y las interacciones bióticas [45].

También son de destacar los estudios, que se han ido desarrollando a lo largo de los últimos años, sobre el **potencial antienvejecimiento** que presentan muchos compuestos extraídos de diversas especies de algas. En un estudio publicado recientemente en la revista *Science* [46], los autores destacan a la Taurina, un micronutriente semi esencial presente en humanos y en una amplia variedad de especies de animales, vegetales, e incluso en algas, como una molécula clave en el envejecimiento. Las principales vías relacionadas con el antienvejecimiento identificadas en animales, incluyen las vías de señalización de las Sirtuinas (SIRT), regulación del metabolismo energético mediante AMPK, autofagia e IGF (proteína que estimula el crecimiento de muchos tipos celulares). Sin embargo, estas vías o rutas no actúan de manera independiente, sino que suelen trabajar de forma integradora y regulan el inicio o el progreso del envejecimiento a través de interacciones cruzadas entre ellas [47].

Las Sirtuinas son proteínas desacetilasas dependientes de NAD^+ que pueden eliminar grupos acetilo de las histonas. Estas, regulan las redes metabólicas para coordinar la respuesta al estrés celular. Específicamente, las sirtuinas controlan la biogénesis y el metabolismo de las mitocondrias, que son centrales eléctricas celulares. La desregulación de las sirtuinas se ha relacionado con trastornos en la función mitocondrial y con diversas enfermedades en humanos, como diabetes, enfermedades cardiovasculares, enfermedades renales, neurodegeneración y cáncer [48]. La primera sirtuina identificada fue la SIR2/SIRT1, descubierta en la levadura *Saccharomyces cerevisiae*, que se asoció con la extensión de la esperanza de vida, observada bajo restricción calórica, en levaduras. Los humanos expresan siete tipos de sirtuinas (SIRT1-7).

Los compuestos moduladores de las sirtuinas han sido previamente estudiados por sus efectos beneficiosos de antienvejecimiento. Asimismo, algunos compuestos bioactivos procedentes de algas o extractos completos de estas, han demostrado poseer habilidades de activación de las sirtuinas. Por ejemplo, Dutot y col., 2012 [49], estudiaron el efecto

inhibidor frente a estrés oxidativo, inflamación y senescencia de cultivos de células epiteliales humanas en presencia de extractos de un alga, *Ascophyllum nodosum*. Encontraron que los extractos al 0,2% de esta alga, estimulaban la activación de la Sirtuina 1 (SIRT1) hasta 2,33 veces más con respecto al control. Otros autores investigaron el efecto del fucosterol, un esterol extraído del alga parda *Ecklonia stolonifera*, sobre la adipogénesis de adipocitos. Encontraron que este compuesto inhibía la adipogénesis de pre-adipocitos 3T3-L1, sugiriéndose a esta molécula, como un nuevo agente para combatir problemas de obesidad [50]. También los polisacáridos sulfatados, como el fucoidano, que se encuentran de forma exclusiva en algas pardas, han demostrado ser moléculas interesantes en este aspecto. Así, Zheng y col., (2018) [51], observaron que la administración de un extracto de fucoidano extraído del alga *Sacharina japónica*, disminuyó los niveles de marcadores de disfunción hepática, así como de triglicéridos y colesterol, en ratones a través de las vías AMPK/SIRT1.

En la **Tabla 1** se detallan más ejemplos de compuestos bioactivos procedentes de algas con propiedades beneficiosas para la salud humana, tales como antioxidantes, antivirales, antiinflamatorias, etc.

Tabla 1. Ejemplos de compuestos procedentes de extractos de algas con propiedades (1) antibacterianas, (2) antivíricas, (3) antifúngicas, (4) antioxidantes, (5) antinflamatorias, y (6) antitumorales.

Compuesto bioactivo	Especie de alga	Efecto / Target	Rereferencia
(1) Actividad antibacteriana			
Ácidos grasos poliinsaturados (PUFAs)	*Gracilaria corticata* *Ulva fasciata* *Enteromorpha compressa*	Bacterias patogénicas de pescado: *Edwardsiella tarda, Vibrio alginolyticus, Pseudomonas fluorescens, P. aeruginosa, Aeromonas hydrophila*	52
Ácidos grasos (Ácido palmítico) Proteinas (aminoácidos) Flavonoides (rutina, quercetiina, kaempherol)	*Ulva reticulata* *Caulerpa occidentalis* *Cladophora socialis* *Dictyota ciliolate* *Gracilaria dendroides*	*Escherichia coli, P. aeruginosa, Stapylococcus aureus, Enterococcus faecalis*	53
Polisacáridos sulfatados	*Sargassum swartzii*	*S. aureus, Proteus vulgaris, E. coli, Bacillus subtilis, Pseudomonas aeroginosa, Salmonella typhi, Shigella flexineri, Klebsiella pneumoniae, E. faecalis, Aeromonas hydrophilla*	54
Carotenoides, alkaloides, favanoides, ácidos grasos, saponinas, aminoácidos, carbohidratos	*Chlorococcum humicola*	Efecto antibacteriano de los pigmentos β-caroteno y Clorofila sobre: *E. coli, P. aeruginosa, B. subtilis, Salmonella typhimurium, K. pneumoniae, Vibreo cholerae, S. aureus*	55

Tabla 1 (Continuación). Ejemplos de compuestos procedentes de extractos de algas con propiedades (1) antibacterianas, (2) antivíricas, (3) antifúngicas, (4) antioxidantes, (5) antiinflamatorias, y (6) antitumorales.

Compuesto bioactivo	Especie de alga	Efecto / Target	Rereferencia
Péptidos	*Tetraselmis suecica*	Efecto bactericida sobre*: Escherichia coli ML35, Salmonella typhimurium ATCC 14028, Pseudomonas aeruginosa ATCC 27853, Bacillus cereus ISP B7/13, Staphylococcus aureus ATCC 25933, Listeria monocytogenes ATCC 19115, Micrococcus luteus ATCC 9341*	56
(2) Actividad antivírica			
Polisacárido	*Laminaria japonica*	H5N1, RSV	57
Carragenanos, Fucoidano	*Chondrus armatus*	Hantavirus	58
Polisacárido	*Laurencia obtuse*	HCV	59
Polisacárido	*Chlorella vulgaris*	HSV-1	60

Tabla 1. (Continuación). Ejemplos de compuestos procedentes de extractos de algas con propiedades (1) antibacterianas, (2) antivíricas, (3) antifúngicas, (4) antitumorales, (5) antiinflamatorias, y (6) antioxidantes.

Compuesto bioactivo	Especie de alga	Efecto / Target	Rereferencia
(3) Actividad antifúngica			
Extractos metanólicos de algas	*Spirulina Platensis, Ulva lactuca, Sargassum denticulatum, Hormophysa triquetra*	4 especies de *Candida*	61
. Extractos metanólicos de algas	*Ulva lactuca, Chlorella vulgaris, Chlorella minutissima, Chlorella protothecoides*	*Aspergillus niger, Alternaria alternata, y Penicillium expansum*	62
(4) Actividad antitumoral			
B-caroteno	*Dunaliella salina*	Actividad in vitro sobre linea cellular PC-3 de cancer de próstata	63
Esteroles	*Nannochloropsis oculate*	Actividad in vitro sobre linea cellular HL-60 de leucemia promielocítica humana	64
Exopolisacáridos	*Chlorella zofingiensis*	Actividad in vitro sobre linea cellular HCT-8 de cáncer de colon	65
C-Ficocianina	*Spirulina platensis*	Actividad in vitro sobre linea cellular MDA-MB-231 de cáncer de mama	66

Tabla 1. (Continuación). Ejemplos de compuestos procedentes de extractos de algas con propiedades (1) antibacterianas, (2) antivíricas, (3) antifúngicas, (4) antitumorales, (5) antinflamatorias, y (6) antioxidantes.

Compuesto bioactivo	Especie de alga	Efecto / Target	Rereferencia
(5) Actividad antiinflamatoria			
Fitosterol: Fucosterol	*Sargassum binderi*	Reducción de la apoptosis en poblaciones de células Sub-G1 Supresión de las vías NF-κB y MAPKs	67
Ácido Fenólico: Ácido Gálico	*Sargassum horneri*	Atenuación de la expresión de ARNm de TLR, citocinas proinflamatorias, quimiocinas derivadas de células epiteliales de pulmón, citocinas proalérgicas TSLP e IL-33 Supresión de la vía MAPK, ERK y JNK	68
Polifenol: Eckol, Dieckol	*Ecklonia cava*	Inhibición de mediadores inflamatorios y citocinas proinflamatorias Reducción de ROS, producción de NO, muerte celular, mortalidad de larvas y bloqueo de branquias larvales en embriones de pez cebra	69

Tabla 1. (Continuación). Ejemplos de compuestos procedentes de extractos de algas con propiedades (1) antibacterianas, (2) antivíricas, (3) antifúngicas, (4) antitumorales, (5) antiinflamatorias, y (6) antioxidantes.

Compuesto bioactivo	Especie de alga	Efecto / Target	Rereferencia
(6) Actividad antioxidante			
Compuestos fenólicos	*Ascophyllum nodosum, Bifurcaria bifurcata, Chlorella vulgaris, Spirulina platensis*	(i) DPPH (ii) TEAC (iii) ORAC (iv) FRAP; (i) 0.8 µmol TE g−1 DW (ii) 15 µmol TE g−1 DW (iii) 31 µmol TE g−1 DW (iv) 0.6 µmol TE g−1 DW	70
Polifenoles, flavonoides, carotenoides	*Scenedesmus sp.*	*(i) DPPH (ii) FRAP; (i) 0.6–3.7 µmol TE g−1 DW (ii) 2.8–47.0 µmol TE g−1 DW*	71
Carotenoids	*Dunaliella salina*	TEAC: 11–1118 µmol TE g−1 extracto	72
Polifenoles, β-glucano, Coenzima-Q10, β-caroteno, fucoxantina	*Isochrysis galbana*	(i) DPPH (ii) TEAC; (i) 0–17 mg AAE L−1 (ii) 52–56 µmol TE g−1 DW	73
Extractos metanólicos	*2 cepas de Nannochloropsis sp., Picochlorum sp., Desmochloris sp.*	(i) DPPH (ii) FCA (iii) CCA; extractos a 1000 µg mL^{-1} (i) <10% inhib. (ii) <25% inhib. (iii) <30% inhib.	74
Carotenos, Polifenoles, Tocoferoles	*Phaeodactylum tricornutum, Nannochloropsis salina, Nannochloropsis limnetica, Chlorella sorokiniana, Dunaliella salina, Desmodesmus sp.*	(i) DPPH (ii) TEAC (iii) FCA (iv) FRAP (v) TAC; (i) 8–14% inhib. (extractos a 250 µg mL^{-1}) (ii) 2.7–24.2 TE g^{-1} (iii) 3–9% quelación (extractos a 250 µg mL^{-1}) (iv) 0.1–0.5 AAE g^{-1} (v) 3.0–8.9 ácido gálico Eq. g^{-1}	75

2. ALGAS MARINAS COMO INGREDIENTE FUNCIONAL EN PRODUCTOS ALIMENTARIOS

El desarrollo económico, cultural y científico de nuestra sociedad ha dado lugar a cambios importantes en nuestra hábitos alimenticios y estilo de vida. Por ejemplo, las dietas en países desarrollados son altamente calóricas, ricas en grasas saturadas y azúcares, mientras que el consumo de carbohidratos complejos y de fibra dietética es baja. Este hecho, junto con una disminución en la actividad física, ha dado lugar a un aumento de problemas de obesidad, y junto con ello, un aumento en la incidencia de enfermedades del corazón, diabetes e hipertensión en la población [76].

El creciente número de artículos científicos publicados en las últimas dos décadas que correlacionan la dieta y algunas enfermedades crónicas, muestra las extraordinarias posibilidades de los alimentos para mejorar nuestra salud [77]. Como consecuencia, hoy en día existe un gran interés entre consumidores y la industria alimentaria sobre productos que pueden promover la salud y el bienestar [78]. Estos alimentos han sido genéricamente denominados como “alimentos funcionales”.

El concepto de alimento funcional se desarrolló a principios de la década de 1980 en Japón, por unos científicos que estudiaban la relación entre nutrición, satisfacción sensorial y “fortificación”, como elementos para favorecer aspectos específicos para la salud [79]. En Europa, en la segunda mitad de la década de 1990, un grupo de trabajo coordinado por la Sección Europea del Instituto Nacional de Ciencias de la Vida (ILSI) y apoyado por el Comisión Europea, fue creado para promover, dentro del IV Programa Marco de la Unión Europea, la acción FUFOSE (Functional Food Science in Europe) para potenciar el estudio científico sobre alimentos funcionales. A partir de este proyecto, se generó una definición de alimento funcional. Así, un alimento puede ser considerado “funcional” si, además de sus efectos nutritivos, tiene un beneficio demostrado para una o más funciones del organismo humano, mejorando el

estado de salud o bienestar o reduciendo el riesgo de enfermedad [80]. Los alimentos funcionales deben tener una serie de características adicionales. como, por ejemplo, la necesidad de eficacia en su acción beneficiosa a las dosis normales consumidas. En el futuro, los alimentos funcionales estarán regulados por la nueva directiva europea aprobada en diciembre de 2006 (Reglamento (CE) 1924/2006 de la Unión Europea Parlamento y del Consejo, 20 de diciembre de 2006: nutrición y declaraciones de propiedades saludables hechas en los alimentos).

Las algas son organismos fotosintéticos, que poseen estructuras reproductivas simples. Estos organismos constituyen un total de 25.000 a 30.000 especies, con una gran diversidad de formas y tamaños, y que pueden existir como organismos microscópicos unicelulares (microalgas) y pluricelulares de gran tamaño (macroalgas). Las algas pueden ser una fuente natural muy interesante de nuevos compuestos con actividad biológica que podrían ser utilizados como ingredientes funcionales. De hecho, algunas algas son organismos que viven en hábitats complejos sometidos a condiciones extremas (por ejemplo, cambios de salinidad, temperatura, nutrientes, irradiación U-Vis), por lo tanto, deben adaptarse rápidamente a las nuevas condiciones ambientales para sobrevivir, produciendo una gran variedad de metabolitos secundarios (biológicamente activos), que no se puede encontrar en otros organismos [81]. Además de su origen natural, otros aspectos importantes relacionados con las algas son su fácil cultivo, su rápido crecimiento (para muchas de las especies) y la posibilidad de controlar la producción de algunos compuestos bioactivos manipulando las condiciones de cultivo. De esta manera, tanto las macroalgas como las microalgas pueden considerarse como reactores genuinos naturales, y, en algunos casos, una buena alternativa a la síntesis química para ciertos compuestos.

La **Tabla 2** enumera varios alimentos que se comercializan actualmente, que contienen microalgas, de acuerdo a su fecha de publicación en la base de datos Mintel [82]. La mayoría de estos productos contienen Espirulina o Chlorella, principalmente

debido a su larga historia de uso. Por ejemplo, en la UE, las microalgas deben comercializarse en virtud del Reglamento sobre nuevos alimentos (Reglamento UE 2015/2283), cuyo objetivo es facilitar que las empresas puedan introducir alimentos novedosos e innovadores en el mercado de la UE con facilidad, pero manteniendo un alto nivel de seguridad alimentaria para los consumidores europeos [83]. De acuerdo con este reglamento, un alimento nuevo se define como aquel que no se ha consumido en un grado significativo en la UE antes del 15 de mayo de 1997. Debido a su larga historia de uso, el acceso al mercado de Espirulina y Chlorella no está sujeto a este reglamento. Ambas especies están reconocidas como "GRAS" por la Administración de Drogas y Alimentos de los Estados Unidos (FDA), que es el acrónimo de la frase "generalmente reconocido como seguro". Asimismo, los nuevos alimentos que contienen biomasa de otras especies de microalgas llegarán pronto al mercado europeo. En efecto, el producto denominado Plancton Marino Veta la Palma® (Fitoplancton Marino S.L., España), que es la biomasa seca de la microalga *Tetraselmis chuii*, ha sido autorizado recientemente por la Autoridad Europea de Seguridad Alimentaria (EFSA) para ser comercializado como Novel Food, de conformidad con el artículo 3, apartado 1, del Reglamento (CE) nº 258/97 [84].

Tabla 2. Productos alimenticios comercializados hasta 2019 que contienen microalgas [82].

Marca	Compañía	Descripción del producto	País de fabricación	Fecha de publicación en MINTEL*	Contenido en microalga	Información adicional
Gullón Vitalday	Galletas Gullón, España	Tortitas de avena y arroz con espirulina	España	Enero 2019	1 %	-
Nutrecentis di Ab	Agricultura Biologica, Italia	Pasta de maíz blanco y spirulina	Italia	Dieciembre 2018	10%	-
Helga	Evasis Edibles, Austria	Galletas de algas con sabor a sal marina	Alemania	Febrero 2019	5%	Contiene Chlorella
Casino Bio	Casino, Francia	Galletas de espirulina y arándanos	Francia	Abril 2019	2,6%	Producto orgánico certificado Ab y UE
Tic tac	Ferrero Iberica, España	Mix de pastillas con sabor a manzana	Portugal	Abril 2019	-	Contiene Espirulina
Happy Planet	Happy Planet Foods, Canada	Batido de frutas verdes con Spirulina y Chlorella.	Canada	Febrero 2019	-	Contiene Espirulina y Chlorella

* Functional Health Ingredients to Watch, www.mintel.com (2019)

Tabla 2 (Continuación). Productos alimenticios comercializados hasta 2019 que contienen microalgas.

Marca	Compañía	Descripción del producto	País de fabricación	Fecha de publicación en MINTEL*	Contenido en microalga	Información adicional
Mavericks	Maverick Makers Snacks, UK	Palitos de pan veganos ricos en fibra y libres de azúcares añadidos	Reino Unido	Febrero 2019	2%	Contiene Espirulina
Ametller Origen	Casa Ametller, España	Albóndigas veganas con fideos de espelta y tofu	España	Diciembre 2018	20%	Contiene fideos elaborados con Spirulina a una concentración del 20%
Simply Raw Protein RAW BA	Merlo's Best, Alemania	Barrita de frutas rica en proteínas con Spirulina y aceite de limon	Alemania	Febrero 2019	5%	Cuenta con el logotipo de la hoja verde de la UE
Próvida	Próvida Produtos Naturais, Portugal	Galletas Bio con matcha y espirulina	Portugal	Febrero 2019	1%	Cuenta con el logotipo de la hoja verde de la UE

* Functional Health Ingredients to Watch, www.mintel.com (2019)

Tabla 2 (Continuación). Productos alimenticios comercializados hasta 2019 que contienen microalgas.

M&M's	Mars Wrigley Confectionery, Irlanda	Pasta con sabor a chocolate y avellanas	Suiza	Abril 2019	-	Contiene Espirulina como colorante natural
Emmy's Organics	Emmy's Organics, USA	Galletas de coco cubiertas de chocolate con menta	Estados Unidos	Mayo 2019	-	Contiene Espirulina orgánica
Frecious Slow Juice	Frecious, Holanda	Zumo de verduras que contiene Chlorella	Holanda	Febrero 2019	2,4%	Cuenta con el logotipo de la hoja verde de la UE

* Functional Health Ingredients to Watch, www.mintel.com (2019)

2.1. Revisión bibliográfica sobre los beneficios de la adición de algas o extractos de algas en alimentos

En los alimentos para consumo humano, las algas han sido mayoritariamente introducidas en la carne, en productos de panadería, queso, pasta, leche, etc. (**Tabla 3**).

La incorporación de las algas, *Enteromorpha*, *Himanthalia elongata*, *Undaria pinnatifida* y *Porphyra umbilicalis* resultaron en cambios en el potencial antioxidante de la carne y los productos a base de cereales [85].

La carne y los productos cárnicos son valiosas fuentes de proteínas y vitaminas, pero contienen una cantidad insuficiente de fibra dietética y una excesiva cantidad de sodio, que puede ser dañina para los humanos. Por lo tanto, la adición de algas como componente funcional puede ayudar a superar problemas tecnológicos asociados con los productos cárnicos con un bajo contenido de sal, incluidas las propiedades de retención de grasa y agua. Algunos intentos se han llevado a cabo para desarrollar sistemas modelo de emulsiones de carne con bajo contenido en grasas y sal, con adición de varios compuestos bioactivos de algas marinas. Así, las algas comestibles Espagueti de mar (*Himanthalia elongata*), Wakame (*Undaria pinnatifida*) y Nori (*Porphyra umbilicalis*) se incorporaron a la carne, lo que resultó en niveles elevados de K, Ca, Mg y Mn. Además, la presencia de alga Nori aumentó los niveles de serina, glicina, alanina, valina, tirosina, fenilalanina y arginina. En cuanto a la adición de Espagueti de mar, esta aumentó el contenido de aminoácidos azufrados en un 20%. También, el enriquecimiento de carne con algas le aportó compuestos polifenólicos solubles, que aumentaron el potencial antioxidante de todo el sistema [86]. Sasaki y col., (2008) [87] estudiaron el efecto de la fucoxantina, el principal pigmento carotenoide del

alga *Undaria pinnatifida*, sobre la peroxidación lipídica y el color de la carne, en carne de pechuga de pollo. Se encontró que la fucoxantina mejoró la apariencia y prolongó el período de usabilidad de este alimento. También se han formulado filetes de ave frescos bajos en grasa y sal con la adición del alga *Himanthalia elongata* (3%) y transglutaminasa microbiana como ligante en frío. En este producto, los efectos de adición de *Himanthalia elongata*, en las propiedades de unión al agua y la textura varió dependiendo de su estado. En estado bruto, debilitó las propiedades de retención de agua, mejorando la consistencia y manipulación de la muestra, mientras que en los productos cocidos mejoró las propiedades de retención de agua y la textura (con un contenido reducido de sal), lo que fue similar a la de una muestra de control con contenido normal de sal [88]. Asimismo, se han adicionado las algas *Himanthalia elongata y Lamina japonica* en salchichas frankfurt [89, 90]. Sin embargo, algunos aspectos, incluyendo la calidad y aceptación organoléptica, aún requieren estudios más detallados [91].

En cuanto a productos de la pesca, Senthil y col., (2005) [92] utilizaron el alga *Eucheuma* como ingrediente de una chuleta de pescado. Los investigadores encontraron que la adición de hasta un 10% de esta alga, no tuvo efectos negativos sobre la apariencia, textura y aceptación sensorial.

También los aceites comestibles han sido enriquecidos con algas. El extracto de algas rojas marinas *Grateloupia filicina* se añadió al ácido linoleico y aceite de pescado. La adición consistió en un 0,01%, un 0,03% y un 0,05%. Los estudios demostraron que la adición inhibía la oxidación del ácido linoleico y del aceite de pescado al nivel de 0,05% [93]. Otro estudio sobre aceite de pescado y ácido linoleico fue realizado por Siriwardhana y col., (2004) [94] quienes analizaron el efecto inhibitorio del extracto metanólico de *Hizikia fusiformis* sobre la peroxidación lipídica. Las algas contienen antioxidantes resistentes al calor y a la radiación UV. Los resultados mostraron que

los antioxidantes de esta alga pueden ser útiles en la prevención de cambios oxidativos en aceites comestibles.

Los productos a base de cereales son comúnmente aceptados, entre otras razones, debido al fácil proceso de preparación, amplio consumo y bajo costo. Sin embargo, tanto su valor nutricional como sus propiedades para la salud pueden verse mejoradas. Este objetivo puede lograrse mediante la adición de algas, ya que son ricas en sustancias bioactivas. La pasta es uno de los productos a base de cereales más versátiles. Desgraciadamente, también se caracteriza por un bajo contenido en proteínas y aminoácidos esenciales. Por eso, la introducción de aditivos ricos en proteínas, como las algas, pueden aumentar la calidad de la pasta. Estudios de adición de las algas comestibles Wakame (*Undaira pinnatifida*, rica en el pigmento fucoxantina) en productos de pasta, concluyeron que la pasta con suplementación de un 10% de algas era aceptable organolépticamente. Además, la presencia de algas ayudó en la interacción entre los gránulos de almidón y la matriz proteica, lo que resultó en una mejor calidad de la pasta [95]. En otro estudio, se adicionaron extractos del alga *Sargassum marginatum* en pasta, mejorando su biofuncionalidad y calidad [96]. También se crearon fideos frescos de huevo chinos con una adición de algas verdes *Monostroma nitidum* [97], dando lugar a mayores rendimientos de cocción en los fideos e intensidades texturales más suaves y esponjosas.

El pan es otro producto a base de cereales que puede ser portador de varios ingredientes bioactivos, incluidas las algas. Así, la calidad del pan se mejoró mediante la adición de algas verdes *Ulva lactuca*, y la adición de un 2,5% de polvo del alga *Laminaria sp.*, mejorando sus propiedades físico-químicas y sensoriales [98].

Goñi y col., (2000) [99] evaluaron el efecto del consumo de alga Nori (*Porphyra tenera*) junto con el consumo de pan blanco en la respuesta glucémica posprandial. El estudio demostró que las algas podrían ser utilizadas, no solo como alimento, sino también

como un componente que aporta al organismo altas cantidades de fibra dietética soluble. La suplementación de pan blanco con algas disminuyó la tasa de hidrólisis de almidón, y también disminuyó la respuesta glucémica a pan blanco en voluntarios sanos del 68% al 100%.

Igualmente, es interesante el uso de moléculas específicas de algas para su uso como ingredientes funcionales en alimentos o como suplementos dietéticos. Es el caso, por ejemplo, de la Fucoxantina [100]. Asimismo, hay autores que han desarrollado suplementos dietéticos a base de Homotaurina, una molécula específica de algas, para el tratamiento de patologías de la retina [101], e incluso patentes en las que se ha desarrollado alimentos suplementados o enriquecidos con Homotaurina [102]. La laminarina y el fucoidano, dos polisacáridos sulfatados únicamente presentes en algas pardas, también han sido usados como ingredientes funcionales en carne de cerdo, evaluándose su potencial antioxidante [103].

En resumen, la creciente incidencia de enfermedades causadas por un estilo de vida intenso y la creciente importancia de la dieta en la vida humana, lleva a la necesidad, cada vez más creciente, de una nutrición natural y beneficiosa para la salud. El valor de la comida se basa, no solo, en la presencia de nutrientes esenciales, sino también en la disponibilidad de otros compuestos bioactivos que influyen en la salud y facilitan la homeostasis del organismo humano. Las algas son una gran fuente de compuestos biológicamente activos, y se pueden utilizar para el desarrollo de alimentos funcionales, además de ser una fuente rica de moléculas antioxidantes naturales y antimicrobianas. La adición de sus extractos naturales, no solo mejoran la calidad de los productos alimenticios, sino que también limitan el uso de conservantes químicos y conllevan numerosos beneficios para la salud.

Teniendo en cuenta la necesidad de innovaciones constantes y de satisfacer los gustos cambiantes de los consumidores, las algas ofrecen muchas posibilidades interesantes para la creación de nuevos productos alimenticios.

En la **Tabla 3** se muestran numerosos ejemplos de los efectos beneficiosos que produce la incorporación de algas o de extractos de algas en alimentos.

Tabla 3. Efecto de la adición de algas marinas comestibles y extractos de algas sobre las propiedades fisicoquímicas y sensoriales de alimentos.

Alimentos	Especie de alga	Cantidad añadida	Efecto	Rereferencia
Pan				
	Kappaphycus alvarezii (Eucheuma)	Alga en polvo: 2-8 %	- La adición de algas aumentó la absorción de agua de los masa y otros parámetros del farinógrafo. - La adición de polvo de algas redujo las propiedades de pegajosidad. - El pan preparado con harina compuesta de algas tuvo mayores valores de firmeza.	**104**
	Fucus vesiculosus	4 %	Se obtuvo un rendimiento mejorado de la masa de trigo y del pan.	**105**
	Myagropsis myagroides	Extracto de alga: 0,5%, 1%, 2%	El recuento microbiano total disminuyó en panes elaborados con un 2% de extracto de alga.	**106**
	Ascophyllum nodosum *Chondrus crispus*	2%, 4%, 6%, 8%	Este studio encontró que la adición de algas a bajas concentraciones (2%, 4%) en el pan, es acceptable sensorialmente para los consumidores. También hubo un aumento del contenido proteico (adicionando *Chondrus crispus*), y de las cenizas y fibra dietética total.	**107**

Tabla 3 (Continuación). Efecto de la adición de algas marinas comestibles y extractos de algas sobre las propiedades fisicoquímicas y sensoriales de alimentos.

Alimento	Especie de alga	Cantidad añadida	Efecto	Rereferencia
Pan	*Chlorella vulgaris y Arthrospira platensis*	Alga en polvo: 1.5 %	La adición de algas aumentó el contenido de carotenos totals y clorofila a	108
	Chlorella vulgaris	Biomasa: 1 %	Los panes adicionados con biomasa de Chlorella, presentaron mayor firmeza y una capacidad antioxidante mayor.	109
Galletas	*Spirulina maxima*	Biomasa microencapsulada: 10 %	El indice de aceptación sensorial de las galletas suplementadas con bioamasa encapsulada fue de casi el 80%. Tenían un 40% mas de proteina. Se pudo declarer un nuevo claim como “Fuente nutricional de hierro”	110
	Spirulina platensis y Dunaliella salina	Biomasa: 1% y 2%	Los resultados muestran que las galletas horneadas con Spirulina añadida eran significativamente más duras y oscuras que los controles, y la adición de *Dunaliella* no afectó el contenido de proteína tanto como la adición de Spirulina, pero el efecto sobre el contenido de humedad fue significativamente positivo. Además, se encontraron valores más altos de contenido fenólico total y actividad antioxidante para las galletas enriquecidas con *Dunaliella*	111

Tabla 3 (Continuación). Efecto de la adición de algas marinas comestibles y extractos de algas sobre las propiedades fisicoquímicas y sensoriales de alimentos.

Alimento	Especie de alga	Cantidad añadida	Efecto	Rereferencia
Carne	Espagueti de mar (*Himanthalia elongata*), Wakame (*Undaria pinnatifida*), y Nori (*Porphyra umbilicalis*)	Extracto de alga en polvo: 5,6%	- La adición de algas mejoró los niveles de Ácidos grasos poliinsaturados n-3 pero redujo el ratio n-6/n-3 - La adición de Nori y Wakame redujo el índice trombogénico - Se incrementaron los niveles de Na, K, Mg y Mn - Las muestras adicionadas con espagueti de mar tuvieron mayores valores de capacidad antioxidante	86
	Espagueti de mar (*Himanthalia elongata*)	10-40% p/p	La adición de algas marinas a las hamburguesas redujo las pérdidas por cocción y aumentó la ternura (≈50%), el nivel de fibra dietética, contenido fenólico total y actividad de eliminación de radicales DPPH. - Los recuentos microbiológicos y la oxidación de lípidos fueron más bajos en fórmulas que contienen algas. - Las fórmulas preparadas con un 40 % de algas marinas tuvieron el nivel más alto en general de aceptabilidad debido a la textura y la mejora de sensación en boca	112
	Wakame (*Undaria pinnatifida*)	Extracto de alga: 200 mg/kg	- La suplementación con fucoxantina mejoró el enrojecimiento y amarillez en la carne de pechuga de pollo molida, e inhibió la peroxidación lipídica en el almacenamiento en refrigeración después de la cocción. - Se descubrió que la fucoxantina es un ingrediente potente para la mejora de la apariencia y vida útil de la carne de pollo y sus productos.	87
	Cystoseira Barbata	0.04% Fucoxanthin	La suplementación de esta alga en salchichas de carne produjo inhibición de la ACE (enzima convertidora de la angiotensina) para la reducción de la presión arterial; mejora del color y de la estabilidad oxidativa.	113

Tabla 3 (Continuación). Efecto de la adición de algas marinas comestibles y extractos de algas sobre las propiedades fisicoquímicas y sensoriales de alimentos.

Alimento	Especie de alga	Cantidad añadida	Efecto	Rereferencia
Carne				
	Chlorella sp.	Biomasa: 1%	Sin efecto sobre la composición proximal, capacidad de retención de agua, adhesividad y cohesividad. Se alteraron los parámetros de color (valores L*, a* y b* más bajos)	114
	Spirulina sp.	Sustitutivo de la proteina de la soja (agente emulsificante)	Incremento en el contenido de amino ácidos totals y en el ratio de amino ácidos esenciales / no esenciales	114
	Spirulina sp.	1% p/p	Los productos adcionados con Spirulina presentaron los mayores contenidos en amino ácidos totals y en el ratio de amino ácidos esenciales / no esenciales	114
Queso				
	Palmaria palmata, Saccharina longicruris	Alga en copos: 2 % Palmaria; 2% Sacharina	Este estudio confirmó el potencial de las algas para combinarse con un alimento fermentado, como un queso, sin causar un impacto negativo en la evolución global del producto. Su presencia podría aumentar la diversidad y el contenido nutricional al mantener el contenido total de sodio. Los quesos de algas agregan fibra comestible, que está ausente en el queso normal.	115
Leche				
	Ascophyllum nodosum y Fucus vesiculosus	Extractos de algas: 0,25; 0,50 %	- Los extractos preparados con agua fueron más aceptables que los extractos etanólicos. - Los extractos de algas marinas eran estables en la leche y tenían diferentes grados de actividad antioxidante antes y después de la digestión in vitro. - La adición de extractos de algas marinas mejoró determinadas características de calidad y vida útil de la leche	116

Tabla 3. (Continuación). Efecto de la adición de algas marinas comestibles y / o extractos de algas sobre las propiedades fisicoquímicas y sensoriales de alimentos.

Alimento	Especie de alga	Cantidad añadida	Efecto	Rereferencia
Leche				
	Nannochloropsis oculate, Porphyridium cruentum, Diacronema vlkianum	Biomasa: 0.1%, 0.2%, 03%	En cuanto a las características sensoriales, las muestras de helado producidas con P. cruentum fueron preferidas más que las otras muestras. El contenido fenólico de las muestras de helado aumentó con el uso de microalgas.	117
	Athrospira platensis.	Biomasa: 0.1%, 0.3%, 0.5%, 1%, 5%, 10%	Incremento en el contenido de ficocianina en todas las formulaciones	118
Arroz				
	Nannochloropsis oceanica	Hierro extraido del alga: 39 mg Fe/kg	Los animales de experimentación alimentados con polvo de arroz suplementado con hierro (extraido del alga), presentaron mejoría en anemia.	119
	Spirulina plantensis	2.5% - 7.5%	Los snacks de arroz suplementados con alga presentaron mayores contenidos en proteina, fibra dietética y actividades antioxidantes	120
Zumo				
	Dunaliella salina y Chlorella vulgaris	Biomasa: 2.5%	Los smoothies suplementados con biomasa de *Dunaliella* presentaron mayor contenido en polifenoles totales y capacidades antioxidantes. Disminución de la carga microbiana con ambas especies. Sensorialmente, los smoothies suplementados con *Dunaliella* fueron preferidos.	121

Tabla 3. (Continuación). Efecto de la adición de algas marinas comestibles y extractos de algas sobre las propiedades fisicoquímicas y sensoriales de alimentos.

Alimento	Especie de alga	Cantidad añadida	Efecto	Rereferencia
Pasta				
	Monostroma nitidum	Extracto de alga en polvo: 3%; 6%	- Los fideos preparados con 6% de algas tuvieron mayor rendimiento de cocción - La adición de algas produjo muestras con mejor resistencia a la fuerza de tracción. - La menor extensibilidad se observó en las muestras que contenía el nivel más alto de algas y pasta de sepia. - Una mayor absorción de agua por parte de las algas, dió lugar a una mayor suavidad e intensidades texturales más esponjosas en los fideos	122
	Monostroma nitidum	Extracto de alga en polvo: 4%, 6%, y 8%	- Se observaron mayores rendimientos de cocción en los fideos. - Se obtuvieron intensidades texturales más suaves y esponjosas. - Los parámetros de textura de los fideos se vieron afectados no solo por la adición de huevos y polvo de algas, sino también por propiedades de cocción	123
	Spirulina platensis	Extracto proteico: 6%, 8%, 10%	La pasta enriquecida con el ectracto proteico mejoró en cuanto a textura, color, y valores nutricionales mas altos (proteinas y minerals).	124
Pakora				
	Enteromorfa	Extracto de polvo de alga: 5%; 7,5%; 10%; 12,5%; 15%	- El aumento en el nivel de algas mejoró el contenido en cenizas, proteína, contenido de fibra dietética total, calcio y hierro. - La adición de algas aumentó el poder reductor y reducción de la actividad de eliminación de radicales libres (DPPH) y el contenido de fenoles totales - La pakora que contenía algas marinas hasta en un 7,5% fue la más aceptable sensorialmente .	125

3. TÉCNICAS ANALÍTICAS EMPLEADAS DE CARACTERIZACIÓN DE COMPUESTOS BIOACTIVOS Y ENSAYOS DE ACTIVIDAD BIOLÓGICA EN ALGAS.

Los últimos años han sido testigos de un mayor interés por los nutracéuticos y los compuestos bioactivos. La posibilidad de preservar la salud consumiendo alimentos beneficiosos específicos o para tratar algunas enfermedades consumiendo los suplementos que contienen componentes naturales, son atractivos tanto para los consumidores como para las industrias alimentarias. A escala de laboratorio, los esfuerzos están dirigidos a extraer nuevas sustancias bioactivas de las matrices alimentarias e identificar y evaluar sus propiedades y / o actividades. Para ello, se buscan enfoques analíticos más rápidos, más baratos y automatizables para recuperar estas moléculas de alto valor. Así, se han utilizado una amplia variedad de técnicas de análisis tanto separativas como no separativas. [126]

Se han utilizado diferentes técnicas separativas para la investigación de compuestos bioactivos, que incluyen cromatografía líquida de alta resolución (HPLC), y cromatografía de gases (GC), pero también otros tipos de enfoques cromatográficos [127]. Asimismo, se han empleado la espectroscopía de resonancia magnética nuclear, espectroscopía infrarroja y espectroscopía Raman [128-140]**.** Sin embargo, las técnicas de elección son la HPLC y la GC, especialmente con detectores de espectrometría de masas en tándem (MS / MS) o MS de alta resolución para la caracterización de analitos. Solo cuando las sustancias ya se conocen es posible utilizar otros detectores, como el detector de matriz de diodos (DAD) o UV-Vis (ultravioleta-visible).

En esta tesis doctoral, se ha empleado la HPLC acoplada a la espectrometría de masas en tándem para la caracterización analítica de compuestos bioactivos. Los ensayos llevados cabo de actividad biológica en algas han sido ensayos de actividad antioxidante, y ensayos de modulación de la Sirtuina 1.

3.1. Técnicas cromatográficas y sistemas de detección

Técnicas cromatográficas

La cromatografía líquida de alta resolución o HPLC (por sus siglas en inglés, High-Performance Liquid Chromatography) es una técnica analítica de separación basada en la capacidad diferencial de los componentes de una muestra para interactuar con una fase móvil y una fase estacionaria. La HPLC se ha convertido en una de las técnicas analíticas más utilizadas en la actualidad debido a su versatilidad, rapidez, precisión y sensibilidad en la separación, identificación y cuantificación de compuestos en una amplia gama de muestras.

La HPLC se basa en el principio de la cromatografía, que se refiere a la separación de los componentes de una muestra mediante su interacción selectiva con una fase estacionaria y una fase móvil. La fase estacionaria es un material sólido o líquido inmovilizado en una columna, mientras que la fase móvil es un disolvente líquido que se mueve a través de la columna. La muestra se inyecta en la columna y se separa en sus componentes individuales según su afinidad por la fase estacionaria y la fase móvil. La separación se logra gracias a la interacción diferencial de los componentes de la muestra con la fase estacionaria y la fase móvil, lo que produce diferentes tiempos de retención en la columna. [141]

En la HPLC, la fase estacionaria es un material sólido o líquido de alta superficie y porosidad, que se encuentra dentro de una columna cromatográfica. La fase móvil es un disolvente líquido que se mueve a través de la columna a alta presión y velocidad constante. El sistema de bomba de la HPLC se utiliza para crear una presión constante y controlada de la fase móvil que fluye a través de la columna. La selección adecuada de la fase estacionaria y la fase móvil es crucial para lograr una separación óptima de los componentes de la muestra.

Los componentes de un sistema HPLC común son: Recipientes para la fase móvil, bomba, inyector de muestra, columna HPLC, detector, y sistema de adquisición de datos. (**Firgura 7**).

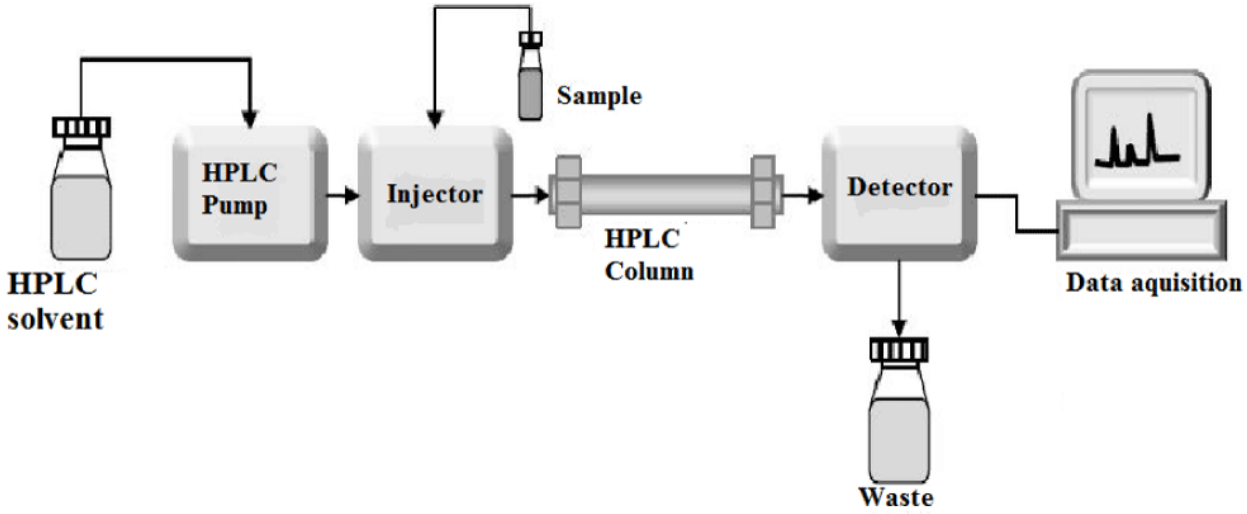


Figura 7. Componentes de un sistema HPLC [142].

La HPLC se puede clasificar en varios tipos según la naturaleza de la fase estacionaria y la fase móvil utilizadas. Estos tipos incluyen la cromatografía de fase normal (NP-HPLC), la cromatografía de fase reversa (RP-HPLC), la cromatografía de exclusión molecular (SEC-HPLC), la cromatografía de afinidad (AC-HPLC) y la cromatografía de intercambio iónico (IC-HPLC), entre otros. Cada tipo de HPLC tiene sus propias ventajas e inconvenientes, lo que permite la selección de la técnica más adecuada para la separación de los componentes de la muestra en cuestión. [142]

Debido a la complejidad de las matrices alimentarias y a la gran variedad estructural de las moléculas que contienen, la separación de compuestos bioactivos y su análisis por HPLC no es una tarea fácil y a menudo requieren tiempos de ejecución prolongados. Sin embargo, en las últimas décadas, la investigación sobre compuestos bioactivos está obteniendo ventajas debido al desarrollo de nuevas tecnologías de fase

estacionaria. De hecho, los materiales de empaquetamiento de estas y el tamaño de sus partículas juegan un papel fundamental en la separación por HPLC. El empleo de materiales de empaquetado cromatográfico de partículas más pequeñas permite la mejora de resolución, eficiencia y sensibilidad del método, porque los picos cromatográficos resultantes son más estrechos y altos [143]. La alta contrapresión resultante se puede aliviar mediante el uso de columnas cortas, que también garantizan bajos volúmenes muertos. Además, la introducción de instrumentos de ultra alto rendimiento (UHPLC-ultra high performance liquid chromatography), capaces de operar en límites de presión de hasta 1500 bar, equipados con sistemas de termostato cada vez más eficientes y reproducibles, permite el funcionamiento con columnas cromatográficas de pequeño diámetro interno (generalmente 2,1 mm d.i.) a un caudal más alto que los instrumentos HPLC clásicos, resolviendo así cientos de compuestos en tiempos de ejecución muy cortos.

La búsqueda de una separación más rápida y eficiente ha dado lugar a varias generaciones de materiales de empaquetamiento con tamaños de partícula cada vez más pequeños (**Figura 8**). Así, el tamaño típico de partícula en los años 1970 para partículas no porosas o totalmente porosas, era de 10 µm; en los años 1980, de 5 µm; en los años 1990, de 3.5 µm, y en los años 2000 a 2006 y en la actualidad, se ha pasado a partículas sub-2µm y superficialmente porosas de tamaño sub-3µm. Sin embargo, los avances en esta tecnología aún no han terminado, y actualmente varios grupos de investigación en el mundo están investigando nuevos materiales (por ejemplo, nano esferas de sílice mesoporosas dendríticas) con tamaños de partícula desafiantes (alrededor de 170 nm).

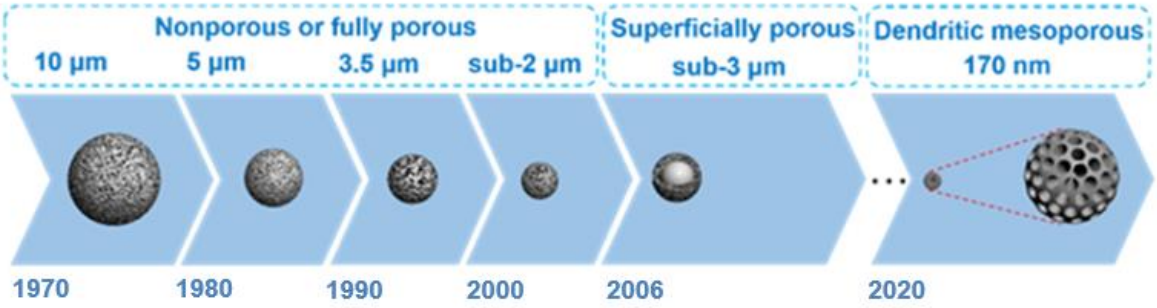


Figura 8. Evolución de partículas de empaquetado de columnas HPLC [143]

Sin embargo, la eficiencia en la separación no solo depende de la tipología de la fase estacionaria, sino que se ve fuertemente afectada por la optimización de los parámetros operativos, como la temperatura de la columna, la composición de la fase móvil y el caudal. [143]

En cuanto al sistema de detección en HPLC, las características que debería reunir un detector ideal serían [144]:

1. Alta sensibilidad y respuesta reproducible y predecible.
2. Responde a todos los solutos o tener una especificidad predecible.
3. Amplio rango dinámico lineal; Respuesta que aumenta linealmente con la cantidad de soluto.
4. Respuesta no afectada por cambios de temperatura y flujo de fase móvil.
5. Respuesta independiente de la fase móvil.
6. No contribuye al ensanchamiento de la banda extra columna.
7. Fiable y cómodo de usar.
8. No destructivo del soluto.
9. Proporciona información cualitativa y cuantitativa sobre el pico detectado.
10. Respuesta rápida.

Sin embargo, no existe ningún detector que reúna todas las características antes mencionadas; de ahí, que existan una amplia variedad de sistemas de detección en función de su aplicación.

Así, los detectores más comúnmente usados en HPLC, junto con sus características y aplicaciones, se pueden enumerar en la **Tabla 4**.

Tabla 4. Detectores en HPLC [144].

Detector	Características	Rango de aplicaciones	Cantidad minima detectable	Rango dinámico lineal	Limitaciones
Uv-Vis/PDA		Selectivo; Universal a bajas longitudes de onda	Nanogramos	10^5	Debe tener un cromóforo; disolventes deben ser transparentes; respuesta muy variable para diferentes solutos
Fluorescencia		Muy selectivo	Picogramos	10^3-10^4	No todos los compuestos emiten fluorescencia; a menudo requiere la formación de derivados; quenching
Electroquímico		Muy selectivo	Femtogramos - Picogramos	10^5	La fase móvil debe ser conductora; susceptible al ruido de fondo y ensuciamiento de electrodos; solo aplicable a los compuestos que pueden ser oxidados o reducidos
Radioactivo		Selectivo	Picogramos	10^3-10^4	Grandes volúmenes en celda de flujo aumentan anchura de pico y disminuyen resolución

Tabla 4 (Continuación). Detectores en HPLC.

Detector	Características	Rango de aplicaciones	Cantidad minima detectable	Rango dinámico lineal	Limitaciones
Conductividad	Detector de elección para cromatografía iónica (iones orgánicos e inorgánicos)	Selectivo	Nanogramos	10^4	Requiere supresión de la conductividad de fondo de la fase móvil; no todos los compuestos se detectan; requiere sistemas y columnas especiales de HPLC
Índice de Refracción	No destructivo; Costo; confiable y fácil de operar	Universal	Microgramos	10^3	Baja sensibilidad; Incompatible con gradiente; intestabilidad (temperatura, flujo)
Light scattering (ELSD)	Detecta la mayoría de los analitos no volátiles; Funciona bien con HPLC en gradiente; Mejor sensibilidad que la detección por RID	Universal	Nanogramos	10^3	Requiere uso tampones volátiles; rango dinámico limitado
Descarga de corona	Máxima sensibilidad entre los detectors universales; Amplio rango dinámico; Detecta cualquier no volátil o semivolátil; Respuesta consistente	Universal	Nanogramos	10^4	Requiere uso de tampones volátiles

Además de los detectores antes mencionados, es de destacar la espectrometría de masas. Esta se ha convertido, en las dos últimas décadas, en la técnica de detección más usada en la gran mayoría de laboratorios del mundo, tanto acoplada a sistemas HPLC, como a GC. John B. Fenn, el creador de la ionización por electrospray para biomoléculas y Premio Nobel de Química en 2002, probablemente dio la respuesta más acertada a qué es la espectrometría de masas: [145]

"*La espectrometría de masas es el arte de medir átomos y moléculas para determinar su peso molecular. Tal información de masa o peso a veces es suficiente, frecuentemente necesaria y siempre útil para determinar la identidad de una especie. Para practicar este arte, se cargan las moléculas de interés, es decir, el analito, y luego se mide cómo responden las trayectorias de los iones resultantes en el vacío a varias combinaciones de campos eléctricos y magnéticos*".

Un espectrómetro de masas estándar consta de cuatro componentes básicos: una entrada de muestra, una fuente de ionización, un analizador de masas, y un detector de iones (**Figura 9**).

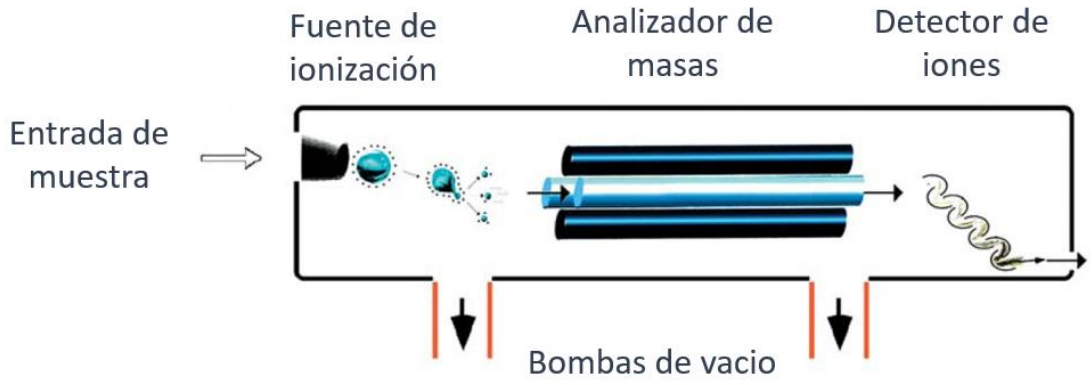


Figura 9. Componentes de un espectrómetro de masas [146].

Algunos instrumentos combinan la entrada de muestras y la fuente de ionización, mientras que otros combinan el analizador de masas y el detector. Sin embargo, todas las moléculas de muestra se someten a los mismos procesos, independientemente de

la configuración del instrumento. Las moléculas de muestra se introducen en el instrumento a través de una entrada de muestra. Una vez dentro del instrumento, las moléculas de la muestra se convierten en iones en la fuente de ionización, antes de ser propulsadas electrostáticamente al analizador de masas. A continuación, los iones se separan según su relación masa/carga (m/z) dentro del analizador de masas. El detector convierte la energía iónica en señales eléctricas, que luego se transmiten a un ordenador. El analizador de masas, el detector y algunas fuentes de ionización funcionan en condiciones de alto vacío para permitir que los iones lleguen al detector sin colisionar con otras moléculas o átomos gaseosos. [146]

Los resultados se presentan en forma de espectro de masas, es decir, una representación en dos dimensiones de la intensidad de la señal (abundancia de especies iónicas, en eje de ordenadas), versus la relación m/z (en eje de abscisas) (**Figura 10**).

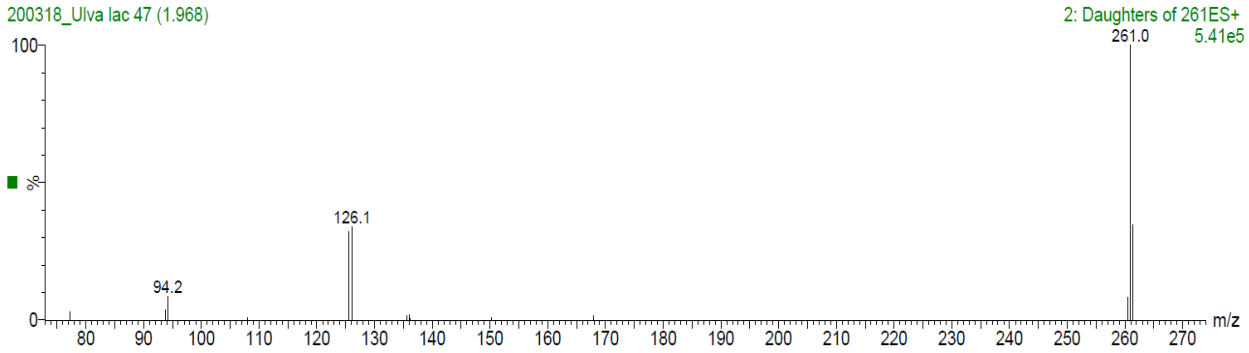


Figura 10. Espectro de masas de la Taurina. (Imagen obtenida en los laboratorios del IRTA mediante la plataforma UPLC-DAD-Triple Cuadrupolo/Masas)

En la actualidad, se pueden encontrar en el mercado una amplia variedad de espectrómetros de masas, que difieren en función tanto del tipo de fuente de

ionización (impacto electrónico-EI, electrospray-ESI, MALDI), como del tipo de analizador de masas (Cuadrupolo o Triple Cuadrupolo-Q/QqQ, tiempo de vuelo-TOF, trampa iónica y trampa iónica lineal-QIT/LTQ, Orbitrap, etc.).

Las características más destacables de los distintos tipos de espectrómetros de masas se resumen en la **Tabla 5**.

Tabla 5. Espectrómetros de masas más comúnmente usados, características y aplicaciones [147]

Instrumento	**Características**				**Aplicaciones**
	Fuente de ionización	**Resolución de masa**	**Sensibilidad**	**Rango dinámico**	
QIT, LTQ	ESI & MALDI	Baja a media	Media	Limitado a medio	Estudios metabolómicos (no dirigidos)
QqQ	ESI	Baja	Alta	Muy amplio	Cuantificación de moléculas a nivel trazas
TOF-TOF	MALDI	Media	Media	Limitado	Proteómica, peptidómica
QTOF	ESI & MALDI	Media	Media	Medio	Estudios metabolómicos (no dirigidos)
LTQ-Orbitrap	ESI & MALDI	Media a alta	Media	Limitado a medio	Estudios metabolómicos (no dirigidos)
FT-ICR	ESI & MALDI	Muy alta	Media	Muy limitado	Composición molecular basada en masa exacta

3.2. Validación de métodos analíticos.

La validación de métodos analíticos es un proceso sistemático que se utiliza para confirmar que un método analítico es adecuado para su uso previsto. Este proceso incluye la evaluación de una serie de parámetros como son la selectividad, precisión, exactitud, linealidad, límite de detección, límite de cuantificación o determinación, robustez, e incertidumbre del método analítico, entre otros.

El término **selectividad** es actualmente preferido por la IUPAC [148] frente al término especificidad. Se refiere a la capacidad del método analítico para cuantificar un analito en presencia de otros analitos, matrices, o potenciales interferentes. La interferencia con la matriz suele solventarse mediante el uso de diferentes técnicas de extracción (extracción en fase sólida, por ejemplo), y cromatografía. En este último caso, para evaluar la selectividad, se suele emplear el término de Resolución, R_S. Esta se determina teniendo en cuenta tanto los tiempos de retención del analito de interés y el tiempo de retención del pico más cercano (t_1 y t_2), como las anchuras de ambos picos cromatográficos (W_1 y W_2), de acuerdo a la siguiente fórmula,

$$R_S = 2x\frac{(t_2 - t_1)}{W_1 + W_2}$$

De acuerdo con la AOAC [149], una resolución cromatográfica de 1,5 será suficiente, considerando el método analítico como selectivo.

La **precisión,** que se define como el grado de concordancia entre los resultados obtenidos de una serie de mediciones repetidas del analito de interés bajo ciertas condiciones, suele englobar tres conceptos:

1. La precisión en condiciones de repetibilidad, RSDr, se obtiene mediante medidas repetidas del mismo analito llevadas a cabo por el mismo laboratorio, analista, equipo, reactivos, etc., en un periodo corto de tiempo.
2. La precisión en condiciones de reproducibilidad, RSD_R, se refiere a las medidas repetidas sobre el mismo analito por diferentes laboratorios, equipos, analistas, días, etc. Representa el valor máximo esperado de precisión del método en cuestión.
3. Y la precisión intermedia, es la medida repetida del analito por el mismo laboratorio, pero en diferentes, días, reactivos, analistas, instrumentos, etc.

El cálculo de este parámetro, según la AOAC [149], tiene cuenta tanto la desviación estándar como la media aritmética de estas medidas repetidas, según la expresión,

$$RSD\% = \frac{s}{x} x100$$

La **exactitud**, se refiere a la proximidad del valor obtenido por el método con respecto a un valor "verdadero" o aceptado. Se suele determinar mediante la "Recuperación", que es la fracción o porcentaje de analito recuperado al final del proceso de extracción del método. De acuerdo con la AOAC, esta prueba de recuperación se debería hacer sobre materiales de referencia certificados (CRFs), comercializados por laboratorios metrológicos, que contienen al analito de interés a una concentración certificada. En caso de no haber disponibilidad de estos CRFs, se pueden utilizar blancos de matriz, a los que se introduce el analito a una concentración conocida. Este valor de recuperación se obtiene de acuerdo a la expresión,

$$R\% = \frac{X_t}{X_v} x100$$

donde X_t, es el valor de concentración obtenida con el método, y X_v, es el valor de concentración "verdadero" o certificado.

La **linealidad**, mide el grado de ajuste de una curva o recta de regresión obtenida mediante diferentes concentraciones de un estándar o patrón analítico del analito de interés. Tiene en cuenta, no solo el coeficiente de correlación R^2 entre las señales analíticas y las concentraciones, sino también otras pruebas estadísticas que miden la bondad del ajuste de calibración o regresión [149].

El **límite de detección** y **cuantificación** de un método de análisis se pueden definir como la cantidad o concentración más pequeñas que se pueden detectar o cuantificar, respectivamente, con una confiabilidad aceptable [149]. Estos límites se suelen determinar estudiando la variabilidad de un blanco. Así, el límite detección es el valor del blanco más tres veces la desviación estándar del blanco ($x_b + 3sd_b$), mientras que el límite de cuantificación es el valor del blanco más diez veces la desviación estándar del blanco ($x_b + 10sd_b$).

La **robustez** de un método analítico se evalúa mediante un experimento específico en el que se introducen pequeñas variaciones de un mínimo de 7 factores del método analítico completo (por ejemplo, volumen de extracción, temperatura incubación, etc.), y se mide en qué grado afectan estas variaciones a la desviación estándar. Uno de los métodos más usados para evaluar la robustez de un método analítico es el método de Youden [150].

Finalmente, la **incertidumbre** de medida, cuya definición metrológica [151] es "*el parámetro asociado con el resultado de una medición que caracteriza la dispersión de valores que podrían atribuirse razonablemente al mensurando*". Se trata de un error "global" del método analítico, que debería ser añadido en cada informe de

resultados de todo laboratorio acreditado bajo ciertas normas de calidad. Tiene en cuenta las incertidumbres (o errores) parciales debidas a múltiples fuentes de error durante todo el proceso analítico, ya sean la incertidumbre debida al uso de balanzas, dispositivos de medidas volumétricas, etc., y la suma de todas estas, da como resultado la llamada incertidumbre expandida.

Existen varias normas y guías de calidad que establecen los requisitos y procedimientos para la validación de métodos de análisis. A continuación, se mencionan algunas de las más importantes:

1. La "Guía de Validación de Métodos Analíticos" de la IUPAC [148] (Unión Internacional de Química Pura y Aplicada, por sus siglas en inglés) es un conjunto de directrices establecidas por la IUPAC para la validación de métodos analíticos utilizados en química y ciencias relacionadas. Esta guía proporciona recomendaciones y pautas sobre los aspectos clave de la validación de métodos analíticos, incluyendo la selección de características de desempeño, la determinación de la selectividad y especificidad del método, la evaluación de la exactitud, precisión, linealidad, límites de detección y cuantificación, así como la estimación de la incertidumbre de los resultados analíticos.
2. AOAC INTERNATIONAL Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [149]: Esta guía publicada por la Asociación Oficial de Químicos Analíticos (AOAC, por sus siglas en inglés) establece los requisitos y procedimientos para la validación de métodos de análisis utilizados en la industria de los suplementos dietéticos y productos botánicos.

3. Norma ISO 17025 [152]: Esta norma internacional establece los requisitos generales para la competencia técnica de los laboratorios de ensayo y calibración. La validación de métodos de análisis es uno de los requisitos clave de la norma ISO 17025.
4. ICH Q2 (R1) Validación de Métodos Analíticos [153]: Esta guía publicada por el Consejo Internacional de Armonización de Requisitos Técnicos para el Registro de Productos Farmacéuticos para Uso Humano (ICH, por sus siglas en inglés) establece los requisitos y procedimientos para la validación de métodos de análisis utilizados en el desarrollo y fabricación de productos farmacéuticos.

En general, estas normas y guías de calidad establecen requisitos y procedimientos específicos para la validación de métodos de análisis en diferentes sectores, y son útiles para garantizar que los resultados obtenidos sean precisos, confiables y reproducibles.

3.3. Ensayos para la determinación de la actividad biológica en algas y en extractos de algas

En las últimas décadas se han ensayado una amplia variedad de actividades biológicas en algas (**Tabla 3**). Las que se han desarrollado en el marco de esta tesis doctoral son las actividades antioxidantes y las de modulación de la Sirtuina 1.

3.3.1 Ensayos de actividad Antioxidante

Las especies reactivas de oxígeno (ROS, en inglés, *reactive oxygen species*) como el anión superóxido ($O_2^{-\cdot}$), hidroxilo ($^{\bullet}OH$), peroxilo ($ROO^{\bullet}$), radicales alcoxilo ($RO^{\bullet}$) y peróxido de hidrógeno (H_2O_2), producidas por luz ultravioleta, radiaciones ionizantes,

procesos metabólicos, etc., pueden atacar macromoléculas biológicas in vivo, lo que puede dar lugar a daños en proteínas, lípidos y ADN, envejecimiento celular y enfermedades originadas por estrés oxidativo [154]. Un antioxidante puede ser definido como "cualquier sustancia que cuando está presente en concentraciones relativamente bajas, en comparación con las del sustrato oxidable, retrasa o inhibe significativamente la oxidación de ese sustrato"[155].

En términos generales, y de acuerdo con las reacciones químicas involucradas, los ensayos de actividad antioxidante se pueden clasificar en 3 grandes grupos:

1. Ensayos "HAT" ("*hydrogen atom transfer*"), de transferencia de protones. Miden la habilidad de una sustancia antioxidante de eliminar radicales libres donando un átomo de hidrógeno. Los HAT se producen de acuerdo a la siguiente reacción,

$$ROO^{\cdot} + {}^{AH}/_{ArOH} \leftrightarrow ROOH + {}^{A^{\cdot}}/_{ArO^{\cdot}}$$

   donde el átomo de hidrógeno (H) de un grupo fenol (ArOH) es transferido a un radical peroxilo, neutralizándolo. Ejemplos de ensayos HAT son a) el ensayo de absorción de radicales de oxígeno (ORAC); b) el ensayo de captura de radicales peroxilo (TRAP); c) el ensayo de capacidad de captación de oxiradicales (TOSC); d) el ensayo del ácido tiobarbitúrico (TBA). [156]

2. Ensayos "SET" ("*single electron transfer*"), de transferencia de un electrón, también llamados de transferencia electrónica (ET, "*electronic transfer*"). Detectan la habilidad de un antioxidante de transferir un electrón para reducir iones metálicos, grupos carbonilo y radicales libres. Estos ensayos siguen las siguientes reacciones,

$$ROO^{\cdot} + {}^{AH}/_{ArOH} \leftrightarrow ROO^{-} + {}^{AH^{\cdot+}}/_{ArOH^{\cdot+}}$$

$${}^{AH^{\cdot+}}/_{ArOH^{\cdot+}} + H_2O \leftrightarrow {}^{A^{\cdot}}/_{ArO^{\cdot}} + H_3O^{+}$$

$$ROO^{-} + H_3O^{+} \leftrightarrow ROOH + H_2O$$

Ejemplos de estos ensayos son a) el ensayo del poder reductor / antioxidante férrico (FRAP); b) el ensayo del poder reductor / antioxidante cúprico (CUPRAC).

3. Ensayos mixtos "HAT/SET", están basados en la eliminación de un cromóforo estable (como el ABTS o DPPH), donde los mecanismos HAT, SET y de transferencia de electrones acoplados a protones (PCET, "*proton-coupled electron transfer*"), dependen de condiciones de reacción como el pH o el tipo de disolvente en que se desarrollan. Estos ensayos mixtos incluyen a) el ensayo de la capacidad antioxidante equivalente de ABTS/Trolox (TEAC, "*trolox equivalent antioxidant capacity"*); b) el ensayo de neutralización del radical DPPH; c) el ensayo de neutralización del radical DMPD. Los 2 primeros, son los ensayos empleados para medir actividades antioxidantes de algas y extractos de algas en esta tesis doctoral.

   3.1. Ensayo ABTS / TEAC.

   El ensayo TEAC fue desarrollado por primera vez por Miller y colaboradores [157]. Mide la capacidad de las sustancias antioxidantes para neutralizar el catión radical estable $ABTS^{\bullet+}$ (ácido 2,2'–azino–bis–(3–etillbenzotiazolin–6–sulfónico), un cromóforo de color verde cuya absorbancia máxima se produce a 734 nm. El grado de decoloración de este catión radical, que se produce en presencia de persulfato de potasio, y es medido también a 734 nm, depende de

la duración de la reacción, de la actividad antioxidante intrínseca, y de la concentración de la muestra (**Figura 11**).

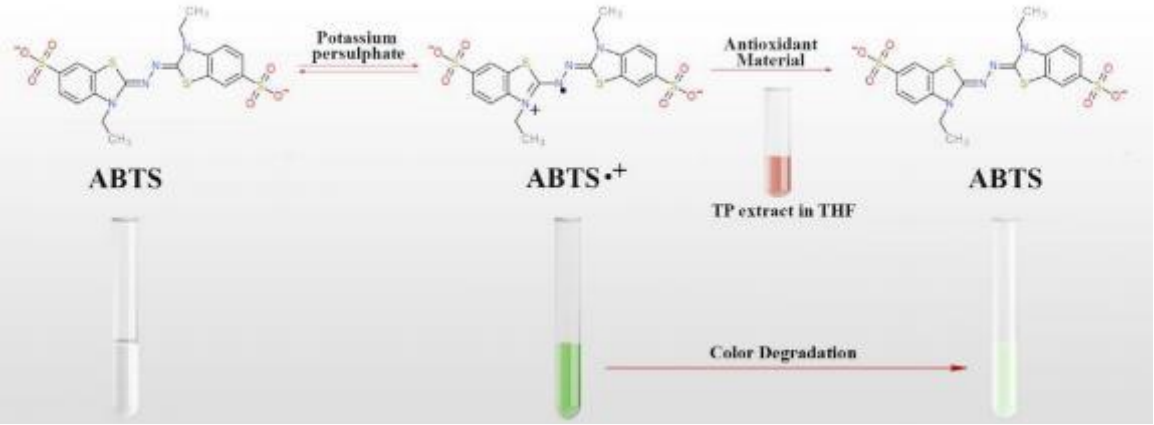


Figura 11. Representación esquemática del método de captación de radicales ABTS. [158]

3.2. Ensayo DPPH.

El ensayo DPPH fue desarrollado por Marsden S. Blois en 1958 [159]. Más tarde, fue ligeramente modificado por numerosos investigadores. Es uno de los ensayos de antioxidantes más utilizados para muestras de plantas y alimentos. Se basa en la donación de electrones por parte de sustancias antioxidantes presentes en la muestra, para neutralizar al radical DPPH (2,2-Difenil-1-Picrilhidrazilo). La reacción de neutralización viene acompañada por cambios en la coloración (de púrpura a amarillo pálido o transparente), leídos a 517 nm., mediante un espectrofotómetro visible o un espectrómetro de resonancia paramagnética electrónica (**Figura 12**).

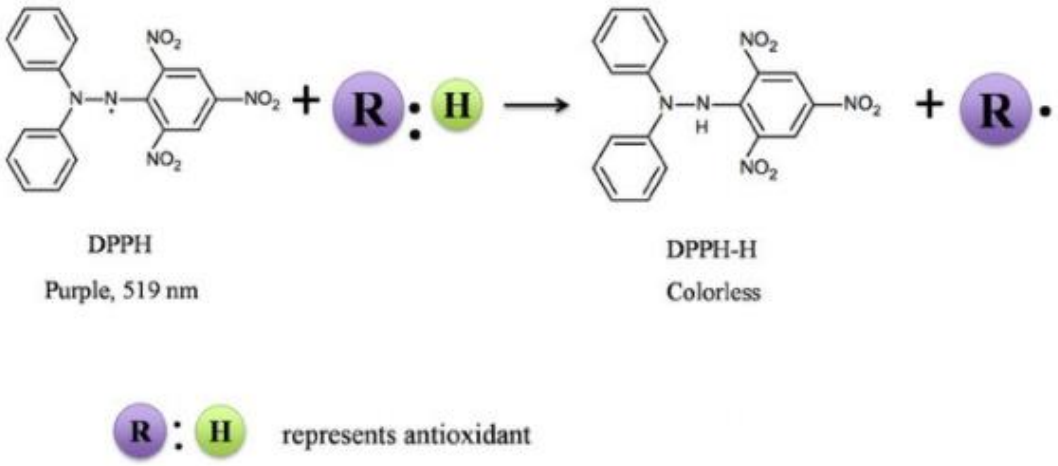


Figura 12. Mecanismo de reacción de 2,2-difenil-1-picrilhidrazilo (DPPH) con antioxidante. R: H = eliminador de radicales antioxidantes; R = radical antioxidante [160].

3.3.2. Ensayos de activación / inhibición de la Sirtuina 1

Las sirtuinas comprenden una familia de proteínas altamente conservadas y que se encuentran en prácticamente todas las especies de mamíferos y bacterias. En mamíferos han sido identificadas hasta 7 tipos (Sirtuina 1 – Sirtuina 7). En un principio se las incluyó en el grupo de las histonas (proteínas unidas al ADN) desacetilasas de clase III dependientes de NAD^+ (eliminan un grupo acetilo de un residuo del aminoácido lisina), pero actualmente se sabe que algunas sirtuinas presentan actividades de desacetilación de otras proteínas no histonas importantes, además de poseer otras actividades más allá de la desacetilación (ADP-ribosil transferasa). Sin embargo, la más caracterizada y relacionada con procesos tales como el control metabólico, la apoptosis, la supervivencia y envejecimiento celulares, etc., es la actividad de desacetilación de histonas. Esta reacción se esquematiza en la

Figura 13, y consiste en la transferencia de un grupo acetilo de una proteína (histona, en este caso), en presencia del cofactor NAD^+, para dar lugar a Nicotinamida y 2´-O-acetil-ADP-ribosa, así como la proteína inicial desacetilada. Actúan como sensores del estado energético y rédox celulares, así como regulan la actividad de enzimas clave y la transcripción de genes en el metabolismo.

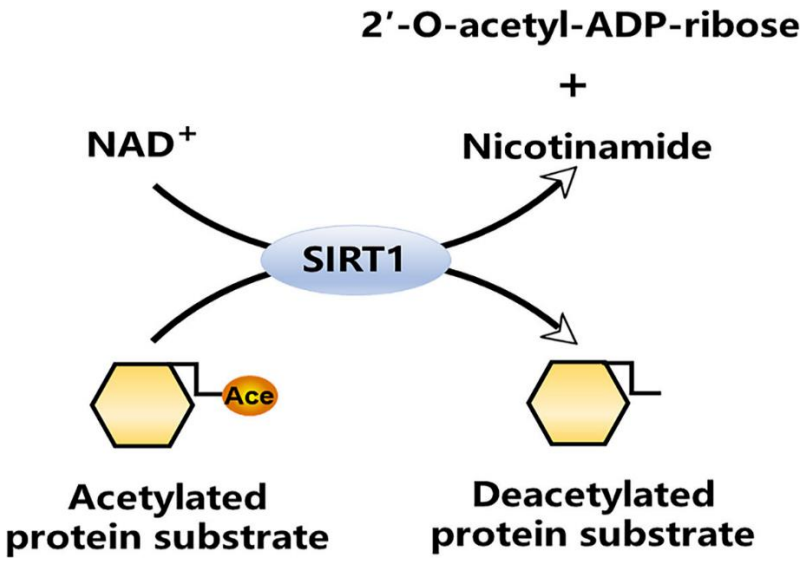


Figura 13. Reacción de desacetilación de la SIRT1 dependiente de NAD^+. [161]

Las sirtuinas también han sido ampliamente estudiadas como potenciales agentes antienvejecimiento y como moléculas íntimamente involucradas en diversas patologías en humanos tales como, diabetes, cáncer, enfermedades inflamatorias y neurodegenerativas [162]. Su localización subcelular es la siguiente (**Figura 14**): la SIRT1 está localizada en núcleo y citosol; SIRT2, en el citosol; la SIRT3, SIRT4, y SIRT5, son proteínas mitocondriales, pero la SIRT3 puede hallarse también en núcleo y citosol, debido a ciertos eventos celulares; la SIRT6 en el núcleo y la SIRT7 en el nucléolo. [163]

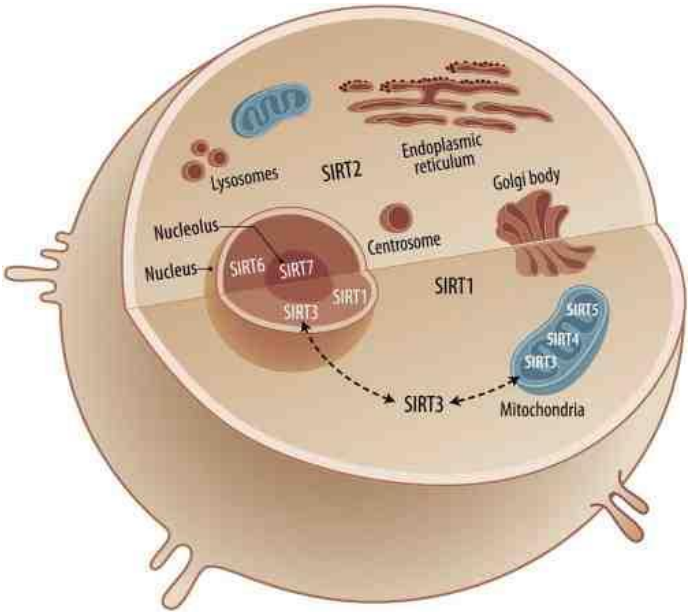


Figura 14. Localización subcelular de las Sirtuinas. [163]

En general, los ensayos para medir actividades enzimáticas se basan en la detección del consumo de sustratos o en la formación de productos. En el caso de las sirtuinas, se han desarrollado muchos ensayos para llevar a cabo el seguimiento, directa o indirectamente, del consumo de péptidos acetilados, de la formación de péptidos desacetilados, la liberación de nicotinamida, etc. Estos ensayos se pueden clasificar en 2 categorías generales [164]:

1. Ensayos sin marcaje ("label-free"), en los que se determinan tanto substrato como producto. Entre estos caben destacar los ensayos basados en espectrometría de masas, y los basados en HPLC. En efecto, mediante métodos adecuados de separación cromatográfica, se puede llevar a cabo el seguimiento de la reacción enzimática, cuantificando tanto el sustrato acetilado, como el producto desacetilado; y mediante la espectrometría de masas, tener

identificados cada uno de los picos correspondientes por medio de su masa, además del tiempo de retención.

2. Ensayos marcados ("labeled"), en los que se determinan únicamente la formación de productos. Entre estos, destacan:
    a. Ensayos con marcaje con radioisótopos. Estos son los primeros ensayos que se utilizaron para medir la actividad de sirtuinas. Se trata de ensayos altamente sensibles con bajos límites de detección, en los que se suelen emplear péptidos acetilados de histonas marcados con ^{3}H, péptidos acetilados de la proteína p53 marcados con ^{14}C, NAD^{+} marcada con ^{32}P, etc. A pesar de tratarse de ensayos muy sensibles, estos han dejado de utilizarse en gran medida, dado su elevado coste y peligros para la salud humana por la manipulación de sustancias radioactivas.
    b. Ensayos con marcaje fluorescente. Estos son los más ampliamente usados por su rapidez, sencillez, y por ser fácilmente miniaturizados y automatizados para el screening de alto rendimiento (=High throughput). La mayoría de estos ensayos se basan en la detección de la formación de péptidos desacetilados o en la formación de nicotinamida. Uno de los más empleados es el llamado Fluor-de-Lys^{TM}. Este se desarrolla en dos pasos, y mide la desacetilación de un péptido de acetil-lisina conjugado con aminometilcumarina (AMC). Este ensayo aprovecha la propiedad de que mientras que la AMC libre emite fluorescencia, su conjugado peptídico no lo hace. Por lo tanto, después del paso de desacetilación, se agrega tripsina para escindir y liberar moléculas de AMC libres de péptidos desacetilados. (**Figura 15**).
    c. Otros ensayos: Elisa, Western – blot (electroforesis).

Dada la relevancia que presentan las sirtuinas desde el punto de vista de la salud humana, es lógico encontrar cada vez más estudios de screening de moléculas de origen tanto natural como artificial, que sean capaces de regular o modular la actividad de estas. Así, varias moléculas, incluidas compuestos fitoquímicos de origen natural, pueden modular la actividad de la SIRT1 [165]. Numerosos estudios han proporcionado evidencia de los efectos protectores de sustancias polifenólicas naturales como el resveratrol, quercetina, curcumina y fisetina, y de sustancias naturales no polifenólicas como la berberina [166]. Asimismo, existen también evidencias científicas, aunque aún escasas, de que otros grupos de moléculas que son específicas de algas, como polisacáridos sulfatados (fucoidano), esteroles (fucosterol), pigmentos (fucoxantina), también tienen la capacidad de modular la SIRT1 de forma significativa. [50, 167, 168]

En el **Capítulo 3** de esta memoria de tesis, se muestra un estudio llevado a cabo de evaluación de actividades biológicas en una amplia variedad de especies de algas tanto comerciales como salvajes.

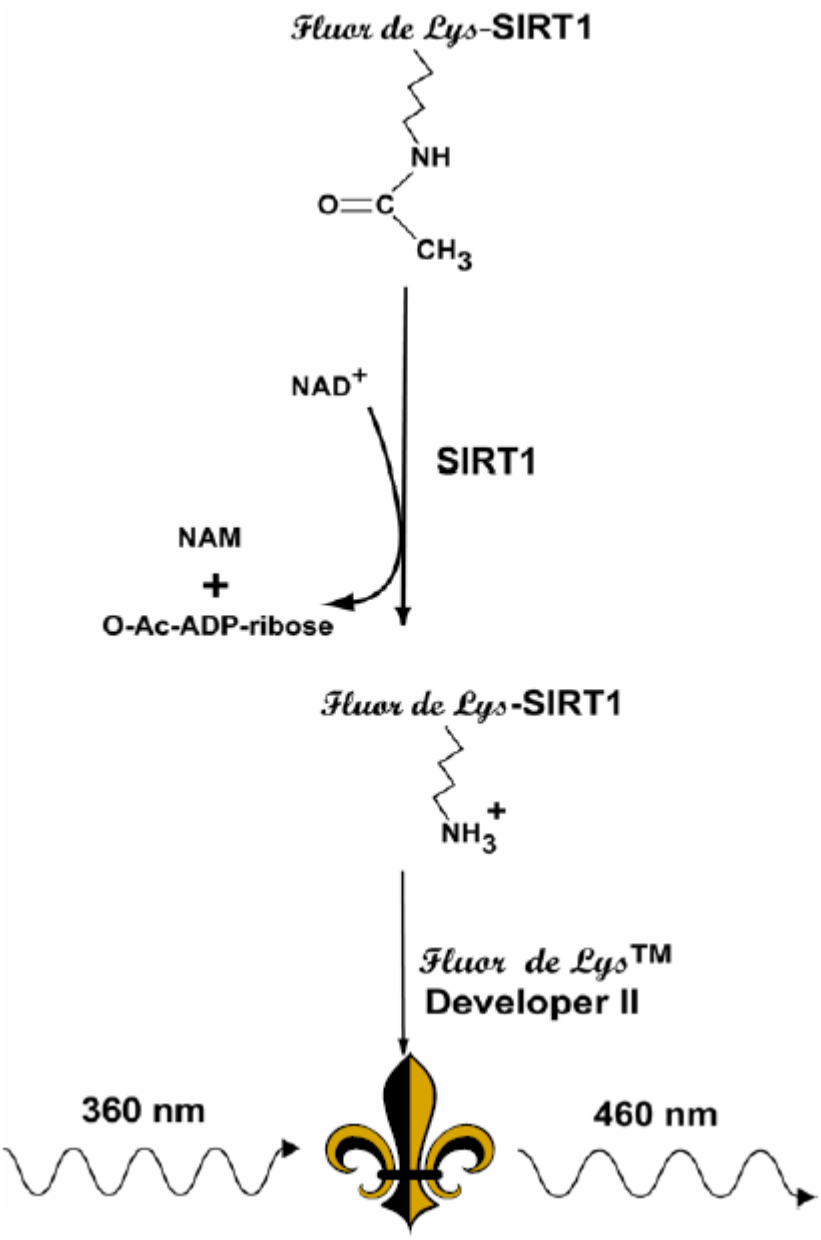


Figura 15. Esquema de la reacción en el ensayo fluorescente de activación de la SIRT1 (Fluor de Lys) [169].

A continuación, se presenta una revisión bibliográfica sobre técnicas analíticas para el Evaluación de compuestos bioactivos en algas y ensayos de actividad biológica en algas.

3.4. Revisión bibliográfica sobre técnicas analíticas para la caracterización de compuestos bioactivos en algas y ensayos de actividad biológica en algas.

En la **Tabla 6** se enumeran las referencias bibliográficas más actuales encontradas, sobre técnicas de caracterización de compuestos bioactivos y sobre ensayos de actividad biológica en algas. En general, las técnicas analíticas más comunes empleadas incluyen la cromatografía líquida de alta resolución (HPLC), la cromatografía de gases (GC), y la detección con espectroscopía de diodos array o Ultravioleta-Visible en combinación con la espectrometría de masas (MS). En menor medida, algunos autores han empleado la cromatografía de fluidos supercríticos para la cuantificación de carotenoides [170], así como técnicas espectroscópicas, como la espectroscopía de resonancia magnética nuclear (RMN), la espectroscopía infrarroja (IR), y la Raman.

La RMN es una de las técnicas más potentes actualmente para la elucidación estructural de moléculas, aunque también puede ser útil como técnica cuantitativa. Se basa en la capacidad que tienen ciertos núcleos (número impar de electrones y/o de neutrones), para absorber energía en el rango de las radiofrecuencias al ser sometidos a un campo magnético [171]. En ciencias de la alimentación, esta técnica ha sido usada para el análisis de una gran diversidad de moléculas, desde lípidos [172], aminoácidos [173], polifenoles [174], terpenos [175], etc. A pesar de tratarse de una técnica con múltiples ventajas, como el hecho de no ser destructiva para la muestra, no invasiva, o el mínimo tratamiento de muestra requerido, su uso aún es limitado debido principalmente al elevado costo del equipamiento, menor sensibilidad comparada con otras técnicas (espectrometría de masas), y la falta de personal cualificado. Aun así, en algas se han encontrado algunos trabajos donde emplean la RMN tanto en estado

líquido como sólido para la determinación estructural y / o cuantificación de lipoproteínas, lípidos [130], de diversos metabolitos [131] etc.

La IR es una técnica espectroscópica bien establecida en el campo del análisis de alimentos, donde las moléculas absorben radiación en el rango del infrarrojo cercano (NIR-near infrared, 750 – 2500 nm), y del infrarrojo medio (MIR-middle infrared, 4000 – 400 cm^{-1}). Se trata, al igual que la RMN, de una técnica no invasiva ni destructiva de muestra, no requiere apenas tratamiento de muestra, es rápida, el equipamiento es relativamente barato, es sensible, y se puede aplicar a una gran variedad de muestras, desde líquidas, sólidas, gaseosas, etc. Como desventajas, se encuentran la dificultad para interpretar espectros de mezclas complejas, o el tratarse de una técnica más apropiada para macroconstituyentes (concentraciones superiores al 0.5%), ya que, por debajo de estos niveles, es difícil distinguir el pico del analito de interés, del resto de picos espectrales. En algas, esta técnica ha sido usada principalmente para analizar lípidos o triglicéridos [136], o diversos metabolitos en análisis no dirigidos [135].

La espectroscopía Raman es una técnica de alta resolución para el análisis químico y estructural de casi cualquier tipo de material orgánico e inorgánico. Se basa en la dispersión Raman, que es la porción de luz dispersada por una muestra, al ser incidida con un haz de luz monocromática. La mayor parte de la luz dispersada por la muestra tendrá la misma energía que la luz incidente, pero una pequeña fracción de esta, tendrá una frecuencia distinta, y esta es la dispersión Raman, que ofrece información estructural de la muestra. En análisis de alimentos, esta técnica ofrece ventajas tales como el no ser invasiva ni destructiva de la muestra, mínima o nula preparación de muestra. Sin embargo, presenta baja reproducibilidad, equipamiento caro, interferencias con muestras que presentan fluorescencia innata [176]. En análisis de

algas, esta técnica ha sido empleada en estudios de carotenoides [137], otros pigmentos [139], y lipidómicos [140].

Además de la identificación de compuestos bioactivos, es importante evaluar la actividad biológica de los compuestos. Los ensayos de actividad biológica en algas pueden ayudar a evaluar la capacidad de los compuestos bioactivos para ejercer un efecto biológico específico y así seleccionar los compuestos más prometedores para su posterior desarrollo.

Los ensayos antioxidantes son particularmente importantes en el estudio de las algas, ya que muchos compuestos bioactivos presentes en las algas tienen propiedades antioxidantes. Los ensayos antioxidantes pueden evaluar la capacidad de los compuestos bioactivos para proteger las células contra los radicales libres y prevenir el daño oxidativo en el cuerpo. Estos ensayos se realizan comúnmente utilizando métodos como la capacidad de absorción de radicales de oxígeno (ORAC) [177], o la capacidad de eliminación de radicales de ácido 2,2-difenil-1-picrilhidracilo (DPPH), la capacidad de neutralizar el catión radical estable $ABTS^{\bullet+}$, y el ensayo del poder reductor / antioxidante férrico (FRAP) [178].

Los ensayos antitumorales también son importantes, ya que algunos compuestos bioactivos presentes en las algas han demostrado tener actividad antitumoral. Los ensayos de actividad biológica pueden evaluar la capacidad de los compuestos para inhibir el crecimiento y la proliferación de células tumorales. Estos ensayos se realizan comúnmente utilizando métodos como la prueba de MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [179].

Aunque hay numerosos estudios, sobre todo en los últimos años, sobre el uso de determinadas metodologías analíticas para la separación, caracterización y / o

cuantificación de diversos compuestos bioactivos en algas, la gran mayoría de estos, usan métodos no validados en algas o productos enriquecidos con algas, lo que puede dar lugar a resultados cuestionables [180]. En la **tabla 6** se enumeran algunos de los trabajos encontrados en la literatura, donde se validan parcial o totalmente los métodos analíticos empleados.

El **Capítulo 1** de esta memoria de tesis, incluye una publicación sobre la optimización y validación de un método analítico de aminoácidos y derivados de ácidos sulfónicos en algas, donde se determinan los principales parámetros de validación analítica. Asimismo, el **Capítulo 2** presenta una publicación donde se ha llevado a cabo un Evaluación de compuestos bioactivos (19 aminoácidos y Taurina, Homotaurina y Hipotaurina) en 26 especies diferentes de algas y se ha validado el método de cuantificación en nuevas matrices, concretamente en 2 tipos de alimentos enriquecidos con algas.

Tabla 6. Listado de métodos analíticos para la evaluación del contenido en compuestos bioactivos y de la actividad biológica de extractos de algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Técnicas analíticas de caracterización de compuestos bioactivos					
Cromatografía líquida (LC/ HPLC)					
	Fase normal	ELSD (light scattering)	Lípidos y ácidos grasos libres	181	-
		ELSD (light scattering)	Lípidos	182	-
		DAD (diodos array), Fluorescencia	Pigmentos	183	-
	Fase reversa	U-Vis	Pigmentos	184	-
		DAD, Masas	Polifenoles	185	+
		UV-Vis	Polifenoles	186	-
		DAD	Polifenoles	187	+
		Masas, DAD	Polifenoles	188	-
		ELSD	Azúcares	189	-
		Masas	Azúcares	190	-
		RID (Índice de refracción)	Azúcares	191	-

Tabla 6 (Continuación)**.** Listado de métodos analíticos de compuestos bioactivos y ensayos de actividad biológica en algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Técnicas analíticas de caracterización de compuestos bioactivos					
Cromatografía líquida (LC/ HPLC)					
	Fase reversa	RID	Azúcares	192	-
		DAD	Florotaninos	193	-
		Masas	Florotaninos	194	-
		DAD, Masas	Florotaninos	195	-
		Masas	Florotaninos	196	-
		Masas	Peptidos	197	-
		Masas	Peptidos	198	-
		DAD	Aminoácidos	199	-
		UV-Vis	Aminoácidos	200	-
		DAD	Aminoácidos	201	+
		UV-Vis	Aminoácidos	202	+

Tabla 6 (Continuación)**.** Listado de métodos analíticos de compuestos bioactivos y ensayos de actividad biológica en algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Técnicas analíticas de caracterización de compuestos bioactivos					
Cromatografía líquida (LC/ HPLC)					
	Fase reversa	DAD	Carotenoides	**203**	-
		DAD-Masas	Carotenoides	**204**	-
		UV-Vis	Carotenoides	**205**	-
		DAD-Masas	Carotenoides	**206**	+
Cromatografía de gases (GC)		Masas	Lípidos	**207**	-
		FID (ionización de llama)	Lípidos	**208**	-
		Masas	Lípidos	**209**	+
		FID	Lípidos	**210**	+
		Masas	Ésteres, Alcoholes, ácidos grasos	**211**	-
		Masas	Ácidos grasos, esteroles, triterpenos	**212**	-

Tabla 6 (Continuación)**.** Listado de métodos analíticos de compuestos bioactivos y ensayos de actividad biológica en algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Técnicas analíticas de caracterización de compuestos bioactivos					
Cromatografía de fluidos supercríticos (SFC)		DAD	Carotenoides	213	+
Espectroscopia de Resonancia magnética nuclear (RMN)	Protón (^{1}H)		Metabolómica (diversos metabolitos)	128	-
			Especies de Arsénico	129	-
			Lipoproteinas, lípidos	130	-
	Carbono (^{13}C)		Diversos metabolitos	131	-
	Estado sólido		Lípidos	132	-
Espectrocopía infraroja			Triglicéridos	133	-

Tabla 6 (Continuación)**.** Listado de métodos analíticos de compuestos bioactivos y ensayos de actividad biológica en algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Técnicas analíticas de caracterización de compuestos bioactivos					
Espectrocopía infrarroja			Diversos metabolitos	134	-
			Diversos metabolitos	135	-
			Lipidos neutros	136	-
Espectroscopía Raman			Carotenoides	137	-
			Pigmentos, lípidos	138	-
			Pigmentos, lípidos	139	-
			Lipidómica	140	-
Ensayos de actividad biológica					
Enzimático			Inhibición de la enzima α-amilasa	214	-

Tabla 6 (Continuación)**.** Listado de métodos analíticos de compuestos bioactivos y ensayos de actividad biológica en algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Ensayos de actividad biológica					
Microbiológico			Ac. Antibacteriana	215	-
Cultivos celulares			Efecto inmunomodulatorio	216	-
ABTS			Antioxidante	218	-
ORAC, FRAP			Antioxidante	177	-
ABTS, DPPH, FRAP			Antioxidante	218	-
ABTS, DPPH, FRAP			Antioxidante	219	-
ABTS, DPPH, FRAP			Antioxidante	178	-
CUPRAC, ABTS			Antioxidante	184	-
Químico			ACE (inhibición de la enzima convertidora de angiotensina)	220	-
Químico			ACE	221	-

Tabla 6 (Continuación)**.** Listado de métodos analíticos de compuestos bioactivos y ensayos de actividad biológica en algas.

Método analítico / Ensayo	Categoría	Detector/es	Compuestos / Actividad	Rereferencia	Validado
Ensayos de actividad biológica					
Fluorimetría			Sirtuina 1	**49**	-
Western-blot			Sirtuina 1	**50**	-
Fluorimetría			Sirtuina 6	**222**	-
Fluorimetría			Sirtuina 1	**223**	-
RT-PCR, Western-blot			Sirtuina 3	**224**	-
Fluorimetría			Sirtuina 1	**225**	-

4. REFERENCIAS

1. Brodie, Juliet & Lewis, Jane. (2007). Unravelling the algae: The past, present, and future of algal systematic. https://doi.org/10.1201/9780849379901

2. Mock, T. (2023). Algal model species for advancing biological sciences. Journal of Phycology, 59(1), 1–3. https://doi.org/10.1111/jpy.13312.

3. Pereira, L. (2021). Macroalgae. Encyclopedia, 1(1), 177–188. https://doi.org/10.3390/encyclopedia1010017.

4. Enamala, M. K., Enamala, S., Chavali, M., Donepudi, J., Yadavalli, R., Kolapalli, B., Aradhyula, T. V., Velpuri, J., & Kuppam, C. (2018). Production of biofuels from microalgae - A review on cultivation, harvesting, lipid extraction, and numerous applications of microalgae. Renewable and Sustainable Energy Reviews, 94(June), 49–68. https://doi.org/10.1016/j.rser.2018.05.012.

5. Škrovánková, Soňa. (2011). Seaweed Vitamins as Nutraceuticals. Advances in food and nutrition research. 64. 357-69. https://doi.org/10.1016/B978-0-12-387669-0.00028-4.

6. Mahadevan, Kritika. (2015). Seaweeds: A sustainable food source. https://doi.org/10.1016/B978-0-12-418697-2.00013-1

7. Ariede, M. B., Candido, T. M., Jacome, A. L. M., Velasco, M. V. R., de Carvalho, J. C. M., & Baby, A. R. (2017). Cosmetic attributes of algae - A review. Algal Research, 25(May), 483–487. https://doi.org/10.1016/j.algal.2017.05.019

8. Garcia-Vaquero, M., Ummat, V., Tiwari, B., & Rajauria, G. (2020). Exploring ultrasound, microwave and ultrasound-microwave assisted extraction technologies to increase the extraction of bioactive compounds and antioxidants from brown macroalgae. Marine Drugs, 18(3), 1–15. https://doi.org/10.3390/md18030172.

9. Hayes, Maria. (2015). Seaweeds: A nutraceutical and health food. 10.1016/B978-0-12-418697-2.00014-3. In Seaweed Sustainability, 1st ed.; Tiwari, B.K., Troy, D.J., Eds.; Academic Press: London, UK, 2015; pp. 365–387.

10. K, I., S, B., M, S., S, B., & T, B. (2013). Evaluation of in vitro antimicrobial property of seaweed (Halimeda tuna) from Tuticorin coast, Tamil Nadu, Southeast coast of India. African Journal of Biotechnology, 12(3), 284–289. https://doi.org/10.5897/ajb12.014.

11. Ganesan, A. R., Tiwari, U., & Rajauria, G. (2019). Seaweed nutraceuticals and their therapeutic role in disease prevention. Food Science and Human Wellness, 8(3), 252–263. https://doi.org/10.1016/j.fshw.2019.08.001.

12. Biris-Dorhoi, E. S., Michiu, D., Pop, C. R., Rotar, A. M., Tofana, M., Pop, O. L., Socaci, S. A., & Farcas, A. C. (2020). Macroalgae—A sustainable source of chemical compounds with biological activities. Nutrients, 12(10), 1–23. https://doi.org/10.3390/nu12103085.

13. Negreanu-Pîrjol, B., Negreanu-Pîrjol, T., Paraschiv, G., Bratu, M., Sîrbu, R., Roncea, F., & Meghea, A. (2011). Physical-chemical characterization of some green and red macrophyte algae from the Romanian Black Sea littoral. Scientific Study and Research: Chemistry and Chemical Engineering, Biotechnology, Food Industry, 12(2), 173–184.

14. EL Boukhari, M. E. M., Barakate, M., Bouhia, Y., & Lyamlouli, K. (2020). Trends in Seaweed Extract Based Biostimulants: Manufacturing Process and. Plants, 23. https://doi.org/10.3390/plants9030359.

15. Barbot, Y. N., Al-Ghaili, H., & Benz, R. (2016). A review on the valorization of macroalgal wastes for biomethane production. Marine Drugs, 14(6). https://doi.org/10.3390/md14060120.

16. Vieira, M. V., Pastrana, L. M., & Fuciños, P. (2020). Microalgae Encapsulation Systems for Food, Pharmaceutical and Cosmetics Applications. Marine Drugs, 18(12). https://doi.org/10.3390/md18120644.

17. Lin, H. Y., & Lin, H. J. (2019). Polyamines in microalgae: Something borrowed, something new. Marine Drugs, 17(1). https://doi.org/10.3390/md17010001.

18. Wei, L., You, W., Gong, Y., El Hajjami, M., Liang, W., Xu, J., & Poetsch, A. (2020). Transcriptomic and proteomic choreography in response to light quality variation reveals key adaption mechanisms in marine Nannochloropsis oceanica. Science of the Total Environment, 720, 137667. https://doi.org/10.1016/j.scitotenv.2020.137667.

19. Borowitzka, Michael. (2018). Microalgae in Medicine and Human Health. Microalgae in Health and Disease Prevention, Academic Press, https://doi.org/10.1016/B978-0-12-811405-6.00009-8.

20. Wan, C., Alam, M. A., Zhao, X. Q., Zhang, X. Y., Guo, S. L., Ho, S. H., Chang, J. S., & Bai, F. W. (2015). Current progress and future prospect of microalgal biomass harvest using various flocculation technologies. Bioresource Technology, 184, 251–257. https://doi.org/10.1016/j.biortech.2014.11.081.

21. Del Mondo, A., Smerilli, A., Sané, E., Sansone, C., & Brunet, C. (2020). Challenging microalgal vitamins for human health. Microbial Cell Factories, 19(1), 1–23. https://doi.org/10.1186/s12934-020-01459-1.

22. Singh, Shailendra & Kaur, Rupali & Bansal, Ajay & Kapur, Suman & Sundaram, Shanthy. (2020). Biotechnological exploitation of cyanobacteria and microalgae for bioactive compounds. 10.1016/B978-0-444-64323-0.00008-4. Elsevier. https://doi.org/10.1016/B978-0-444-64323-0.00008-4

23. Charles, C. N., Msagati, T., Swai, H., & Chacha, M. (2019). Microalgae: An alternative natural source of bioavailable omega-3 DHA for promotion of mental health in East Africa. Scientific African, 6, 4–7. https://doi.org/10.1016/j.sciaf.2019.e00187.

24. Ramos-vega, A., Angulo, C., Ba, B., & Monreal-escalante, E. (2021). Microalgae-made vaccines against infectious diseases. 58(June). https://doi.org/10.1016/j.algal.2021.102408.

25. Biesalski, H., Ph, D., Dragsted, L. O., Ph, D., Elmadfa, I., D, M., Ph, D., Schrenk, D., Ph, D., Grossklaus, R., D, M., Mu, M., Walter, P., D, M., Weber, P., & Ph, D. (2009). Bioactive compounds: Definition and assessment of activity. 25, 1202–1205. https://doi.org/10.1016/j.nut.2009.04.023.

26. Srivastava, N., Singh, A., Kumari, P., Nishad, J. H., Gautam, V. S., Yadav, M., Bharti, R., Kumar, D., & Kharwar, R. N. (2021). Natural bioactive compounds. Academic Press. https://doi.org/10.1016/B978-0-12-820655-3.00021-5.

27. Rico, M., González, A.G., Santana-Casiano, M., González-Dávila, M., Pérez-Almeida, N., de Tangil, M.S. (2017). Production of Primary and Secondary Metabolites Using Algae. In: Tripathi, B., Kumar, D. (eds) Prospects and Challenges in Algal Biotechnology. Springer, Singapore. https://doi.org/10.1007/978-981-10-1950-0_12.

28. Wang, Y., Tibbetts, S., & McGinn, P. (2021). Microalgae as Sources of High-Quality Protein for Human Food and Protein Supplements. Foods, 10(12), 3002. https://doi.org/10.3390/foods10123002.

29. Van Dijk, M., Morley, T., Rau, M. L., & Saghai, Y. (2021). A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. Nature Food, 2(7), 494–501. https://doi.org/10.1038/s43016-021-00322-9.

30. O'connor, J., Garcia-Vaquero, M., Meaney, S., & Tiwari, B. K. (2022). Bioactive Peptides from Algae: Traditional and Novel Generation Strategies, Structure-Function Relationships, and Bioinformatics as Predictive Tools for Bioactivity. Marine Drugs, 20(5). https://doi.org/10.3390/md20050317.

31. Magdugo, Rexie P., Nolwenn Terme, Marie Lang, Hugo Pliego-Cortés, Christel Marty, Anicia Q. Hurtado, Gilles Bedoux, and Nathalie Bourgougnon. (2020). An Analysis of the Nutritional and Health Values of Caulerpa racemosa (Forsskål) and Ulva fasciata (Delile)—Two Chlorophyta Collected from the Philippines. Molecules 25, no. 12: 2901. https://doi.org/10.3390/molecules25122901

32. Casas-Arrojo, V., Arrojo Agudo, M. de los Á., Cárdenas García, C., Carrillo, P., Pérez Manríquez, C., Martínez-Manzanares, E., & Abdala Díaz, R. T. (2022). Antioxidant, Immunomodulatory and Potential Anticancer Capacity of Polysaccharides (Glucans) from Euglena gracilis G.A. Klebs. Pharmaceuticals, 15(11). https://doi.org/10.3390/ph15111379.

33. Hans, N., Malik, A., & Naik, S. (2021). Antiviral activity of sulfated polysaccharides from marine algae and its application in combating COVID-19: Mini review. Bioresource technology reports, 13, 100623. https://doi.org/10.1016/j.biteb.2020.100623.

34. Ismail, M. M., Alotaibi, B. S., & EL-Sheekh, M. M. (2020). Therapeutic uses of red macroalgae. Molecules, 25(19), 1–14. https://doi.org/10.3390/molecules25194411.

35. Udayan, A., Pandey, A. K., Sharma, P., Sreekumar, N., & Kumar, S. (2021). Emerging industrial applications of microalgae: challenges and future perspectives. Systems Microbiology and Biomanufacturing, 1(4), 411–431. https://doi.org/10.1007/s43393-021-00038-8.

36. Moloudizargari, M., Mortaz, E., Asghari, M. H., Adcock, I. M., Redegeld, F. A., & Garssen, J. (2018). Effects of the polyunsaturated fatty acids, EPA and DHA, on hematological malignancies: A systematic review. Oncotarget, 9(14), 11858–11875. https://doi.org/10.18632/oncotarget.24405.

37. Lopes, D., Melo, T., Rey, F., Meneses, J., Monteiro, F. L., Helguero, L. A., Abreu, M. H., Lillebø, A. I., Calado, R., & Domingues, M. R. (2020). Valuing Bioactive Lipids from Green, Red and Brown Macroalgae from Aquaculture, to Foster Functionality and Biotechnological Applications. Molecules, 25(17), 1–18. https://doi.org/10.3390/molecules25173883.

38. Monla, R. Al, Dassouki, Z., Kouzayha, A., Salma, Y., Gali-Muhtasib, H., & Mawlawi, H. (2020). The cytotoxic and apoptotic effects of the brown algae colpomenia sinuosa are mediated by the generation of reactive oxygen species. Molecules, 25(8), 1–14. https://doi.org/10.3390/molecules25081993.

39. Alassali, A., & Cybulska, I. (2015). Methods for Upstream Extraction and Chemical Characterization of Secondary Metabolites from Algae Biomass.

Advanced Techniques in Biology & Medicine, 04(01). https://doi.org/10.4172/2379-1764.1000163

40. Pangestuti R, Kim SK. (2011). Biological activities and health benefit effects of natural pigments derived from marine algae. Journal of Functional Foods. 3(4):255-266

41. Suleria HAR, Osborne S, Masci P, Gobe G. (2015). Marine-based nutraceuticals: An innovative trend in the food and supplement industries. Marine Drugs. 13:6336-6351

42. Plaza M, Cifuentes A, et al. (2008). In the search of new functional food ingredients from algae. Trends in Food Science & Technology. 19(1):31-39

43. Plaza M, Herrero M, et al. (2009). Innovative natural functional ingredients from microalgae. Journal of Agricultural and Food Chemistry. 57(16):7159-7170

44. Suleria HR, Butt MS, Anjum FM, Arshad M, Khalid N. (2013). Aqueous garlic extract mitigates hypercholesterolemia and hyperglycemia; rabbit experimental modelling. Annals of Nutrition and Metabolism. 63:271

45. Stengel DB, Connan SN, et al. (2011). Algal chemodiversity and bioactivity: Sources of natural variability and implications for commercial application. Biotechnology Advances. 29(5):483-501

46. Singh, P., Gollapalli, K., Mangiola, S., Schranner, D., Yusuf, M. A., Chamoli, M., Shi, S. L., Lopes Bastos, B., Nair, T., Riermeier, A., Vayndorf, E. M., Wu, J. Z., Nilakhe, A., Nguyen, C. Q., Muir, M., Kiflezghi, M. G., Foulger, A., Junker, A., Devine, J., … Yadav, V. K. (2023). Taurine deficiency as a driver of aging. Science (New York, N.Y.), 380(6649), eabn9257. https://doi.org/10.1126/science.abn9257

47. Pan, H., & Finkel, T. (2017). Key proteins and pathways that regulate lifespan. Journal of Biological Chemistry, 292(16), 6452–6460. https://doi.org/10.1074/jbc.R116.771915.

48. He, J. L., Wang, T. S., & Wang, Y. P. (2021). Sirtuins and mitochondrial dysfunction. Sirtuin Biology in Cancer and Metabolic Disease: Cellular

Pathways for Clinical Discovery, 79–89. https://doi.org/10.1016/B978-0-12-822467-0.00007-3.

49. Dutot, M., Fagon, R., Hemon, M., & Rat, P. (2012). Antioxidant, anti-inflammatory, and anti-senescence activities of a phlorotannin-rich natural extract from Brown Seaweed Ascophyllum nodosum. Applied Biochemistry and Biotechnology, 167(8), 2234–2240. https://doi.org/10.1007/s12010-012-9761-1.

50. Lee, J. H., Jung, H. A., Kang, M. J., Choi, J. S., & Kim, G. Do. (2017). Fucosterol, isolated from Ecklonia stolonifera, inhibits adipogenesis through modulation of FoxO1 pathway in 3T3-L1 adipocytes. Journal of Pharmacy and Pharmacology, 69(3), 325–333. https://doi.org/10.1111/jphp.12684.

51. Zheng, Y., Liu, T., Wang, Z., Xu, Y., Zhang, Q., & Luo, D. (2018). Low molecular weight fucoidan attenuates liver injury via SIRT1/AMPK/PGC1α axis in db/db mice. International Journal of Biological Macromolecules, 112, 929–936. https://doi.org/10.1016/j.ijbiomac.2018.02.072.

52. Choudhury, S., Sree, A., Mukherjee, S. C., Pattnaik, P., & Bapuji, M. (2005). In Vitro Antibacterial Activity of Extracts of Selected Marine Algae and Mangroves against Fish Pathogens. Asian Fisheries Science, 18(3). https://doi.org/10.33997/j.afs.2005.18.3.009.

53. Al-Saif, S. S. A. llah, Abdel-Raouf, N., El-Wazanani, H. A., & Aref, I. A. (2014). Antibacterial substances from marine algae isolated from Jeddah coast of Red sea, Saudi Arabia. Saudi Journal of Biological Sciences, 21(1), 57–64. https://doi.org/10.1016/j.sjbs.2013.06.001.

54. Vijayabaskar, P., Vaseela, N., & Thirumaran, G. (2012). Potential antibacterial and antioxidant properties of a sulfated polysaccharide from the brown marine algae Sargassum swartzii. Chinese Journal of Natural Medicines, 10(6), 421–428. https://doi.org/10.1016/S1875-5364(12)60082-X.

55. Bhagavathy, S., Sumathi, P., & Jancy Sherene Bell, I. (2011). Green algae Chlorococcum humicola- a new source of bioactive compounds with antimicrobial activity. Asian Pacific Journal of Tropical Biomedicine, 1(SUPPL. 1), S1. https://doi.org/10.1016/S2221-1691(11)60111-1.

56. Guzmán, F., Wong, G., Román, T., Cárdenas, C., Alvárez, C., Schmitt, P., Albericio, F., & Rojas, V. (2019). Identification of Antimicrobial Peptides from the Microalgae Tetraselmis suecica (Kylin) Butcher and Bactericidal Activity Improvement. Marine Drugs, 17(8), 1–19. https://doi.org/10.3390/md17080453.

57. Cao, Y. guang, Hao, Y., Li, Z. hui, Liu, S. tao, & Wang, L. xin. (2016). Antiviral activity of polysaccharide extract from Laminaria japonica against respiratory syncytial virus. Biomedicine and Pharmacotherapy, 84, 1705–1710. https://doi.org/10.1016/j.biopha.2016.10.082.

58. Pavliga, S. N., Kompanets, G. G., & Tsygankov, V. Y. (2016). The Experimental Research (In Vitro) of Carrageenans and Fucoidans to Decrease Activity of Hantavirus. Food and Environmental Virology, 8(2), 120–124. https://doi.org/10.1007/s12560-016-9233-9.

59. Gheda, S. F., El-Adawi, H. I., & El-Deeb, N. M. (2016). Antiviral profile of brown and red seaweed polysaccharides against hepatitis c virus. Iranian Journal of Pharmaceutical Research, 15(3), 483–491.

60. Santoyo, S., Plaza, M., Jaime, L., Ibañez, E., Reglero, G., & Señorans, F. J. (2010). Pressurized liquid extraction as an alternative process to obtain antiviral agents from the edible microalga Chlorella vulgaris. Journal of Agricultural and Food Chemistry, 58(15), 8522–8527. https://doi.org/10.1021/jf100369h.

61. Musbah, H., Abouelkhair, W., Yousef, S., Moustafa, E., & Hasan, A. (2019). Evaluación of Antifungal Activities of Five Algal Crude Extracts. Journal of Scientific Research in Science, 36(1), 318–338. https://doi.org/10.21608/jsrs.2019.57633.

62. Vehapi, M., Koçer, A. T., Yılmaz, A., & Özçimen, D. (2020). Investigation of the antifungal effects of algal extracts on apple-infecting fungi. Archives of Microbiology, 202(3), 455–471. https://doi.org/10.1007/s00203-019-01760-7.

63. Jayappriyan, K. R., Rajkumar, R., Venkatakrishnan, V., Nagaraj, S., & Rengasamy, R. (2013). In vitro anticancer activity of natural β-carotene from Dunaliella salina EU5891199 in PC-3 cells. Biomedicine and Preventive Nutrition, 3(2), 99–105. https://doi.org/10.1016/j.bionut.2012.08.003.

64. Sanjeewa, K. K. A., Fernando, I. P. S., Samarakoon, K. W., Lakmal, H. H. C., Kim, E. A., Kwon, O. N., Dilshara, M. G., Lee, J. B., & Jeon, Y. J. (2016). Anti-inflammatory and anti-cancer activities of sterol rich fraction of cultured marine microalga nannochloropsis oculata. Algae, 31(3), 277–287. https://doi.org/10.4490/algae.2016.31.6.29.

65. Zhang, J., Liu, L., Ren, Y., & Chen, F. (2019). Characterization of exopolysaccharides produced by microalgae with antitumor activity on human colon cancer cells. International Journal of Biological Macromolecules, 128, 761–767. https://doi.org/10.1016/j.ijbiomac.2019.02.009.

66. Jiang, L., Zhao, X., Xu, J., Li, C., Yu, Y., Wang, W., & Zhu, L. (2019). The Protective Effect of Dietary Phytosterols on Cancer Risk: A Systematic Meta-Analysis. Journal of Oncology, 2019. https://doi.org/10.1155/2019/7479518.

67. Fernando, I. P. S., Jayawardena, T. U., Kim, H. S., Lee, W. W., Vaas, A. P. J. P., De Silva, H. I. C., Abayaweera, G. S., Nanayakkara, C. M., Abeytunga, D. T. U., Lee, D. S., & Jeon, Y. J. (2019). Beijing urban particulate matter-induced injury and inflammation in human lung epithelial cells and the protective effects of fucosterol from Sargassum binderi (Sonder ex J. Agardh). Environmental Research, 172(February), 150–158. https://doi.org/10.1016/j.envres.2019.02.016.

68. Herath, K. H. I. N. M., Kim, H. J., Lee, J. H., Je, J. G., Yu, H. S., Jeon, Y. J., Kim, H. J., & Jee, Y. (2021). Sargassum horneri (Turner) C. Agardh containing polyphenols attenuates particulate matter-induced inflammatory response by blocking TLR-mediated MYD88-dependent MAPK signaling pathway in MLE-12 cells. Journal of Ethnopharmacology, 265(March 2020), 113340. https://doi.org/10.1016/j.jep.2020.113340

69. Sanjeewa, K. K. A., Fernando, I. P. S., Kim, H. S., Jayawardena, T. U., Ryu, B. M., Yang, H. W., Ahn, G., Lee, W. W., & Jeon, Y. J. (2020). Dieckol: an algal polyphenol attenuates urban fine dust-induced inflammation in RAW 264.7 cells via the activation of anti-inflammatory and antioxidant signaling pathways. Journal of Applied Phycology, 32(4), 2387–2396. https://doi.org/10.1007/s10811-019-01964-w.

70. Agregán, R., Munekata, P., Franco, D., Carballo, J., Barba, F., & Lorenzo, J. (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(2), 33. https://doi.org/10.3390/medicines5020033.

71. Bulut, O., Akın, D., Sönmez, Ç., Öktem, A., Yücel, M., & Öktem, H. A. (2019). Phenolic compounds, carotenoids, and antioxidant capacities of a thermo-tolerant Scenedesmus sp. (Chlorophyta) extracted with different solvents. Journal of Applied Phycology, 31(3), 1675–1683. https://doi.org/10.1007/s10811-018-1726-5.

72. Herrero, M., Jaime, L., Martín-Álvarez, P. J., Cifuentes, A., & Ibáñez, E. (2006). Optimization of the extraction of antioxidants from Dunaliella salina microalga by pressurized liquids. Journal of Agricultural and Food Chemistry, 54(15), 5597–5603. https://doi.org/10.1021/jf060546q.

73. Matos, J., Cardoso, C., Gomes, A., Campos, A. M., Falé, P., Afonso, C., & Bandarra, N. M. (2019). Bioprospection of: Isochrysis galbana and its potential as a nutraceutical. Food and Function, 10(11), 7333–7342. https://doi.org/10.1039/c9fo01364d.

74. Pereira, H., Custódio, L., Rodrigues, M. J., De Sousa, C. B., Oliveira, M., Barreira, L., Neng, N. D. R., Nogueira, J. M. F., Alrokayan, S. A., Mouffouk, F., Abu-Salah, K. M., Ben-Hamadou, R., & Varela, J. (2015). Biological activities and chemical composition of methanolic extracts of selected autochthonous microalgae strains from the Red Sea. Marine Drugs, 13(6), 3531–3549. https://doi.org/10.3390/md13063531.

75. Safafar, H., Wagenen, J. Van, Møller, P., & Jacobsen, C. (2015). Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater. Marine Drugs, 13(12), 7339–7356. https://doi.org/10.3390/md13127069.

76. Geslain-Lane´elle, C. (2006). Conference on nutrition and health claims. Bologna: Noviembre.

77. Palanca, V., Rodríguez, E., Señoráns, J., & Reglero, G. (2006). Bases científicas para el desarrollo de productos cárnicos funcionales con actividad biológica combinada. Nutricion Hospitalaria, 21(2), 199–202.

78. Sloan, E. (1999). The new market: foods for the not-so-healthy. Food Technology, 53, 54e60.

79. Arai, S. (1996). Studies on functional foods in japan-state of the art. Bioscience, Biotechnology and Biochemistry, 60(1), 9–15. https://doi.org/10.1271/bbb.60.9

80. Diplock, A.T., Aggett, P.J., Ashwell, M., Bornet, F., Fern, E.B. and Roberfroid, M.B. (1999), "Scientific concepts of functional foods in Europe: consensus document", British Journal of Nutrition, Vol. 81, pp. S1-S27.

81. Carlucci, M. J., Scolaro, L. A., & Damonte, E. B. (1999). Inhibitory action of natural carrageenans on herpes simplex virus infection of mouse astrocytes. Chemotherapy, 45(6), 429–436. https://doi.org/10.1159/000007236

82. MINTEL, Functional Health Ingredients to Watch, www.mintel.com (2019), pp.1–31.

83. EU, Regulation (EU) 2015/2283 of the European Parliament and of the council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001, Off. J. Eur. Union 327 (2015) 1–22.

84. AECOSAN, Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition on a request for initial assessment for marketing of the marine microalgae Tetraselmis chuii under Regulation (EC) No 258/97 on novel foods and novel food ingredients, Revista del Comite Científico 25 (2017) 1–10.

85. Gupta, S., & Abu-Ghannam, N. (2011). Recent developments in the application of seaweeds or seaweed extracts as a means for enhancing the safety and quality attributes of foods. Innovative Food Science and Emerging Technologies, 12(4), 600–609. https://doi.org/10.1016/j.ifset.2011.07.004

86. López-López, I., Bastida, S., Ruiz-Capillas, C., Bravo, L., Larrea, M. T., Sánchez-Muniz, F., Cofrades, S., & Jiménez-Colmenero, F. (2009a). Composition and antioxidant capacity of low-salt meat emulsion model systems containing edible seaweeds. Meat Science, 83(3), 492–498. https://doi.org/10.1016/j.meatsci.2009.06.031

87. Sasaki, K., Ishihara, K., Oyamada, C., Sato, A., Fukushi, A., Arakane, T., Motoyama, M., Yamazaki, M., & Mitsumoto, M. (2008). Effects of fucoxanthin addition to ground chicken breast meat on lipid and colour stability during chilled storage, before and after cooking. Asian-Australasian Journal of Animal Sciences, 21(7), 1067–1072. https://doi.org/10.5713/ajas.2008.70670

88. Cofrades, S., López-López, I., Ruiz-Capillas, C., Triki, M., & Jiménez-Colmenero, F. (2011). Quality characteristics of low-salt restructured poultry with microbial transglutaminase and seaweed. Meat Science, 87(4), 373–380. https://doi.org/10.1016/j.meatsci.2010.11.014

89. López-López, I., Cofrades, S., & Jiménez-Colmenero, F. (2009b). Low-fat frankfurters enriched with n-3 PUFA and edible seaweed: Effects of olive oil and chilled storage on physicochemical, sensory and microbial characteristics. Meat Science, 83(1), 148–154. https://doi.org/10.1016/j.meatsci.2009.04.014

90. Kim, H. W., Choi, J. H., Choi, Y. S., Han, D. J., Kim, H. Y., Lee, M. A., Kim, S. Y., & Kim, C. J. (2010). Effects of sea tangle (Lamina japonica) Powder on quality characteristics of breakfast sausages. Korean Journal for Food Science of Animal Resources, 30(1), 55–61. https://doi.org/10.5851/kosfa.2010.30.1.55

91. Pina-Pérez, M. C., Rivas, A., Martínez, A., & Rodrigo, D. (2017). Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food. Food Chemistry, 235, 34–44. https://doi.org/10.1016/j.foodchem.2017.05.033

92. Senthil, A., Mamatha, B. S., & Mahadevaswamy, M. (2005). Effect of using seaweed (eucheuma) powder on the quality of fish cutlet. International Journal of Food Sciences and Nutrition, 56(5), 327–335. https://doi.org/10.1080/09637480500224205

93. Athukorala, Y., Lee, K.-W., Shahidi, F., Hheu, M.S., Kim, H.-T., Lee, J.-S. and JeonN, Y.-J. (2003), Antioxidant Efficacy of extracts o fan edible red algae (*Grateoloupia filicina*) in linoleic acid and fish oil. Journal of Food Lipids, 10: 313-327. https://doi.org/10.1111/j.1745-4522.2003.tb00024.x

94. Siriwardhana, N., Jeon, Y., Kim, S.H., Ha, J., Heo, S., & Lee, K. (2004). Enzymatic Hydrolysis for Effective Extraction of Antioxidative Compounds from Hizikia fusiformis. Algae, 19, 59-68. https://doi.org/10.4490/ALGAE.2004.19.1.059

95. Prabhasankar, P., Ganesan, P., Bhaskar, N., Hirose, A., Stephen, N., Gowda, L. R., Hosokawa, M., & Miyashita, K. (2009a). Edible Japanese seaweed, wakame (Undaria pinnatifida) as an ingredient in pasta: Chemical, functional and structural evaluation. Food Chemistry, 115(2), 501–508. https://doi.org/10.1016/j.foodchem.2008.12.047

96. Prabhasankar, P., Ganesan, P., & Bhaskar, N. (2009b). Influence of Indian brown seaweed (Sargassum marginatum) as an ingredient on quality, biofunctional, and microstructure characteristics of pasta. Food Science and Technology International, 15(5), 471–479. https://doi.org/10.1177/1082013209350267

97. Chang, H. C., & Wu, L. C. (2008). Texture and quality properties of Chinese fresh egg noodles formulated with green seaweed (Monostroma nitidum) powder. Journal of Food Science, 73(8), 398–404. https://doi.org/10.1111/j.1750-3841.2008.00912.x

98. Cofrades, S., Serdaroğlu, M., & Jiménez-Colmenero, F. (2013). Design of healthier foods and beverages containing whole algae. Functional Ingredients from Algae for Foods and Nutraceuticals, 609–633. https://doi.org/10.1533/9780857098689.4.609

99. Goñi, Isabel & Valdivieso, Laura & Garcia-Alonso, Alejandra. (2000). Nori seaweed consumption modifies glycemic response in healthy volunteers. Nutrition Research - NUTR RES. 20. 1367-1375. 10.1016/S0271-5317(00)80018-4.

100. Din, N. A. S., Mohd Alayudin, 'Ain Sajda, Sofian-Seng, N. S., Rahman, H. A., Mohd Razali, N. S., Lim, S. J., & Wan Mustapha, W. A. (2022). Brown Algae as Functional Food Source of Fucoxanthin: A Review. Foods, 11(15), 1–35. https://doi.org/10.3390/foods11152235

101. Cammalleri, M., Monte, M. D., Amato, R., Bagnoli, P., & Rusciano, D. (2020). A dietary combination of forskolin with homotaurine, spearmint and B vitamins protects injured retinal ganglion cells in a rodent model of

hypertensive glaucoma. Nutrients, 12(4), 1–19. https://doi.org/10.3390/nu12041189

102. Kong, Xianqi & Bellini, Francesco. (2010). Homotaurine- supplemented and /or enriched edible material, methods of preparation and uses.

103. Moroney, N. C., O'Grady, M. N., Lordan, S., Stanton, C., & Kerry, J. P. (2015). Seaweed polysaccharides (laminarin and fucoidan) as functional ingredients in pork meat: An evaluation of anti-oxidative potential, thermal stability and bioaccessibility. Marine Drugs, 13(4), 2447–2464. https://doi.org/10.3390/md13042447

104. Mamat, H., Matanjun, P., Ibrahim, S., Siti, S. F., Abdul Hamid, M., & Rameli, A. S. (2014). The effect of seaweed composite flour on the textural properties of dough and bread. Journal of Applied Phycology, 26(2), 1057–1062. https://doi.org/10.1007/s10811-013-0082-8

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

106. Lee, S., Kim, K., Kim, T., Park, N., & Korea, S. (2010). 빵의 저장성 증진 및 품질 향상 효과. August 2014.

107. Lamont, T., & McSweeney, M. (2021). Consumer acceptability and chemical composition of whole-wheat breads incorporated with brown seaweed (Ascophyllum nodosum) or red seaweed (Chondrus crispus). Journal of the Science of Food and Agriculture, 101(4), 1507–1514. https://doi.org/10.1002/jsfa.10765

108. Igual, M., Uribe-Wandurraga, Z. N., García-Segovia, P., & Martínez-Monzó, J. (2022). Microalgae-enriched breadsticks: Analysis for vitamin C, carotenoids, and chlorophyll a. Food Science and Technology International, 28(1), 26–31. https://doi.org/10.1177/1082013221990252

109. Nunes, M. C., Graça, C., Vlaisavljević, S., Tenreiro, A., Sousa, I., & Raymundo, A. (2020). Microalgal cell disruption: Effect on the bioactivity and

rheology of wheat bread. Algal Research, 45(December 2019). https://doi.org/10.1016/j.algal.2019.101749

110. Paula da Silva, S., Ferreira do Valle, A., & Perrone, D. (2021). Microencapsulated Spirulina maxima biomass as an ingredient for the production of nutritionally enriched and sensorially well-accepted vegan biscuits. Lwt, 142(January), 110997. https://doi.org/10.1016/j.lwt.2021.110997

111. Şahin, O. I. (2020). Functional and sensorial properties of cookies enriched with SPIRULINA and DUNALIELLA biomass. Journal of Food Science and Technology, 57(10), 3639–3646. https://doi.org/10.1007/s13197-020-04396-4

112. Cox, S., & Abu-Ghannam, N. (2013). Enhancement of the phytochemical and fibre content of beef patties with Himanthalia elongata seaweed. International Journal of Food Science and Technology, 48(11), 2239–2249. https://doi.org/10.1111/ijfs.12210

113. Sellimi, S., Ksouda, G., Benslima, A., Nasri, R., Rinaudo, M., Nasri, M., & Hajji, M. (2017). Enhancing colour and oxidative stabilities of reduced-nitrite turkey meat sausages during refrigerated storage using fucoxanthin purified from the Tunisian seaweed Cystoseira barbata. Food and Chemical Toxicology, 107, 620–629. https://doi.org/10.1016/j.fct.2017.04.001

114. Marti-Quijal, F. J., Zamuz, S., Tomašević, I., Gómez, B., Rocchetti, G., Lucini, L., Remize, F., Barba, F. J., & Lorenzo, J. M. (2019). Influence of different sources of vegetable, whey and microalgae proteins on the physicochemical properties and amino acid profile of fresh pork sausages. Lwt, 110(October 2018), 316–323. https://doi.org/10.1016/j.lwt.2019.04.097

115. Hell, A., Labrie, S., & Beaulieu, L. (2018). Effect of seaweed flakes addition on the development of bioactivities in functional Camembert-type cheese. International Journal of Food Science and Technology, 53(4), 1054–1064. https://doi.org/10.1111/ijfs.13681

116. O'Sullivan, A. M., O'Callaghan, Y. C., O'Grady, M. N., Waldron, D. S., Smyth, T. J., O'Brien, N. M., & Kerry, J. P. (2014). An examination of the potential of seaweed extracts as functional ingredients in milk. International Journal of Dairy Technology, 67(2), 182–193. https://doi.org/10.1111/1471-0307.12121

117. Durmaz, Yaşar & Kiliçli, Mahmut & Toker, Omersaid & Konar, Nevzat & Palabiyik, Ibrahim & Tamtürk, Faruk. (2020). Using spray-dried microalgae in ice cream formulation as a natural colorant: Effect on physicochemical and functional properties. Algal Research. 47. https://doi.org/10.1016/j.algal.2020.101811.

118. Pan-utai, W., & Atkonghan, J. (2020). Current Research in Nutrition and Food Science, Effect of Arthrospira Microalga Fortification on Physicochemical Properties of Yogurt. 08(2). https://dx.doi.org/10.12944/CRNFSJ.8.2.19

119. Bhatnagar RS, Miller DD, Padilla-Zakour OI, Lei XG. Supplemental Microalgal Iron Helps Replete Blood Hemoglobin in Moderately Anemic Mice Fed a Rice-Based Diet. Nutrients. (2020) Jul 27;12(8):2239. https://doi.org/10.3390/nu12082239

120. Melo, G. B. de., Farago, C. V.., Escher, G. B.., Granato, D.., Bolanho, B. C.., & Danesi, E. D. G. (2021). Extruded rice-based snack with Spirulina platensis and mixed flour of orange and peach palm by-products. Research, Society and Development, 10(5), e41310515142. https://doi.org/10.33448/rsd-v10i5.15142

121. Sahin, O. I., & Ozturk, B. Microalgal biomass -- a bio-based additive: evaluation of green smoothies during storage. (2021). International Food Research Journal, 28(2), 309–316. https://doi.org/10.47836/ifrj.28.2.11

122. Chang, H. C., Chen, H. H., & Hu, H. H. (2011). Textural changes in fresh egg noodles formulated with seaweed powder and full or partial replacement of cuttlefish paste. Journal of Texture Studies, 42(1), 61–71. https://doi.org/10.1111/j.1745-4603.2010.00268.x

123. Chang, H. C., & Wu, L. C. (2008). Texture and quality properties of Chinese fresh egg noodles formulated with green seaweed (Monostroma nitidum) powder. Journal of Food Science, 73(8), 398–404. https://doi.org/10.1111/j.1750-3841.2008.00912.x

124. Ram, M. M., Estrada-beristain, C., Metri-ojeda, J., Alexa, P., & Baigts-allende, D. K. (2021). Spirulina platensis Protein as Sustainable Ingredient for Nutritional Food Products Development. https://doi.org/10.3390/su13126849

125. Mamatha, B. S., Namitha, K. K., Senthil, A., Smitha, J., & Ravishankar, G. A. (2007). Studies on use of Enteromorpha in snack food. Food Chemistry, 101(4), 1707–1713. https://doi.org/10.1016/j.foodchem.2006.04.032

126. La Barbera, G., Capriotti, A. L., Cavaliere, C., Montone, C. M., Piovesana, S., Samperi, R., Zenezini Chiozzi, R., & Laganà, A. (2017). Liquid chromatography-high resolution mass spectrometry for the analysis of phytochemicals in vegetal-derived food and beverages. *Food Research International*, *100*(34), 28–52. https://doi.org/10.1016/j.foodres.2017.07.080

127. Bruni, R., Brighenti, V., Caesar, L. K., Bertelli, D., Cech, N. B., & Pellati, F. (2018). Analytical methods for the study of bioactive compounds from medicinally used Echinacea species. *Journal of Pharmaceutical and Biomedical Analysis*, *160*, 443–477. https://doi.org/10.1016/j.jpba.2018.07.044

128. Chauton, M. S., & Størseth, T. F. (2007). HR MAS NMR Spectroscopy of Marine Microalgae, Part 1: Classification and Metabolite Composition from HR MAS 1H NMR Spectra and Multivariate Analysis. *Modern Magnetic Resonance*, 947–951. https://doi.org/10.1007/1-4020-3910-7_109

129. Arora, N., Dubey, D., Sharma, M., Patel, A., Guleria, A., Pruthi, P. A., Kumar, D., Pruthi, V., & Poluri, K. M. (2018). NMR-Based Metabolomic Approach to Elucidate the Differential Cellular Responses during Mitigation of Arsenic (III, V) in a Green Microalga. *ACS Omega*, *3*(9), 11847–11856. https://doi.org/10.1021/acsomega.8b01692

130. Holmes, M. V., Millwood, I. Y., Kartsonaki, C., Hill, M. R., Bennett, D. A., Boxall, R., Guo, Y., Xu, X., Bian, Z., Hu, R., Walters, R. G., Chen, J., Ala-Korpela, M., Parish, S., Clarke, R. J., Peto, R., Collins, R., Li, L., & Chen, Z. (2018). Lipids, Lipoproteins, and Metabolites and Risk of Myocardial Infarction and Stroke. *Journal of the American College of Cardiology*, *71*(6), 620–632. https://doi.org/10.1016/j.jacc.2017.12.006

131. Nami, F., Ferraz, M. J., Bakkum, T., Aerts, J. M. F. G., & Pandit, A. (2022). Real-Time NMR Recording of Fermentation and Lipid Metabolism Processes in Live Microalgae Cells. *Angewandte Chemie - International Edition*, *61*(14), 1–6. https://doi.org/10.1002/anie.202117521

132. Poulhazan, A., Arnold, A. A., Warschawski, D. E., & Marcotte, I. (2020). Solid-state NMR study of microalgal membranes and cell walls. *Solid-State NMR*, 4-1-4–23. https://doi.org/10.1088/978-0-7503-2532-5ch4

133. Miglio, R., Palmery, S., Salvalaggio, M., Carnelli, L., Capuano, F., & Borrelli, R. (2013). Microalgae triacylglycerols content by FT-IR spectroscopy. *Journal of Applied Phycology*, *25*(6), 1621–1631. https://doi.org/10.1007/s10811-013-0007-6

134. Manjunatha, S. S., & Girisha, S. T. (2021). Characterization of microalgal biomass through fourier transforms infrared (FT-IR) spectroscopy. *International Journal of Botany Studies*, *6*(1), 57–60.

135. Scarsini, M., Thurotte, A., Veidl, B., Amiard, F., Niepceron, F., Badawi, M., Lagarde, F., Schoefs, B., & Marchand, J. (2021). Metabolite Quantification by Fourier Transform Infrared Spectroscopy in Diatoms: Proof of Concept on Phaeodactylum tricornutum. *Frontiers in Plant Science*, *12*(November). https://doi.org/10.3389/fpls.2021.756421

136. Al-Shikaili, T. Y., Thomasson, J. A., Ge, Y., Brown, L., & Brown, J. (2022). FTIR transmission spectroscopy for measurement of algae neutral lipids. *Agricultural Engineering International: CIGR Journal*, *24*(4), 111–118

137. Jehlička, J., Edwards, H. G. M., Osterrothová, K., Novotná, J., Nedbalová, L., Kopecký, J., Němec, I., & Oren, A. (2014). Potential and limits of Raman spectroscopy for carotenoid detection in microorganisms: Implications for astrobiology. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, *372*(2030). https://doi.org/10.1098/rsta.2014.0199

138. Wei, X., Jie, D., Cuello, J. J., Johnson, D. J., Qiu, Z., & He, Y. (2014). Microalgal detection by Raman microspectroscopy. *TrAC - Trends in Analytical Chemistry*, *53*, 33–40. https://doi.org/10.1016/j.trac.2013.09.012

139. Pinto, R., Vilarinho, R., Carvalho, A. P., Moreira, J. A., Guimarães, L., & Oliva-Teles, L. (2021). Raman spectroscopy applied to diatoms (microalgae, Bacillariophyta): Prospective use in the environmental diagnosis of freshwater ecosystems. *Water Research*, *198*, 117102. https://doi.org/10.1016/j.watres.2021.117102

140. Wu, H., Volponi, J. V., Oliver, A. E., Parikh, A. N., Simmons, B. A., & Singh, S. (2011). In vivo lipidomics using single-cell Raman spectroscopy. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(9), 3809–3814. https://doi.org/10.1073/pnas.1009043108

141. Lee, T. D. (2011). Introduction to Modern Liquid Chromatography, Third Edition. *Journal of the American Society for Mass Spectrometry*, *22*(1), 196–196. https://doi.org/10.1007/s13361-010-0021-8

142. Abdu Hussen, A. (2022). High-Performance Liquid Chromatography (HPLC): A review. *Annals of Advances in Chemistry*, *6*(1), 010–020. https://doi.org/10.29328/journal.aac.1001026

143. Guo, Z., Chen, Y., Wang, S., Pang, J., & Liu, Z. (2021). Dendritic Mesoporous Silica Nanospheres: Toward the Ultimate Minimum Particle Size for Ultraefficient Liquid Chromatographic Separation. *ACS Applied Materials and Interfaces*, *13*(19), 22970–22977. https://doi.org/10.1021/acsami.1c03985

144. Swartz, M. (2010). HPLC detectors: A brief review. *Journal of Liquid Chromatography and Related Technologies*, *33*(9–12), 1130–1150. https://doi.org/10.1080/10826076.2010.484356

145. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1990). ChemInform Abstract: Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *ChemInform*, *21*(5). https://doi.org/10.1002/chin.199005359

146. Siuzdak, G. (2004). An Introduction to Mass Spectrometry Ionization: An Excerpt from The Expanding Role of Mass Spectrometry in Biotechnology, 2nd ed.; MCC Press: San Diego, 2005. *JALA: Journal of the Association for Laboratory Automation*, *9*(2), 50–63. https://doi.org/10.1016/j.jala.2004.01.004

147. Uchida, Y., Tachikawa, M., Obuchi, W., Hoshi, Y., Tomioka, Y., Ohtsuki, S., & Terasaki, T. (2013). A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: Application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. *Fluids and Barriers of the CNS*, *10*(1), 1. https://doi.org/10.1186/2045-8118-10-21

148. Thompson, M., Ellison, S. L. R., & Wood, R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). Pure and Applied Chemistry, 74(5), 835–855. https://doi.org/10.1351/pac200274050835

149. AOAC International, (2002). "AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals," Association of Official Analytical Chemists, Arlington.

150. Youden W.J., Steiner E.H. (1975). Statistical Manual of the AOAC—Association of the Official Analytical Chemists, AOAC-I, Washington DC.

151. JCGM 2008:100. (2008). Evaluation of measurement data — Guide to the expression of uncertainty in measurement. International Organization for Standardization Geneva ISBN, September, 134. http://www.bipm.org/en/publications/guides/gum.html

152. ISO/IEC 17025:2017 General Requirements for The Competence of Testing dan Calibration Laboratories. International Organization for Standardization. https://www.iso.org/obp/ui/#iso:std:iso-iec:17025:ed-3:v1:en

153. ICH Q2(R1). 2005. Validation of analytical procedures: Text and methodology. Current step 4 version, November 2005. https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf

154. Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., & Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC technical report). *Pure and Applied Chemistry*, *85*(5), 957–998. https://doi.org/10.1351/PAC-REP-12-07-15

155. Halliwell B, Gutteridge JM. (1995). The definition and measurement of antioxidants in biological systems. Free Radic Biol Med. Jan;18(1):125-6. https://doi.org/10.1016/0891-5849(95)91457-3

156. Prior, R. L., 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*(10), 4290–4302. https://doi.org/10.1021/jf0502698

157. Miller, N. J., Rice-Evans, C., & Davies, M. J. (1993). A new method for measuring antioxidant activity. *Biochemical Society Transactions*, *21*(2), 1992. https://doi.org/10.1042/bst021095s

158. ÜSTÜNDAŞ, M., YENER, H. B., & HELVACI, Ş. Ş. (2018). Parameters Affecting Lycopene Extraction from Tomato Powder and Its Antioxidant Activity. Anadolu University Journal of Science and Technology-A Applied Sciences and Engineering, July, 1–1. https://doi.org/10.18038/aubtda.363140

159. Blois, M. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. Nature. 181, 1199–1200. https://doi.org/10.1038/1811199a0

160. Liang, N., & Kitts, D. D. (2014). Antioxidant property of coffee components: Assessment of methods that define mechanism of action. Molecules, 19(11), 19180–19208. https://doi.org/10.3390/molecules191119180

161. Yang, Y., Liu, Y., Wang, Y., Chao, Y., Zhang, J., Jia, Y., Tie, J., & Hu, D. (2022). Regulation of SIRT1 and Its Roles in Inflammation. *Frontiers in Immunology*, *13*(March), 1–16. https://doi.org/10.3389/fimmu.2022.831168

162. Wu, Q. J., Zhang, T. N., Chen, H. H., Yu, X. F., Lv, J. Le, Liu, Y. Y., Liu, Y. S., Zheng, G., Zhao, J. Q., Wei, Y. F., Guo, J. Y., Liu, F. H., Chang, Q., Zhang, Y. X., Liu, C. G., & Zhao, Y. H. (2022). The sirtuin family in health and disease. *Signal Transduction and Targeted Therapy*, *7*(1). https://doi.org/10.1038/s41392-022-01257-8

163. Alhazzazi, T. Y., Kamarajan, P., Verdin, E., & Kapila, Y. L. (2011). SIRT3 and cancer: Tumor promoter or suppressor? *Biochimica et Biophysica Acta - Reviews on Cancer*, *1816*(1), 80–88. https://doi.org/10.1016/j.bbcan.2011.04.004

164. Li, Y., Liu, T., Liao, S., Li, Y., Lan, Y., Wang, A., Wang, Y., & He, B. (2015). A mini-review on Sirtuin activity assays. *Biochemical and Biophysical Research Communications*, *467*(3), 459–466. https://doi.org/10.1016/j.bbrc.2015.09.172

165. Miceli, M., Bontempo, P., Nebbioso, A., & Altucci, L. (2014). Natural compounds in epigenetics: A current view. *Food and Chemical Toxicology*, *73*, 71–83. https://doi.org/10.1016/j.fct.2014.08.005

166. McCubrey, J. A., Lertpiriyapong, K., Steelman, L. S., Abrams, S. L., Yang, L. V., Murata, R. M., Rosalen, P. L., Scalisi, A., Neri, L. M., Cocco, L., Ratti, S., Martelli, A. M., Laidler, P., Dulinska-Litewka, J., Rakus, D., Gizak, A., Lombardi, P., Nicoletti, F., Candido, S., … Cervello, M. (2017). Effects of resveratrol, curcumin, berberine and other nutraceuticals on aging, cancer development, cancer stem cells and microRNAs. *Aging*, *9*(6), 1477–1536. https://doi.org/10.18632/aging.101250

167. Zheng, Y., Liu, T., Wang, Z., Xu, Y., Zhang, Q., & Luo, D. (2018). Low molecular weight fucoidan attenuates liver injury via SIRT1/AMPK/PGC1α axis in db/db mice. *International Journal of Biological Macromolecules*, *112*, 929–936. https://doi.org/10.1016/j.ijbiomac.2018.02.072

168. Chang, Y. H., Chen, Y. L., Huang, W. C., & Liou, C. J. (2018). Fucoxanthin attenuates fatty acid-induced lipid accumulation in FL83B hepatocytes through regulated Sirt1/AMPK signaling pathway. *Biochemical and Biophysical Research Communications*, *495*(1), 197–203. https://doi.org/10.1016/j.bbrc.2017.11.022

169. https://www.enzolifesciences.com/BML-AK500/fluor-de-lys-hdac-fluorometric-activity- assay-kit/

170. Abrahamsson, V., Rodriguez-Meizoso, I., & Turner, C. (2012). Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography. *Journal of Chromatography A*, *1250*, 63–68. https://doi.org/10.1016/j.chroma.2012.05.069

171. Hatzakis, E. (2019). Nuclear Magnetic Resonance (NMR) Spectroscopy in Food Science: A Comprehensive Review. *Comprehensive Reviews in Food Science and Food Safety*, *18*(1), 189–220. https://doi.org/10.1111/1541-4337.12408

172. Tsiafoulis, C. G., Papaemmanouil, C., Alivertis, D., Tzamaloukas, O., Miltiadou, D., Balayssac, S., Malet-Martino, M., & Gerothanassis, I. P. (2019). NMR-based metabolomics of the lipid fraction of organic and conventional bovine milk. *Molecules*, *24*(6), 1–18. https://doi.org/10.3390/molecules24061067

173. Belton, P. S., Delgadillo, I., Holmes, E., Nicholls, A., Nicholson, J. K., & Spraul, M. (1996). Use of high-field 1H NMR spectroscopy for the analysis

of liquid foods. *Journal of Agricultural and Food Chemistry*, *44*(6), 1483–1487. https://doi.org/10.1021/jf950640z

174. Charisiadis, P., Exarchou, V., Troganis, A. N., & Gerothanassis, I. P. (2010). Exploring the "forgotten" -OH NMR spectral region in natural products. *Chemical Communications*, *46*(20), 3589–3591. https://doi.org/10.1039/b927256a

175. Dais, P., Plessel, R., Williamson, K., & Hatzakis, E. (2017). Complete1H and13C NMR assignment and31P NMR determination of pentacyclic triterpenic acids. *Analytical Methods*, *9*(6), 949–957. https://doi.org/10.1039/c6ay02565j

176. Wang, K., Li, Z., Li, J., & Lin, H. (2021). Raman spectroscopic techniques for nondestructive analysis of agri-foods: A state-of-the-art review. *Trends in Food Science and Technology*, *118*(PA), 490–504. https://doi.org/10.1016/j.tifs.2021.10.010

177. Harnedy, P. A., O'Keeffe, M. B., & FitzGerald, R. J. (2017). Fractionation and identification of antioxidant peptides from an enzymatically hydrolysed Palmaria palmata protein isolate. *Food Research International*, *100*(May), 416–422. https://doi.org/10.1016/j.foodres.2017.07.037

178. Urrea-Victoria, V., Furlan, C. M., dos Santos, D. Y. A. C., & Chow, F. (2022). Antioxidant potential of two Brazilian seaweeds in response to temperature: Pyropia spiralis (red alga) and Sargassum stenophyllum (brown alga). *Journal of Experimental Marine Biology and Ecology*, *549*(January), 1–8. https://doi.org/10.1016/j.jembe.2022.151706

179. Tavares-Carreón, F., De La Torre-Zavala, S., Arocha-Garza, H. F., Souza, V., Galán-Wong, L. J., & Avilés-Arnaut, H. (2020). In vitro anticancer activity of methanolic extract of Granulocystopsis sp., a microalgae from an oligotrophic oasis in the Chihuahuan desert. *PeerJ*, *2020*(3), 1–21. https://doi.org/10.7717/peerj.8686

180. Lane, M., Van Wychen, S., Politis, A., & Laurens, L. M. L. (2021). A data-driven comparison of commercially available testing methods for algae characterization. *Algal Research*, *53*, 102134. https://doi.org/10.1016/j.algal.2020.102134

181. Donot, F., Strub, C., Fontana, A., Jouy, N., Delbes, C., Gunata, Z., & Schorr-Galindo, S. (2016). Rapid analysis and quantification of major neutral lipid species and free fatty acids by HPLC-ELSD from microalgae. *European Journal of Lipid Science and Technology*, *118*(10), 1550–1556. https://doi.org/10.1002/ejlt.201500545

182. Jones, J., Manning, S., Montoya, M., Keller, K., & Poenie, M. (2012). Extraction of algal lipids and their analysis by HPLC and mass spectrometry. *JAOCS, Journal of the American Oil Chemists' Society*, *89*(8), 1371–1381. https://doi.org/10.1007/s11746-012-2044-8

183. Somasundaram, S. (2017). Separation of Algal Pigments By Thin Layer Chromatography (Tlc) and High Performance Liquid Chromatography. *World Journal of Pharmaceutical Reasearch*, *6*(16), 1276–1284. https://doi.org/10.20959/wjpr201716-10328

184. Yalçln, S., Karakaş, Ö., Okudan, E. S., Başkan, K. S., Çekiç, S. D., & Apak, R. (2021). HPLC Detection and Antioxidant Capacity Determination of Brown, Red and Green Algal Pigments in Seaweed Extracts. *Journal of Chromatographic Science*, *59*(4), 325–337. https://doi.org/10.1093/chromsci/bmaa107

185. Rajauria, G. (2018). Optimization and validation of reverse phase HPLC method for qualitative and quantitative assessment of polyphenols in seaweed. *Journal of Pharmaceutical and Biomedical Analysis*, *148*, 230–237. https://doi.org/10.1016/j.jpba.2017.10.002

186. Aminina, N. M., Karaulova, E. P., Vishnevskaya, T. I., Yakush, E. V., Kim, Y. K., Nam, K. H., & Son, K. T. (2020). Characteristics of Polyphenolic Content in Brown Algae of the Pacific Coast of Russia. *Molecules*, *25*(17), 1–13. https://doi.org/10.3390/molecules25173909

187. Sánchez-Bonet, D., García-Oms, S., Belda-Antolí, M., Padrón-Sanz, C., Lloris-Carsi, J. M., & Cejalvo-Lapeña, D. (2021). RP-HPLC-DAD determination of the differences in the polyphenol content of Fucus vesiculosus extracts with similar antioxidant activity. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1184*(October). https://doi.org/10.1016/j.jchromb.2021.122978

188. Zhong, B., Robinson, N. A., Warner, R. D., Barrow, C. J., Dunshea, F. R., & Suleria, H. A. R. (2020). LC-ESI-QTOF-MS/MS characterization of seaweed

phenolics and their antioxidant potential. *Marine Drugs*, *18*(6). https://doi.org/10.3390/md18060331

189. Chirapart, A., Praiboon, J., Puangsombat, P., Pattanapon, C., & Nunraksa, N. (2014). Chemical composition and ethanol production potential of Thai seaweed species. *Journal of Applied Phycology*, *26*(2), 979–986. https://doi.org/10.1007/s10811-013-0235-9

190. Ortiz-Tena, J. G., Rühmann, B., Schieder, D., & Sieber, V. (2016). Revealing the diversity of algal monosaccharides: Fast carbohydrate fingerprinting of microalgae using crude biomass and showcasing sugar distribution in Chlorella vulgaris by biomass fractionation. *Algal Research*, *17*, 227–235. https://doi.org/10.1016/j.algal.2016.05.008

191. Dahiya, S., Chowdhury, R., Kumar, P., Ghosh, S., & Srinivasan, A. (2022). Recovery of Sugar and Nutrients from Algae and Colocasia esculenta (Taro) Leaves Using Chemical Hydrolysis. *Sustainability (Switzerland)*, *14*(24). https://doi.org/10.3390/su142416383

192. Tassakka, A. C. M. A. R., Sulfahri, Iqbal Djawad, M., Kasmiati, K., & Zaenab, S. (2021). Biosugar production from marine algae Kappaphycus alvarezii by acid hydrolysis. *IOP Conference Series: Earth and Environmental Science*, *860*(1). https://doi.org/10.1088/1755-1315/860/1/012078

193. Shakambari, G., Ashokkumar, B., & Varalakshmi, P. (2015). Phlorotannins from Brown Algae: inhibition of advanced glycation end products formation in high glucose induced Caenorhabditis elegans. 53(June), 371–379.

194. Melanson, J. E., & Mackinnon, S. L. (n.d.). *Chapter 16*. *1308*, 253–266. https://doi.org/10.1007/978-1-4939-2684-8

195. Lopes, G., Barbosa, M., Vallejo, F., Gil-Izquierdo, Á., Andrade, P. B., Valentão, P., Pereira, D. M., & 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*(May 2017), 113–120. https://doi.org/10.1016/j.algal.2017.11.025

196. Sardari, R. R. R., Prothmann, J., Gregersen, O., & Turner, C. (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.

197. Zhao, S. (2016). Purification and Identification of Antioxidant Peptides from Enzymatic Hydrolysate of Spirulina platensis. *26*, 1216–1223.

198. Sun, K., Gao, M., Wang, Y., Li, X., Wang, P., & Wang, B. (2022). Antioxidant Peptides from Protein Hydrolysate of Marine Red Algae Eucheuma cottonii: Preparation, Identification, and Cytoprotective Mechanisms on H_2O_2 Oxidative Damaged HUVECs. *13*(April), 1–14. https://doi.org/10.3389/fmicb.2022.791248

199. Santiago-díaz, P., Rico, M., Rivero, A., & Santana-casiano, M. (2022). Bioactive Metabolites of Microalgae from Canary Islands for Functional Food and Feed Uses. https://doi.org/10.1002/cbdv.202200230

200. Hassan, M. U., Islam, M. M., Wang, R., Guo, J., Luo, H., Chen, F., & Li, X. (2020). Glutamine application promotes nitrogen and biomass accumulation in the shoot of seedlings of the maize hybrid ZD958. *Planta*, *251*(3), 1–15. https://doi.org/10.1007/s00425-020-03363-9

201. Araya, M., García, S., Rengel, J., Pizarro, S., & Álvarez, G. (2021). Determination of free and protein amino acid content in microalgae by HPLC-DAD with pre-column derivatization and pressure hydrolysis. *Marine Chemistry*, *234*(May). https://doi.org/10.1016/j.marchem.2021.103999

202. Cuchiaro, H., & Laurens, L. M. L. (2019). *Total Protein Analysis in Algae via Bulk Amino Acid Detection: Optimization of Amino Acid Derivatization after Hydrolysis with O - Phthalaldehyde 3 - Mercaptopropionic Acid (OPA-3MPA)*. https://doi.org/10.1021/acs.jafc.9b00884

203. Aluc, Y. (2018). Determination of carotenoids in two algae species from the saline water of Kapulukaya reservoir by HPLC Journal of Liquid Chromatography & Related Determination of carotenoids in two algae species from the saline water of Kapulukaya reservoir by HPLC. January. https://doi.org/10.1080/10826076.2017.1418376

204. Fernandes, S., Petry, F. C., Mercadante, A. Z., Jacob-lopes, E., & Zepka, L. Q. (2020). *Current Research in Food Science HPLC-PDA-MS / MS as a strategy to characterize and quantify natural pigments from microalgae*. *3*, 100–112. https://doi.org/10.1016/j.crfs.2020.03.009

205. Jin, G., Liu, Y., Xue, S., Meng, Y., Yan, J., Yang, F., Guo, Z., & Zhu, J. (2019). Determination of Three Carotenoids in Microalgae by Matrix Solid - Phase Dispersion Extraction and High - Performance Liquid Chromatography. *Chromatographia*, *82*(11), 1593–1601. https://doi.org/10.1007/s10337-019-03795-w

206. Grujić, V.J.; Todorović, B.; Kranvogl, R.; Ciringer, T.; Ambrožič-Dolinšek, J. Diversity and Content of Carotenoids and Other Pigments in the Transition from the Green to the Red Stage of Haematococcus pluvialis Microalgae Identified by HPLC-DAD and LC-QTOF-MS. (2022). Plants, 11, 1026. https://doi.org/10.3390/plants11081026

207. Sarpal, A. S., Teixeira, C. M. L. L., Silva, P. R. M., Lima, G. M., Silva, S. R., Monteiro, T. V, Cunha, V. S., & Daroda, R. J. (2015). *Determination of lipid content of oleaginous microalgal biomass by NMR spectroscopic and GC – MS techniques*. 3799–3816. https://doi.org/10.1007/s00216-015-8613-6

208. Kurnia, Dewi & Yuliantini, Anne & Cendana, Irwan & Nurachman, Z. (2019). Fatty Acid Analysis of Marine Microalgae Chlorella vulgaris in Modified Medium Used GC-FID. Journal of Physics: Conference Series. https://doi.org/1338. 012007. 10.1088/1742-6596/1338/1/012007

209. Avula SGC, Belovich JM, Xu Y. (2017). Determination of fatty acid methyl esters derived from algae Scenedesmus dimorphus biomass by GC-MS with one-step esterification of free fatty acids and transesterification of glycerolipids. J Sep Sci. May;40(10):2214-2227. doi: https://doi.org/10.1002/jssc.201601336

210. Dillon, G. P., Yiannikouris, A., Brandl, W., Cardinall, C., Yuan, W., & Moran, C. A. (2019). *Analytical Method Assessment for the Determination of DHA and Fatty Acids Present in Unextracted Aurantiochytrium limacinum Biomass*. 469–483. https://doi.org/10.4236/fns.2019.104035

211. Silva, M.; Kamberovic, F.; Uota, S.T.; Kovan, I.-M.; Viegas, C.S.B.; Simes, D.C.; Gangadhar, K.N.; Varela, J.; Barreira, L. (2022). Microalgae as Potential Sources of Bioactive Compounds for Functional Foods and Pharmaceuticals. Appl. Sci., 12, 5877. https://doi.org/10.3390/app12125877

212. Teixeira, F.S.; Vidigal, S.S.M.P.; Pimentel, L.L.; Costa, P.T.; Tavares-Valente, D.; Azevedo-Silva, J.; Pintado, M.E.; Fernandes, J.C.; Rodríguez-

Alcalá, L.M. (2021). Phytosterols and Novel Triterpenes Recovered from Industrial Fermentation Coproducts Exert In Vitro Anti-Inflammatory Activity in Macrophages. Pharmaceuticals, 14, 583. https://doi.org/10.3390/ph14060583

213. Abrahamsson, V., Rodriguez-Meizoso, I., & Turner, C. (2012). Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography. *Journal of Chromatography A*, *1250*, 63–68.https://doi.org/10.1016/j.chroma.2012.05.069

214. Admassu, H., Gasmalla, M. A. A., Yang, R., & Zhao, W. (2018). Identification of Bioactive Peptides with α-Amylase Inhibitory Potential from Enzymatic Protein Hydrolysates of Red Seaweed (Porphyra spp). *Journal of Agricultural and Food Chemistry*, *66*(19), 4872–4882. https://doi.org/10.1021/acs.jafc.8b00960

215. Beaulieu, L., Bondu, S., Doiron, K., Rioux, L. E., & Turgeon, S. L. (2015). Characterization of antibacterial activity from protein hydrolysates of the macroalga Saccharina longicruris and identification of peptides implied in bioactivity. *Journal of Functional Foods*, *17*, 685–697. https://doi.org/10.1016/j.jff.2015.06.026

216. Cian, R. E., Hernández-Chirlaque, C., Gámez-Belmonte, R., Drago, S. R., Sánchez de Medina, F., & Martínez-Augustin, O. (2018). Green alga Ulva spp. hydrolysates and their peptide fractions regulate cytokine production in splenic macrophages and lymphocytes involving the TLR4-NFκB/MAPK pathways. *Marine Drugs*, *16*(7). https://doi.org/10.3390/md16070235

217. Frazzini, S., Scaglia, E., Dell'anno, M., Reggi, S., Panseri, S., Giromini, C., Lanzoni, D., Rossi, C. A. S., & Rossi, L. (2022). Antioxidant and Antimicrobial Activity of Algal and Cyanobacterial Extracts: An In Vitro Study. *Antioxidants*, *11*(5). https://doi.org/10.3390/antiox11050992

218. Polo, L. K., & Chow, F. (2022). Variation of antioxidant capacity and antiviral activity of the brown seaweed Sargassum filipendula (Fucales, Ochrophyta) under UV radiation treatments. *Applied Phycology*, *3*(1), 260–273. https://doi.org/10.1080/26388081.2022.2030653

219. Tolpeznikaite, E., Starkute, V., Zokaityte, E., Ruzauskas, M., Pilkaityte, R., Viskelis, P., Urbonaviciene, D., Ruibys, R., Rocha, J. M., & Bartkiene, E. (2022). Effect of solid-state fermentation and ultrasonication processes on

antimicrobial and antioxidant properties of algae extracts. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.990274

220. Sun, S., Xu, X., Sun, X., Zhang, X., Chen, X., & Xu, N. (2019). Preparation and identification of ACE inhibitory peptides from the marine macroalga Ulva intestinalis. *Marine Drugs*, *17*(3), 1–17. https://doi.org/10.3390/md17030179

221. Zheng, Y., Zhang, Y., & San, S. (2020). Efficacy of a novel ACE-inhibitory peptide from Sargassum maclurei in hypertension and reduction of intracellular endothelin-1. *Nutrients*, *12*(3), 1–14. https://doi.org/10.3390/nu12030653

222. Li, R., Sun, X., Shu, Y., Wang, Y., Xiao, L., Wang, Z., Hu, X., Kermode, A. G., & Qiu, W. (2017). Serum CCL20 and its association with SIRT1 activity in multiple sclerosis patients. Journal of Neuroimmunology, 313(August), 56–60. https://doi.org/10.1016/j.jneuroim.2017.10.013

223. Shokri Afra, H., Zangooei, M., Meshkani, R., Ghahremani, M. H., Ilbeigi, D., Khedri, A., Shahmohamadnejad, S., Khaghani, S., & Nourbakhsh, M. (2019). Hesperetin is a potent bioactivator that activates SIRT1-AMPK signaling pathway in HepG2 cells. Journal of Physiology and Biochemistry, 75(2), 125–133. https://doi.org/10.1007/s13105-019-00678-4

224. Wang, T., Zhu, M., & He, Z. Z. (2016). Low-Molecular-Weight Fucoidan Attenuates Mitochondrial Dysfunction and Improves Neurological Outcome After Traumatic Brain Injury in Aged Mice: Involvement of Sirt3. *Cellular and Molecular Neurobiology*, *36*(8), 1257–1268. https://doi.org/10.1007/s10571-015-0323-2

225. Yang, Y., Fu, W., Chen, J., Olashaw, N., Zhang, X., Nicosia, S. V., Bhalla, K., & Bai, W. (2007). SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. Nature Cell Biology, 9(11), 1253–1262. https://doi.org/10.1038/ncb1645

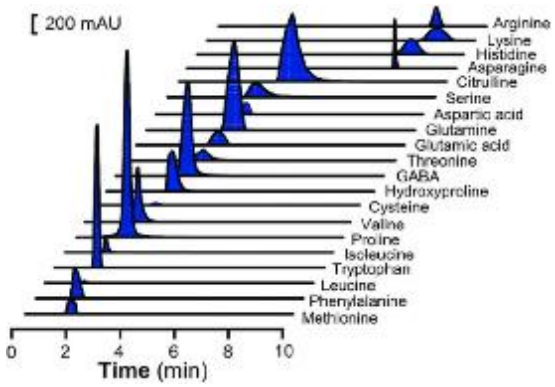
PARTE EXPERIMENTAL

BLOQUE 1

CARACTERIZACIÓN DE COMPUESTOS BIOACTIVOS

CAPÍTULO 1

Método validado de cromatografía líquida de ultra alto rendimiento con detección mediante diodos array acoplado a espectrometría de masas de triple cuadrupolo e ionización por electro spray para la cuantificación simultánea de taurina, homotaurina, hipotaurina y aminoácidos en macro- y microalgas



AUTORES**:** Carlos Terriente-Palacios [a, b], Isabel Diaz [a], Massimo Castellari [a]

TITULO**: A validated ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and triplequadrupole mass spectrometry method to simultaneously quantify taurine, homotaurine, hypotaurine and amino acids in macro- and microalgae**

REFERENCIA**:** DOI: 10.1016/j.chroma.2018.12.058. Volume 1589, 29 March 2019, Pages 83-92. ISSN: 0021-9673

REVISTA: **JOURNAL OF CHROMATOGRAPHY A**

EDITORIAL: ELSERVIER

AFILIACIONES:
[a] IRTA Food Industries – Finca Camps I Armet s/n, 17121 Monells, Girona, Spain
[b] Escuela Internacional de Doctorado UNED - Calle Bravo Murillo 38, 28015 Madrid, Spain

LUGAR DE PUBLICACIÓN: NETHERLANDS

FECHA DE PUBLICACIÓN: **2019**

FACTOR DE IMPACTO: PUBLICADO (JCR-2019) **4.049**

Biochemical Research Methods	POSICIÓN: 13/78 (JCR-2019)	PRIMER TERCIO (1º CUARTIL)
Analytical Chemistry	POSICIÓN: 17/87 (JCR-2019)	PRIMER TERCIO (1º CUARTIL)

Journal of Chromatography A, 1589 (2019) 83–92

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A validated ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry method to simultaneously quantify taurine, homotaurine, hypotaurine and amino acids in macro- and microalgae

Carlos Terriente-Palacios[a,b,*], Isabel Diaz[a], Massimo Castellari[a]

[a] IRTA Food Industries – Finca Camps I Armet s/n, 17121 Monells, Girona, Spain
[b] Escuela Internacional de Doctorado UNED - Calle Bravo Murillo 38, 28015 Madrid, Spain

a b s t r a c t

A fast and reliable method for the simultaneous quantification of Taurine, Homotaurine, Hypotaurine and 19 amino acids in algae samples by Ultra-performance liquid chromatography coupled with diodearray and tandem mass spectrometry (UHPLC–DAD-MS/MS) was optimized and validated. Target compounds were chromatographically resolved in less than 15 min. (ESI)-MS/MS electrospray ionization and pure analytical standards were used to confirm the identity of all analytes, while quantitation was carried out with diode array detection. Validation parameters of the method were satisfactory: Resolution of peak pairs was always higher than 1.55; all analytical curves showed R^2> 0.99, with working ranges between 0.04 mg/g to 33.1 mg/g and 9.13 mg/g to 107 mg/g and the Lack-of-fit test was not significant. The intra and inter-day precision of the method (expressed as relative standard deviation) were lower than 6% and recovery values ranged between 95% and 105%. The method was demonstrated to be robust to small deliberate

variations of seven variables such sample weight, volume of hydrolysis reagent, hydrolysis time and temperature, derivatization time, column temperature and flow rate. The mean expanded uncertainty for all the target compounds were 0.7 mg/g with a coverage factor of 2. Method Limits of detection and quantification varied from $0.005*10^{-3}$ mg/g to $0.11*10^{-3}$ mg/g and $0.01*10^{-3}$ mg/g to $0.22*10^{-3}$ mg/g respectively, allowing the routine determination of these bioactive compounds in algae extracts. Therefore, the method was successfully applied for the quantitative determination of the 22 target compounds in five seaweed commercial samples. Relevant compounds were quantified for the first time in the five algae species, namely: i) Taurine in Gracilaria longissima and Chlorella spp., ii) Gamma-aminobutyric acid in G. longissima and L. japonica, iii) Hydroxyproline in G. longissima, Ulva lactuca, Porphyra spp., and L. japonica and iv) Homotaurine and Hypotaurine in the five species studied.

Keywords: Homotaurine; Hypotaurine; Taurine; Amino acids; Algae; UPLC-DAD-MS/MS

1. Introduction

Amino acids (AAs) are the main constituents of proteins and act as precursors for nucleic acids, hormones, vitamins, and otherimportant molecules. Thus, an adequate supply of dietary protein and amino acids is essential to maintain cellular integrity and function, as well as a healthy state at different stages of life [1,2].

In the latest years, the interest for the algae in the food market has been increasing due to the many positive nutritional properties and health benefits, including their protein fraction. However, there is still a limited knowledge of nutritional composition across algal species, geographical regions, seasons, all of which can substantially affect their dietary value [3]. The protein content of seaweed and microalgae can vary greatly depending on different factors as specie, environmental growth conditions (geographic area, season, temperature, light, available nutrients, etc.) and stage of algal life cycle [4]. As an example, the protein content of brown algae species (e.g. *Laminaria japonica* and *Undaria pinnatifida*) is relatively low, about 7–16% on dry weight basis [5]. In contrast, red algae (e.g. *Palmaria palmata* and *Porphyra tenera*) contain 21–47% protein on dry weight basis [6], and fresh-water micro-algae, as *Chlorella vulgaris*, can reach concentrations of protein up to 58% on dry weight basis [7]. High concentrations of Arginine, Asparagine and Glutamic acid are generally found in many seaweed species [6], but algae protein contain also high proportions of all essential amino acids (EAAs) [8], and, in some algae species (e.g., Porphyra sp.), EAAs concentration compares extremely well with that of soy and egg protein [6,8]. Additionally, other amino acids (e.g. Hydroxyproline, Ornithine and Citrulline), amino acid-like compounds, such as Gamma-aminobutyric acid (GABA), and mycosporine like amino acids have been occasionally found in seaweed species [9]. GABA is a non-protein amino acid, considered a potent bioactive compound, which has been widely studied because of its numerous physiological functions and positive effects on many metabolic disorders.

One of the most important is the hypotensive effect that has been demonstrated in animals and in human intervention trials [10]. In the past, Hydroxyproline (Hyp) has been considered to have little nutritional significance, but it is now recognized as a substrate for the synthesis of glycine, pyruvate, and glucose, and an oxidants scavenger which may regulate the redox state of cells [11,12]. Algae can also be a source of sulfonic acid derivatives, like Taurine, Hypotaurine, and Homotaurine (**Fig. 1**), which may play important roles in human and animal health, due to their properties to prevent neurodegenerative diseases.

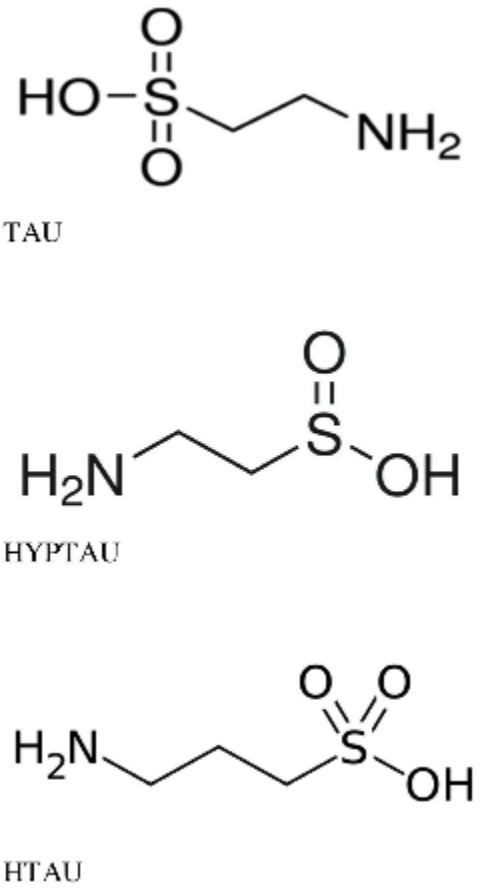


Figure 1. Chemical structure of Taurine, Hypotaurine and Homotaurine.

Taurine (2-aminoethanesulfonic acid) is an amino acid–like compound widely distributed in animals and an essential nutrient in some species. It is involved in the regulation of neuroendocrine functions and nutrition [13], and can show anti-obesity effects in humans [14]. Taurine can effectively prevent glutamate-induced neuronal injury in cultured neurons [15], may play an important role in inflammation associated with oxidative stress [16], and can protect against H2O2-induced cell injury in PC12 cell cultures [17].

Homotaurine (3-Amino-propanesulfonic acid), which can be found in the market as "tramiprosate" (Alzhemed^{TM}), is a small molecule that is naturally present in different species of marine red algae [18]. This compound (an analog of GABA), has been demonstrated to have a neuroprotective effect and has been evaluated as a possible therapeutic agent for Alzheimer's disease [19]. Both in vitro and in vivo models, tramiprosate provide a relevant neuroprotective effect, by preventing the formation of Aβ fibrils and the β-sheet conformation and plaque formation in TgCRND8 mice [20]. Moreover, recent studies have demonstrated positive and significant effects of Homotaurine on the reduction of hippocampal volume loss, on the reduction of global cognitive decline in Apo ε4 allele carriers, and on decline in memory function [21,22].

Hypotaurine (2-aminoethanesulfinic acid), a non-proteinogenic cysteine-oxoform and an intermediate in the biosynthesis of Taurine found in some species of green algae, shows a strong free radical detoxifying action as well as other healthy properties such as antihypertensive and hypocholesterolemic [23]. Fontana et al. [24] pointed out that Hypotaurine is a strong antioxidant in vivo, and a protective agent preventing damage from oxidizing and nitrating agents under physiological conditions, while Araki et al. [25] showed that Hypotaurine may exhibit cytoprotective effect against H_2O_2-induced cell damage by scavenging hydroxyl radicals in placental trophoblast cells.

Analysis of amino acids and sulfonic acid derivatives in algae has been typically carried out by chromatographic methods and hyphenated techniques with pre and post column derivatization, such as ion exchange chromatography [26–29] and high-performance liquid chromatography with UV or fluorescence detection [30].

Typical derivatizing agents include, ortho-phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC), 1-fluoro-2, 4-dinitrobenzene, 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide, dansyl and dabsyl chloride. Anyway, many derivatizing agents suffer from some limitation; OPA cannot react with secondary amino acids such as proline, FMOC-Cl is fluorescent by itself, and may give rise to disubstituted derivatization products with Tyrosine and Histidine, and Dansyl and Dabsyl chloride reactions proceed very slowly especially with Proline. On the contrary, formation of PITC derivatives is rapid and complete, with both primary and secondary amino acids [31].

Despite the extensive literature available about total amino acid profile in algae, data are scattered among the multiple possible species, and there is limited information regarding the content of sulfonic acid derivatives and GABA and Hyp.

For instance, several authors quantified Taurine and main amino acids in some green, red and brown algae species [27,32,33], or quantified only Taurine and Homotaurine by HPLC with fluorescence detection in several non-commercial marine macro algae [30]. Hypotaurine was detected by UPLC-MS/MS, but not quantified, in a metabolomic study including red, brown and green algae [34], as well as by NMR in the green alga Ulva lactuca [35]. GABA and Hyp have been previously quantified at low or even trace amounts in several red, green and brown algal species by colorimetric and chromatographic methods, but only in two works both compounds were considered [32,36,37,50].

So, to the best of our knowledge, there is no published analytical procedure allowing the simultaneous quantification of the amino acid profile (including GABA and Hyp) and the main sulfonic acid derivatives (Taurine, Hypotaurine and Homotaurine) in algae samples. Hence, the aim of this work was to develop and validate a fast and reproducible analytical method to simultaneously quantitate the main amino acids plus Taurine, Hypotaurine and Homotaurine in algae samples by UHPLC-DAD-MS/MS.

2. Materials and methods

2.1. Chemicals and reagents.

Acetonitrile (ACN) and methanol (MeOH) were HPLC gradient-grade (Merck KGaA (Darmstadt, Germany). Perchloric (60%) and hydrochloric acid (37%) were from J.T. Baker (NJ, United States). Sigma-Aldrich Chemie (Sant Quentin Fallavier, France) provided formic acid, ammonium acetate, ammonium formate, phenyl isothiocyanate (PITC), triethylamine (TEA), and pure standards for 19 amino acids, Taurine, Hypotaurine and Homotaurine. Ultrapure water was obtained with a Milli-Q system from Millipore (Bedford,MA, USA).Single stock solutions were prepared for each of the 22 target compounds [Histidine (His), Hypotaurine (Hyptau), Hydroxyproline (Hyp), Taurine (Tau), Homotaurine (HTau), Arginine (Arg), Serine (Ser), Glycine (Gly), Aspartic acid (Asp), Glutamic acid (Glu), Cysteine (Cys), Threonine (Thr), Proline (Pro), Alanine (Ala), Gamma aminobutyric acid (GABA), Lysine (Lys), Tyrosine (Tyr), Methionine(Met), Valine (Val), Isoleucine (Ile), Leucine (Leu), and Phenylalanine (Phe)] by dissolving the corresponding pure standards in 0.1 M HCl. Calibration working solutions were prepared by mixing suitable volumes of each stock solution in 0.1 M HCl, to obtain the following calibration levels for each compound: 0.1 mM, 0.5 mM, 1 mM,1.5 mM and 2.5 mM.

2.2. Algae samples.

Five different species of algae, including macroalgae (red algae *Porphyra* and *Gracilaria*, green algae *Ulva lactuca*, brown algae *Laminaria japonica*) and green microalga *Chlorella*, were purchased in their dehydrated form in commercial establishments in Girona (Spain). All samples, except microalgae *Chlorella*, were in the form of flakes or sheets, so it was necessary to reduce the sample size before extraction, by using a mixer mill (Retsch GmbH & Co, KGGermany). The powdered samples were stored at ambient temperature under dry and dark conditions.

2.3. Amino acid extraction and derivatization.

The samples were processed following the method of Campanella et al. [38] with some modifications. Briefly, for the quantitation of the total amino acids, 10 mg of seaweed simple were placed in 15 mL falcon tubes and 1 mL of 8 M perchloric acid was added. Hydrolysis was carried out for 24 h at 110°C. After cooling at room temperature, the samples were filtered through 0.2 µm membrane syringe filters (GMP filter membranes, MerckKGaA, Darmstadt, Germany), and then derivatized. The derivatization was carried out following the method of Zheng et al. [39], with some modifications. Sample extracts or calibration solutions (40 µL) were pipetted into 10 mL polypropylene tubes and dried under nitrogen at 60◦C. The dried sample was resuspended with 40 µL of a methanol-water-TEA solution (2:2:1, v/v/v), dried again under nitrogen at 60◦C, added with 40 µL of a methanol-water-TEA-PITC solution (7:1:1:1, v/v/v/v), and vigorously mixed. The derivatization was performed for 20 min at ambient temperature, and then the excess reagent was evaporated under nitrogen at 60°C.The derivatized samples were redissolved with 24 µL of mobile phase B and 226 µL of mobile phase A, centrifuged at 11,000 × g for 5 min, filtered through a Thomson Single Step Standard Filter Vials (Thomson Instrument Company, CA, USA), and injected into the UHPLC system (4 µL).

2.4. Chromatographic analysis.

The chromatographic system consisted of an Acquity UPLC® (Waters, Milford, MA, USA), equipped with a diode array detector (Acquity PDA detector, Waters, Milford, MA, USA), an electrospray (ESI) as a source of ionization and a triple quadrupole mass spectrometer (Acquity TQD, Waters, Milford, MA, USA) operated at unit mass resolution. The system was controlled by MassLynx 4.1 software (Waters, Milford, MA, USA).

Four columns (100 mm x 2.1 mm i.d.), packed with different stationary phases, were tested, namely: i) Charged Surface Hybrid particle with C18 reversed phase (1.7 µm, CSH-C18), ii) Phenyl-Hexyl reversed phase (1.7 µm, CSH-PH), iii) Ethylene Bridged Hybrid particle with C18 reversed-phase (1.7 µm, BEH-C18), and iv) High Strength Silica particle with trifunctional C18 alkyl phase bonded (1.8 µm, HSS-T3) (Waters, Milford, MA, USA).

Optimization of the chromatographic performances was carried out by modifying: i) the percentage of organic modifiers (methanol or acetonitrile) in the mobile phase, ii) the pH modifiers (ammonium acetate, ammonium formate, formic acid) in the mobile phase, iii) the flow rate and the gradient elution program, and iv) the column temperature.

Electrospray interface (ESI) was operated in the positive mode; the source temperature was fixed at 135°C, the capillary voltage was set at 3.0 kV and the desolvation temperature was set at 350°C. The cone gas (nitrogen) flow rate was 350 L/h and cone voltage was set at 30 V. MS experiments were carried out in "Scan" mode to obtain m/z values of the molecular ions. MS/MS experiments in "Daughter Ions" mode were also performed, to obtain the fragmentation patterns of molecular ions. The collision energies varied between 10 and 20 eV (**Supplementary material, Table A1**). The gas used in the collision cell was argon at a flow rate of 0.1 mL/min.

Identity of the peaks in the sample extracts was confirmed by comparing their retention times, UV spectra, MS and MS/MS spectra with the corresponding data obtained from pure standards.

Quantitation of the target compounds was done based on an external calibration curve and taking into account the sample dilution during the extraction and derivatization steps. Calibration curve was made by injecting derivatized amounts of pure standards in the range from 0.1 mM to 2.5 mM, and by plotting the signal obtained from the diode array detector at $\lambda = 254$ nm versus the corresponding concentrations.

Table A1. Precursor/products ions and parameters for Daughter-MS/MS experiments.

Amino acid	Retention time	MW	PITC-Amino acid derivative $[M+H]^+$ (m/z)	Main Fragments (m/z)	Cone voltage eV	Collision energy eV
His	1.67	155	291	156, 109	30	20
Hyptau	1.78	109	245	110, 94	30	10
Hyp	1.88	131	268	132, 56	30	20
Tau	1.96	125	261	126, 94	30	20
Htau	2.12	139	275	140, 94	30	20
Arg	2.27	174	310	175	30	20
Ser	2.85	105	241	106, 88	30	20
Gly	3.31	75	211	76	30	20
Asp	3.52	132	268	133, 115, 89	30	20
Glu	3.74	147	283	148, 129, 102, 83	30	20
Thr	3.88	118	254	119, 102, 85	30	20
Cys	4.46	121	257	122, 74	30	20
Pro	4.92	115	251	116, 84	30	10
Ala	5.05	89	225	90	30	10
GABA	5.37	103	239	136, 128, 104, 86	30	20
Lys	7.23	147	283	148, 101	30	10
Tyr	7.88	181	317	182, 165, 146, 90	30	20
Met	8.91	149	285	150, 132, 104, 77	30	20
Val	9.49	117	253	118, 72	30	10
Ileu	12.23	131	267	132, 86, 75	30	10
Leu	12.33	131	267	132, 86	30	20
Phe	12.78	165	301	166, 120, 82	30	10

2.5. Method validation.

The whole protocol of analysis was validated in terms of selectivity, accuracy (precision, trueness), linearity and working range, robustness / ruggedness, uncertainty and detection and quantification limits according to Harmonized guidelines for single laboratory validation of methods of analysis (IUPAC Technical Report), ICH, AOAC, EURACHEM and GUM [40–45].Selectivity is the ability to unequivocally assess the target analyte in the presence of other analytes, matrices or other potentially interfering materials that may be expected to be present in the matrix or sample. Peak resolution for each targeted analyte (Rs) was calculated as a function of both the absolute separation distance expressed as retention times (minutes) of the two peaks, t_{R1} and t_{R2}, and the peak widths at half height, $W1_{1/2}$ and $W2_{1/2}$, of the analyte and nearest peak

Eq. (1).

$$R_S = 1.18 \times \left(\frac{t_{R2} - t_{R1}}{W1_{1/2} + W2_{1/2}} \right)$$

AOAC International has recommended that a suitable Rs value to obtain a usable separation of two peaks is at least 1.5. The linearity was assessed by checking the following parameters: coefficient of determination (R2), residual value of replicates, and Lack-of-fit (LoF) test significance. This test, recommended by the IUPAC validation guidelines [40], measure if the regression model fits the data. The extent of deviation of the points from the line caused by random scatter of the points was estimated by the mean sum of squares of random error (MSS_{error}). This was compared to the extent of

deviation of the points from the line caused by mismatch of the calibration model (mean sum of squares due to lack of fit MSS_{LOF};

Eq. (2).

$$F = \frac{MSS_{LOF}}{MSS_{error}} = \frac{\frac{\sum \left(\bar{y}_{i-\hat{y}_i}\right)^2}{n-2}}{\frac{\sum (y_i - \bar{y}_i)^2}{n(p-1)}}$$

When the $F_{calculated}$ was lower than $F_{tabulated}$, the model was considered to fit the data. The linear ranges were assessed by injecting calibration working solutions of pure compounds at different concentrations, ranging from 0.1 mM to 10.0 mM. The instrument limit of detection (ILOD) and the instrument limit of quantification (ILOQ) were calculated as 3.3/b and 10 /b, respectively, where "σ" is the Residual Standard Deviation of the Calibration Curve ($S_{x/y}$) and "b" is the slope of regression line from the calibration curves of each compound. The Breush-Pagan test, to establish the presence or absence of heteroscedasticity, was also applied. The method limits of detection and quantification (MLOD and MLOQ, respectively) were estimated from ILOD and ILOQ taking into account the dilution factor and the mass fraction of each sample. The accuracy of a measurement result describes how close the result is to its true value and includes the effect of both precision and trueness (expressed in the form of bias). Precision, which relates to the repeatability and / or reproducibility condition of the measurement "getting the same measurement each time", was estimated as both intra-day repeatability (RSD_r) and inter-day reproducibility (RSD_R). RSD_r was calculated by analysing six spiked samples in the same day (n = 6), while RSD_R was assessed by analysing six spiked samples on three different days during the same week (n = 18).

Precision was expressed by relative standard deviation (RSD%) of the measurements and calculated from Eq. (3).

$$RSD\% = \left(\frac{s}{X}\right) \times 100$$

Where “s” is standard deviation of replicates and “X” is the arithmetic mean of the measurements. The repeatability standard deviation varies with concentration, C, that is expressed as a mass fraction. The predicted acceptable value, RSD_r, for each concentration is proximate to the value recommended by the FDA Guidelines for the Validation of Chemical Methods for the Food Program, or can be calculated using the Horwitz equation as follows [40]:

$$RSDr\ (\%) = 2 * C^{-0.15}$$

The acceptable values for repeatability are between ½ and 2 times the calculated values. Trueness (or bias) describes the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value. As no commercial Certified Reference Material was available, spiked samples were analysed to evaluate bias.

Accuracy of the method was assessed by analyzing a Chlorella sample spiked before hydrolysis with known amounts of pure standards at three levels (0.1, 5.0 and 10.0 mM), to cover the working range of the method. Three sample replicates for each spiking level (n = 9) were prepared by adding the suitable volume of the standard solution, allowing the samples to settle for 30 min, and then carrying out the hydrolysis, extraction and derivatization procedures as described above. The robustness or ruggedness of an analytical method is the resistance to change in the results when minor changes are made from the experimental conditions described in the procedure.

Robustness was tested by deliberately introducing small changes into the procedure and examining the effect on the results following the work described by Youden et al. [46], which suggested variations of selected factors at once.

Robustness of the method was determined on the basis of independent assays of a Chlorella sample, following a fractional factorial design obtained by taking into account seven factors (simple weight, volume of hydrolysis reagent, hydrolysis time, hydrolysis temperature, derivatization time, column temperature and flow rate), each of them with two levels (nominal value/alternate value), for a total of eight different combinations analyzed in duplicate (n = 16) (*Supplementary material*, **Table A2**). Once quantified each amino acid, the difference D_i and the standard deviation of the difference SD_i were calculated

Eq. (5).

$$SD_i = \sqrt{2x \sum \left(\frac{D_i^2}{7} \right)}$$

D_i= (difference between the mean concentration obtained with the factor at nominal value and the mean concentration obtained with the factor at alternate value). When SD_i is significantly lower (significance level of 0.05) than the standard deviation of the method carried out under within-laboratory reproducibility conditions (RSD_r) can be concluded there is no global effect of the factors on the result, and the method can be considered robust.

Table A2. Fractional factorial design. Youden robustness experiment.

Minor Changes using the Youden Method								
Sample Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	C	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g
Results	H	I	J	K	L	M	N	O

Minor Changes using the Youden Method								
Sample Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A/a	11 mg	11 mg	11 mg	11 mg	9 mg	9 mg	9 mg	9 mg
B/b	1.1 ml	1.1 ml	0.9 ml	0.9 ml	1.1 ml	1.1 ml	0.9 ml	0.9 ml
C/c	26 h	22 h	26 h	22 h	26 h	22 h	26 h	22 h
D/d	120°C	120°C	100 °C	100 °C	100 °C	100 °C	120°C	120°C
E/e	25 min	15 min	25 min	15 min	15 min	25 min	15 min	25 min
F/f	35 °C	25 °C	25 °C	35 °C	35 °C	25 °C	25 °C	35 °C
G/g	0.45 ml/min	0.25 ml/min	0.25 ml/min	0.45 ml/min	0.25 ml/min	0.45 ml/min	0.45 ml/min	0.25 ml/min
Results	H	I	J	K	L	M	N	O

Variables						
		sample processing			chromatography	
1 **sample weight**	2 **vol. HClO4**	3 **hydrolysis time**	4 **hydrolysis temperature**	5 **derivatization time**	6 **column temperature**	7 **flow rate**
A= 11 mg	B=1.1 ml	C= 26 h	D= 120ºC	E= 25 mins	F =35 ºC	G =0.45 ml/min
a = 9mg	b = 0.9 ml	c= 22 h	d= 100ºC	e= 15 mins	f= 25 ºC	g =0.35 ml/min

Calculation for Robustness for factor A/a:

Differences:

$$D_{A/a} = \left(\frac{H+I+J+K}{4}\right) - \left(\frac{L+M+N+O}{4}\right)$$

Standard deviation of differences:

$$SD_i = \sqrt{2x\sum\left(\frac{D_i^2}{7}\right)}$$

The uncertainty estimation was carried out using GUM Workbench 1.3 TrainMiC software package (Metrodata GmbH) [44]. Among the possible sources of uncertainty (*Ishikawa diagram*, *Supplementary material*, **Fig. 1**), those arising from balances and volumetric measuring devices are covered by the precision and recovery studies since all these instruments are controlled under UNE-EN-ISO/IEC 9001. Sample homogeneity and calibration uncertainties are included in the precision uncertainty because various replicates from the same sample were analyzed and standards were injected each day of analysis. The purity of amino acid standards is given by the manufacturer, but the contribution is so small that could be neglected. So, the expanded uncertainty was estimated using the in-house validation data (precision and trueness).

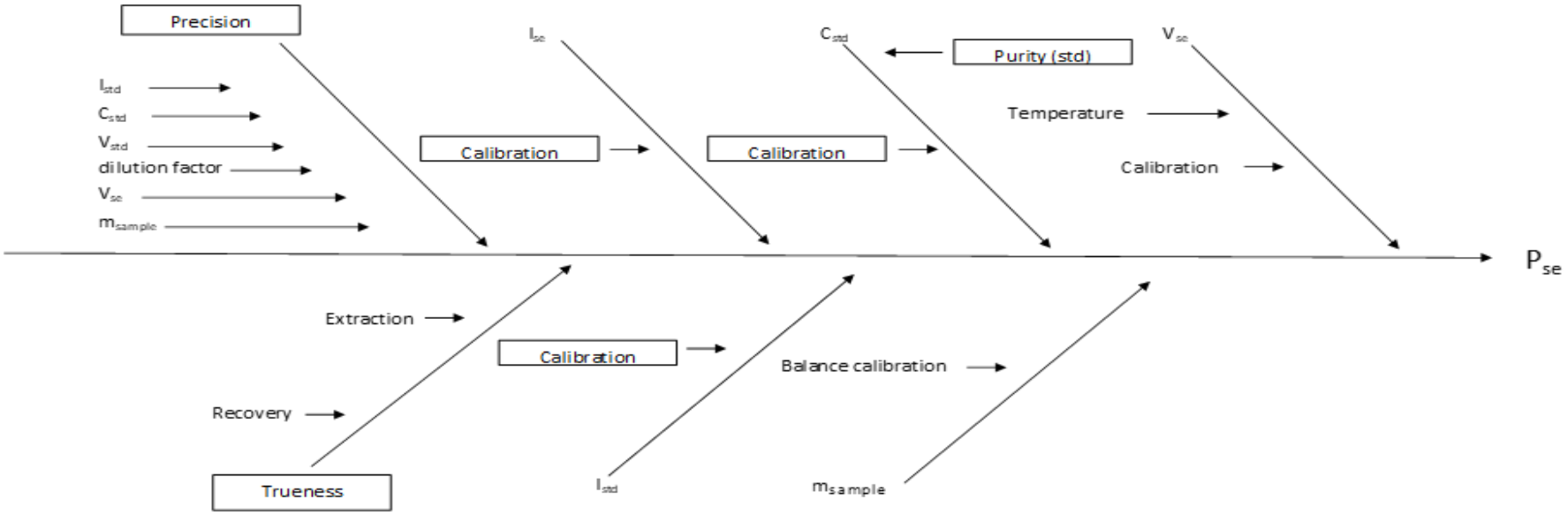


Fig. 1. Ishikawa diagram of the procedure of amino acids determination in algae samples by UPLC-DAD-MS/MS method.

* I_{se} = Peak intensity of the sample extract

P_{se} = Mass fraction of amino acid in the sample

I_{std} = Peak intensity of the amino acids standard

m_{sample} = Mass of the sample

m_{std} = Mass concentration of the amino acid standard

V_{se} = Final volume of the extract

V_{std} = Volume of the amino acid standard

3. Results and discussion.

3.1. Method development.

Different chromatographic conditions were explored, by varying column stationary phase, mobile phase composition and gradient elution profile, to reach a suitable chromatographic separation of all the target compounds in a short time with a mobile phase compatible with both DAD and MS detection. Ammonium acetate, ammonium formate and formic acid, which are volatile and may improve amino acids separation as well as peak shape in UPLC chromatography [47,48] were employed as pH modifiers, while ACN was preferred as organic modifier because provided better peak shapes than methanol. The flow rate was set at 0.4 mL/min and the column temperature was maintained at 30°C.

Preliminary trials showed that mobile phases A (7.5 mmol/L ammonium formate, 7.5 mmol/L ammonium acetate and 0.075% formic acid in aqueous solution) and B (1 mmol/L ammonium formate, 1 mmol/L ammonium acetate and 0.075% formic acid in acetonitrile) gave the better chromatographic performances with all the columns.

On the other hand, stationary phases other than BEH needed larger and more complex elution programs to separate some critical peak pairs, while modifications of the mobile phases were limited, to allow the MS detection. Finally, to show the different behaviour of the four columns, the same elution program was used. Elution was carried out by varying the proportion of the mobile phases A and B; the program started with an isocratic elution with 11% B until 1.3 min., then the percentage of B was increased up to 32% at 15 min. with a linear gradient. Afterwards, the columns were washed with 80% B for one min. and re-equilibrated to the initial conditions for 2 min.

Fig. 2 shows the capacity factor (k) of the 22 target compounds eluted with the four different columns under the same conditions. The BEH-C18 showed a stronger

retention ability as well as better resolution of critical pairs under the same condition, allowing a satisfactory chromatographic separation of all the compounds including Tau, Htau and Hyptau. CSH-PH showed poor resolution, while CSH-C18 could not separate specific pairs of amino acids such as HTau/Arg, Thr/Cys and Ala/GABA. HSS-T3 is ideally suited for the enhanced retention of polar compounds and metabolites by reversed-phase LC, nevertheless His and Hyp nearly coeluted, and critical pairs His/Hyptau, Thr/Cys, Pro/Ala and Leu/Ile were not resolved. Therefore, method validation was carried out only with the BEH-C18 column, which gave the best performances.

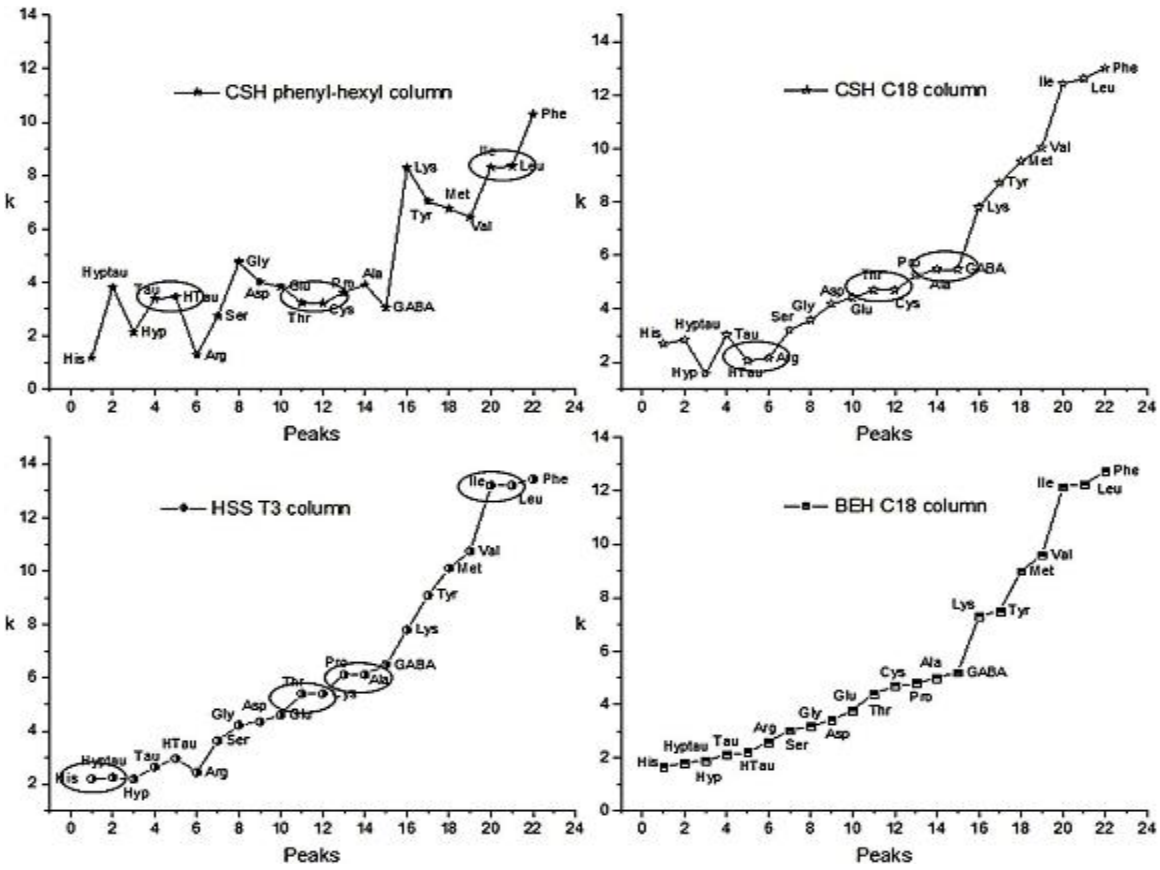


Fig. 2. Elution profile of 4 chromatography columns (CSH phenyl - hexyl 1.7 µm, 2.1 mm × 100 mm; CSH C18 1.7 µm, 2.1 mm × 100 mm; HSS T3 1.8 µm, 2.1 mm × 100 mm and BEH C18 1.7 µm, 2.1 mm × 100 mm). (*) Ovals show co-elution or low resolution of critical peak pairs. His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys

(Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Phenylalanine).

Fig. 3 shows typical chromatographic separations of the 22 target compounds, for a standard solution and an algae extract, with the BEH-C18 column. The high efficiency of the UPLC column allowed a complete separation of Hyptau, Tau, Htau and 19 amino acids derivatized with PITC within 13 min with a reasonable resolution of all the critical peak pairs. This result can be considered satisfactory, taking into account that chromatographic separation of PITC amino acid derivatives is extremely challenging. Other authors, operating with conventional HPLC [49], underlined that the mixture of Ser, His, Glu, Thr, and Arg, as well as Tyr and Leu, could not be completely separated, or reported the incomplete separation of a mixture of Asp, Ser, and Hyp, and of Tyr and Leu. Zheng et al. [39] separated PITC derivatives of 15 amino acids in a total run of 28 min by using UHPLC-ESI-MS.

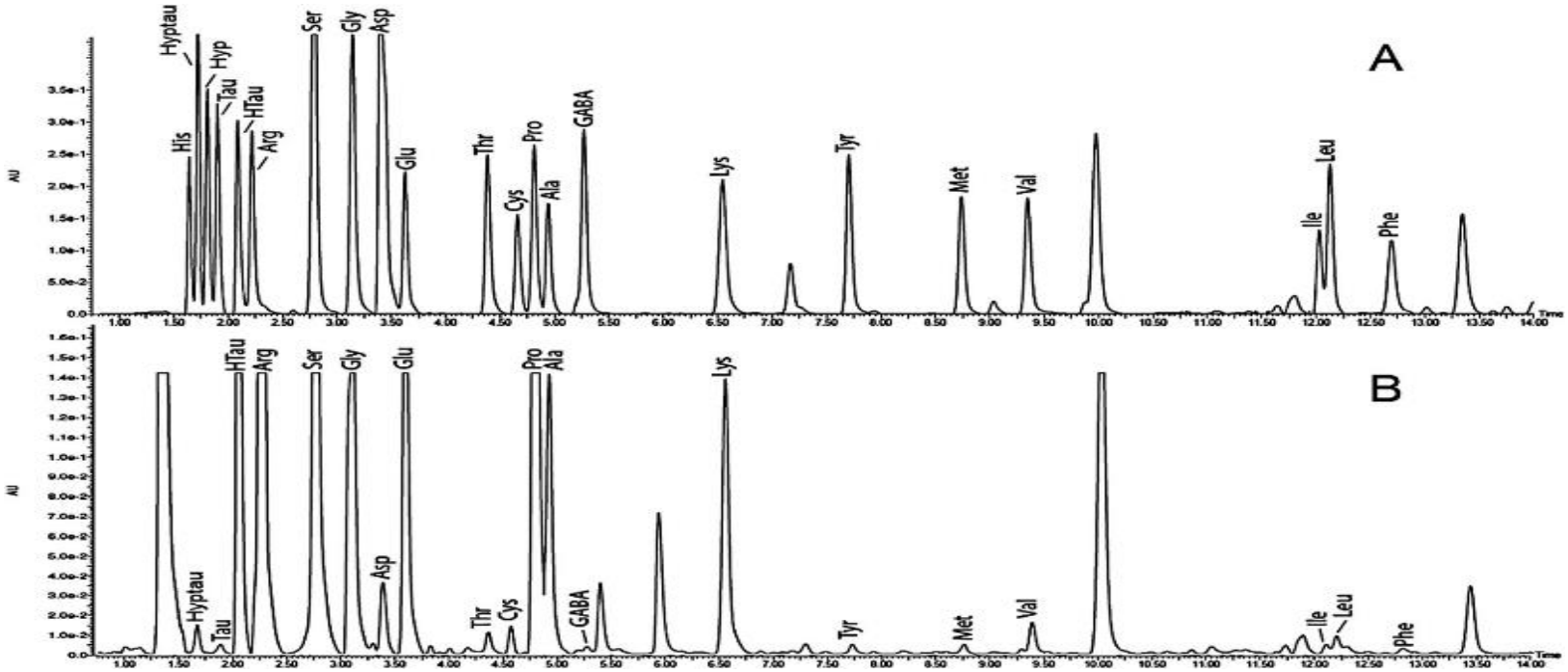


Fig. 3. DAD chromatograms of an amino acid standard mixture (A) and a total amino acids profile in Ulva lactuca (B). His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxyproline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Phenylalanine).

In our study the quantification was performed with the DAD, nevertheless experiments with MS/MS in "daughter" mode were performed to support the identification of the peaks, especially in the case of the three sulfonic acid derivatives. **Fig. 4** shows the MS/MS spectra of Hyptau and Htau PITC derivatives, and their corresponding peaks found in an Ulva lactuca extract. In each spectrum can be recognized the molecular ion of the PITC derivative (Hyptau m/z = 245 and Htau m/z = 275) and the typical fragmentation pattern which includes, in all cases, the molecular mass of each compound (Hyptau m/z = 110 and Htau m/z = 140).

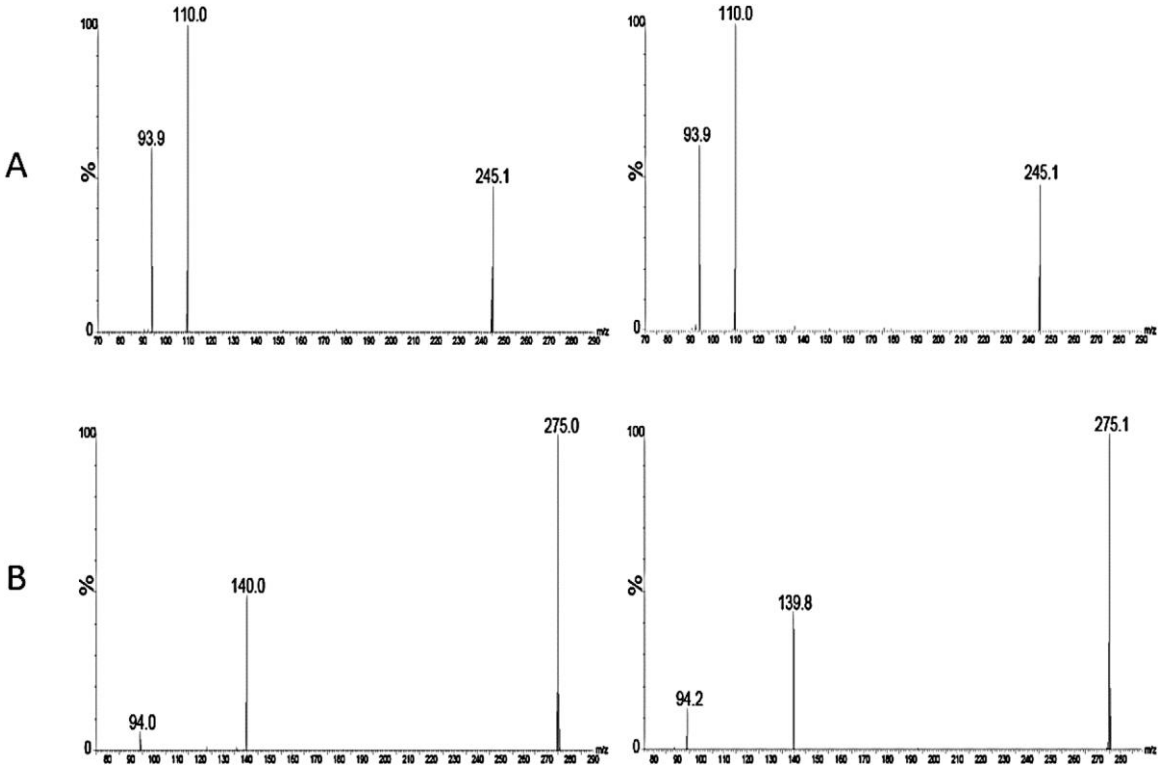


Fig. 4. Daughter ion spectra for Hyptau (A) and Htau (B) from a standard solution (left), and from an Ulva lactuca sample (right) respectively.

3.2. Validation parameters.

The results of method validation and performance parameters are summarized in **Table 1**.

The selectivity expressed as resolution of peak pairs (RS) for all the target compounds was ranged between 2.07–26.9 and 1.55–40.5 in standard solution and samples, respectively (*Supplementary material* **Table A3**). Satisfactory chromatographic separation (Rs > 1.5) was achieved for all the amino acid pairs. Calibrations showed R^2 value always higher than 0.994 for all the compounds and fulfilled the homoscedasticity criterion, and the residual standard deviation approach could be applied. Therefore, $F_{calculated}$ values were lower than $F_{tabulated}$ in the Lack of fit test; so the calibration model fitted well with the data for all the 22 compounds.

Table 1. Validation results for the analysis of total amino acids with the proposed method. His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Phenylalanine).

Amino acid	RT (min)	Regression equation[a]	LoF[b]	R^2	Precision (RSD %)		Youden robustness test	MLOD	MLOQ	% Recovery (n = 9)
					Intraday (n = 6)	Interday (n = 6, in 3 days)	SDi (%) [c]	(μg/g)	(μg/g)	(mean ± SD)
His	1.67	$y = 2.1 \cdot 10^2 x - 33.2$	0.25	0.996	1.2	3.6	1.1	0.043	0.085	99.3 ± 3.2
Hyptau	1.78	$y = 7.5 \cdot 10^2 x - 11.8$	0.32	0.998	1.0	4.7	0.2	0.115	0.220	99.4 ± 2.5
Hyp	1.88	$y = 5.7 \cdot 10^2 x + 17.7$	0.24	0.999	1.2	2.4	0.1	0.042	0.084	98.6 ± 2.1
Tau	1.96	$y = 2.7 \cdot 10^2 x + 28.1$	0.45	0.999	1.9	3.2	0.1	0.036	0.073	103.1 ± 3.4
HTau	2.12	$y = 5.0 \cdot 10^2 x + 73.5$	0.48	0.999	1.0	1.9	0.1	0.066	0.131	97.2 ± 1.6
Arg	2.27	$y = 6.6 \cdot 10^2 x + 19.8$	0.12	0.999	2.7	4.0	3.5	0.040	0.078	96.7 ± 1.4
Ser	2.85	$y = 1.4 \cdot 10^2 x + 60.3$	0.54	0.999	2.8	3.0	2.3	0.022	0.043	97.9 ± 1.9
Gly	3.31	$y = 1.7 \cdot 10^2 x + 22.1$	0.24	0.999	1.2	1.7	1.3	0.005	0.010	99.2 ± 5.5
Asp	3.52	$y = 8.7 \cdot 10^2 x + 12.3$	0.36	0.998	2.8	3.3	1.9	0.036	0.071	98.3 ± 6.1
Glu	3.74	$y = 7.1 \cdot 10^2 x - 24.74$	0.24	0.999	2.1	5.4	5.2	0.026	0.052	102.2 ± 2.4
Cys	3.88	$y = 4.7 \cdot 10^2 x - 11.29$	0.28	0.996	2.5	3.2	1.4	0.063	0.119	95.4 ± 1.9
Thr	4.46	$y = 7.9 \cdot 10^2 x + 16.35$	0.29	0.998	1.5	3.2	0.9	0.039	0.078	97.6 ± 1.6
Pro	4.92	$y = 9.4 \cdot 10^2 x - 31.41$	0.27	0.999	1.3	2.2	1.9	0.022	0.044	96.6 ± 2.6
Ala	5.05	$y = 7.0 \cdot 10^2 x + 60.39$	0.27	0.997	2.2	4.0	2.1	0.033	0.064	99.1 ± 7.7
GABA	5.37	$y = 4.5 \cdot 10^2 x + 44.53$	0.24	0.999	3.3	4.5	0.1	0.042	0.084	98.2 ± 2.3
Lys	7.23	$y = 5.4 \cdot 10^2 x - 16.33$	0.44	0.998	4.7	5.8	4.6	0.042	0.084	96.1 ± 3.1
Tyr	7.88	$y = 1.0 \cdot 10^2 x + 40.09$	0.41	0.998	2.1	2.5	0.5	0.048	0.094	104.8 ± 0.9
Met	8.91	$y = 8.4 \cdot 10^2 x + 10.46$	0.47	0.999	3.4	3.6	0.4	0.026	0.053	97.4 ± 5.4
Val	9.49	$y = 9.0 \cdot 10^2 x + 53.91$	0.21	0.999	1.7	3.3	2.3	0.021	0.041	103.2 ± 2.8
Ile	12.23	$y = 1.8 \cdot 10^2 x + 12.65$	0.23	0.997	2.4	3.7	3.5	0.092	0.179	103.4 ± 6.3
Leu	12.33	$y = 1.6 \cdot 10^2 x + 11.29$	0.28	0.998	2.6	4.8	0.3	0.070	0.131	99.7 ± 3.8
Phe	12.78	$y = 8.0 \cdot 10^2 x + 63.01$	0.44	0.999	3.1	5.1	0.6	0.090	0.173	99.6 ± 3.1

[a] y = signal intensity; x = compound concentration.
[b] LoF = lack of fit test (Ftab: 2.69).
[c] S_{Di} = standard deviation of differences.

Table A3. Results of selectivity test on standard stock solution and samples.

Amino acid	Peak Resolution, RS			
Peak pairs	Reference std	*Chlorella*	*Porphyra*	*Laminaria*
His / Hyptau	2.16	2.12	2.36	2.60
Hyptau / Hyp	2.15	4.05	3.25	3.54
Hyp / Tau	2.36	2.36	2.07	4.86
Tau / Htau	3.78	2.53	4.25	4.02
Htau / Arg	2.21	2.87	3.30	2.53
Arg / Ser	4.03	6.84	6.73	7.08
Ser / Gly	3.10	4.83	5.43	5.64
Gly / Asp	2.25	1.97	2.48	3.39
Asp / Glu	2.88	1.73	1.85	2.07
Glu / Thr	2.07	1.65	1.72	1.18
Thr / Cys	4.56	3.87	3.89	3.15
Cys / Pro	4.18	2.66	3.54	3.88
Pro / Ala	2.56	1.97	1.92	3.07
Ala / GABA	4.20	2.70	2.36	2.60
GABA / Lys	10.45	10.9	8.04	9.10
Lys / Tyr	2.79	2.88	2.26	2.93
Tyr / Met	4.96	6.40	4.43	4.05
Met / Val	2.85	3.89	3.11	2.21
Val / Ileu	23.0	19.1	18.8	14.7
Leu / le	2.36	1.57	1.77	1.52
Ile / Phe	2.95	3.70	4.52	4.18

The linear range was initially tested between 0.1 mM–2.5 mM. Preliminary analysis of samples gave the need to extent the upper limit of the calibration curve to 10 mM for several amino acids. So the working range for all the amino acids was finally established between 0.1 mM–10.0 mM (0.04 mg/g and 98.3 mg/g) (*Supplementary material*, **Table A4**).

The method limits of detection and quantification were comprised between $0.005*10^{-3}$ mg/g to $0.11*10^{-3}$ mg/g and $0.01*10^{-3}$ mg/g to $0.22*10^{-3}$ mg/g respectively, for all the target compounds. Gly was detected at the lowest detection and quantification limits. Hyptau was detected at the highest detection and

quantification limits. Precision, expressed as RSD%, ranged from 1.0% to 4.7%, (intra-day) and from 1.7% to 5.8% (inter-day). The results indicated that there is no remarkable variability in precision at different concentrations measured on the same or in different days. Method recoveries for overall amino acids at different concentrations were found within the range of 95% and 105%. These results indicate that the bias due to the effects of operating on the added analyte, which was conducted independently on different days, were very small. The results largely achieved the accepted value of recovery at certain analyte concentration levels recommended by the IUPAC Technical report [40]. Regarding the robustness of the method, all the SD_i values were lower than the relative standard deviation of within lab reproducibility meaning that the method is robust for the 7 factors studied (**Table 1**). However, there are specific variables that contribute to a larger variation of D_i. For instance, variations in volume of perchloric acid and temperature of hydrolysis have both the major differences for all the amino acids while hydrolysis and derivatization times have the minor effect.

Results for standard and expanded uncertainty are summarized in *Supplementary materi*al **Table A5**. Expanded uncertainty was calculated for a level of confidence of approximately 95% considering a coverage factor of 2, because when assuming infinite degrees of freedom, t-Student distribution tends to a normal distribution. As shown in **Table A5**, the values of uncertainty for each amino acid due to within lab reproducibility study, u (RSD_R), ranged between 0.17 mg/g – 3.95 mg/g, where Met and Arg represented the lowest and highest uncertainty of method precision, respectively. Therefore, the uncertainty of method recovery ranged between 0.07 mg/g to 2.75 mg/g. Hence, the precision is the largest contribution to the measurement uncertainty. Since this component is derived from the overall variability in the method, further experiments would be needed to show where improvements could be

made. Finally, the expanded uncertainty for all the 22 compounds was ranged between 0.001 mg/g to 2.5 mg/g, and the mean expanded uncertainty was 0.7 mg/g.

Table A5. Uncertainty results.

Amino acid	Pse $_{(mg/g)}$ #	u(RSD_R) [%] *	u(rec) [%] **	u (Pse) [%] **	Coverage factor	Coverage
His	22.9	2.95	1.68	0.78	2	95% (t-table 95,45%)
Hyptau	0.55	1.43	0.72	0.01	2	95% (t-table 95,45%)
Hyp	0.24	0.52	0.22	0.001	2	95% (t-table 95,45%)
Tau	0.66	3.21	2.75	0.03	2	95% (t-table 95,45%)
Htau	0.76	3.24	0.51	0.02	2	95% (t-table 95,45%)
Arg	50.2	3.95	2.39	2.30	2	95% (t-table 95,45%)
Ser	54.0	1.22	0.90	0.83	2	95% (t-table 95,45%)
Gly	36.0	1.47	0.85	0.59	2	95% (t-table 95,45%)
Asp	98.2	1.48	0.18	1.40	2	95% (t-table 95,45%)
Glu	89.1	2.94	1.19	2.50	2	95% (t-table 95,45%)
Thr	30.5	2.28	0.29	0.69	2	95% (t-table 95,45%)
Cys	35.0	1.26	0.95	0.54	2	95% (t-table 95,45%)
Pro	35.3	2.50	0.78	0.92	2	95% (t-table 95,45%)
Ala	96.8	1.27	0.83	1.50	2	95% (t-table 95,45%)
GABA	18.5	3.27	1.01	0.63	2	95% (t-table 95,45%)
Lys	1.97	0.30	0.07	0.01	2	95% (t-table 95,45%)
Tyr	12.8	0.58	0.32	0.09	2	95% (t-table 95,45%)
Met	0.77	0.17	1.13	0.01	2	95% (t-table 95,45%)
Val	10.3	3.54	1.02	0.38	2	95% (t-table 95,45%)
Ile	32.1	3.75	0.22	1.20	2	95% (t-table 95,45%)
Leu	33.2	3.75	0.68	0.38	2	95% (t-table 95,45%)
Phe	9.14	1.50	0.82	0.15	2	95% (t-table 95,45%)

$Pse_{(mg/g)}$ = amino acid concentration (mg/g)

* $u(RSD_R)$ = standard uncertainty for within lab reproducibility

** $u(rec)$ = standard uncertainty for recovery

*** $u(Pse)$ = Expanded uncertainty

Although several papers can be found about the analysis of total amino acids in algae, few of them include results of validation studies. Considering only published methods which provide data about validation, the overall chromatographic performances of our method are similar or better, especially the higher number of compounds simultaneously quantified and the shorter total runtime. For instance, Sanchez-Machado et al. [50] reported the separation of 17 amino acids in algae with a total run of 35 min., with RSD% values ranging between 1.3% and 3.8% and estimated instrument limits of detection ranged between 6.9 ng/mL and 13 ng/mL. Other authors [30] developed an HPLC-FLD method to separate and quantitate only Tau and Htau in a total run of 20 min. In this case the RSD% and recovery values were comprised between 2% and 6% and 94% and 110%, respectively and instrument limits of detection for Tau and Htau were 30 ng/mL and 15 ng/mL, respectively. Campanella et al [38] described an HPLC - UV method to analyze 18 amino acids in algae samples in a total run of 30 min with recovery values ranging between 87% and 102%. Besides, almost all the non-validated methods found in the literature [26–29, 32, 33] has been performed by using automated amino acids analyzers with longer run times and less compounds quantified. Finally, none of them include Hyptau, Tau and Htau in a single chromatographic run. (*Supplementary material*, **Table A6**).

Table A6. Comparative chromatographic performances of total amino acids in algae.

	Validated methods									Non-validated methods						
HPLC parameters	Our results			[49]		[30]			[38]	[26]	[27]	[28]	[29]	[32]	[33]	[50]
Detection mode	DAD-MS/MS			DAD		FLD			DAD	Automated amino acid analyzer						DAD
Total Run time (min)	18			35		20			30	102						
No amino acids	22			17		2			18	18	11	21	17	13	20	21
	MLOD*	RSD	Recovery	ILOD**	RSD	ILOD**	RSD	Recovery	Recovery							
amino acid	ng/g	%	%	ng/mL	%	ng/mL	%	%	range (%)							
His	43	1.2	99	12	2.8											
Hyptau	115	1.0	99													
Hyp	42	1.2	99													
Tau	36	1.9	103			30	3.9	101								
HTau	66	1.0	97			15	4.0	98								
Arg	40	2.7	97	10	2.5											
Ser	22	2.8	98	8.1	1.7											
Gly	5.0	1.2	99	13	2.9											
Asp	36	2.8	98	8.9	2.0											
Glu	26	2.1	102	7.5	2.0											
Cys	63	2.5	95						(87 - 102)							
Thr	39	1.5	98	6.9	2.2											
Pro	22	1.3	97	8.3	2.9											
Ala	33	2.2	99	1.4	2.7											
GABA	42	3.3	98													
Lys	42	4.7	96	8.5	1.7											
Tyr	48	2.1	105	7.6	3.1											
Met	26	3.4	97	8.9	3.9											
Val	21	1.7	103	6.9	2.8											
Ile	92	2.4	103	7.8	1.3											
Leu	70	2.6	100	7.9	2.3											
Phe	90	3.1	100	13	2.5											

* Method limit of detection

** Instrument limit of detection

3.3. Analysis of commercial algae samples.

Once validated, the method was used to quantify the levels of 19 amino acids, Tau, Htau and Hyptau in five commercial samples of different algae species. Concentrations of each compound are listed in **Table 2**.

One of the main results of this study is that some relevant compounds were quantified for the first time in the five algae species, namely: i) Tau in *G. longissima* and *Chlorella spp*., ii) GABA in *G. longissima* and *L. japonica*, iii) Hyp in *G. longissima*, *Ulva lactuca*, *Porphyra spp*., and *L. japonica* and iv) Htau and Hyptau in the five species studied. Comparative results from other authors regarding the amino acid content in algae are summarized in *Supplementary material*, **Tables A7–A10**.

The overall concentration of EAAs are in good agreement with other studies, which were carried out with *L. japonica*, *Chlorella spp*.and *Porphyra spp*. [26–28]. Mc Cusker et al. [27] reported a content of 37.54 mg/g of EAAs in *Porphyra spp*., which also agrees with our results (**Table 2**). As a rule, the levels of NEAAs found in the five commercial samples were consistent with previously published data, reporting high levels of glutamic and aspartic acids in *Chlorella spp*., *Porphyra spp*. and *Laminaria japonica* [26–29]. We detected GABA at significant amounts in *Chlorella spp*. (18.49 mg/g) and *Porphyra spp*. (5.90 mg/g). Concentrations of Hyp were generally lower, and only in the case of *U. lactuca* reached 0.95 mg/g (**Table 2**). As previously outlined, there is a substantial lack of information about the presence of GABA and Hyp in algae. Anyway, Eun-Sun Hwang et al. [32] found 0.31 mg/g of GABA in *Porphyra tenera*, which agrees with our results in *Porphyra spp*. In contrast, Brown et al. [51] reported lower contents of GABA and Hyp in *Chlorella spp*. strains than in our study. The concentrations of Tau agreed with previous Works highlighting the occurrence of this sulfonic acid derivative mostly in red algae species (**Table 2**) [27, 28, 32].

With the proposed method, Hyptau was detected and quantified in the five algae species at concentrations ranging from 0.55 mg/g (*Chlorella spp*.) to 0.19 mg/g (*L. japonica*), while the amount of Htau varied between 4.26 mg/g (*U. lactuca*) and 0.18 mg/g (*L. japonica*) (**Table 2**). Studies about the content of sulfonic acid derivatives in algae has been overlooked, so the comparison with previous works is limited by the substantial lack of data for many species. For instance, Mehdinia et al. [30] quantified Tau and Htau in several marine macroalgae and outlined levels between 0.009 mg/g and 2.5 mg/g for Tau and from 0.0003 mg/g to 0.7 mg/g for Htau. In other previous studies, Hyptau was detected in the green alga *Ulva lactuca*, but authors did not report quantitative data [23, 34, 35].

Table 2. Concentrations (mg/g dry weight) of amino acids and sulfonic acid derivatives in the five algae samples included in the study (values are means of n = 3 independent determinations ± standard deviation).

	Gracilaria longissima (red)	*Ulva lactuca* (green)	*Chlorella spp.* (green)	*Porphyra spp.* (red)	*Laminaria japonica* (brown)
Ile	4.11 ± 0.04	4.47 ± 0.31	3.82 ± 0.18	5.72 ± 0.22	3.87 ± 0.04
Leu	8.59 ± 0.07	8.42 ± 0.58	10.10 ± 0.74	12.08 ± 0.40	7.90 ± 0.40
Lys	11.63 ± 0.19	17.24 ± 0.39	21.21 ± 0.17	10.54 ± 0.21	3.95 ± 0.07
Met	0.17 ± 0.01	1.17 ± 0.07	0.77 ± 0.01	1.88 ± 0.04	6.68 ± 0.92
Phe	7.81 ± 0.12	6.51 ± 0.17	6.92 ± 0.07	8.79 ± 0.34	6.80 ± 0.04
Thr	4.44 ± 0.16	10.77 ± 0.10	30.51 ± 1.23	3.33 ± 0.09	2.78 ± 0.02
Val	11.34 ± 0.07	18.76 ± 1.18	10.26 ± 0.92	11.76 ± 0.21	4.13 ± 0.06
Arg	3.12 ± 0.16	3.17 ± 0.27	2.15 ± 0.07	4.41 ± 0.08	3.34 ± 0.07
His	1.02 ± 0.07	7.26 ± 1.28	22.96 ± 1.29	1.24 ± 0.04	8.90 ± 0.46
[Ω] ΣEAA	52.23 ± 4.23	77.72 ± 6.03	108.72 ± 10.43	59.73 ± 4.25	48.35 ± 2.22
Ala	2.78 ± 0.19	15.05 ± 0.84	96.80 ± 1.44	19.62 ± 1.04	4.42 ± 0.18
Tyr	2.38 ± 0.10	1.62 ± 0.06	12.84 ± 0.83	12.20 ± 0.37	3.52 ± 0.19
Asp	86.46 ± 1.61	29.15 ± 1.36	98.18 ± 3.42	39.02 ± 1.22	14.01 ± 0.28
Cys	1.49 ± 0.07	1.40 ± 0.08	4.01 ± 0.13	2.93 ± 0.18	0.71 ± 0.01
Glu	18.15 ± 0.27	33.08 ± 0.85	89.14 ± 1.73	26.63 ± 0.20	3.72 ± 0.19
Gly	13.31 ± 0.71	14.83 ± 0.25	15.97 ± 0.87	18.78 ± 1.03	5.83 ± 0.37
Pro	9.38 ± 0.91	78.02 ± 1.25	35.28 ± 1.23	18.61 ± 1.27	2.23 ± 0.15
Ser	12.83 ± 0.15	12.27 ± 0.18	13.98 ± 0.75	19.72 ± 1.27	10.21 ± 0.26
GABA	2.56 ± 0.11	0.86 ± 0.04	18.49 ± 1.48	5.90 ± 0.10	0.45 ± 0.03
Hyp	0.08 ± 0.01	0.95 ± 0.01	0.24 ± 0.01	0.09 ± 0.01	0.04 ± 0.01
[#] ΣNEAA	149.42 ± 25.87	187.23 ± 23.85	384.93 ± 39.94	163.49 ± 11.66	45.14 ± 4.50
Hyptau	0.24 ± 0.01	0.35±.016	0.55 ± 0.02	0.21 ± 0.01	0.19 ± 0.01
Tau	13.03 ± 0.71	0.17 ± 0.02	0.66 ± 0.04	6.30 ± 0.12	0.05 ± 0.01
HTau	0.19 ± 0.01	4.26 ± 0.01	0.76 ± 0.06	0.51 ± 0.03	0.18 ± 0.03
[$] ΣSAD	13.46 ± 7.40	4.78 ± 2.31	1.97 ± 0.11	7.02 ± 3.43	0.42 ± 0.08

([Ω]) ΣEAA – Sum of essencial amino acids; (#) ΣNEAA – Sum of non-essential amino acids; ([$]) ΣSAD – Sum of Tau, Hyptau and Htau. His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Pheylalanine).

Table A7. Comparative results in *Porphyra sp*. Values expressed as mg/g d.w. *data has been converted from g/16 g N units to mg/g d.w. taking into account that 16 g N (nitrogen) correspond to approximately 100 g protein (Food composition Data, FAO, 2003) and considering the protein content (in g / 100 g d.w.) in the algal sample.

Porphyra spp. (red)	*Our results*	*Dawczynski et al. [28] **	*Mišurcová et al. [26] **	*Sanchez-machado et al. [49]*	*McCusker et al. [27]*
Ile	5.72	8.37	9.10	4.60	3.51
Leu	12.1	14.9	15.2	7.10	6.16
Lys	10.5	13.2	10.3	7.70	5.18
Met	1.88	4.86	8.34	1.60	1.66
Phe	8.79	8.91	11.3	16.6	4.25
Thr	3.33	14.3	13.1	9.70	3.69
Val	11.7	14.0	14.6	7.20	4.79
Arg	4.41	15.9	19.5	7.60	6.93
His	1.24	7.02	5.24	8.30	1.27
ΣEAA	59.7	102	107	70.4	37.4
Ala	19.6	16.7	18.1	14.5	
Tyr	12.2	9.18	7.67	4.40	
Asp	39.0	22.9	27.2	11.5	
Cys	2.93	3.24	7.56		
Glu	26.6	27.5	28.9	12.7	
Gly	18.8	13.7	14.9	9.40	
Pro	18.6	9.45	9.72	8.40	
Ser	19.7	10.8	12.4	6.70	
GABA	5.90				
Hyp	0.09				
ΣNEAA	163	114	127	67.6	
Total	223	215	233	138	
Hyptau	0.21				
Tau	6.30	11.6			1.22
HTau	0.51				
ΣESAD	7.02				

Table A8. Comparative results in *Laminaria japonica*. Values expressed as mg/g d.w. *data has been converted from g/16 g N units to mg/g d.w. taking into account that 16 g N (nitrogen) correspond to approximately 100 g protein (Food composition Data, FAO, 2003) and considering the protein content (in g / 100 g d.w.) in the algal sample.

Laminaria japonica (brown)	*Our results*	*Dawczynski et al. [28] **	*Mišurcová et al. [26] **	*McCusker et al. [27]*
Ile	3.87	1.70	1.58	4.34
Leu	7.90	3.09	2.78	8.39
Lys	3.95	2.46	2.02	9.97
Met	6.68	0.57	1.25	2.17
Phe	6.80	2.02	1.76	5.48
Thr	2.78	2.21	2.22	7.01
Val	4.13	2.39	2.37	6.68
Arg	3.34	2.08	2.09	5.42
His	8.90	1.39	0.77	2.21
ΣEAA	48.3	17.9	16.8	51.7
Ala	4.42	3.59	3.83	
Tyr	3.52	1.07	0.9	
Asp	14.0	7.88	5.32	
Cys	0.71	0.76	1.34	
Glu	3.72	14.9	9.69	
Gly	5.83	2.52	2.38	
Pro	2.23	1.95	3.18	
Ser	10.2	2.08	1.85	
GABA	0.45			
Hyp	0.04			
ΣNEAA	45.1	34.8	28.5	
Total	93.5			
Hyptau	0.19			
Tau	0.06	0.19		0.02
HTau	0.18			
ΣESAD	0.42			

Table A9. Comparative results in *Ulva lactuca* Values expressed as mg/g d.w.

Ulva lactuca (green)	*Our results*	*McCusker et al. [27]*
Ile	4.47	9.26
Leu	8.42	16.8
Lys	17.2	11.6
Met	1.17	4.47
Phe	6.51	11.7
Thr	10.8	14.3
Val	18.8	16.3
Arg	3.17	16.0
His	7.26	4.52
%EAA	77.8	105
Ala	15.1	
Tyr	1.62	
Asp	29.1	
Cys	1.40	
Glu	33.1	
Gly	14.8	
Pro	78.0	
Ser	12.3	
GABA	0.86	
Hyp	0.95	
%NEAA	187	
Total	265	
Hyptau	0.35	
Tau	0.17	0.01
HTau	4.26	
ΣSAD	4.78	

Table A10. Comparative results in *Chlorella sp*. Values expressed as mg/g d.w.

Chlorella sp. (brown)	*Our results*	*Kent et al. [29]*	*Brown et al. [50]*
Ile	3.82	44.0	42.0
Leu	10.1	92.0	74.0
Lys	21.2	88.9	61.0
Met	0.77	22.3	23.0
Phe	6.92	54.7	58.0
Thr	30.5	47.4	53.0
Val	10.3	61.0	63.0
Arg	2.15	71.5	69.0
His	23.0	24.3	19.0
%EAA	109	506	462
Ala	96.8	47.4	85.0
Tyr	12.8	41.6	42.0
Asp	98.2	93.6	
Cys	4.01	4.35	8.70
Glu	89.1	128	
Gly	16.0	53.8	60.0
Pro	35.3	47.8	
Ser	14.0	40.4	49.0
GABA	18.5		8.10
Hyp	0.24		1.70
%NEAA	385	457	255
Total	494	963	717
Hyptau	0.55		
Tau	0.66		
HTau	0.76		
Σ%AD	1.97		

4.

Conclusions.

In this study, a validated UPLC-DAD-MS/MS method is proposed to simultaneously quantify 19 amino acids and three sulfonic acid derivatives (Hyptau, Tau and Htau), which have been demonstrated to have interesting bioactive functions, with a short chromatographic run (15 min). To the best of our knowledge, it is the first time that these amino acids and sulfonic acid derivatives are separated and quantified in a single chromatographic run. Both chromatographic performances and validation parameters were satisfactory in terms of resolution of critical peaks pairs, linearity, working

range, LOD, LOQ, accuracy, precision and robustness, indicating that the method is suitable for the routinely assessment of the target compounds in algae sample at trace levels. Moreover, the measurement uncertainty of the entire analytical method is reported. The major contribution to uncertainty arises from precision study and expanded uncertainties of amino acids ranged from 0.001 mg/g o 2.50 mg/g. Our method is based on sample derivatization with PITC and DAD detection; a protocol that can be easily implemented for routine analysis of algae samples. Furthermore, the fast and simultaneous profiling of both amino acids and sulfonic acid derivatives makes the proposed method very useful for high throughput Evaluación purposes, when the occurrence and concentration of these bioactive molecules should be assessed in a wide number of different algae species. The analyses of five commercial edible algae with the proposed method gave results that were generally in good agreement with other studies reporting the amino acid content of algal samples. Notwithstanding, with the method developed in the present work we quantified for the first time: i) Tau in *Gracilaria longissima* and *Chlorella spp*., ii) GABA in *Gracilaria longissima* and *Laminaria japo*nica, iii) Hyp in *Gracilaria longissima*, *Ulva lactuca*, *Porphyra spp*., and *Laminaria japonica*, and v) Hyptau and Htau in the five species included in this study.

Acknowledgements.

This research was supported by the CERCA programme/Generalitat de Catalunya and ALGAE project “Retos Colaboracion” (2016, RTC-2016-5183-2).

References

[1] J.J. Otten, J.P. Hellwig, L.D. Meyers, Dietary Reference Intakes: The Essential Guide to Nutrient Requirements, 2006, http://dx.doi.org/10.1016/S0002-8223(02)90177-X.

[2] WHO/FAO/UNU Expert Consultation, Protein and Amino Acid Requirements in Human Nutrition, World Health Organ. Tech. Rep. Ser., 2007, doi:ISBN 92 4120935 6.

[3] M.L. Wells, P. Potin, J.S. Craigie, J.A. Raven, S.S. Merchant, K.E. Helliwell, A. G. Smith, M.E. Camire, S.H. Brawley, Algae as nutritional and functional food sources: revisiting our understanding, J. Appl. Phycol. (2017), http://dx.doi.org/10.1007/s10811-016-0974-5.

[4] S. Mabeau, J. Fleurence, Seaweed in food products: biochemical and nutritional aspects, Trends Food Sci. Technol. 4 (1993) 103–107, http://dx.doi.org/10.1016/0924-2244(93)90091-N.

[5] P. Rupérez, F. Saura-Calixto, Dietary fibre and physicochemical properties of edible Spanish seaweeds, Eur. Food Res. Technol. (2001), http://dx.doi.org/10.1007/s002170000264.

[6] J. Fleurence, Seaweed proteins: biochemical, nutritional aspects and potential uses, Trends Food Sci. Technol. (1999), http://dx.doi.org/10.1016/S0924-2244(99)00015-1.

[7] E.W. Becker, Micro algae as a source of protein, Biotechnol. Adv. (2007),http://dx.doi.org/10.1016/j.biotechadv.2006.11.002.

[8] A.V. Galland-Irmouli, J. Fleurence, R. Lamghari, M. Luc¸ on, C. Rouxel, O. Barbaroux, J.P. Bronowicki, C. Villaume, J.L. Guéant, Nutritional value of proteins from edible seaweed Palmaria palmata (Dulse), J. Nutr. Biochem. (1999), http://dx.doi.org/10.1016/S0955-2863(99)00014-5.

[9] P.A. Harnedy, R.J. Fitzgerald, Bioactive proteins, peptides, and amino acids from macroalgae, J. Phycol. (2011), http://dx.doi.org/10.1111/j.1529-8817.2011.00969.x.

[10] M. Diana, J. Quílez, M. Rafecas, Gamma-aminobutyric acid as a bioactive compound in foods: a review, J. Funct. Foods (2014), http://dx.doi.org/10.1016/j.jff.2014.07.004.

[11] J.M. Phang, S.P. Donald, J. Pandhare, Y. Liu, The metabolism of proline, a stress substrate, modulates carcinogenic pathways, Amino Acids (2008), http://dx.doi.org/10.1007/s00726-008-0063-4.

[12] J.M. Phang, W. Liu, O. Zabirnyk, Proline metabolism and microenvironmental stress, Annu. Rev. Nutr. (2010), http://dx.doi.org/10.1146/annurev.nutr.012809.104638.

[13] H. Yu, Z. Guo, S. Shen, W. Shan, Effects of taurine on gut microbiota and metabolism in mice, Amino Acids (2016), http://dx.doi.org/10.1007/s00726-016-2219-y.

[14] S. Murakami, Role of taurine in the pathogenesis of obesity, Mol. Nutr. FoodRes. 59 (2015) 1353–1363, http://dx.doi.org/10.1002/mnfr.201500067.

[15] J.Y. Wu, H. Prentice, Role of taurine in the central nervous system, J. Biomed.Sci. (2010), http://dx.doi.org/10.1186/1423-0127/7-S1-S1.

[16] J. Marcinkiewicz, E. Kontny, Taurine and inflammatory diseases, Amino Acids46 (2014) 7–20, http://dx.doi.org/10.1007/s00726-012/361-4.

[17] C.L. Pan, G.S. Giraldo, H. Prentice, J.Y. Wu, Taurine protection of PC12 cells against endoplasmic reticulum stress induced by oxidative stress, J. Biomed.Sci. (2010), http://dx.doi.org/10.1186/1423-0127-17-S1-S17, Artn S17r.

[18] K. Ito, K. Miyazawa, F. Matsumoto, Amino acid composition of the ethanolic extractives from 31 species of marine red algae, Hiroshima DaigakuSui-Chikusangakubu KiyoJ. (1977).

[19] L. Ricciardi, F. De Nigris, A. Specchia, A. Fasano, Homotaurine in Parkinson'sdisease, Neurol. Sci. (2015), http://dx.doi.org/10.1007/s10072-015-2201-6.

[20] F. Gervais, J. Paquette, C. Morissette, P. Krzywkowski, M. Yu, M. Azzi, D.Lacombe, X. Kong, A. Aman, J. Laurin, W.A. Szarek, P. Tremblay, Targetingsoluble A peptide with Tramiprosate for the treatment of brain amyloidosis,Neurobiol.Aging(2007),http://dx.doi.org/10.1016/j.neurobiolaging.2006.02.015.

[21] G. Spalletta, L. Cravello, W. Gianni, F. Piras, M. Iorio, C. Cacciari, A.R. Casini, C.Chiapponi, G. Sancesario, C. Fratangeli, M.D. Orfei, C. Caltagirone, F. Piras, Homotaurine effects on hippocampal volume loss and episodic memory inamnestic mild cognitive impairment, J. Alzheimers Dis. (2016), http://dx.doi.org/10.3233/JAD-150484.

[22] A. Martorana, C. Motta, G. Koch, M. Massaia, S. Mondino, I. Raniero, A. Vacca, F.Di Lorenzo, G. Cavallo, E. Oddenino, E. Pavanelli, M. Maniscalco, V. Montano,A. Mastropietro, N.C. Bellia, E. Ciravegna, M. La Rocca, E. Vitale, F. Lorico, G.Levi della Vida, Effect of homotaurine in patients with cognitive impairment:results from an Italian observational retrospective study, J. Gerontol.Geriatrics (2018).

[23] V. Gupta, R.S. Thakur, C.R.K. Reddy, B. Jha, Central metabolic processes ofmarine macrophytic algae revealed from NMR based metabolome analysis,RSC Adv. 3 (2013) 7037, http://dx.doi.org/10.1039/c3ra23017a.

[24] M. Fontana, L. Pecci, S. Duprè, D. Cavallini, Antioxidant properties of sulfinates: protective effect of hypotaurine on peroxynitrite-dependent damage, Neurochem. Res. 29 (2004) 111–116, http://dx.doi.org/10.1023/B:NERE.0000010439.99991.cf.

[25] H. Araki, D. Mariam, T. Nishimura, Y. Sugita, K. Higuchi, M. Tomi, E.Nakashima, Cytoprotective properties of hypotaurine by scavenging hydroxyl radicals in placental trophoblast cells, Placenta 34 (2018) A46, http://dx.doi.org/10.1016/j.placenta.2013.06.140.

[26] L. Miˇsurcová, F. Buˇnka, J. Vávra Ambroˇzová, L. Machˆu, D. Samek, S. Kráˇcmar, Amino acid composition of algal products and its contribution to RDI, FoodChem. (2014), http://dx.doi.org/10.1016/j.foodchem.2013.11.040.

[27] S. McCusker, P.R. Buff, Z. Yu, A.J. Fascetti, Amino acid content of selected plant, algae and insect species: a search for alternative protein sources for use in pet foods, J. Nutr. Sci. (2014), http://dx.doi.org/10.1017/jns.2014.33.

[28] C. Dawczynski, G. Jahreis, C. Dawczynski, R. Schubert, G. Jahreis, Amino acids, fatty acids, and dietary fibre in edible seaweed products, Food Chem. 103 (2007)891–899, http://dx.doi.org/10.1016/j.foodchem.2006.09.041.

[29] M. Kent, H.M. Welladsen, A. Mangott, Y. Li, Nutritional evaluation of Australian microalgae as potential human health supplements, PLoS One(2015), http://dx.doi.org/10.1371/journal.pone.0118985.

[30] A. Mehdinia, S. Rostami, S. Dadkhah, N.S. Fumani, Simultaneous Evaluation of homotaurine and taurine in marine macro-algae using liquid chromatography–fluorescence detection, J. Iran. Chem. Soc. 14 (2017)2135–2142, http://dx.doi.org/10.1007/s13738-017/150-9.

[31] R. Checa-Moreno, E. Manzano, G. Mirón, L.F. Capitán-Vallvey, Revisitation of the phenyl isothiocyanate-derivatives procedure for amino acid determination by HPLC-UV, J. Sep. Sci. (2008), http://dx.doi.org/10.1002/jssc.200800363.

[32] E.S. Hwang, K.N. Ki, H.Y. Chung, Proximate composition, amino acid, mineral, and heavy metal content of dried laver, Prev. Nutr. Food Sci. (2013), http://dx.doi.org/10.3746/pnf.2013.18.2.139.

[33] K.G. Bogolitsyn, P.A. Kaplitsin, A.S. Pochtovalova, Amino-acid composition of arctic brown algae, Chem. Nat. Compd. (2014), http://dx.doi.org/10.1007/s10600-014-0831-1.

[34] I. Belghit, J.D. Rasinger, S. Heesch, I. Biancarosa, N. Liland, B. Torstensen, R.Waagbø, E.J. Lock, C.G. Bruckner, In-depth metabolic profiling of marine macroalgae confirms strong biochemical differences between brown, red and green algae, Algal Res. (2017), http://dx.doi.org/10.1016/j.algal.2017.08.001.

[35] V. Gupta, H.R. Kushwaha, Metabolic regulatory oscillations in intertidal green seaweed Ulva lactuca against tidal cycles, Sci. Rep. 7 (2017), http://dx.doi.org/10.1038/s41598-017/5994-2.

[36] E.J. Lewis, E.A. Gonzalves, Amino acid contents of some marine algae from Bombay, New Phytol. 59 (1960) 109–115, http://dx.doi.org/10.1111/j.1469-8137.1960.tb06208.x.0.45.

[37] Y. Cao, J. Duan, J. Guo, S. Guo, J. Zhao, Rapid determination of nucleosides, nucleobases and free amino acids in brown seaweeds using ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry, J.Appl. Phycol. (2014), http://dx.doi.org/10.1007/s10811-013-0079-3.

[38] L. Campanella, M.V. Russo, P. Avino, Free and total amino acid composition in blue-green algae, Ann. Chim. (Paris) (2002).

[39] G. Zheng, W. Jin, P. Fan, X. Feng, Y. Bai, T. Tao, L. Yu, A novel method for detecting amino acids derivatized with phenyl isothiocyanate by high-performance

liquid chromatography-electrospray ionization mass spectrometry, Int. J. Mass Spectrom. 392 (2015) 1–6, http://dx.doi.org/10.1016/j.ijms.2015.08.004.

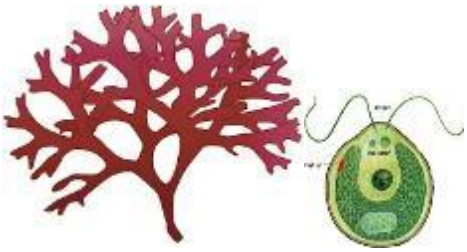
[40] M. Thompson, S.L.R. Ellison, R. Wood, Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report),PureAppl,Chem.(2002),http://dx.doi.org/10.1351/pac200274050835

[41] ICH, ICH Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2(R1), 2005 http://www.ich.org/fileadmin/PublicWebSite/ICH Products/Guidelines/Quality/Q2 R1/Step4/Q2 R1 Guideline.pdf.

[42] AOAC International, Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals, AOAC Int. (2002), http://dx.doi.org/10.1074/jbc.M200778200.

CAPÍTULO 2

Niveles de taurina, hipotaurina, homotaurina y perfiles de aminoácidos en macroalgas y microalgas comerciales y en productos alimentarios enriquecidos con algas



Main pathway:

Methionine

⬇

Cysteine

⬇

Cysteine sulphonic acid

⬇

Cysteine sulphonic acid decarboxylase

⬇

Hypotaurine

⬇

NAD dependent hypotaurine oxidase

⬇

Taurine

AUTORES**:** Carlos Terriente-Palacios [a, b], Massimo Castellari [a]

TITULO**: Levels of taurine, hypotaurine and homotaurine, and amino acids profiles in selected commercial seaweeds, microalgae, and algae-enriched food products**

REFERENCIA**:** DOI: 10.1016/j.foodchem.2021.130770. Volume 368, 30 January 2022, 130770. ISSN: 0308-8146

REVISTA: **FOOD CHEMISTRY**

EDITORIAL: ELSERVIER

AFILIACIONES:
[a] IRTA Food Industries – Finca Camps I Armet s/n, 17121 Monells, Girona, Spain
[b] Escuela Internacional de Doctorado UNED - Calle Bravo Murillo 38, 28015 Madrid, Spain

LUGAR DE PUBLICACIÓN: UNITED KINGDOM

FECHA DE PUBLICACIÓN: **2022**

FACTOR DE IMPACTO: PUBLICADO (JCR-2021) **8.795**

FOOD SCIENCE & TECHNOLOGY	POSICIÓN: 8/144 (JCR-2021)	PRIMER TERCIO (1º CUARTIL)
NUTRITION & DIETETICS	POSICIÓN: 6/90 (JCR-2021)	PRIMER TERCIO (1º CUARTIL)
CHEMISTRY, APPLIED	POSICIÓN: 6/73 (JCR-2021)	PRIMER TERCIO (1º CUARTIL)

Food Chemistry 368 (2022) 130770

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Levels of taurine, hypotaurine and homotaurine, and amino acids profiles in selected commercial seaweeds, microalgae, and algae-enriched food products

Carlos Terriente-Palacios [a,b], Massimo Castellari [a,*]

[a] Institute of Agrifood Research and Technology (IRTA) - Food Industries, Finca Camps i Armet s/n, 17121 Monells, Girona, Spain
[b] Escuela Internacional de Doctorado UNED - Calle Bravo, Murillo 38, 28015 Madrid, Spain

Abstract

Amino acids and sulfonic acid derivatives (Taurine-Tau; Hypotaurine-HypTau; Homotaurine-HTau) of 26 different species of commercial macroalgae, microalgae and 10 algae-enriched food products from the market were quantified in a single chromatographic run. Tau and analogues were predominantly distributed in red species followed by green and brown species. *Palmaria palmata*, *Gracilaria longissima* and *Porphyra* sp. were the species with the highest content of Tau and total sulfonic acid derivatives (TAD). Notwithstanding, relatively high concentrations of HTau were found in green algae *Ulva lactuca* and *G. vermicullophyla* as well as in the brown algae *Undaria pinnatifida*. HTau and HypTau were found at lower concentrations than Tau in all species, except in *Ulva lactuca*. The samples with the highest protein content were the green species *Chlorella vulgaris*, *Nannochloropsis*, and *Afanizomenon-flos aquae*, followed by the red algae *Gracilaria longissima* and *Gracilaria vermicullophyla*.

Samples of pasta formulated with algae ingredients showed the highest levels of sulfonic acid derivatives, evidencing that these products can provide levels of TAD comparable to those found in foods of animal origin.

This study provides, for the first time, quantitative information regarding the distribution of sulfonic acid derivatives and total amino acids in multiple algae species as well as the nutritional impact of the inclusion of algae ingredients in commercial food matrices.

Highlights

-Taurine, Hypotaurine, Homotaurine were quantified in 26 algae species and 20 food samples

-Red algae species showed the higher concentrations of Tau and total sulfonic acid derivatives

-Pasta formulated with algae showed the highest levels of sulfonic acid derivatives

Keywords

Taurine, Homotaurine, Hypotaurine, algae, protein, total amino acids.

Introduction

Nowadays alternative protein sources are constantly explored to secure the future food and protein demand, due to the estimation that the world needs to close a 70 percent "food gap" between the crop calories available in 2006 and the expected calorie demand in 2050 (Godfray et al., 2010). Seaweeds and microalgae are gaining more and more attention since they have high levels of protein (up to 70 % in several microalgal species) and an interesting amino acids profile. Hence, the levels of essential amino acids (EAA) in seaweeds are comparable to those defined by the FAO/WHO for dietary proteins (Černá, 2011). In microalgae, the levels of amino acids such as isoleucine, valine, lysine, tryptophan, methionine, threonine and histidine are similar or greater than those found in protein-rich sources such as eggs and soybean (Koyande et al., 2019). Otherwise, seaweeds or macroalgae are rich in the amino acids responsible for the umami flavour, *i.e.*, glutamic acid, aspartic acid, glycine and alanine. However, when compared with other protein-rich food sources, seaweeds are limited by their low levels of lysine, threonine, tryptophan, cysteine, and methionine. The amino acid score and the essential amino acid index, which describe the nutritional properties of the protein, are higher in red seaweed than those in brown and green seaweeds. Instead, brown seaweeds have been reported to contain higher levels of acidic amino acids as compared to red and green seaweeds (Tiwari & Troy, 2015).

Considering the high protein content and the health benefits associated with algae bioactive compounds, the incorporation of algae or algae extracts into food products is receiving a keen interest in recent years. For instance, Prabhasankar et al., (2009) developed a pasta product with the addition of 10 % of wakame (*Undaria pinnatifida*) as an ingredient and found increased level of total protein and amino acids threonine,

isoleucine, lysine and methionine if compared with pasta without algae supplementation. The green microalgae *Nannochloropsis oculata* was used as a functional ingredient in cookies and pasta to increase the content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in these products. Other Authors (Babuskin et al., 2014; El-Baz et al., 2017; Fradique et al., 2020) reformulated pasta products with *Chlorella vulgaris*, *Spirulina platensis* and/or *Dunaliella sp.* powder to improve their protein, fat and ash content. Batista et al., (2019) reformulated wheat crackers products with different microalgae species such *Spirulina platensis*, *Chlorella vulgaris*, *Tetraselmis sp.* and *Phaeodactylon sp*. and observed that, by adding a 6 % of Spirulina and Chlorella extracts, the protein content was significantly higher than in control crackers.

In addition to the high protein and essential amino acid content, some algae species may contain uncommon bioactive molecules, as the sulfonic acid derivatives taurine (Tau), its precursor hypotaurine (HypTau) and the homologue homotaurine (HTau), which may contribute to modulate several diseases and can provide protection against free radicals and heavy metals (Colovic et al., 2018).

Tau (2-aminoethanosulfonic acid), a conditionally essential amino acid for humans and essential for animals, is gaining attention for its several implications in human diseases. For instance, Wójcik et al., (2010) identified Tau as a preventive factor for coronary heart disease. Tau has recently been pointed out as a promising new therapeutic agent in the treatment of diseases affecting the muscles, the central nervous and the cardiovascular systems as well as against cancer and other metabolic disorders such diabetes type 2 (Gossai et al., 2009; Schaffer et al., 2018). Hatab et al., (2019) found that Tau showed potent antitumor activities to control hepatocellular carcinoma. Tau was also demonstrated to play a protective function in different neurodegenerative models for Parkinson's, Alzheimer's and Huntington's diseases (Jakaria et al., 2019).

HTau, also known as Tramiprosate, it is a low-molecular weight sulfonic compound capable of binding to the β-peptides Lys16, Lys28, and Asp23 of Aβ42 (the pathological element typical of Alzheimer's and of brain aging) in its soluble form. Tramiprosate has been shown safe and effective in several neurocognitive disorders, particularly Alzheimer's Disease and Mild cognitive impairment, where the results are consistent with a disease-modifying effect (Manzano et al., 2020). HTau also has a direct effect on neuronal activity; thanks to its affinity with GABA A receptors, it modulates cortical inhibitory activity by reducing the response of neurons to excitatory stimuli to glutamate. HTau, as a safe blood-brain barrier permeable GABAA-R-specific agonist, ameliorates disease status in mouse models of multiple sclerosis (Tian et al., 2018). Gossai et al., (2009) compared Tau, HTau and HypTau for their ability to modify several indices of oxidative stress and membrane damage associated with type 2 diabetes and found these 3 molecules equiprotective against diabetes-induced alterations in enzymatic and non-enzymatic indices of oxidative stress and against membrane susceptibility to oxidative damage (Gossai et al., 2009).

HypTau is the direct precursor of Tau which may regulate nociceptive transmission in acute, inflammatory, and neuropathic pain in rat models by activating glycinergic neurons in the spinal cord, and it may be a promising candidate for treating various pain states (Hara et al., 2012). HypTau and Tau have anti-inflammatory and antioxidant functions and are important to stimulate immune cell metabolism (Ali et al., 2019). Association of Tau and HypTau with adaptive immune cells and platelets suggests the importance of these amino acids in triggering immunity in the setting of Hepatitis C virus (HCV) infection. Recently, Wan et al., (2020) found that HypTau may regulates a variety of metabolic pathways and leads to the upregulation of some age-related genes to extend lifespan.

In the context of the quantification of these sulfonic acid derivatives available in literature, Kawasaki et al. (2017) reported the content of free amino acids and Tau in

29 Japanese algae species. The Authors found the higher Tau contents in red algae species. Furthermore, Medhdinia et al., (2017) quantified HTau and Tau in 8 algae species from coastal zones from the Persian Gulf and found greater amounts of these molecules in 3 red species, *i.e.*, *Hypnea boergesenii*, *Gracilaria corticate*, and *Gracilaria pygmaea*. McCusker et al. (2014) quantified Tau and essential amino acids (EAA) in 18 algae species and found higher content of Tau in red species. Hwang et al., (2013) reported the Tau content of two Porphyra species – *P. Tenera* and *P. Haitanensis*, while Bogolitsyn et al. (2014) studied the amino acid and Tau composition in 4 species of artic brown algae. Cao et al., (2014) quantified Tau content in 24 brown algae species from China and found Tau only in five species, with levels ranging between 0.64 mg/100 g d.w. and 3.66 mg/100 g d.w.

Anyway, to the best of our knowledge, any study quantified the three sulfonic acid derivatives simultaneously, so, considering the significant health implications related to the ingestion of these molecules, there is still a lack of information about the presence of these compounds in many algae species. Furthermore, there are no data available about the content of Tau, HTau and HypTau in commercial food products including algae ingredients in their formulation.

So, the main scope of this work was to provide information about the levels of Tau, HypTau and HTau in different species of algae as well as in algae-containing pasta and crackers available in the market, by using a validated UHPLC-DAD-MS/MS (Terriente-Palacios et al., 2019). Besides, their amino acid profile, total protein content, Essential Amino Acid Index (EAAI), Limiting Amino Acid (LAA) and Essential to Non-Essential amino acid ratio (EAA/NEAA) were also assessed to evaluate the impact of the reformulation with algae ingredients in the final product.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH) were HPLC gradient-grade (Merck KGaA (Darmstadt, Germany). Perchloric (60%) and hydrochloric acid (37%) were from J.T. Baker (NJ, United States). Sigma-Aldrich Chemie (Sant Quentin Fallavier, France) provided formic acid, ammonium acetate, ammonium formate, phenyl isothiocyanate (PITC), triethylamine (TEA), Trichloroacetic acid (TCA), NaK tartrate tetrahydrate, Folin-Ciocalteu's phenol reagent, Bovine serum albumin, Na_2CO_3, NaOH and pure standards for the target 19 amino acids, Tau, HypTau and HTau. Ultrapure water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA).

Single stock solutions were prepared for each of the 22 target compounds [Histidine (His), HypTau, Hydroxyproline (Hyp), Tau, HTau, Arginine (Arg),Serine (Ser), Glycine (Gly), Aspartic acid (Asp), Glutamic acid (Glu),Cysteine (Cys), Threonine (Thr), Proline (Pro), Alanine (Ala), Gamma aminobutyric acid (GABA), Lysine (Lys), Tyrosine (Tyr), Methionine(Met), Valine (Val), Isoleucine (Ile), Leucine (Leu), and Phenylalanine (Phe)] by dissolving the corresponding pure standards in 0.1 M HCl.

2.2. Algae and food sampling

Twenty-six different species of algae (including fresh and dried seaweeds and microalgae), ten algae-enriched food products and ten food products of the same categories not containing algae ingredients were purchased in commercial establishments in Spain, France, Japan, and Germany (**Table S1**, *Supplementary material*). Three different batches were acquired for each product. Dried products were homogenized with a mixer mill (Retsch GmbH & Co, KG Germany). Fresh products were freeze dried (LYOMICRON 55 – Cool vacuum Technologies, Barcelona - Spain) and homogenized as previously described (Terriente-Palacios et

al., 2019). The powdered samples were stored at ambient temperature under dry and dark conditions and analysed within one month.

Table S1. Product information recovered from the label.

Algae sp./Composition	Sample	Product description	Form	Country	Protein Content (g/100 g d.w.)
		Algae			
Condrus crispus	Chc	Dried Irish Moss	Dried	Spain	10
Gracillaria longissima	Gl	Gracillaria	Fresh	Spain	34.2
Mastocarpus stellatus	Ms	Organic Irish Moss	Dried	Spain	10.8
Gracillaria sp.	Gsp	Red Ogonori	Fresh	Spain	not specified
Palmaria palmata	Pp	Dulse	Dried	Spain	16.9
Porphyra sp.	Po	Nori	Dried	Spain	30.5
Gigartina pistillata	Gp	Fresh Gigartina	Fresh	Spain	not specified
Eisenia byciclis	Eb	Arame	Dried	Japan	10.1
Himanthalia elongata	He	Sea Spaguetti bio	Dried	Spain	9.3
Hizikia fusiformis	Hf	Iziki seaweed	Dried	Spain	26.2
Laminaria japonica	Lj	Kombu	Dried	Spain	19.6
Laminaria ochroleuca	Lo	Kombu bio	Dried	Spain	7.1
Undaria pinnatifida	Up	Wakame	Dried	Spain	11.9
Fucus vesiculosus	Fv	Fucus	Capsules	Spain	10.4
Ascophyllum nodosum	An	Kelp	Dried	Spain	5.7
Odontella aurita	Oa	Odontella	Capsules	France	14.4
Enteromorpha intestinalis	Ei	Green Aonori	Fresh	Japan	not specified
Caulerpa lentillifera	Cl	Green Caviar	Dried	Germany	12.6
Codium sp.	Csp	Barnacle seaweed	Dried	Spain	10.9
Ulva lactuca	Ul	Sea Lettuce	Dried	Germany	26.8
Nanochloropsis	Nsp	Nanochloropsis	Dried	Spain	46.4
Chlorella vulgaris	Cv	Chlorella	Dried	Spain	54.7
Dunaliella salina	Ds	Dunaliella	Dried	Spain	18.5
Tetraselmis chuii	Tch	Holofit TetraSod	Capsules	Spain	not specified
Afanizomenon flos-aquae	Afa	Klamath eco	Dried	Spain	34.8
Spirulina platensis	Sp	Spìrulina	Dried	Spain	37.9
		Pasta			
Nori seaweed (Porphyra spp., 4.44%)	NN	Noodles with Nori	Dried	Spain	16
Sea lettuce seaweed (Ulva spp., 2.4%)	NU	Noodles with Ulva	Dried	Spain	12
Wakame seaweed (Undaria pinnatifida, 2.1%)	NW	Noodles with Wakame	Dried	Spain	12
Spirulina (4%)	FS	Fusilli with Spirulina	Dried	France	8
Spirulina (1.5%), Fucus (1.5%)	SS	Spaghetti with seaweeds	Dried	Spain	14
without algae	P1	Spaghetti	Dried	Spain	12
without algae	P2	Noodles	Dried	Spain	12
without algae	P3	Pasta spirals	Dried	Spain	12
without algae	P4	Noodles	Dried	Spain	12
without algae	P5	Noodles	Dried	Spain	12
		Crackers			
Sea spaguetti seaweed (Himanthalia elongata, 5%)	CH	Rice crackers with organic seaweed.	Dried	Spain	8.2

Oatmeal, whole wheat flour, sesame, Atlantic Wakame seaweed (Undaria pinnatifida, 1.5%)	CW	Crackers with seaweed, oatmeal and sesame	Dried	Spain	11.5
Sea lettuce seaweed (Ulva spp, 1.5%)	CU	Crackers with seaweed, tomatoe and chia seeds	Dried	Spain	14.3
Italian spirulina (2.6%)	CS	Wholemeal crackers Spirulina eco	Dried	Spain	6.5
Roasted nori seaweed (62%)	CN	Seaveg crispies	Dried	United Kingdom	30
without algae	C1	Brown rice crackers	Dried	Spain	8
without algae	C2	Brown rice crackers	Dried	Spain	7
without algae	C3	Salad crackers	Dried	Belgium	7.8
without algae	C4	Crackers with olive oil	Dried	Spain	11
without algae	C5	Flaxseed, Sesame and Quinoa crackers	Dried	Spain	13.2

2.3. Quantification of amino acids and sulfonic acid derivatives.

Algae and food samples were processed as previously described (Terriente-Palacios et al., 2019). Briefly, 10 mg of seaweed sample were hydrolysed with 1 mL of 8 M perchloric for 24 h at 110 °C. After cooling at room temperature, the samples were filtered through 0.2 µm membrane syringe filters (GMP filter membranes, Merck KGaA, Darmstadt, Germany), and then derivatized with a methanol-water-TEA-PITC solution (7:1:1:1, v/v/v/v). The derivatized samples were re-dissolved with 24 µL of mobile phase B and 226 µL of mobile phase A, centrifuged at 11,000 × g for 5 min, and filtered through a Single Step Standard Filter Vials (Thomson Instrument Company, CA, USA).

Four µL of the sample were injected into the chromatographic system, consisting of an Acquity UPLC® equipped with a PDA detector, an electrospray (ESI) as a source of ionization operated in the positive mode, and a TQD triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The system was controlled by MassLynx 4.1 software (Waters, Milford, MA, USA). Chromatographic separation was carried out on BEH-C_{18} ,1.7 µm, 100 mm x 2.1 mm i.d. (Waters, Milford, MA, USA). Source

temperature was fixed at 135 ºC, the capillary voltage was set at 3.0 kV and the desolvation temperature was set at 350 ºC. The cone gas (nitrogen) flow rate was 350 L/h and cone voltage was set at 30 V. MS experiments were carried out in "Scan" mode to obtain *m/z* values of the molecular ions. MS/MS experiments in "Daughter Ions" mode were also performed, to obtain the fragmentation patterns of molecular ions. The collision energies varied between 10 and 20 eV. The gas used in the collision cell was argon at a flow rate of 0.1 mL/min. Peak identity was confirmed by comparing their retention times, UV spectra, MS and MS/MS spectra with the corresponding data obtained from pure standards.

Quantitation of the target compounds was done based on an external calibration curve and considering the sample dilution during the extraction and derivatization steps. Calibration curves were made for each target compound by injecting derivatized amounts of pure standards at different concentrations in the range from 0.1 mM to 10.0 mM and by plotting the signal obtained from the diode array detector at □=254 nm versus the corresponding concentrations. Three replicates of analysis were carried out for each sample.

Even if the method was already validated in algae samples, additional validation was carried out for the new food matrices (pasta and crackers), according to harmonized guidelines for single-laboratory validation of methods of analysis (Thompson et al., 2002; AOAC International 2002).

The precision of the method (RSD_r%), was calculated from Eq. 1

$$RSD\% = \left(\frac{s}{x}\right) x\ 100$$

(Eq. 1)

under repeatable conditions by spiking one pasta and one cracker sample without algae supplementation with a solution containing a mix of the 22 standards of the target compounds at three concentration levels (0.1 mM, 5 mM and 10 mM); six replicates of analysis were carried out on the same day. Similarly, the intermediate precision was calculated by analysing the above spiked samples at three concentration levels along 6 days (RSD_R%).

Trueness was evaluated by analysing recovery of the 22 target compounds in a pasta and a cracker sample spiked before hydrolysis with known amounts of pure standards at three levels (0.1, 5.0 and 10.0 mM). Three spiked sample replicates were analysed for each spiking level. Linearity was assessed based on the external calibration curves by checking the working range, the coefficient of determination (R^2), the residual value of replicates, and the Lack-of-fit (LoF) test significance (Thompson et al., 2002). The instrument limit of detection (ILOD) and the instrument limit of quantification (ILOQ) were calculated as 3.3 σ/b and 10 σ /respectively, where "σ" is the Residual Standard Deviation of the Calibration Curve (Sx/y) and "b" is the slope of regression line from the calibration curves of each compound. The Breush-Pagan test, to establish the presence or absence of heteroscedasticity, was also applied. The method limits of detection (MLOD) and quantification (MLOQ) were estimated from ILOD and ILOQ considering the dilution factor and the mass fraction of each sample.

2.4. Total Protein.

Protein extraction was carried out by following Slocombe et al., (2013) with minor modifications. Five mg of sample were re-suspended in 200 µL 24 % (w/v) TCA and, after homogenization by agitation, were incubated for 15 minutes in a water bath at 95 ºC, and then allowed to cool at room temperature. After addition of 600 µL MilliQ water, the samples were centrifuged at 15,000 g for 20 min at 4 ºC (Microcentrifuge 5415 R, Eppendorf AG, Hamburg Germany) and their supernatants discarded. The

pellets were re-suspended in 0.5 mL Lowry Reagent D and incubated 10 min at 55 ºC. Samples were then cooled at room temperature, spun at 15,000 g for 20 min and the supernatant retained. For protein quantification, a stock of Lowry Reagent D was made up daily in a 48:1:1 ratio (v/v/v) of Lowry Reagents A, B and C. A suitable volume (up to 50 µL) of the protein extract was put in individual 1.5 mL microfuge tubes, followed by 950 µL of Lowry Reagent D followed by immediate mixing. After incubation for 10 min at room temperature, 0.1 mL of the 0.2 N Folin-Ciocalteu phenol reagent was added to each tube and mixed immediately. Absorbance was read at 600 nm (1800 UV-Vis spectrophotometer, Shimadzu Co, Madison, WI) using UVProbe™ software (Shimadzu Co, Madison, WI) after 30 minutes at room temperature. Calibration curves were prepared for each assay with a bovine serum albumin (BSA) stock solution (200 mg/mL) and using a polynomial line of best fit generated in Microsoft Excel 365. Three replicates of analysis were carried out for each sample.

2.5. Essential Amino Acid Index (EAAI), limiting amino acid (LAA) and ratio of Essential to Non-Essential Amino Acids (EAA/NEAA).

The Essential Amino Acid Index (EAAI) (Friedman et al., 1996), a measure of the protein nutritional quality, was calculated with the following formula:

$$EAAI = \sqrt[n]{CS1x\ CS2\ x\ CS3\ x\ \ldots\ldots CSn}$$

(Eq. 2)

where CS1, CS2, …CSn are the chemical scores of each essential amino acid, calculated as,

$$Chemical\ score = \frac{EAA\ in\ sample\ protein\ (mg\ g^{-1})}{EAA\ in\ standard\ protein\ (mg\ g^{-1})} \times 100$$

(Eq. 3)

where "EAA in sample protein" indicates the concentration of a given essential amino acid in the test protein and "EAA in standard protein" represents the concentration of the same essential amino acid in a reference protein, in this case egg protein (FAO/WHO, 2007). From CS, the limiting amino acid (LAA) for each algae sample was determined as the EAA in sample protein which showed the greatest difference in concentration from the same EAA in the standard protein.

The ratio of Essential to Non-Essential Amino Acids (EAA/NEAA) was determined considering EAA as the sum of the concentrations of His, Arg, Thr, Lys, Met, Val, Ile, Leu and Phe, and NEAA as the sum of the concentrations of Hyp, Ser, Gly, Asp, Glu, Cys, Pro, Ala, GABA and Tyr.

To estimate the variations of EAA and NEAA between products enriched with algae and controls, the percentile variations of the Essential Amino Acid Index (% ΔEAAI) and of the ratio of Essential to Non-Essential Amino Acids (% ΔEAA/NEAA) were calculated as follow:

$$\%\,\Delta\text{EAAI} = \left(\frac{EAAI_P - EAAI_C}{EAAI_C}\right) \times 100$$

(Eq. 4)

were,

$EAAI_P$ is the Essential amino acid index of a given product enriched with algae,

$EAAI_C$ is the mean EAAI value of the corresponding control food category

$$\%\Delta\, EAA/NEAA = \left(\frac{(EAA/NEAA)_P - (EAA/NEAA)_C}{(EAA/NEAA)_C}\right) \times 100$$

(Eq. 5)

where $(EAA/NEAA)_P$ is the ratio of Essential to Non-Essential Amino Acids of a given product enriched with algae, $(EAA/NEAA)_C$ is the mean ratio of Essential to Non-Essential Amino Acids of the corresponding control food category.

2.6. Statistical analysis

Statistical analyses were all performed using the software Minitab® version 19.2, 2019 (Minitab Inc., State College, PA, USA). The Kolmogorov-Smirnov test was applied to verify whether the distribution of the variables was normal ($p< 0.05$). The Kruskal-Wallis non-parametric test was applied. Mann-Whitney pairwise

comparisons of median values were also done. Differences were considered significant at $p< 0.05$.

3. Results and discussion

3.1. UHPLC method validation for pasta & crackers

Precision (RSD_r%), ranged from 1.0 % to 4.4 % in pasta and between 1.0% to 4.3 % in crackers. Intermediate precision (RSD_R%) ranged from 1.0 % to 4.7 % in pasta and between 1.0 % to 5.1 % in crackers (**Table S2, Table S3**, *Supplementary material*). Recovery values for the 22 compounds were comprised within 85.5 % and 107.0 % in pasta and within 85.5 % and 106.0 % in crackers (**Table S4**, *Supplementary material)*. These results fulfil the acceptable values of precision and recovery recommended by the IUPAC Technical report. Calibration curves showed R^2 value always higher than 0.997 for all the compounds and satisfied the homoscedasticity criterion, and the residual standard deviation approach could be applied (Terriente-Palacios et al., 2019). The linear range was initially tested between 0.1 mM–2.5 mM. Preliminary analysis of samples indicated the need to extent the upper limit of the calibration curve to 10 mM for several amino acids. So, the working range was confirmed for all the amino acids and was finally established between 0.1 mM–10.0 mM.

The MLOD and MLOQ were from 0.01 mg/kg d.w. to 0.11 mg/kg d.w. and from 0.04 mg/kg d.w. to 0.31 mg/kg d.w. respectively, for all the target compounds. Glycine showed the lowest detection and quantification limits while HypTau the higher ones (**Table S5**, *Supplementary material*).

Table S2. Results for precision (RSD_r%) in pasta and cracker matrices at three different spiking concentrations (0.1 mM, 5 mM and 10 mM).

	RSD_r (%) n=6							
	Pasta				Crackers			
Amino acid	0.1 mM	5 mM	10 mM	Mean	0.1 mM	5 mM	10 mM	Mean
Hyptau	1.30	2.01	1.88	1.73	3.71	2.36	3.27	3.11
Tau	2.40	1.93	2.03	2.12	3.91	1.57	2.52	2.67
Htau	3.42	2.08	4.45	3.32	1.15	2.19	2.28	1.87
His	3.01	2.90	4.48	3.46	2.27	2.54	2.75	2.52
Arg	3.10	1.90	3.01	2.67	3.45	1.05	2.46	2.32
Thr	3.80	3.07	1.83	2.90	3.88	1.03	2.24	2.38
Lys	2.70	2.56	3.09	2.78	1.47	2.50	1.35	1.77
Met	2.01	2.62	3.51	2.71	1.89	3.29	3.96	3.05
Val	2.84	3.18	1.23	2.42	1.76	2.81	1.45	2.01
Ileu	2.94	3.58	4.29	3.60	3.21	2.84	2.46	2.84
Leu	2.57	2.72	3.98	3.09	4.23	3.56	1.61	3.13
Phe	2.22	2.27	3.09	2.53	3.90	2.22	1.12	2.41
Hyp	4.02	2.85	2.32	3.06	4.31	4.02	4.03	4.12
Ser	3.51	1.15	3.54	2.73	3.74	2.15	2.23	2.71
Gly	1.19	1.22	1.29	1.23	2.86	2.87	4.46	3.40
Asp	3.65	3.04	1.05	2.58	1.54	2.34	3.44	2.44
Glu	4.12	2.32	1.64	2.69	2,00	3.38	2.22	2.53
Cys	3.22	1.58	2.23	2.34	1.45	3.12	1.19	1.92
Pro	3.34	4.34	3.62	3.77	1.35	4.32	1.92	2.53
Ala	1.33	2.44	1.44	1.74	3.62	2.95	2.93	3.17
GABA	3.96	2.36	1.05	2.46	2.59	3.50	2.97	3.02
Tyr	2.96	4.04	2.95	3.32	1.37	3.01	2.84	2.41

Table S3. Results for intermediate precision (RSD_R%) in pasta and cracker matrices at three different spiking concentrations (0.1 mM, 5 mM and 10 mM).

	RSD_R (%) n=6							
	Pasta				Crackers			
Amino acid	0.1 mM	5 mM	10 mM	Mean	0.1 mM	5 mM	10 mM	Mean
Hyptau	1.58	4.21	1.53	2.44	3.45	1.91	4.46	3.27
Tau	4.29	3.51	2.41	3.40	3.94	3.22	3.52	3.56
Htau	1.95	4.15	2.18	2.76	2.60	1.67	3.78	2.68
His	3.21	1.73	3.82	2.92	2.01	4.12	2.71	2.95
Arg	4.02	4.44	4.21	4.22	3.70	2.32	1.01	2.34
Thr	2.19	3.85	3.52	3.19	1.17	3.22	1.31	1.90
Lys	4.05	2.74	2.59	3.13	2.28	1.40	1.55	1.74
Met	1.84	1.27	3.99	2.37	4.36	3.77	4.01	4.05
Val	3.99	2.70	4.25	3.65	4.37	1.22	4.20	3.26
Ileu	3.08	4.23	4.01	3.77	2.36	2.43	1.35	2.05
Leu	3.65	3.87	3.87	3.80	2.44	2.32	2.08	2.28
Phe	2.69	2.38	2.44	2.50	3.97	1.66	3.42	3.02
Hyp	4.41	3.56	3.99	3.99	3.40	3.34	3.33	3.36
Ser	2.06	2.55	2.98	2.53	3.35	3.29	2.35	3,00
Gly	3.86	1.19	3.84	2.96	3.32	3.77	3.89	3.66
Asp	3.76	1.02	3.61	2.80	2.13	3.72	1.21	2.35
Glu	3.74	2.75	3,00	3.16	4.23	3.10	1.71	3.01
Cys	1.86	2.03	2.94	2.28	3.94	4.40	2.79	3.71
Pro	3.92	2.08	2.54	2.85	3.09	2.48	4.21	3.26
Ala	2.77	1.73	2.84	2.45	2.44	1.45	1.58	1.82
GABA	4.19	1.47	1.67	2.44	1.12	1.83	2.55	1.83
Tyr	3.18	4.47	1.34	3,00	3.79	1.31	1.35	2.15

Table S4. **Recovery** values in pasta and cracker matrices (mean ± standard deviation, n=3) at 3 different spiking concentrations (0.05 mM, 0.5 mM and 2.5 mM).

	Recovery (%) n=6							
	Pasta				Crackers			
Amino acid	0,05 mM	0,5 mM	2,5 mM	Mean	0,05 mM	0,5 mM	2,5 mM	Mean
Hyptau	92.4 ± 0.81	105 ± 1.70	99.9 ± 0.42	102	95.2 ± 1.43	91.2 ± 0.55	97.5 ± 1.45	94.6
Tau	93.2 ± 0.52	103 ± 2.45	91.4 ± 0.99	95.9	106 ± 1.04	97.1 ± 1.28	92.8 ± 0.73	98.6
Htau	90.1 ± 2.25	95.9 ± 1.52	91.2 ± 0.90	92.4	93.5 ± 0.82	88.2 ± 0.77	101 ± 1.26	94.2
His	92.1 ± 0.91	93.0 ± 2.41	95.1 ± 2.41	93.4	96.0 ± 1.61	97.6 ± 0.23	94.7 ± 0.12	96.1
Arg	90.1 ± 1.01	91.4 ± 1.38	94.2 ± 0.21	91.9	102 ± 1.11	93.0 ± 2.16	101 ± 1.94	98.7
Thr	96.4 ± 1.65	94.5 ± 0.19	93.9 ± 0.75	94.9	103 ± 1.92	99.5 ± 0.15	102 ± 2.05	102
Lys	85.5 ± 0.16	98.2 ± 1.28	100 ± 2.58	94.6	97.3 ± 0.68	104 ± 1.34	98.5 ± 2.32	99.9
Met	93.9 ± 0.92	92.5 ± 1.47	93.8 ± 2.56	93.4	87.9 ± 2.13	104 ± 2.32	91.8 ± 2.23	94.6
Val	95.8 ± 0.88	101 ± 1.95	100 ± 0.64	98.9	88.0 ± 2.58	96.2 ± 0.39	94.5 ± 2.35	92.9
Ileu	90.8 ± 1.22	95.1 ± 2.54	92.3 ± 1.22	92.7	105 ± 0.92	102 ± 2.15	102 ± 0.68	103
Leu	90.8 ± 2.01	104 ± 0.25	96.4 ± 1.44	97.1	85.5 ± 1.83	89.9 ± 2.42	102 ± 1.84	92.5
Phe	94.0 ± 1.05	100 ± 0.22	99.7 ± 0.14	97.9	93.4 ± 1.54	100 ± 0.11	94.4 ± 1.35	95.9
Hyp	93.1 ± 0.62	93.4 ± 1.27	95.0 ± 1.57	93.8	104 ± 1.02	87.1 ± 0.12	92.6 ± 0.83	94.6
Ser	107 ± 1.95	102 ± 0.57	101 ± 2.42	103	88.2 ± 1.75	100 ± 0.90	101 ± 2.02	96.4
Gly	102 ± 1.23	100 ± 0.43	101 ± 2.42	101	90.9 ± 2.43	100 ± 0.82	103 ± 1.94	98.0
Asp	88.7 ± 0.22	92.0 ± 1.59	99.4 ± 1.82	93.4	86.1 ± 2.08	103 ± 0.81	96.2 ± 0.25	95.1
Glu	91.5 ± 1.11	95.9 ± 0.52	93.2 ± 0.97	93.5	98.8 ± 0.11	96.1 ± 0.82	98.5 ± 0.82	97.8
Cys	86.9 ± 1.20	101 ± 2.00	100 ± 1.83	96.0	102 ± 0.19	94.8 ± 1.90	101 ± 0.52	99.3
Pro	102 ± 0.52	87.1 ± 1.01	97.3 ± 0.62	95.9	97.2 ± 0.10	91.5 ± 1.63	99.0 ± 0.94	95.9
Ala	102 ± 0.85	90.1 ± 0.62	94.0 ± 2.54	95.4	103 ± 1.32	102 ± 2.16	92.3 ± 1.35	99.1
GABA	95.3 ± 1.52	101 ± 1.82	91.9 ± 0.82	96.1	97.9 ± 0.55	90.7 ± 1.25	96.7 ± 0.52	95.1
Tyr	85.5 ± 0.98	93.8 ± 1.83	97.1 ± 1.59	92.1	98.9 ± 0.53	93.3 ± 2.12	97.9 ± 1.01	96.7

Table S5. Linearity, explained by regression equation and correlation coefficient (R^2), limit of detection (LOD) and limit of quantification (LOD) expressed as mg/Kg d.w. in pasta and cracker matrices.

*Terriente et al. 2019

Amino acids	Regression equation*	R^2*	Pasta / Crackers	
			MLOD	MLOQ
			mg / kg d.w.	
Hyptau	$y = 7.2 * 10^2 x - 10.6$	0.999	0.11	0.29
Tau	$y = 2.3 * 10^2 x - 27.0$	0.999	0.03	0.12
Htau	$y = 4.8 * 10^2 x - 74.6$	0.999	0.06	0.13
His	$y = 2.7 * 10^2 x - 40.3$	0.999	0.04	0.14
Arg	$y = 6.1 * 10^2 x - 21.0$	0.999	0.04	0.13
Thr	$y = 7.2 * 10^2 x - 11.6$	0.999	0.03	0.11
Lys	$y = 4.3 * 10^2 x - 11.4$	0.999	0.04	0.15
Met	$y = 8.2 * 10^2 x - 12.3$	0.998	0.02	0.08
Val	$y = 9.9 * 10^2 x - 53.4$	0.999	0.02	0.07
Ileu	$y = 1.3 * 10^2 x - 20.3$	0.998	0.09	0.30
Leu	$y = 1.1 * 10^2 x - 10.4$	0.999	0.07	0.23
Phe	$y = 8.7 * 10^2 x - 58.4$	0.999	0.09	0.28
Hyp	$y = 4.1 * 10^2 x - 22.2$	0.998	0.04	0.11
Ser	$y = 1.3 * 10^2 x - 75.6$	0.999	0.02	0.08
Gly	$y = 2.2 * 10^2 x - 32.4$	0.999	0.01	0.04
Asp	$y = 8.7 * 10^2 x - 14.5$	0.999	0.03	0.11
Glu	$y = 7.0 * 10^2 x - 26.4$	0.999	0.02	0.08
Cys	$y = 4.7 * 10^2 x - 10.1$	0.998	0.06	0.21
Pro	$y = 10.4 * 10^2 x - 32.3$	0.999	0.02	0.08
Ala	$y = 6.6 * 10^2 x - 55.4$	0.998	0.03	0.12
GABA	$y = 4.0 * 10^2 x - 42.7$	0.999	0.04	0.13
Tyr	$y = 1.4 * 10^2 x - 41.4$	0.999	0.04	0.09

3.2. Quantification of sulfonic acid derivatives.

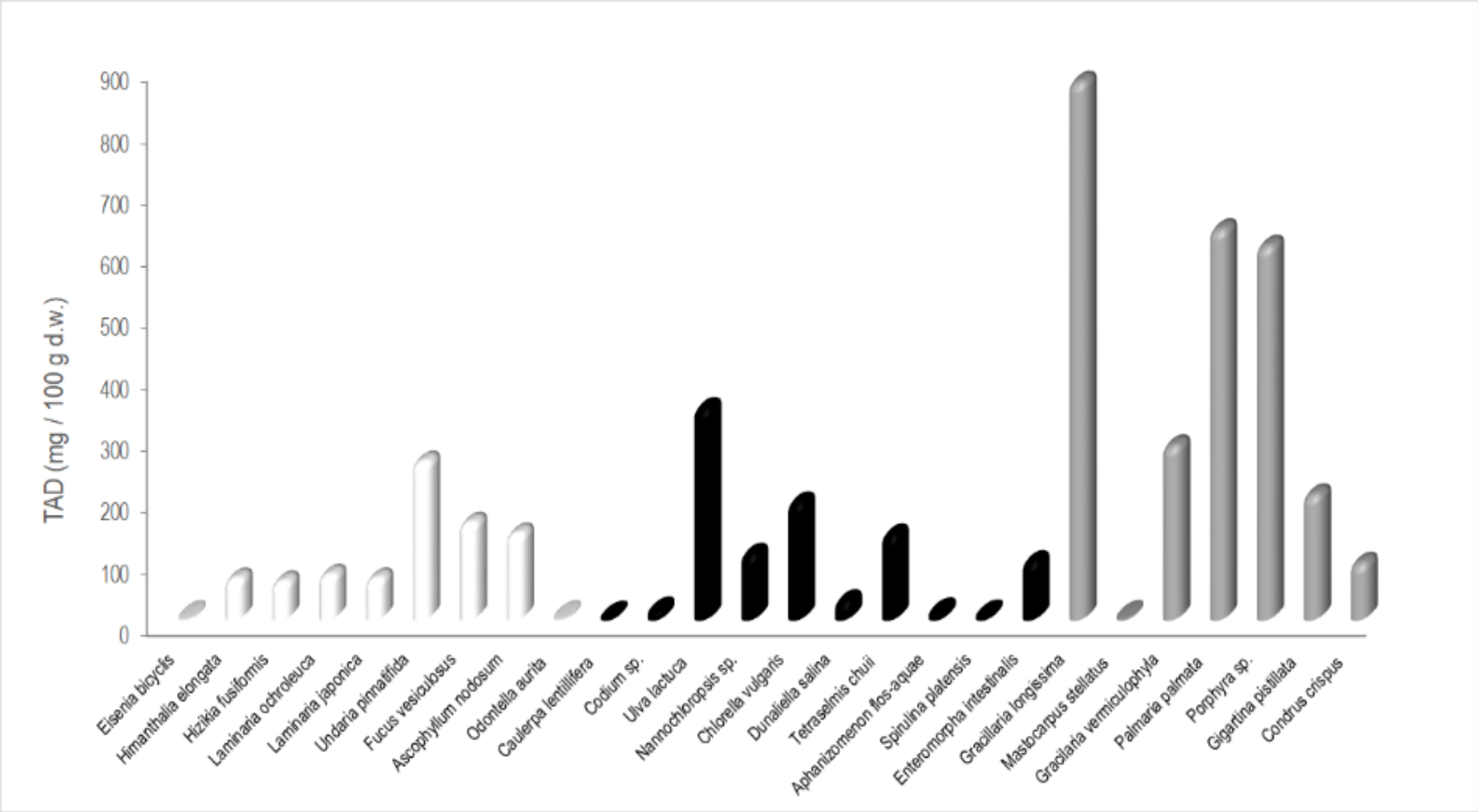
Concentrations of HypTau, Tau and HTau are listed in **Table 1**, while **Figure 1** shows the sum of the three sulfonic acid derivatives (TAD) in the 26 algae species. The higher concentrations of Tau, HTau and TAD were found in red algae, with values of TAD ranging between 2.52 mg/100 g d.w. and 868 mg/100 g d.w., followed by green and brown algae with TAD concentrations up to 339 mg/100 g d.w. and 252 mg/100 g d.w., respectively. Among all the species, *Gracilaria longissima*, *Palmaria palmata* and *Porphyra sp* were those with the highest contents of TAD. No significant differences were found among the three groups for the HypTau content.

Table 1. Concentration of Taurine. Homotaurine and Hypotaurine (mg/100 g d.w.), Sum of sulfonic acid derivatives (TAD, mg/ 100 g d.w), Protein content (g/100 g d.w.), Essential to Non – Essential amino acid ratio (EAA/NEAA), Essential amino acid Index (EAAI) and Limiting amino acid (LAA) in the 26 algae species.

Each value is the mean of *n*=3 independent determinations ± standard deviation)
nd: not detected, lower than the limit of detection
Small letters on the same column indicate differences among median at p<0.05
Algae specie as indicated in the label of the commercial product

Algae species*	Taurine	Homotaurine	Hypotaurine	TAD	Protein	EEA/NEAA	EAAI	LAA
	mg / 100 g d.w.				g / 100 d.w.			
Red algae								
Condrus crispus	84.5 ± 0.31	3.45 ± 0.05	nd	87.9	12.8 ± 0.03	0.77 ± 0.01	123 ± 0.01	Met
Gracilaria longissima	842 ± 0.12	12.2 ± 0.11	14.6 ± 0.10	868	34.6 ± 0.07	1.20 ± 0.02	172 ± 0.02	Met
Mastocarpus stellatus	0.58 ± 0.01	1.69 ± 0.13	0.25 ± 0.05	2.52	11.0 ± 0.01	0.51 ± 0.02	106 ± 0.04	Lys
Gracilaria vermicullophyla	227 ± 0.94	50.1 ± 0.81	1.85 ± 0.07	279	35.1 ± 0.06	0.41 ± 0.02	84.8 ± 0.02	Leu
Palmaria palmata	555 ± 0.10	67.2 ± 0.11	7.56 ± 0.02	630	17.8 ± 0.02	0.56 ± 0.02	112 ± 0.02	Met
Porphyra sp.	527 ± 0.95	70.2 ± 0.53	5.81 ± 0.08	603	31.0 ± 0.07	0.64 ± 0.01	134 ± 0.03	Phe
Gigartina pistillata	185 ± 0.17	14.4 ± 0.21	nd	199	28.1 ± 0.01	1.14 ± 0.01	185 ± 0.01	Met
Median	**227 a**	**14.4 a**	**1.85**	**279 a**	**28.1 a**	**0.64**	**129**	
Brown algae								
Eisenia byciclis	0.88 ± 0.07	0.57 ± 0.01	0.62 ± 0.05	2.07	10.4 ± 0.01	0.63 ± 0.01	104 ± 0.03	Met
Himanthalia elongata	0.28 ± 0.05	0.28 ± 0.05	0.20 ± 0.09	0.76	9.20 ± 0.06	0.56 ± 0.01	95.9 ± 0.02	Leu
Hizikia fusiforme	57.0 ± 0.01	0.38 ± 0.05	0.33 ± 0.04	57.7	22.5 ± 0.05	0.49 ± 0.02	99.6 ± 0.02	Leu
Laminaria japonica	65.0 ± 0.02	2.05 ± 0.04	1.60 ± 0.11	68.6	22.9 ± 0.02	0.41 ± 0.04	81.4 ± 0.04	Phe
Laminaria ochroleuca	57.0 ± 0.06	3.50 ± 0.06	2.19 ± 0.01	62.4	6.90 ± 0.01	0.50 ± 0.01	36.8 ± 0.01	Lys
Undaria pinnatifida	195 ± 0.09	56.7 ± 0.09	0.67 ± 0.01	252	13.5 ± 0.03	0.98 ± 0.03	137 ± 0.01	Leu
Fucus vesiculosus	152 ± 0.08	0.96 ± 0.08	0.21 ± 0.05	153	10.7 ± 0.02	0.55 ± 0.01	85.3 ± 0.01	Leu
Ascophyllum nodosum	135 ± 0.01	0.61 ± 0.01	nd	136	8.80 ± 0.03	0.96 ± 0.01	134 ± 0.01	Met
Odontella aurita	1.36 ± 0.01	0.36 ± 0.01	0.30 ± 0.01	2.02	15.1 ± 0.01	1.20 ± 0.01	121 ± 0.04	Met
Median	**62.0 ab**	**0.61 b**	**0.33**	**62.7 b**	**11.0 b**	**0.55**	**134**	
Green algae								
Enteromorpha intestinalis	30.8 ± 0.02	60.0 ± 0.05	nd	90.8	9.28 ± 0.05	0.83 ± 0.01	169 ± 0.05	Met
Caulerpa lentillifera	nd	0.60 ± 0.05	0.14 ± 0.02	0.74	13.2 ± 0.04	0.51 ± 0.02	99.2 ± 0.03	Lys
Codium sp.	12.1 ± 0.05	Nd	nd	12.1	11.4 ± 0.05	0.96 ± 0.01	143 ± 0.03	Val
Ulva lactuca	158 ± 0.14	159 ± 0.05	22.1 ± 0.02	339	24.1 ± 0.02	0.85 ± 0.02	168 ± 0.02	Met
Nannochloropsis	101 ± 0.51	2.05 ± 0.07	0.16 ± 0.01	103	40.0 ± 0.02	0.64 ± 0.04	79.9 ± 0.02	Met
Chlorella vulgaris	138 ± 0.12	10.5 ± 0.01	37.9 ± 0.01	186	52.2 ± 0.03	0.67 ± 0.02	152 ± 0.02	Ile
Dunaliella salina	26.2 ± 0.02	0.96 ± 0.08	0.20 ± 0.03	27.3	17.0 ± 0.05	0.94 ± 0.01	100 ± 0.02	Met
Tetraselmis chuii	132 ± 0.12	1.82 ± 0.02	0.03 ± 0.04	134	13.4 ± 0.03	0.79 ± 0.01	122 ± 0.01	Phe
Afanizomenon flos-aquae	10.1 ± 0.2	Nd	Nd	10.1	36.4 ± 0.04	0.76 ± 0.01	139 ± 0.04	Val
Spirulina platensis	4.12 ± 0.03	121 ± 0.02	0.12 ± 0.01	125	31.7 ± 0.08	0.47 ± 0.02	105 ± 0.04	Met
Median	**28.5 bc**	**1.94 ab**	**0.14**	**97.0 ab**	**20.6 a**	**0.78**	**141**	

Figure 1. Sum of Taurine, Hypotaurine and Homotaurine, (TAD) mg/100 g d.w. in the 26 algae species.



Tau was the predominant sulfonic acid derivative in all the algal species, except for the green algae *Ulva lactuca*, which showed similar concentrations of HTau and Tau. As it was pointed out in other studies (Tevatia et al., 2015; Kawasaki et al., 2017), the high content of Tau in red macroalgae and some green microalgae species may be related with their growing environment and the hypothesis that this compound is involved in osmoregulation. Our findings agree (**Table S9**, *Supplementary material*) with the levels of Tau found by Vieira et al., (2018) in almost all the species, except for the samples of *Palmaria palmata* and *Porphyra sp* which showed lower contents of this compound.

In the case of *Porphyra sp* our results are in the middle of the range reported by other Authors (Dawczynski et al., 2007; McCusker S. et al., 2014) and are lower than those found by Hwang et al., (2013). Our results for the specie *Gracilaria vermiculophylla* ("Ogonori") are in good agreement with the data reported in two previous works (Mehdinia et al., 2017; Kawasaki et al., 2017), while in the case of *Eisenia arborea, Laminaria spp*., and *Ulva spp*., McCusker et al., (2014) reported lower concentrations of Tau, ranging between 1 mg/100 g d.w. and 7 mg/100 g d.w.

It should be underlined that relatively high levels of Tau have been found in products of animal origin, such as mussels and clams, with concentrations of 655 mg/100 g d.w. and 240 mg/100 g d.w. respectively; beef and lamb meat contain lower values around 40 mg/100 g d.w. (Lourenço R. & Camilo M. E., 2002). Considering the results obtained in this study, some species of algae, such as *Gracilaria longissima* (842 mg/100 g d.w.), *Palmaria palmata* (555 mg/100 g d.w.) and *Porphyra sp.* (527 mg/100 g d.w.), can be considered one of the best alimentary sources of Tau.

Regarding the concentration of HTau, red algae species showed the higher median concentration; *Porphyra sp., Palmaria palmata* and *Gracilaria vermicullophyla*, had the highest concentrations in agreement with the scarce data available in literature

about this compound (Mehdinia et al., 2017; Kawasaki et al., 2017), while brown algae samples contained the lowest amounts among the species included in this study (**Table 1**).

Notwithstanding, relatively high concentrations of HTau were found in green algae *Ulva lactuca* (159 mg/100 g d.w.) and *G. vermicullophyla* (60.00 mg/100 g d.w.) as well as in the brown algae *Undaria pinnatifida* (56,7 mg/100 g d.w.). We already reported (Terriente-Palacios et al., 2019) high levels of this compound in a distinct sample of *Ulva lactuca*. In the case of *G. vermicullophyla* and *U. pinnatifida* is the first time that HTau is quantified in these species. Even though more data need to be gathered, our findings seem to indicate that relatively high levels of HTau can be found not only in red algae species but also in green and brown species.

HypTau was present in almost all the products analyzed but in smaller quantities than those found for the other two sulfonic acid derivatives, with concentrations ranging from 0.03 mg/100 g d.w. (*Tetraselmis sp.*) to 37.9 mg/100 g d.w. (*Chlorella vulgaris*). Previously HypTau was detected, but not quantified, only in *Ulva lactuca* (Gupta et al., 2017), while Tevatia et al., (2015) found 0.02 mg/100 g d.w. of HypTau in *Tetraselmis sp*., which is consistent with our result for this specie. So, quantitative information about the content of HypTau is provided for the first time for most of the seaweed and microalgae species included in our study.

Table S9. Tau, HTau and HypTau content in algae samples from literature.

Concentrations expressed in mg/100 g d.w.

Algae specie	Tau	HTau	HypTau	Reference
Chondrus crispus	78.1			Vieira, E. F., et al, 2018
Gracilaria vermicullophyla	≈ 250 ≈ 200	≈ 40		Mehdinia, A., et al, 2017 Kawasaki, A., et al. 2017
Palmaria palmata	201.6			Vieira, E. F., et al, 2018
Porphyra sp	1160 396 122 979; 646			Dawczynski, C., et al, 2007 Vieira, E. F., et al, 2018 McCusker, S., et al, 2014 Hwang, E. S., et al, 2013
Eisenia bicyclis	7			McCusker, S., et al, 2014
Himanthalia elongata	63.2			Vieira, E. F., et al, 2018
Hizikia fusiformis	69.6 ≈ 50			Dawczynski, C., et al, 2007 Kawasaki, A., et al. 2017
Laminaria japonica	19 70.7 ≈2			Dawczynski, C., et al, 2007 Vieira, E. F., et al, 2018 McCusker, S., et al, 2014
Laminaria ochroleuca	≈2			McCusker, S., et al, 2014
Undaria pinnatifida	19.8 165.1			Dawczynski, C., et al, 2007 Vieira, E. F., et al, 2018
Fucus vesiculosus	143.9			Vieira, E. F., et al, 2018
Ascophyllum nodosum	162.5			Vieira, E. F., et al, 2018
Codium sp	≈10			Kawasaki, A., et al. 2017
Ulva lactuca	171.4 ≈1			Vieira, E. F., et al, 2018 McCusker, S., et al, 2014
Tetraselmis chuii	68.7		0.02	Tevatia et al., 2015

The content of HypTau, Tau and HTau in pasta and crackers products including or not (Control) some algae is presented in **Table 2**. Any of the three sulfonic acid derivatives were found at detectable level in the Control products. On the contrary, all pasta and crackers products with algae addition contained at least one of the three sulfonic acid derivatives at detectable levels.

Thus, within pasta products the TAD content ranged between 0.19 mg/100 g d.w and 23.1 mg/100 g d.w., while in cracker samples TAD ranged between 0.75 mg/100 g d.w. and 159 mg/100 g d.w. It should be underlined that "Crackers with Porphyra" showed the highest content of Tau, which is in line with the high content of this compound observed in the raw algae sample (**Table 1**).

As expected, lower amounts of TAD were found in products reformulated with low percentages of algae inclusion or with algae species relatively poor in sulfonic acid derivatives, as is the case of "Noodles with wakame" (2.1 % *Undaria pinnatifida*) or "Crackers with Wakame" (1.5 % *Undaria pinnatifida*).

Table 2. **Concentration of sulfonic acid derivatives (mg/100 g d.w.),** Sum of sulfonic acid derivatives (TAD, g/ 100 g d.w) **Protein content (g/100 g d.w.), Essential to Non – Essential amino acid ratio (EAA/NEAA), Essential amino acid Index (EAAI) and Limiting amino acid (LAA) in algae-enriched food products and controls.** (Each value is the mean of n=3 independent determinations ± standard deviation). nd: not detected, lower than the limit detection). Small letters on the same column indicate significant differences between the median within the same food category. Product description and algae specie as indicated in the label of the commercial product

Product description*	Taurine	Homotaurine	Hypotaurine	TAD	Protein	EEA/NEAA	EAAI	LAA
	mg / 100 g d.w.				g / 100 d.w.			
Pasta + Algae								
(NN) Noodles with Nori	20.8 ± 0.01	2.15 ± 0.03	0.14 ± 0.01	23.1	12.9 ± 0.01	0.50 ± 0.03	97.5 ± 0.01	Met
(NU) Noodles with Ulva	13.9 ± 0.02	2.94 ± 0.02	0.10 ± 0.01	16.9	11.8 ± 0.05	0.49 ± 0.02	95.9 ± 0.02	Met
(NW) Noodles with Wakame	2.82 ± 0.03	1.08 ± 0.02	0.06 ± 0.00	3.96	11.4 ± 0.04	0.49 ± 0.04	97.1 ± 0.02	Met
(FS) Fusilli with Spirulina	3.45 ± 0.04	11.9 ± 0.03	0.19 ± 0.03	0.19	26.4 ± 0.03	0.47 ± 0.01	98.6 ± 0.01	Met
(SS) Spaghetti with seaweeds	17.7 ± 0.03	1.21 ± 0.01	0.09 ± 0.02	3.47	10.2 ± 0.05	0.50 ± 0.03	94.0 ± 0.01	Met
Median	**6.82 a**	**2.15 a**	**0.10 a**	**11.6 a**	**10.9**	**0.49**	**97.1**	
Pasta Control								
(P1) Spaghetti	nd	nd	nd	nd	10.1 ± 0.07	0.48 ± 0.01	97.7 ± 0.01	Met
(P2) Noodles	nd	nd	nd	nd	10.4 ± 0.06	0.47 ± 0.03	94.9 ± 0.01	Met
(P3) Pasta spirals	nd	nd	nd	nd	10.0 ± 0.05	0.50 ± 0.03	90.6 ± 0.02	Met
(P4) Noodles	nd	nd	nd	nd	10.9 ± 0.02	0.52 ± 0.02	90.3 ± 0.01	Met
(P5) Noodles	nd	nd	nd	nd	10.2 ± 0.01	0.50 ± 0.01	97.2 ± 0.01	Met
Median	**<0.003 b**	**<0.006 b**	**<0.01 b**	**<0.01 b**	**10.2**	**0.50**	**94.9**	
Crackers + Algae								
(CH) Crackers with Himanthalia	2.58 ± 0.02	2.07 ± 0.00	0.06 ± 0.00	4.71	10.4 ± 0.01	0.51 ± 0.01	97.9 ± 0.03	Lys
(CW) Crackers with Wakame	2.20 ± 0.01	0.75 ± 0.00	nd	0.75	12.3 ± 0.05	0.50 ± 0.01	97.1 ± 0.02	Lys
(CU) Crackers with Ulva	4.90 ± 0.02	nd	nd	4.90	13.2 ± 0.04	0.50 ± 0.02	97.2 ± 0.02	Lys
(CS) Crackers with Spirulina	2.21 ± 0.03	0.70 ± 0.00	nd	2.91	12.9 ± 0.03	0.50 ± 0.01	97.5 ± 0.01	Lys
(CP) Crackers with Porphyra	159 ± 2.18	0.01 ± 0.00	0.09 ± 0.00	159	24.4 ± 0.05	0.52 ± 0.02	101.3 ± 0.01	Lys
Median	**3.74 a**	**0.70 a**	**<0.02 a**	**4.71 a**	**12.9 a**	**0.50**	**97.5**	
Crackers + Control								
(C1) Brown rice crackers	nd	nd	nd	nd	7.81 ± 0.07	0.49 ± 0.02	97.1 ± 0.01	Lys
(C2) Brown rice crackers	nd	nd	nd	nd	7.67 ± 0.06	0.49 ± 0.01	97.1 ± 0.01	Lys
(C3) Salad crackers	nd	nd	nd	nd	8.01 ± 0.05	0.51 ± 0.03	96.7 ± 0.01	Lys
(C4) Crackers with olive oil	nd	nd	nd	nd	10.9 ± 0.02	0.51 ± 0.01	97.4 ± 0.01	Lys
(C5) Flaxseed. Sesame crackers	nd	nd	nd	nd	12.8 ± 0.01	0.51 ± 0.03	97.9 ± 0.01	Lys
Median	**<0.003 b**	**<0.006 b**	**<0.01 b**	**<0.01 b**	**8.00 b**	**0.51**	**97.1**	

3.3. Total protein and Amino acids indexes.

Table 1 shows the total protein content and amino acid indexes in the 26 algae samples (see also **Table S6 Supplementary material)**. Our results for the total protein content agreed with the values provided in the product label for 15 over 26 samples; in 4 samples the protein content was not specified in the label and in 7 samples (*Chondrus crispus*, *Himanthalia elongata*, *Hizikia fusiforme*, *Laminaria japonica*, *Undaria pinnatifida*, *Ascophyllum nodosum* and *Spirulina platensis*) the differences were higher than 10 %.

Red and green algae showed the highest total protein contents; the green microalgae *Chlorella vulgaris*, *Nannochloropsis sp*. and *Afanizomenon flos-aquae* exhibited the higher total protein content (52.2 g/100 g d.w., 40.0 g/100 g dw. and 36.4 g/100 d.w. respectively), followed by the red algae *Gracilaria vermiculophylla* and Gracilaria *longissima*. These protein contents are in line with the results of other Authors (Vieira et al., 2018) and with studies indicating that the protein content in brown algae is generally lower than in green and red species (Olson et al., 2020).

The EAA/NEAA ratio was in the range of 0.41 and 1.20 in both red and brown algae, and between 0.47 and 0.96 in green algae samples (Table 1). The highest EAA/NEAA ratio (1.20) was found in red seaweed *Gracilaria longissima* and in the brown microalgae *Odontella aurita*, indicating that these two algae species can be considered a good source of essential amino acids. In the case of the samples of *U. pinnatifida* (0.96) and *Porphyra sp*. (0.64) the results are in good agreement with the estimation of Mišurcová et al., (2014).

Other Authors reported EAA/NEAA ratios from 1.06 to 2.17 in commercial algae products, *i.e., Porphyra sp*. and *Ulva spp*., respectively (Vieira et al., 2018) and from 0.5 to 0.8 in products from the species *Porphyra sp*., *Laminaria sp*. and *Undaria pinnatifida* (Dawczynski et al., 2007; Mišurcová et al., 2014). In this case the differences could be partially associated to the amino acids included in the calculation.

The essential amino acid index (EAAI) was in the range of 84.8–185 in red algae samples, 79.9-169 in brown algae and 36.8–137 for green algae; the highest EAAI were found in the samples of the red seaweeds *Gigartina pistillata*, and *Gracilaria longissima*. These values are comparable with those estimated by Vieira et al., (2018) and Mišurcová et al., (2014). Methionine was the limiting amino acid (LAA) in 12 samples over 26, followed by lysine and leucine, in agreement with data available in literature (Dawczynski et al., 2007; Mišurcová et al., 2014; Vieira et al., 2018). Since EAAI is closely related with the biological quality of the protein, these data confirm that algae, and more specifically certain red algae species, could be a source of high-quality protein.

Total protein content and amino acid indexes in pasta and crackers products including or not (Control) algae are listed in **Table 2** (see also **Table S7 and S8 Supplementary material**).

In general, our results for the total protein content were in good agreement with what indicated in the label of pasta and cracker products, except for the products “Noodles with Nori” (-19 % than declared in the label) and “Crackers with Spirulina” (-44 % than declared in the label).

In general, and as pointed out by other authors (Prabhasankar et al., 2009; Fradique et al., 2010; Rodríguez De Marco et al., 2018), the higher the percentage of seaweed addition, the higher the protein content in the final product (*e.g.*, "Fusilli with Spirulina" with 4% of Spirulina, and "Crackers with Porphyra with 62 % of *Porphyra sp*.). Interestingly, if compared to the corresponding "Control" category, the inclusion of algae ingredients increased significantly the total protein content only in crackers but did not significantly ($p<0.05$) modify neither the EEA/NEAA ratio nor the EAAI in both pasta and crackers samples. Besides, the use of algae ingredients did not modify limiting amino acid (LAA), which was always Methionine in pasta and Lysine in crackers.

Table S6. Protein content (g/100 g d.w.) and Essential (EAA) and non-essential (NEEA) amino acids (g/100 g protein) in the 26 algae species. Chc (*Chondrus crispus*); Gl (*Gracilaria longissima*); Ms (*Mastocarpus stellatus*); Gsp (*Gracilaria sp*); Eb (*Eisenia bicyclys*); He (*Himanthalia elongate*); Hf (*Hizikia fusiformis*); Lj (*Laminaria japonica*); Ei (*Enteromorpha intestinalis*); Cl (*Caulerpa lentillifera*); Csp (*Codium sp*); Ul (*Ulvalactuca*); Nsp (*Nanochloropsis sp*).

	Red algae				**Brown algae**				**Green algae**				
	Chc	Gl	Ms	Gsp	Eb	He	Hf	Lj	Ei	Cl	Csp	Ul	Nsp
Protein	12.8 ± 0.03	34.6 ± 0.07	10.9 ± 0.01	35.1 ± 0.06	10.3 ± 0.01	11.0 ± 0.06	22.5 ± 0.05	22.8 ± 0.02	9.28 ± 0.05	13.1 ± 0.04	11.4 ± 0.05	24.1 ± 0.02	40.0 ± 0.02
His	1.98 ± 0.03	4.09 ± 0.05	2.29 ± 0.03	1.27 ± 0.05	2.35 ± 0.01	10.5 ± 0.04	1.97 ± 0.01	4.20 ± 0.04	2.76 ± 0.02	2.03 ± 0.01	3.75 ± 0.02	3.15 ± 0.03	1.02 ± 0.02
Arg	5.98 ± 0.03	9.70 ± 0.05	4.01 ± 0.03	11.0 ± 0.05	5.26 ± 0.01	1.86 ± 0.04	7.83 ± 0.01	5.09 ± 0.04	4.16 ± 0.02	3.06 ± 0.01	4.11 ± 0.02	7.03 ± 0.03	2.12 ± 0.02
Thr	3.30 ± 0.03	8.12 ± 0.02	4.48 ± 0.02	4.35 ± 0.03	5.38 ± 0.01	3.72 ± 0.03	4.99 ± 0.01	4.85 ± 0.01	3.29 ± 0.02	2.42 ± 0.01	2.92 ± 0.02	8.45 ± 0.01	3.59 ± 0.02
Lys	17.7 ± 0.03	5.15 ± 0.02	13.0 ± 0.02	2.96 ± 0.03	3.21 ± 0.02	7.97 ± 0.03	5.87 ± 0.01	4.46 ± 0.01	7.62 ± 0.02	0.74 ± 0.01	6.31 ± 0.02	8.82 ± 0.01	5.44 ± 0.02
Met	0.43 ± 0.03	1.20 ± 0.02	0.89 ± 0.02	14.7 ± 0.03	5.54 ± 0.01	0.86 ± 0.03	4.68 ± 0.01	0.89 ± 0.01	2.69 ± 0.02	1.98 ± 0.01	8.61 ± 0.02	1.70 ± 0.01	0.22 ± 0.02
Val	6.72 ± 0.03	6.68 ± 0.02	4.77 ± 0.02	3.76 ± 0.03	4.82 ± 0.01	6.72 ± 0.03	5.41 ± 0.01	2.16 ± 0.01	12.1 ± 0.02	8.94 ± 0.01	14.7 ± 0.02	6.53 ± 0.01	11.8 ± 0.02
Ileu	9.12 ± 0.03	5.53 ± 0.02	4.48 ± 0.02	11.9 ± 0.03	3.77 ± 0.01	1.65 ± 0.03	4.17 ± 0.01	4.84 ± 0.01	7.08 ± 0.02	5.21 ± 0.01	5.09 ± 0.02	5.20 ± 0.01	14.2 ± 0.02
Leu	9.67 ± 0.03	10.2 ± 0.02	6.35 ± 0.02	1.12 ± 0.03	7.89 ± 0.01	3.69 ± 0.03	12.3 ± 0.01	6.24 ± 0.01	11.3 ± 0.02	8.33 ± 0.01	8.19 ± 0.02	9.74 ± 0.01	6.23 ± 0.02
Phe	5.79 ± 0.03	8.09 ± 0.02	5.07 ± 0.02	4.18 ± 0.03	4.07 ± 0.01	1.59 ± 0.03	4.43 ± 0.01	0.27 ± 0.01	6.99 ± 0.02	5.15 ± 0.01	5.39 ± 0.02	5.43 ± 0.01	0.64 ± 0.02
Hyp	0.30 ± 0.03	1.47 ± 0.05	0.66 ± 0.03	0.18 ± 0.05	0.14 ± 0.01	1.85 ± 0.04	0.35 ± 0.01	0.51 ± 0.04	0.18 ± 0.02	0.24 ± 0.01	0.31 ± 0.02	3.28 ± 0.03	0.10 ± 0.02
Ser	11.6 ± 0.03	4.85 ± 0.02	4.89 ± 0.02	5.52 ± 0.03	4.62 ± 0.01	6.48 ± 0.03	3.68 ± 0.01	3.15 ± 0.01	4.92 ± 0.02	6.41 ± 0.01	4.04 ± 0.02	23.5 ± 0.01	12.7 ± 0.02
Gly	13.3 ± 0.03	5.80 ± 0.02	8.44 ± 0.02	24.7 ± 0.03	5.29 ± 0.01	4.00 ± 0.03	6.43 ± 0.01	12.4 ± 0.01	5.60 ± 0.02	7.37 ± 0.01	6.62 ± 0.02	5.57 ± 0.01	9.18 ± 0.02
Asp	7.59 ± 0.03	8.80 ± 0.02	10.5 ± 0.02	4.16 ± 0.03	9.78 ± 0.01	17.5 ± 0.03	15.5 ± 0.01	18.7 ± 0.01	8.65 ± 0.02	17.6 ± 0.01	5.71 ± 0.02	8.18 ± 0.01	10.5 ± 0.02
Glu	10.5 ± 0.03	9.18 ± 0.02	8.95 ± 0.02	8.83 ± 0.03	21.0 ± 0.01	10.0 ± 0.03	23.3 ± 0.01	21.5 ± 0.01	15.5 ± 0.02	17.2 ± 0.01	14.7 ± 0.02	6.51 ± 0.01	13.7 ± 0.02
Cys	9.18 ± 0.03	0.62 ± 0.02	3.50 ± 0.02	2.63 ± 0.03	6.71 ± 0.01	3.83 ± 0.03	3.52 ± 0.01	2.39 ± 0.01	8.35 ± 0.01	0.29 ± 0.01	1.07 ± 0.02	1.49 ± 0.01	1.83 ± 0.02
Pro	8.06 ± 0.03	5.01 ± 0.02	4.87 ± 0.02	6.21 ± 0.03	3.25 ± 0.01	2.18 ± 0.03	4.15 ± 0.01	10.0 ± 0.01	10.8 ± 0.02	8.22 ± 0.01	4.92 ± 0.02	4.85 ± 0.01	5.95 ± 0.02
Ala	3.49 ± 0.02	5.84 ± 0.02	8.31 ± 0.02	9.73 ± 0.03	6.25 ± 0.01	16.8 ± 0.03	9.78 ± 0.01	9.38 ± 0.01	10.0 ± 0.02	11.5 ± 0.01	5.83 ± 0.02	5.68 ± 0.01	11.6 ± 0.02
GABA	3.39 ± 0.03	1.41 ± 0.02	15.6 ± 0.02	6.01 ± 0.03	0.54 ± 0.01	3.85 ± 0.03	1.85 ± 0.01	1.12 ± 0.01	0.75 ± 0.02	1.09 ± 0.01	1.78 ± 0.02	1.38 ± 0.01	1.10 ± 0.02
Tyr	2.58 ± 0.03	6.10 ± 0.02	3.59 ± 0.02	10.8 ± 0.03	2.06 ± 0.01	2.45 ± 0.03	2.08 ± 0.01	1.70 ± 0.01	4.89 ± 0.04	4.36 ± 0.01	4.03 ± 0.02	5.17 ± 0.01	4.10 ± 0.02
Total Aas	131	108	115	134	102	108	122	114	128	112	108	122	116

Table S6 (continued). Pp (*Palmaria palmata*); Po (*Porphyra sp*); Gp (*Gigartina pistillata*); Lo (*Laminaria ochroleuca*); Up (*Undaria pinnatifida*); Fv (*Fucus vesiculosus*); An (*Ascophyllum nodosum*); Oa (*Odontella aurita*); Cv (*Chlorella vulgaris*); Ds (*Dunaliella salina*); Tch (*Tetraselmis chuii*); Afa (*Afanizonemon flos-aquae*); Sp (Spirulina pacifica).

	Red algae			**Brown algae**					**Green algae**				
	Pp	Po	Gp	Lo	Up	Fv	An	Oa	Cv	Ds	Tch	Afa	Sp
Protein	17.8 ± 0.02	31.0 ± 0.07	28.1 ± 0.01	6.90 ± 0.01	13.4 ± 0.03	10.6 ± 0.02	8.80 ± 0.03	15.1 ± 0.01	52.2 ± 0.03	17.0 ± 0.05	13.4 ± 0.03	36.4 ± 0.04	31.7 ± 0.08
His	1.62 ± 0.04	2.19 ± 0.05	1.69 ± 0.03	2.15 ± 0.01	1.85 ± 0.01	2.17 ± 0.02	4.07 ± 0.02	1.04 ± 0.02	2.58 ± 0.02	1.31 ± 0.04	5.03 ± 0.02	1.67 ± 0.01	1.54 ± 0.01
Arg	6.15 ± 0.04	7.27 ± 0.05	7.88 ± 0.03	1.66 ± 0.01	10.0 ± 0.01	3.63 ± 0.02	2.43 ± 0.02	1.21 ± 0.02	8.17 ± 0.02	0.94 ± 0.04	3.70 ± 0.02	7.87 ± 0.01	4.73 ± 0.01
Thr	5.37 ± 0.02	6.23 ± 0.02	10.4 ± 0.02	0.95 ± 0.01	12.5 ± 0.03	4.64 ± 0.04	7.48 ± 0.03	4.54 ± 0.02	4.20 ± 0.02	3.54 ± 0.04	5.18 ± 0.02	7.12 ± 0.01	1.72 ± 0.01
Lys	4.51 ± 0.02	5.68 ± 0.02	13.5 ± 0.02	1.69 ± 0.01	10.0 ± 0.03	11.8 ± 0.04	12.3 ± 0.03	17.7 ± 0.02	7.88 ± 0.02	5.79 ± 0.04	6.91 ± 0.02	8.06 ± 0.01	4.60 ± 0.01
Met	0.89 ± 0.02	1.93 ± 0.02	1.73 ± 0.02	0.12 ± 0.01	1.32 ± 0.03	0.42 ± 0.04	0.97 ± 0.03	0.37 ± 0.02	3.53 ± 0.02	0.51 ± 0.04	1.80 ± 0.02	1.33 ± 0.01	1.52 ± 0.01
Val	5.42 ± 0.02	6.53 ± 0.02	14.7 ± 0.02	3.04 ± 0.01	3.94 ± 0.03	4.79 ± 0.04	7.73 ± 0.03	4.00 ± 0.02	5.38 ± 0.02	3.97 ± 0.04	10.3 ± 0.02	2.07 ± 0.01	4.56 ± 0.01
Ileu	3.51 ± 0.02	4.38 ± 0.02	5.24 ± 0.02	1.81 ± 0.01	7.52 ± 0.03	2.26 ± 0.04	5.13 ± 0.03	21.9 ± 0.02	3.52 ± 0.02	18.0 ± 0.04	5.37 ± 0.02	6.95 ± 0.01	3.80 ± 0.01
Leu	6.17 ± 0.02	6.90 ± 0.02	7.89 ± 0.02	3.26 ± 0.01	1.88 ± 0.03	3.88 ± 0.04	6.39 ± 0.03	15.0 ± 0.02	8.56 ± 0.02	14.4 ± 0.04	9.73 ± 0.02	8.96 ± 0.01	8.55 ± 0.01
Phe	5.18 ± 0.02	3.12 ± 0.02	5.90 ± 0.02	0.46 ± 0.01	4.57 ± 0.03	1.48 ± 0.04	2.85 ± 0.03	5.13 ± 0.02	5.08 ± 0.02	3.67 ± 0.04	0.43 ± 0.02	6.88 ± 0.01	5.55 ± 0.01
Hyp	0.31 ± 0.04	0.05 ± 0.03	1.85 ± 0.01	0.34 ± 0.05	0.19 ± 0.01	0.64 ± 0.02	0.15 ± 0.02	0.36 ± 0.02	0.58 ± 0.02	0.16 ± 0.04	1.35 ± 0.02	0.07 ± 0.01	1.54 ± 0.01
Ser	12.7 ± 0.02	17.6 ± 0.01	5.03 ± 0.04	6.76 ± 0.02	14.5 ± 0.03	5.14 ± 0.04	4.83 ± 0.03	2.80 ± 0.02	21.4 ± 0.02	5.73 ± 0.04	6.01 ± 0.02	18.8 ± 0.01	22.5 ± 0.01
Gly	11.8 ± 0.02	10.6 ± 0.01	4.96 ± 0.04	5.70 ± 0.02	7.57 ± 0.03	10.2 ± 0.04	4.28 ± 0.03	17.7 ± 0.02	7.18 ± 0.02	7.65 ± 0.04	6.58 ± 0.02	6.60 ± 0.01	6.29 ± 0.01
Asp	12.1 ± 0.02	6.40 ± 0.01	7.14 ± 0.04	2.60 ± 0.02	9.90 ± 0.03	13.1 ± 0.04	9.64 ± 0.03	3.22 ± 0.02	9.53 ± 0.02	9.83 ± 0.04	10.8 ± 0.02	9.87 ± 0.01	11.0 ± 0.01
Glu	10.2 ± 0.02	14.3 ± 0.01	11.4 ± 0.04	2.14 ± 0.02	11.2 ± 0.03	12.4 ± 0.04	15.4 ± 0.03	9.05 ± 0.02	11.1 ± 0.02	12.3 ± 0.04	12.1 ± 0.02	14.6 ± 0.01	16.9 ± 0.01
Cys	3.72 ± 0.02	2.40 ± 0.01	2.40 ± 0.04	1.98 ± 0.02	1.36 ± 0.03	1.13 ± 0.04	1.02 ± 0.03	0.12 ± 0.02	1.64 ± 0.02	3.09 ± 0.04	1.06 ± 0.02	0.42 ± 0.01	1.70 ± 0.01
Pro	8.56 ± 0.02	4.72 ± 0.01	12.0 ± 0.04	4.31 ± 0.02	3.06 ± 0.03	7.61 ± 0.04	5.47 ± 0.03	11.3 ± 0.02	5.81 ± 0.02	7.38 ± 0.04	5.78 ± 0.02	6.49 ± 0.01	5.06 ± 0.01
Ala	7.53 ± 0.02	4.87 ± 0.01	15.5 ± 0.04	5.19 ± 0.02	3.97 ± 0.03	10.4 ± 0.04	5.61 ± 0.03	1.38 ± 0.02	8.18 ± 0.02	6.88 ± 0.04	10.7 ± 0.02	8.76 ± 0.01	8.64 ± 0.01
GABA	0.87 ± 0.02	2.54 ± 0.01	2.37 ± 0.04	0.76 ± 0.02	1.83 ± 0.03	0.74 ± 0.04	3.39 ± 0.03	10.4 ± 0.02	3.60 ± 0.02	1.19 ± 0.04	2.59 ± 0.02	0.51 ± 0.01	0.98 ± 0.01
Tyr	1.58 ± 0.02	5.28 ± 0.01	0.45 ± 0.04	0.42 ± 0.02	0.93 ± 0.03	2.18 ± 0.04	1.71 ± 0.03	2.92 ± 0.02	3.76 ± 0.02	1.31 ± 0.04	4.38 ± 0.02	0.71 ± 0.01	3.27 ± 0.01
Total Aas	108	113	130	45	108	99	101	131	122	108	110	118	115

Table S7. Essential amino acids in pasta and crackers samples with and without algae addition.

Product	His	Arg	Thr	Lys	Met	Val	Ileu	Leu	Phe
mg / 100 g					**Pasta**				
Noodles with Porphyra (NN)	0.16 ± 0.01	0.53 ± 0.01	0.31 ± 0.02	0.35 ± 0.02	0.05 ± 0.01	0.48 ± 0.01	0.38 ± 0.01	0.82 ± 0.01	0.54 ± 0.02
Noodles with Ulva (NU)	0.15 ± 0.02	0.52 ± 0.02	0.31 ± 0.01	0.33 ± 0.01	0.05 ± 0.01	0.47 ± 0.02	0.38 ± 0.01	0.83 ± 0.02	0.54 ± 0.02
Noodles with Wakame (NW)	0.14 ± 0.01	0.53 ± 0.01	0.32 ± 0.01	0.34 ± 0.01	0.06 ± 0.01	0.48 ± 0.01	0.38 ± 0.01	0.82 ± 0.01	0.54 ± 0.01
Fusilli with Spirulina (FS)	0.11 ± 0.02	0.53 ± 0.03	0.32 ± 0.03	0.32 ± 0.03	0.06 ± 0.03	0.48 ± 0.03	0.38 ± 0.03	0.95 ± 0.03	0.64 ± 0.01
Spaghetti with seaweeds (SS)	0.14 ± 0.02	0.52 ± 0.02	0.31 ± 0.02	0.33 ± 0.02	0.05 ± 0.02	0.47 ± 0.02	0.38 ± 0.02	0.82 ± 0.02	0.54 ± 0.02
Spaguetti (P1)	0.14 ± 0.05	0.53 ± 0.05	0.31 ± 0.02	0.34 ± 0.02	0.06 ± 0.02	0.48 ± 0.02	0.39 ± 0.02	0.81 ± 0.02	0.53 ± 0.01
Noodles (P2)	0.13 ± 0.04	0.55 ± 0.04	0.31 ± 0.04	0.34 ± 0.03	0.05 ± 0.03	0.50 ± 0.03	0.38 ± 0.01	0.81 ± 0.03	0.54 ± 0.03
Pasta Spirals (P3)	0.12 ± 0.01	0.51 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.04 ± 0.03	0.45 ± 0.01	0.35 ± 0.01	0.82 ± 0.01	0.55 ± 0.01
Noodles (P4)	0.15 ± 0.04	0.51 ± 0.04	0.30 ± 0.01	0.33 ± 0.01	0.03 ± 0.01	0.47 ± 0.01	0.37 ± 0.01	0.84 ± 0.01	0.55 ± 0.01
Noodles (P5)	0.14 ± 0.05	0.52 ± 0.05	0.31 ± 0.02	0.32 ± 0.02	0.07 ± 0.02	0.45 ± 0.02	0.40 ± 0.02	0.83 ± 0.02	0.53 ± 0.01
mg / 100 g					**Crakers**				
Crackers Himanthalia (CH)	0.25 ± 0.01	0.38 ± 0.01	0.29 ± 0.01	0.22 ± 0.01	0.16 ± 0.01	0.44 ± 0.01	0.33 ± 0.01	0.66 ± 0.01	0.48 ± 0.01
Crackers Wakame (CW)	0.24 ± 0.01	0.38 ± 0.02	0.29 ± 0.02	0.19 ± 0.02	0.16 ± 0.02	0.44 ± 0.01	0.34 ± 0.02	0.66 ± 0.02	0.48 ± 0.03
Crackers Ulva (CU)	0.24 ± 0.01	0.38 ± 0.01	0.28 ± 0.02	0.21 ± 0.01	0.16 ± 0.01	0.44 ± 0.01	0.33 ± 0.01	0.66 ± 0.01	0.47 ± 0.01
Crackers Spirulina (CS)	0.24 ± 0.03	0.38 ± 0.03	0.28 ± 0.03	0.21 ± 0.03	0.16 ± 0.03	0.44 ± 0.03	0.34 ± 0.03	0.66 ± 0.03	0.48 ± 0.03
Crackers Nori (CN)	0.74 ± 0.02	0.79 ± 0.02	0.33 ± 0.02	0.52 ± 0.02	0.36 ± 0.02	1.76 ± 0.02	0.74 ± 0.02	1.16 ± 0.02	1.88 ± 0.02
Brown rice crackers (C1)	0.24 ± 0.05	0.38 ± 0.04	0.29 ± 0.02	0.19 ± 0.02	0.16 ± 0.02	0.44 ± 0.02	0.34 ± 0.02	0.66 ± 0.02	0.48 ± 0.02
Brown rice crackers (C2)	0.24 ± 0.04	0.39 ± 0.01	0.28 ± 0.03	0.19 ± 0.03	0.15 ± 0.03	0.44 ± 0.03	0.34 ± 0.02	0.65 ± 0.03	0.48 ± 0.02
Salad crackers (C3)	0.24 ± 0.01	0.38 ± 0.02	0.28 ± 0.01	0.19 ± 0.01	0.16 ± 0.01	0.44 ± 0.01	0.33 ± 0.01	0.66 ± 0.01	0.48 ± 0.01
Crackers with olive oil (C4)	0.24 ± 0.04	0.38 ± 0.04	0.29 ± 0.01	0.19 ± 0.01	0.16 ± 0.01	0.44 ± 0.01	0.34 ± 0.01	0.66 ± 0.01	0.48 ± 0.01
Crackers sesame and quinoa (C5)	0.24 ± 0.05	0.38 ± 0.05	0.29 ± 0.02	0.19 ± 0.02	0.16 ± 0.02	0.44 ± 0.02	0.34 ± 0.02	0.67 ± 0.02	0.49 ± 0.02

Table S8. Non-essential amino acids in pasta and crackers samples with and without algae addition.

Product	Hyp	Ser	Gly	Asp	Glu	Cys	Pro	Ala	GABA	Tyr
mg / 100 g					**Pasta**					
Noodles with Nori (NN)	0.13 ± 0.01	0.51 ± 0.01	0.41 ± 0.01	0.47 ± 0.01	3.16 ± 0.01	0.09 ± 0.01	1.24 ± 0.01	0.35 ± 0.01	0.80 ± 0.01	0.26 ± 0.01
Noodles with Ulva (NU)	0.13 ± 0.01	0.51 ± 0.02	0.42 ± 0.02	0.48 ± 0.02	3.25 ± 0.02	0.09 ± 0.01	1.23 ± 0.02	0.35 ± 0.02	0.81 ± 0.02	0.26 ± 0.02
Noodles with Wakame (NW)	0.13 ± 0.01	0.51 ± 0.02	0.41 ± 0.01	0.41 ± 0.01	3.15 ± 0.01	0.09 ± 0.00	1.23 ± 0.01	0.35 ± 0.01	0.81 ± 0.01	0.26 ± 0.01
Fusilli with Spirulina (FS)	0.13 ± 0.02	0.52 ± 0.03	0.41 ± 0.03	0.50 ± 0.03	3.28 ± 0.03	0.09 ± 0.01	1.24 ± 0.03	0.35 ± 0.03	0.81 ± 0.02	0.26 ± 0.01
Spaghetti with seaweeds (SS)	0.13 ± 0.01	0.51 ± 0.01	0.40 ± 0.02	0.47 ± 0.02	3.18 ± 0.02	0.09 ± 0.00	1.23 ± 0.02	0.35 ± 0.02	0.81 ± 0.01	0.26 ± 0.00
Spaguetti (P1)	0.12 ± 0.01	0.50 ± 0.01	0.41 ± 0.02	0.47 ± 0.02	3.33 ± 0.02	0.09 ± 0.02	1.19 ± 0.02	0.35 ± 0.02	0.88 ± 0.02	0.27 ± 0.02
Noodles (P2)	0.13 ± 0.03	0.50 ± 0.02	0.40 ± 0.03	0.46 ± 0.03	3.44 ± 0.03	0.09 ± 0.00	1.25 ± 0.03	0.35 ± 0.03	0.82 ± 0.03	0.26 ± 0.03
Pasta Spirals (P3)	0.11 ± 0.01	0.51 ± 0.01	0.41 ± 0.01	0.48 ± 0.01	2.90 ± 0.01	0.10 ± 0.01	1.25 ± 0.01	0.33 ± 0.01	0.81 ± 0.01	0.25 ± 0.01
Noodles (P4)	0.14 ± 0.04	0.51 ± 0.01	0.41 ± 0.01	0.46 ± 0.01	2.66 ± 0.01	0.10 ± 0.01	1.28 ± 0.01	0.33 ± 0.01	0.75 ± 0.01	0.29 ± 0.01
Noodles (P5)	0.14 ± 0.01	0.51 ± 0.02	0.40 ± 0.02	0.47 ± 0.02	3.15 ± 0.02	0.09 ± 0.02	1.19 ± 0.02	0.35 ± 0.02	0.72 ± 0.02	0.21 ± 0.02
mg / 100 g					**Crackers**					
Crackers Himanthalia (CH)	0.10 ± 0.01	0.56 ± 0.01	0.40 ± 0.01	0.52 ± 0.01	2.82 ± 0.01	0.20 ± 0.01	1.08 ± 0.01	0.31 ± 0.01	0.20 ± 0.01	0.30 ± 0.01
Crackers Wakame (CW)	0.10 ± 0.02	0.56 ± 0.02	0.41 ± 0.02	0.51 ± 0.02	2.72 ± 0.02	0.20 ± 0.01	1.08 ± 0.02	0.30 ± 0.02	0.21 ± 0.02	0.30 ± 0.01
Crackers Ulva (CU)	0.10 ± 0.02	0.56 ± 0.01	0.40 ± 0.01	0.52 ± 0.01	2.78± 0.01	0.20 ± 0.01	1.08 ± 0.01	0.30 ± 0.01	0.21 ± 0.02	0.29 ± 0.01
Crackers Spirulina (CS)	0.10 ± 0.01	0.57 ± 0.03	0.40 ± 0.01	0.51 ± 0.03	2.73 ± 0.03	0.20 ± 0.02	1.08 ± 0.03	0.31 ± 0.03	0.20 ± 0.03	0.29 ± 0.03
Crackers Nori (C1)	0.10 ± 0.01	0.57 ± 0.02	0.42 ± 0.02	0.54 ± 0.02	2.85 ± 0.02	0.21 ± 0.02	1.10 ± 0.02	0.31 ± 0.02	0.20 ± 0.02	0.29± 0.02
Brown rice crackers (C2)	0.09 ± 0.01	0.57 ± 0.02	0.41 ± 0.02	0.51 ± 0.02	2.71 ± 0.02	0.20 ± 0.02	1.04 ± 0.02	0.31 ± 0.02	0.40 ± 0.02	0.30 ± 0.02
Brown rice crackers (C3)	0.11 ± 0.03	0.55 ± 0.02	0.40 ± 0.03	0.50 ± 0.03	2.71 ± 0.03	0.20 ± 0.01	1.08 ± 0.03	0.30 ± 0.03	0.27 ± 0.03	0.30 ± 0.03
Salad crackers (C4)	0.09 ± 0.01	0.57 ± 0.01	0.40 ± 0.01	0.48 ± 0.01	2.69 ± 0.01	0.21 ± 0.01	1.08 ± 0.01	0.29 ± 0.01	0.13 ± 0.01	0.29 ± 0.01
Crackers with olive oil (C5)	0.10 ± 0.02	0.57 ± 0.01	0.41 ± 0.01	0.52 ± 0.01	2.75 ± 0.01	0.20 ± 0.01	1.10 ± 0.01	0.30 ± 0.01	0.09 ± 0.01	0.29 ± 0.01
Crackers sesame and quinoa (C5)	0.10 ± 0.01	0.55 ± 0.02	0.39 ± 0.02	0.52 ± 0.02	2.70 ± 0.02	0.22 ± 0.03	1.11 ± 0.02	0.31 ± 0.02	0.12 ± 0.02	0.30 ± 0.02

Percentual variations of EAAI and EAA/NEAA ratio in specific products vs. the corresponding "Control" food categories are represented **in Figure 2 and Figure 3**. Two commercial pasta samples including algae in their formulation, i.e., "Noodles with Porphyra" (NN) and "Fusilli with Spirulina" (FS) exhibited an improvement of their EAAI and EAA/NEAA ratio close to or higher than 5 % compared to the corresponding control products (**Figure 2**). In one product (SS) the variations were lower than 1 %, in one product (NU) the EAAI increased but the EAA/NEAA ratio was unchanged and in one product (NW) the EAAI increased but the EAA/NEAA ratio decreased if compared to the corresponding "Control" category. In the case of the commercial crackers, only one sample (CN, Crackers with 62 % Nori) showed an improvement of both EAAI and EAA/NEAA parameters if compared to the corresponding control products (**Figure 3**). The other samples had limited variations of these parameters (lower than 1 %).

So, it seems that products with the highest percentages of algae addition provided not only a significant improvement of their total protein content compared with the products without algae addition, but also increased the nutritional quality of the protein fraction.

Figure 2. Percentual variations of the Essential Amino Acid Index (% Δ EAAI) and of the Essential to Non-Essential Amino Acid ratio (% Δ EAA/NEAA) for pasta products enriched with different algae species in comparison to the control samples; SS (Spaghetti with 3% seaweeds), FS (Fusilli with 4% spirulina), NW (Noodles with 2.1% wakame), NU (Noodles with 2.4% ulva), NN (Noodles with 4.4% nori).

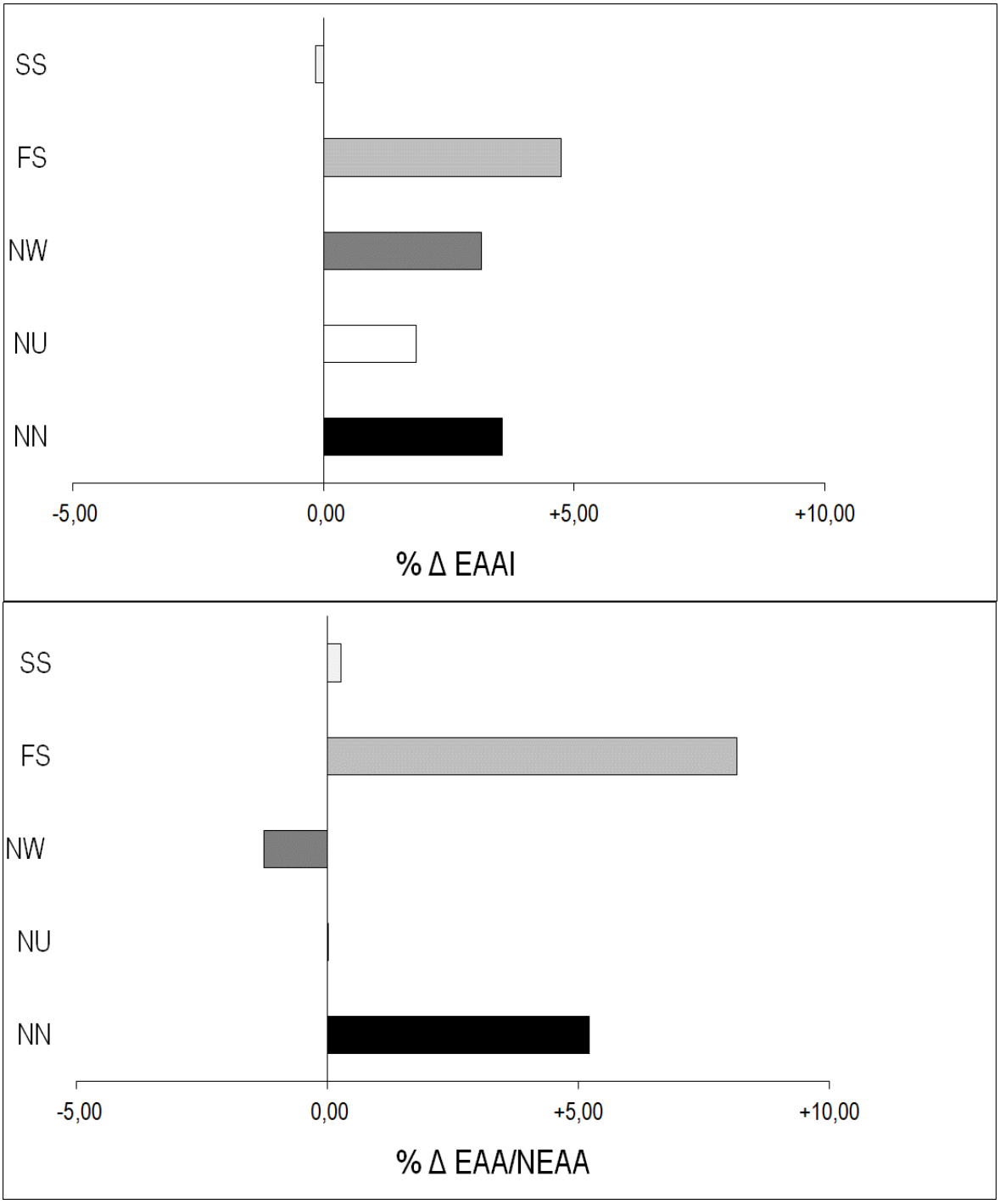
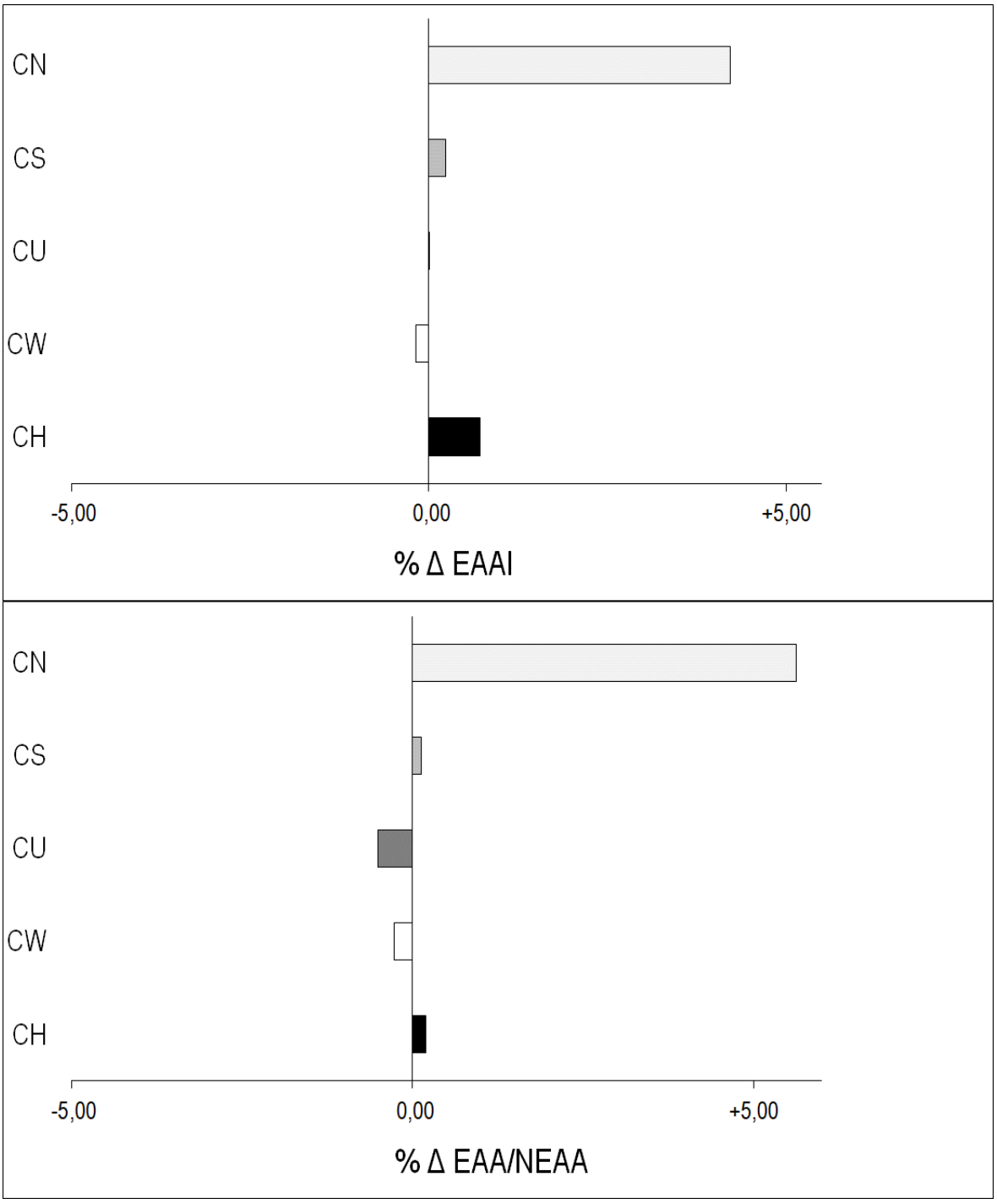


Figure 3. Percentual variations of the Essential Amino Acid Index (% □ EAAI) and of the Essential to Non-Essential Amino Acid ratio (% □ EAA/NEAA) for crackers products enriched with different algae species in comparison to the control samples. CN (Crackers with 62 % nori), CS (Crackers with 2.6 % spirulina), CU (Crackers with 1.5 % ulva), CW (Crackers with 1.5 % wakame), CH (Crackers with 5 % himanthalia).



4. Conclusion

An UHPLC-DAD-MS/MS analytical method to quantify the composition of Taurine, Hypotaurine, Homotaurine and main amino acids in algae was further validated in two food matrices, pasta and crackers.

The distribution of the target compounds was assessed in 26 samples of macroalgae and microalgae available on the market, as well as in 20 samples of commercial pasta and crackers products including or not algae as ingredient. All the algae species included in this study contained at least one of the three target sulfonic acid derivatives. Red algae species stand out for their high content in Taurine and Homotaurine, being the species belonging to the genus *Gracilaria* those which presented the highest concentration. Hypotaurine was also found in almost all species, but at lower concentrations than the other two compounds.

It is important to highlight that, to the best of our knowledge, we report for the first time the concentration of Tau in 9 algae species, i.e. *Gracilaria longissima*, *Mastocarpus stellatus*, *Gigartina pistillata*, *Eisenia bicyclis*, *Odontella aurita*, *Enteromospha intestinalis*, *Codium sp*, *Dunaliella salina* and *Aphanizomenon flos-aquae*, while HTau and HypTau were quantified first ever in almost all the species except in *Gracilaria* sp, *Porphyra* sp, *Laminaria japónica*, *Ulva Lactuca*, *Chlorella vulgaris* and *Tetraselmis chuii* (Tevatia et al., 2015; Terriente-Palacios et al., 2019).

Commercial pasta and crackers reformulated with different species of algae contained at least one of the three target sulfonic acid derivatives at detectable levels. In some case, i.e., “Crackers with Porphyra” or “Noodles with Nori” the total sulfonic acid derivatives reached concentration comparable to those found in foods of animal origin. Inclusion of seaweeds and microalgae also contributed to significantly increase the total protein content in crackers if compared to

commercial products of the same category, and increased other nutritional properties of the protein fraction (EEA/NEAA, EAA) in products with the higher percentage of algae addition (*e.g.*, "Fusilli with 4% spirulina", "Noodles with 4.4% Nori" and "Crackers with 62 % Nori). Nevertheless, these effects may differ according to the algae species and the amount used in the formulation.

Acknowledgements

This research was supported by the CERCA programme/Generalitat de Catalunya and by the "ALGAE" project "Retos Colaboracion" (2016, RTC-2016-5183-2).

References

Ali, R., Quinn, G., Hill, K., Zhang, G., Townsend, E., Koh, C., Rampertaap, S., Rosenzweig, S., Levy, E., Kleiner, D., Etzion, O., & Heller, T. (2019). Sa1528 – Emerging Role of Hypotaurine and Taurine Amino Acids in Chronic Hepatitis C Associated Chronic Liver Disease Before and After Hcv Clearance. *Gastroenterology*. https://doi.org/10.1016/s0016-5085(19)40076-0

AOAC International (2002). Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals, AOAC Int., http://dx.doi.org/10.1074/jbc.M200778200.

Babuskin, S., Krishnan, K. R., Babu, P. A. S., Sivarajan, M., & Sukumar, M. (2014). Functional foods enriched with marine microalga Nannochloropsis oculata as a source of ω-3 fatty acids. *Food Technology and Biotechnology*.

Batista, A. P., Niccolai, A., Bursic, I., Sousa, I., Raymundo, A., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae as functional ingredients in savory food products: Application to wheat crackers. *Foods*. https://doi.org/10.3390/foods8120611

Bogolitsyn, K. G., Kaplitsin, P. A., & Pochtovalova, A. S. (2014). Amino-acid composition of arctic brown algae. *Chemistry of Natural Compounds*. https://doi.org/10.1007/s10600-014-0831-1

Cao, Y., Duan, J., Guo, J., Guo, S., & Zhao, J. (2014). Rapid determination of nucleosides, nucleobases and free amino acids in brown seaweeds using ultra-performance liquid chromatography coupled with triple quadrupole mass

spectrometry. *Journal of Applied Phycology*. https://doi.org/10.1007/s10811-013-0079-3

Černá, M. (2011). Seaweed proteins and amino acids as nutraceuticals. In *Advances in Food and Nutrition Research*. https://doi.org/10.1016/B978-0-12-387669-0.00024-7

Colovic, M. B., Vasic, V. M., Djuric, D. M., & Krstic, D. Z. (2018). Sulphur-containing Amino Acids: Protective Role Against Free Radicals and Heavy Metals. *Current Medicinal Chemistry*. https://doi.org/10.2174/0929867324666170609075434

Dawczynski, C., Schubert, R., & Jahreis, G. (2007). Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2006.09.041

El-Baz, F. K., Abdo, S. M., & Hussein, A. M. S. (2017). Microalgae Dunaliella salina for use as Food Supplement to improve Pasta Quality. *Int. J. Pharm Sci Rev*.

Fradique, Ḿonica, Batista, A. P., Nunes, M. C., Gouveia, L., Bandarra, N. M., & Raymundo, A. (2010). Incorporation of Chlorella vulgaris and Spirulina maxima biomass in pasta products. Part 1: Preparation and evaluation. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.3999

Friedman, M. (1996). Nutritional Value of Proteins from Different Food Sources. A Review. In *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf9400167

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., & Toulmin, C. (2010).

Food security: The challenge of feeding 9 billion people. In *Science*. https://doi.org/10.1126/science.1185383

Gossai, D., & Lau-Cam, C. A. (2009). The effects of taurine, taurine homologs and hypotaurine on cell and membrane antioxidative system alterations caused by type 2 diabetes in rat erythrocytes. *Advances in Experimental Medicine and Biology*. https://doi.org/10.1007/978-0-387-75681-3_37

Gupta, V., & Kushwaha, H. R. (2017). Metabolic regulatory oscillations in intertidal green seaweed Ulva lactuca against tidal cycles. *Scientific Reports*. https://doi.org/10.1038/s41598-017-15994-2

Hara, K., Nakamura, M., Haranishi, Y., Terada, T., Kataoka, K., & Sata, T. (2012). Antinociceptive effect of intrathecal administration of hypotaurine in rat models of inflammatory and neuropathic pain. *Amino Acids*. https://doi.org/10.1007/s00726-011-1094-9

Hatab, H. M., Abdel Hamid, F. F., Soliman, A. F., Al-Shafie, T. A., Ismail, Y. M., & El-Houseini, M. E. (2019). A combined treatment of curcumin, piperine, and taurine alters the circulating levels of IL-10 and miR-21 in hepatocellular carcinoma patients: A pilot study. *Journal of Gastrointestinal Oncology*. https://doi.org/10.21037/jgo.2019.03.07

Hwang, E. S., Ki, K. N., & Chung, H. Y. (2013). Proximate composition, amino acid, mineral, and heavy metal content of dried laver. *Preventive Nutrition and Food Science*. https://doi.org/10.3746/pnf.2013.18.2.139

Jakaria, M., Azam, S., Haque, M. E., Jo, S. H., Uddin, M. S., Kim, I. S., & Choi, D. K. (2019). Taurine and its analogs in neurological disorders: Focus on therapeutic potential and molecular mechanisms. In *Redox Biology*. https://doi.org/10.1016/j.redox.2019.101223

Kawasaki, A., Ono, A., Mizuta, S., Kamiya, M., Takenaga, T., & Murakami, S. (2017). The taurine content of Japanese seaweed. *Advances in Experimental Medicine and Biology*. https://doi.org/10.1007/978-94-024-1079-2_88

Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y., Chu, D. T., & Show, P. L. (2019). Microalgae: A potential alternative to health supplementation for humans. In *Food Science and Human Wellness*. https://doi.org/10.1016/j.fshw.2019.03.001

Lourenço, R., & Camilo, M. E. (2002). Taurine: A conditionally essential amino acid in humans? An overview in health and disease. In *Nutricion Hospitalaria*.

Manzano, S., Agüera, L., Aguilar, M., & Olazarán, J. (2020). A Review on Tramiprosate (Homotaurine) in Alzheimer's Disease and Other Neurocognitive Disorders. In *Frontiers in Neurology*. https://doi.org/10.3389/fneur.2020.00614

McCusker, S., Buff, P. R., Yu, Z., & Fascetti, A. J. (2014). Amino acid content of selected plant, algae and insect species: a search for alternative protein sources

for use in pet foods. *Journal of Nutritional Science*. https://doi.org/10.1017/jns.2014.33

Mehdinia, A., Rostami, S., Dadkhah, S., & Fumani, N. S. (2017). Simultaneous Evaluation of homotaurine and taurine in marine macro-algae using liquid chromatography–fluorescence detection. *Journal of the Iranian Chemical Society*. https://doi.org/10.1007/s13738-017-1150-9

Mišurcová, L., Buňka, F., Vávra Ambrožová, J., Machů, L., Samek, D., & Kráčmar, S. (2014). Amino acid composition of algal products and its contribution to RDI. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2013.11.040

Olsson, J., Toth, G. B., & Albers, E. (2020). Biochemical composition of red, green and brown seaweeds on the Swedish west coast. *Journal of Applied Phycology*. https://doi.org/10.1007/s10811-020-02145-w

Prabhasankar, P., Ganesan, P., Bhaskar, N., Hirose, A., Stephen, N., Gowda, L. R., Hosokawa, M., & Miyashita, K. (2009). Edible Japanese seaweed, wakame (Undaria pinnatifida) as an ingredient in pasta: Chemical, functional and structural evaluation. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2008.12.047

FAO Join. (2007). WHO Technical Report Series Protein and Amino Acid Requirements in Human Nutrition. *WHO Int*.

Rodríguez De Marco, E., Steffolani, M. E., Martínez, M., & León, A. E. (2018). The use of Nannochloropsis sp. as a source of omega-3 fatty acids in dry pasta: chemical, technological and sensory evaluation. *International Journal of Food Science and Technology*. https://doi.org/10.1111/ijfs.13609

Schaffer, S., & Kim, H. W. (2018). Effects and mechanisms of taurine as a therapeutic agent. In *Biomolecules and Therapeutics*. https://doi.org/10.4062/biomolther.2017.251

Slocombe, S. P., Ross, M., Thomas, N., McNeill, S., & Stanley, M. S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2012.10.163

Terriente-Palacios, C., Diaz, I., & Castellari, M. (2019). A validated ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry method to simultaneously quantify taurine, homotaurine, hypotaurine and amino acids in

macro- and microalgae. *Journal of Chromatography A*. https://doi.org/10.1016/j.chroma.2018.12.058

Tevatia, R., Allen, J., Rudrappa, D., White, D., Clemente, T. E., Cerutti, H., Demirel, Y., & Blum, P. (2015). The taurine biosynthetic pathway of microalgae. *Algal Research*. https://doi.org/10.1016/j.algal.2015.02.012

Thompson, M., Ellison, S. L. R., & Wood, R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure and Applied Chemistry*. https://doi.org/10.1351/pac200274050835

Tian, J., Dang, H., Wallner, M., Olsen, R., & Kaufman, D. L. (2018). Homotaurine, a safe blood-brain barrier permeable GABAA-R-specific agonist, ameliorates disease in mouse models of multiple sclerosis. *Scientific Reports*. https://doi.org/10.1038/s41598-018-32733-3

Tiwari, B. K., & Troy, D. J. (2015). Seaweed Sustainability: Food and Non-Food Applications. In *Seaweed Sustainability: Food and Non-Food Applications*. https://doi.org/10.1016/C2013-0-12836-X

Vieira, E. F., Soares, C., Machado, S., Correia, M., Ramalhosa, M. J., Oliva-teles, M. T., Paula Carvalho, A., Domingues, V. F., Antunes, F., Oliveira, T. A. C., Morais, S., & Delerue-Matos, C. (2018). Seaweeds from the Portuguese coast as a source of proteinaceous material: Total and free amino acid composition profile. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2018.06.145

Wan, Q. L., Fu, X., Meng, X., Luo, Z., Dai, W., Yang, J., Wang, C., Wang, H., & Zhou, Q. (2020). Hypotaurine promotes longevity and stress tolerance: Via the stress response factors DAF-16/FOXO and SKN-1/NRF2 in Caenorhabditis elegans. *Food and Function*. https://doi.org/10.1039/c9fo02000d

Wójcik, O. P., Koenig, K. L., Zeleniuch-Jacquotte, A., Costa, M., & Chen, Y. (2010). The potential protective effects of taurine on coronary heart disease. In *Atherosclerosis*. https://doi.org/10.1016/j.atherosclerosis.2009.06.002

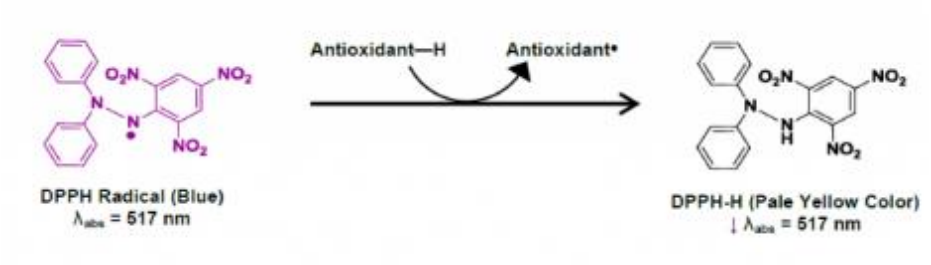
BLOQUE 2

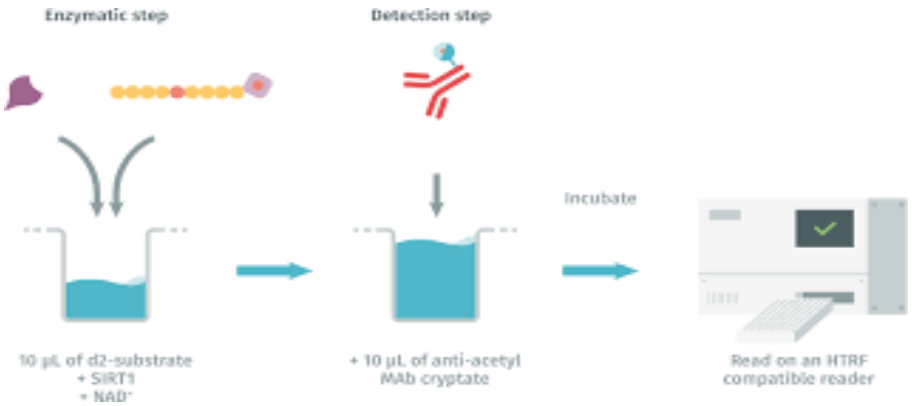
MEDIDA DE LA ACTIVIDAD BIOLÓGICA DE EXTRACTOS DE ALGAS

CAPÍTULO 3

El contenido de Taurina, Homotaurina, GABA y aminoácidos hidrofóbicos influye en las actividades "in vitro" antioxidantes y de modulación de la SIRT1 de hidrolizados enzimáticos proteicos de algas

Algae Protein hydrolysates





AUTORES: **Carlos Terriente-Palacios** [a, b], Susana Rubino Campoy [a], María Hortòs [a], César Peteiro [c], Massimo Castellari [a]

TITULO: **Taurine, Homotaurine, GABA and hydrophobic amino acids content influences "in vitro" antioxidant and SIRT1 modulation activities of enzymatic protein hydrolysates from algae**

REFERENCIA: https://doi.org/10.1038/s41598-022-25130-4

REVISTA: **Nature – Scientific Reports**

EDITORIAL: NATURE

AFILIACIONES:
[a] IRTA Food Industries – Finca Camps I Armet s/n, 17121 Monells, Girona, Spain
[b] Escuela Internacional de Doctorado UNED - Calle Bravo Murillo 38, 28015 Madrid, Spain
[c] Spanish Institute of Oceanography of the Spanish National Research Council (IEO, CSIC), Oceanographic Center of Santander, Marine Culture Units "El Bocal", Seaweeds Center, Barrio Corbanera s/n., 39012 Monte, Santander (Spain)

LUGAR DE PUBLICACIÓN: NETHERLANDS

FECHA DE PUBLICACIÓN: **2022**

FACTOR DE IMPACTO: PUBLICADO (JCR-2022) **6.475**.

FOOD SCIENCE & TECHNOLOGY	POSICIÓN: 9/143 (JCR-2022)	PRIMER TERCIO (1º CUARTIL)

Abstract

Prevention and control of diseases and delaying the signs of ageing are nowadays one of the major goals of biomedicine. Sirtuins, a family of NAD^+ dependent deacylase enzymes, could be pivotal targets of novel preventive and therapeutic strategies to achieve such aims. SIRT1 activating and inhibiting compounds, such as polyphenols and bioactive peptides, have been proposed to be involved in the development of many human diseases. The objective of this work was to assess and compare the antioxidant and SIRT1 modulation activities of enzymatic protein hydrolysates (EPHs) from a wide number of algae species (24 commercial samples and 12 samples harvested of the Atlantic coast of northern Spain). High antioxidant activities were observed in EPHs from red and green seaweed species. Moreover, 19 samples exhibited SIRT1 activation, while EPHs from the 16 samples were SIRT1 inhibitors. Pearson's correlation test and Principal Component Analysis revealed significant correlations between (1) total peptide and hydrophobic amino acid content in EPHs and their antioxidant activities, and (2) concentrations of taurine, homotaurine, and amino acid gamma aminobutyric acid in EPHs and their SIRT1 modulation activities.

Keywords

Protein hydrolysate, DPPH, ABTS, Sirtuins, Activators, Inhibitors, Seaweed, Microalgae

Introduction

The ocean covers more than 70% of the earth's surface, and therefore marine biodiversity is an essential part of the global system since a large part of human food consumption is derived from marine resources, and global food demands are continually rising. Due to the complex habitats in which they live, marine organisms can produce a wide variety of secondary metabolites that cannot be found elsewhere. Hence, sponges, algae, and bryozoans, among many other marine organisms, are important sources of bioactive compounds with interesting properties like antioxidant, antimicrobial, anticancer, antihypertensive, and anti-inflammatory activities [1,2].

Protein hydrolysates from macro-and microalgae have been reported by various authors as possible therapeutic agents due to their antimicrobial and antiviral [3,4], anticancer[5], antioxidant[6-9], and immunoregulatory functions[10]. Additionally, a close relationship has been observed between the antioxidant capacity of the protein hydrolysates and their hydrophobic and/or aromatic amino acid content[11–14]. Heo et al.[7] observed pronounced anti-oxidative effects in water-soluble, enzymatic extracts of seven species of marine edible brown seaweed from South Korea's coasts. In this study, protease extracts of Ecklonia cava scavenged DPPH free radicals more effectively than other algal extracts. Other authors[8,15] found proteases more effective than carbohydrases in enhancing the recovery of hydrophilic antioxidant compounds from the seaweeds *Palmaria palmata* and *Undaria pinnatifida*, providing extracts with the greatest scavenging activity against DPPH and peroxyl radicals. Besides, Hu et al.[9], observed that large hydrolysis times with a neutral protease enhanced the antioxidant activities of protein extracts from the microalgae *Schizochytrium limacinum.*

Sirtuins are a class of signaling NAD+ dependent proteins that may remove acetyl groups from histones[16]. The first identified sirtuin was the Silent Information Regulator 2 (SIR2/SIRT1), discovered in Saccharomyces cerevisiae, which was associated with the lifespan extension observed under dietary restriction in yeast[17]. Mice and humans express seven sirtuins (SIRT1-7) which possess either mono-ADP ribosyl-transferase or deacetylase activity.

It seems that SIRT1 and SIRT6 have a synergistic association in the spatiotemporal regulation of the DNA damage response and DNA repair mechanisms[18]. Several studies have shown that sirtuins can modulate cell response to oxidative or genotoxic stresses and play an important role in regulating sphingolipid metabolism[19,20], as well as protecting against oxidative stress-mediated pathological processes such as ischemia-reperfusion, cardiac damage, arterial wall remodeling, inflammation, vascular aging, atherosclerosis, and brain senescence[21,22]. In addition, sirtuins are important for neural connectivity and synaptic plasticity; during aging, a deficit of SIRT1 activity has been directly associated with defined neurophysiological and neuropathological mechanisms of cognitive decline and metabolic dysfunction, and it has been stated that SIRT1 overexpression in certain neurons in the brain increases lifespan[23].

In recent years, there has been a growing interest in the discovery of novel natural or synthetic SIRT1 modulators. Hence, SIRT1 activators (Liriopesides B, Schisandrin A) and inhibitors (3,5-dicaffeoylquinic acid, esculetin) have been previously identified in traditional medicine products[24].

In streptozotocin-induced diabetic rats treated with horsetail extracts (Equisetum arvense L.), Hegedus et al.,[25] discovered increased SIRT1 levels in cardiomyocytes

as well as anti-diabetic and cardioprotective effects. Huang et al.,[26] identified Ginsenoside Rc, an active ingredient of Panax ginseng, as a potent SIRT1 activator that promotes energy metabolism to improve cardio- and neuroprotective functions. However, no information has been found regarding seaweeds or microalgae protein hydrolysates extracts with sirtuin activation or inhibition capabilities.

Due to these multiple effects on metabolic functions, the identification of suitable sirtuin activating compounds (STACs) is a research field of great interest. Several classes of plant and seaweed polyphenols may increase SIRT1 activity[27,28]. Resveratrol (3, 5, 4′-trihydroxystilbene), a polyphenol found in grapes and red wines, is one of the most effective SIRT1 activators[29]. Taurine (2-aminoethanesulfonic acid, Tau), Homotaurine (Htau), and the amino acid gamma aminobutyric acid (GABA), have been recently proposed as natural SIRT1 activators with possible therapeutic benefits for metabolic, age-related, and neurological disorders[30-32]. Besides, phlorotannin-rich natural extract from the brown seaweed *Ascophyllun nodosum* and polyphenol extract from *Ecklonia cava* have been studied for their ability to increase SIRT1 activity[33,34]. Other studies, on the other hand, have shown that inhibiting or decreasing SIRT1 can inhibit cancer cell proliferation[35,36]. Furthermore, some studies have found that peptides and protein fractions can modulate sirtuin activities, which can slow the aging process[37-39].

Tau, a β-amino acid found in very high concentration in most cells, has been pointed out as a promising new therapeutic agent in the treatment of several diseases affecting the muscles, the central nervous and the cardiovascular systems as well as with potent antitumor activities to control, e.g., hepatocellular carcinoma[40–42]. In addition, Tau was also demonstrated to improve the antioxidant defence networks by scavenging free radicals, maintaining the integrity of the electron-transport chain of mitochondria, inhibiting the activities of ROS-producing enzymes, etc.[43]. Htau, an

amine sulfonate compound and an analog of GABA naturally found in several algae species, has been previously described as a potent neuroprotector and as a possible therapeutic agent for Alzheimer's disease, Parkinson's disease, and Mild cognitive impairment[44]. Finally, GABA is a potent inhibitory neurotransmitter in the central nervous system in animals and a powerful protector against stress in plants and microorganisms. In mammals, interesting health implications have been described such as epilepsy, depression and cancer, and in neural diseases such as schizophrenia, Parkinson's disease, Alzheimer's disease, and Huntington´s disease[45].

The aim of this work was to assess and compare the antioxidant and SIRT1 modulation activities of enzymatic protein hydrolysates from 36 samples of different algae species, being for most of these algal species the first time that their protein hydrolysates have been assessed for these activities. Additionally, possible correlations between Taurine, homotaurine and GABA levels in the EPHs, with their antioxidant and / or SIRT1 modulation capabilities have been evaluated.

Results and Discussion

Composition of Algae protein hydrolysates.

Protein content. The protein content in algae samples and in their corresponding EPHs is summarized in Table 1. The green algae group, especially the microalgae *Aphanizomenon flos-aquae*, *Spirulina platensis*, *Chlorella vulgaris*, and *Auxenochlorella pyrenoidosa*, showed the highest total protein concentration in both dried samples and in their protein hydrolysates (EPHs). These results agree with previous studies [44], which indicate microalgae represent a good source of high-quality protein for human food consumption.

The recovery of the protein fraction in algae hydrolysates was comprised of between 31.4 % and 68.8 %, with no significant differences between algae groups. These results are in line with Hu et al. [9], who obtained enzymatic protein hydrolysates from microalgae species with protein recoveries ranging between 40 % and 60 %.

Table 1. Protein content (in the algae samples and in their enzymatic protein hydrolysates - EPHs) and protein recovery (mean of n=3 independent determinations ± std. dev.). Different small letters in the same column indicate significant differences between medians of the algae groups ($p < 0.05$).

	Protein (g / 100 g d.w.)		Protein Recovery
	Algae	EPHs	%
Red Algae			
Porphyra sp.	31.0 ± 1.25	15.6 ± 0.41	50.3 ± 0.47
Gigartina pistillata	28.1 ± 2.65	15.7 ± 0.63	55.9 ± 0.65
Chondrus crispus	12.8 ± 1.05	6.19 ± 1.25	48.4 ± 0.98
Mastocarpus stellatus	11.0 ± 1.33	5.10 ± 1.27	46.4 ± 1.08
Palmaria palmata	17.8 ± 1.21	10.4 ± 0.47	58.4 ± 1.65
Gelidium corneum	9.42 ± 0.58	5.41 ± 0.69	57.4 ± 1.33
Plocammium cartilagineum	10.2 ± 0.66	6.41 ± 0.98	62.8 ± 2.22
Centroceras clavulatum	11.1 ± 1.65	7.03 ± 1.65	63.3 ± 2.25
Halopithys incurva	13.5 ± 1.87	7.41 ± 1.74	54.9 ± 2.39
Median	**12.8** [b]	**7.03** [b]	**55.9** [a]
Green Algae			
Aphanizomenon flos-aquae	36.4 ± 1.55	21.2 ± 1.41	58.2 ± 1.65
Caulerpa lentillifera	13.2 ± 1.63	7.74 ± 1.66	58.6 ± 1.54
Codium sp.	10.8 ± 1.74	5.87 ± 1.87	54.3 ± 1.74
Dunaliella salina	18.1 ± 1.04	11.8 ± 2.08	65.0 ± 2.55
Arthrospira platensis	33.0 ± 1.10	22.7 ± 2.56	68.8 ± 2.14
Chlorella vulgaris	50.3 ± 2.02	30.5 ± 2.44	60.6 ± 2.05
Tetraselmis chui	14.2 ± 2.23	9.60 ± 1.65	67.6 ± 1.32
Auxenochlorella pyrenoidosa	36.0 ± 2.85	23.9 ± 2.39	66.4 ± 1.33
Ulva lactuca	26.3 ± 1.95	15.4 ± 2.77	58.5 ± 1.22
Enteromorpha intestinalis	9.28 ± 1.33	6.35 ± 2.41	68.5 ± 2.85
Codium decorticatum	9.35 ± 1.47	5.41 ± 1.65	57.9 ± 1.96
Median	**18.1** [a]	**11.8** [a]	**65.0** [a]
Brown Algae			
Ascophyllum nodosum	8.30 ± 1.54	4.13 ± 1.65	49.7 ± 1.36
Sargassum fusiforme	21.2 ± 2.22	7.83 ± 2.36	36.9 ± 1.52
Eisenia bicyclis	12.0 ± 2.47	8.11 ± 1.41	67.6 ± 3.25
Laminaria ochroleuca	8.41 ± 2.69	4.57 ± 1.44	54.3 ± 1.69
Himanthalia elongata	10.2 ± 1.36	3.20 ± 2.41	31.4 ± 2.66
Undaria pinnatifida	12.4 ± 1.55	7.83 ± 3.33	63.1 ± 2.45
Odontella aurita	14.3 ± 1.87	6.58 ± 3.08	46.0 ± 2.74
Fucus vesiculosus	11.8 ± 2.52	6.95 ± 2.52	58.9 ± 2.52
Bifurcaria bifurcata	10.9 ± 2.05	6.72 ± 3.10	61.6 ± 2.41
Fucus guiryi	13.5 ± 2.07	9.11 ± 2.15	67.5 ± 1.65
Pelvetia canaliculata	7.56 ± 1.35	4.92 ± 3.25	65.1 ± 1.66
Halopteris scoparia	8.65 ± 1.66	5.15 ± 2.05	59.5 ± 2.65
Gongolaria baccata	12.7 ± 1.65	6.45 ± 2.55	50.8 ± 2.85
Cladostephus spongiosus	7.44 ± 2.36	4.78 ± 2.98	64.2 ± 3.05
Ericaria selaginoides	13.2 ± 1.23	8.49 ± 2.12	64.3 ± 1.05
Nannochloropsis sp.	37.0 ± 1.05	23.8 ± 1.52	64.3 ± 2.55
Median	**11.9** [b]	**6.65** [b]	**60.6** [a]

Peptide concentration. EPHs of red and green species showed the highest peptide content (**Table 2**); among the 36 samples, *Aphanizomenon flos-aquae*, *Chlorella vulgaris*, and *Enteromorpha intestinalis* showed peptide concentrations higher than 2.8 mg glutathione equivalent/mL.

Amino acid profiles. The analysis of the amino acid profiles showed that red and green algae EPHs had the highest content in hydrophobic amino acids. Green algae EPHs contained significantly more GABA than the red and brown groups, while EPHs from both green and brown algae showed the highest contents in Tau (Table 2, and Table S1, *Supplementary material*). *Caulerpa lentillifera* and *Codium sp*. samples provided the EPHs with the highest amounts of Tau, Htau, and GABA. Considering the significance and health implications that these molecules have, it is worthwhile to quantify their content in protein hydrolysates. Liaset et al. and Lassoued et al., highlighted the high levels of Tau concentrations in fish protein hydrolysates [45,46], while Evache et al. [47] stated that seaweed protein hydrolysates could have significantly higher levels of Tau compared to other vegetal sources. However, in the scientific literature there is a substantial lack of information about the Tau, Htau and GABA content in enzymatic protein hydrolysates from algae.

Table 2. Peptide, sum of hydrophobic amino acids (SHA), Taurine (Tau), Homotaurine (Htau), and Gamma-aminobutyric acid (GABA) content in enzymatic

protein hydrolysates (EPH) from the 36 algae species studied (mean of n=3 independent determinations ± std. dev.). Different small letters in the same column indicate significant differences between medians of the algae groups ($p < 0.05$).

	Peptide	SHA	Tau	Htau	GABA
Red Algae	mg Glu eq/mL		g /100 g d.w.		
Porphyra sp.	2.32 ± 0.14	21.5 ± 2.13	0.35 ± 0.02	0.06 ± 0.01	1.25 ± 0.08
Gigartina pistillata	2.05 ± 0.13	20.7 ± 3.65	0.38 ± 0.02	0.05 ± 0.00	1.14 ± 0.01
Chondrus crispus	2.11 ± 0.09	21.8 ± 1.52	0.25 ± 0.01	0.04 ± 0.00	1.05 ± 0.11
Mastocarpus stellatus	1.98 ± 0.21	20.0 ± 2.54	0.14 ± 0.01	0.04 ± 0.01	0.88 ± 2.74
Palmaria palmata	1.98 ± 0.21	28.4 ± 2.74	0.06 ± 0.01	0.02 ± 0.01	0.35 ± 0.02
Gelidium corneum	2.58 ± 0.19	48.2 ± 2.96	0.03 ± 0.01	0.01 ± 0.01	0.39 ± 0.01
Plocammium Cartilagineum	2.64 ± 0.11	33.0 ± 3.05	0.04 ± 0.01	0.02 ± 0.01	0.29 ± 0.06
Centroceras clavulatum	2.14 ± 0.10	44.9 ± 1.74	0.02 ± 0.04	0.00 ± 0.01	0.19 ± 0.05
Halopithys incurva	2.81 ± 0.04	47.0 ± 2.65	0.04 ± 0.01	0.02 ± 0.01	0.37 ± 0.03
Median	**2.14** [a]	**28.4** [a]	**0.06**[b]	**0.02**[a]	**0.39**[b]
Green Algae					
Aphanizomenon flos-aquae	2.83 ± 0.14	26.7 ± 2.52	0.24 ± 0.01	0.04 ± 0.00	1.05 ± 0.08
Caulerpa lentillifera	2.28 ± 0.10	28.7 ± 2.74	0.39 ± 0.02	0.06 ± 0.00	1.28 ± 0.09
Codium sp.	1.84 ± 0.05	9.90 ± 2.04	0.38 ± 0.01	0.06 ± 0.00	1.27 ± 0.05
Dunaliella salina	2.13 ± 0.06	21.5 ± 3.12	0.35 ± 0.00	0.04 ± 0.00	0.91± 0 .03
Arthrospira platensis	2.60 ± 0.04	26.6 ± 3.63	0.35 ± 0.03	0.05 ± 0.00	0.83 ± 0.05
Chlorella vulgaris	2.87 ± 0.15	26.5 ± 3.55	0.31 ± 0.00	0.04 ± 0.00	0.94 ± 1.05
Tetraselmis chui	1.62 ± 0.17	13.1 ± 2.55	0.25 ± 0.05	0.05 ± 0.00	0.63 ± 0.20
Auxenochlorella pyrenoidosa	2.43 ± 0.09	26.8 ± 2.74	0.27 ± 0.00	0.04 ± 0.00	0.72 ± 0.92
Ulva lactuca	2.38 ± 0.17	14.9 ± 1.85	0.03 ± 0.00	0.02 ± 0.00	0.37 ± 0.03
Enteromorpha intestinalis	2.81 ± 0.13	45.5 ± 1.96	0.26 ± 0.01	0.05 ± 0.01	0.82 ± 0.01
Codium decorticatum	1.67 ± 0.15	16.9 ± 1.47	0.00 ± 0.00	0.01 ± 0.00	0.18 ± 001
Median	**2.38** [a]	**21.5** [a]	**0.27**[a]	**0.04**[a]	**0.88**[a]
Brown Algae					
Ascophyllum nodosum	1.51 ± 0.04	4.70 ± 0.05	0.19 ± 0.00	0.05 ± 0.00	0.68 ± 0.02
Sargassum fusiforme	1.70 ± 0.14	5.00 ± 0.55	0.25 ± 0.00	0.04 ± 0.00	0.72 ± 0.01
Eisenia bicyclis	1.86 ± 0.16	6.60 ± 0.41	0.23 ± 0.00	0.03 ± 0.00	0.74 ± 0.00
Laminaria ochroleuca	2.19 ± 0.18	27.2 ± 3.52	0.25 ± 0.01	0.05 ± 0.00	0.85 ± 0.01
Himanthalia elongata	1.69 ± 0.25	9.00 ± 1.55	0.23 ± 0.01	0.05 ± 0.00	0.65 ± 0.06
Undaria pinnatifida	1.73 ± 0.16	14.4 ± 1.47	0.19 ± 0.05	0.04 ± 0.00	0.64 ± 0.08
Odontella aurita	1.80 ± 0.08	27.2 ± 1.98	0.19 ± 0.01	0.04 ± 0.00	1.12 ± 0.13
Fucus vesiculosus	1.83 ± 0.27	18.6 ± 1.85	0.21 ± 0.01	0.03 ± 0.00	0.51 ± 0.03
Bifurcaria bifurcata	2.12 ± 0.20	31.3 ± 3.74	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Fucus guiryi	1.62 ± 0.05	4.60 ± 3.14	0.12 ± 0.00	0.01 ± 0.00	0.27 ± 0.02
Pelvetia canaliculata	1.72 ± 0.14	5.50 ± 3.85	0.07 ± 0.00	0.02 ± 0.00	0.09 ± 0.01
Halopteris scoparia	1.82 ± 0.10	4.60 ± 2.52	0.04 ± 0.00	0.01 ± 0.00	0.25 ± 0.12
Gongolaria baccata	1.88 ± 0.16	4.61 ± 2.15	0.06 ± 0.00	0.02 ± 0.00	0.11 ± 0.10
Cladostephus spongiosus	2.01 ± 0.18	9.81 ± 2.19	0.05 ± 0.00	0.01 ± 0.00	0.11 ± 0.01
Ericaria selaginoides	1.55 ± 0.21	5.42 ± 1.14	0.06 ± 0.01	0.02 ± 0.00	0.39 ± 0.01
Nannochloropsis sp.	2.22 ± 0.27	27.1 ± 1.11	0.15 ± 0.06	0.03 ± 0.00	0.49 ± 0.05
Median	**1.83** [b]	**5.50** [b]	**0.17**[a]	**0.03**[a]	**0.50**[b]

Table S1. Amino acid profiles of 36 algal protein hydrolysates. Data are expressed as g / 100 g protein. Each value is the mean of n = 3 independent determinations ± standard deviation.
* EAA= essential amino acid; NEAA= non-essential amino acids

Amino acids	*Porphyra sp.*	*Gigartina pistillata*	*Chondrus crispus*	*Enteromorpha intestinalis*	*Mastocarpus stellatus*	*Palmaria palmata*	*Gelidium corneum*	*Plocamium cartilagineum*	*Centroceras clavulatum*	*Halopithys incurva*
His	1.05 ± 0.15	0.41 ± 0.03	0.87 ± 0.18	1.64 ± 0.65	1.38 ± 0.34	1.46 ± 0.21	0.48 ± 0.05	1.85 ± 0.08	1.58 ± 0.06	1.33 ± 0.06
Thr	4.35 ± 0.21	4.70 ± 0.04	1.88 ± 0.02	2.72 ± 0.13	2.23 ± 0.01	4.23 ± 0.65	5.40 ± 0.06	5.23 ± 1.41	4.53 ± 0.71	3.29 ± 0.19
Lys	4.45 ± 0.32	6.23 ± 0.25	11.1 ± 0.87	5.66 ± 0.75	8.29 ± 0.08	10.7 ± 1.36	5.07 ± 1.96	5.08 ± 0.32	5.31 ± 1.94	6.45 ± 1.54
Met	1.02 ± 0.07	0.44 ± 0.10	0.09 ± 0.01	0.56 ± 0.08	0.55 ± 0.07	0.86 ± 0.09	3.51 ± 0.04	2.10 ± 0.12	2.67 ± 0.01	2.89 ± 0.84
Val	4.03 ± 0.21	6.94 ± 2.12	3.22 ± 0.72	9.97 ± 0.34	3.28 ± 0.69	4.50 ± 0.86	13.9 ± 1.30	4.60 ± 0.25	8.14 ± 0.91	7.75 ± 1.73
Ileu	2.98 ± 0.34	1.87 ± 0.17	4.09 ± 0.46	6.09 ± 1.39	2.84 ± 0.36	4.37 ± 0.61	7.20 ± 0.89	4.60 ± 0.23	6.62 ± 0.89	7.09 ± 0.60
Leu	5.23 ± 0.14	3.63 ± 0.58	5.58 ± 0.90	10.7 ± 1.18	4.45 ± 0.41	5.20 ± 0.48	10.6 ± 0.44	8.84 ± 1.70	14.4 ± 2.52	16.6 ± 1.79
Phe	2.02 ± 0.11	2.32 ± 0.89	3.22 ± 0.26	5.52 ± 0.15	3.55 ± 0.52	4.75 ± 1.73	5.20 ± 0.62	5.81 ± 0.95	6.07 ± 1.78	4.75 ± 0.31
∑EAA	25.1	26.5	30.0	43.5	26.6	29.4	49.5	42.0	46.6	47.3
Hyptau	0.03 ± 0.01	nd	nd	nd	0.01 ± 2.12	0.01 ± 2.36	nd	nd	nd	nd
Hyp	0.02 ± 0.00	nd	0.03 ± 0.00	0.05 ± 0.00	0.10 ± 0.03	0.10 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Tau	0.35 ± 0.02	0.38 ± 0.02	0.24 ± 0.01	0.26 ± 0.01	0.14 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.04	0.04 ± 0.01
Htau	0.06 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.004 ± 0.01	0.02 ± 0.01
Arg	4.97 ± 0.12	3.39 ± 0.50	2.54 ± 0.07	2.27 ± 0.07	2.29 ± 0.14	4.94 ± 0.63	6.58 ± 1.20	5.27 ± 0.38	7.00 ± 1.02	6.78 ± 0.72
Ser	13.3 ± 0.24	2.21 ± 0.11	6.88 ± 1.17	3.50 ± 0.69	2.35 ± 1.01	10.7 ± 1.50	4.01 ± 0.36	6.00 ± 1.39	4.16 ± 0.21	4.68 ± 0.16
Gly	6.81 ± 0.34	2.17 ± 0.26	7.97 ± 0.19	4.17 ± 0.62	5.34 ± 0.15	10.6 ± 1.27	4.55 ± 0.72	6.75 ± 0.14	4.16 ± 1.51	4.68 ± 1.25
Asp	5.32 ± 0.18	2.94 ± 0.52	4.23 ± 1.50	6.73 ± 0.41	6.47 ± 1.69	9.81 ± 0.94	8.62 ± 1.08	13.4 ± 0.55	9.80 ± 1.15	8.93 ± 1.03
Glu	10.0 ± 0.33	5.09 ± 1.54	4.85 ± 1.62	12.1 ± 2.02	5.86 ± 0.51	8.01 ± 1.60	11.4 ± 0.26	12.8 ± 0.46	10.0 ± 1.80	8.55 ± 0.61
Cys	1.15 ± 0.04	nd	3.22 ± 1.76	0.29 ± 0.64	1.32 ± 0.51	2.98 ± 0.59	0.03 ± 0.00	1.17 ± 0.38	1.30 ± 0.09	0.97 ± 0.01
Pro	2.72 ± 0.24	5.41 ± 1.39	4.78 ± 0.95	7.81 ± 0.15	3.14 ± 0.05	7.67 ± 1.66	4.89 ± 0.94	4.01 ± 0.33	3.49 ± 0.87	5.11 ± 0.33
Ala	2.31 ± 0.32	7.30 ± 0.16	1.48 ± 0.91	7.52 ± 0.73	5.93 ± 1.15	6.38 ± 0.42	5.41 ± 0.04	7.99 ± 0.61	5.58 ± 0.32	6.27 ± 0.83
GABA	1.25 ± 0.08	1.14 ± 0.01	1.05 ± 0.11	0.88 ± 0.01	0.82 ± 2.74	0.35 ± 0.02	0.39 ± 0.01	0.29 ± 0.06	0.19 ± 0.05	0.37 ± 0.03
Tyr	3.55 ± 0.17	0.04 ± 0.01	0.82 ± 0.02	4.15 ± 0.06	2.19 ± 0.17	1.01± 0.07	2.75 ± 0.22	3.05 ± 0.04	3.49 ± 0.09	2.73 ± 0.90
∑NEAA	51.9	30.1	38.1	56.2	36.0	62.7	48.7	60.9	49.2	49.2
Total Amino acids	77.0	56.7	68.2	99.1	62.6	98.9	100	99.0	95.5	99.4

Table S1. Amino acid profiles of 36 algal protein hydrolysates (continued). Data are expressed as g / 100 g protein. Each value is the mean of n = 3 independent determinations ± standard deviation.

* EAA= essential amino acid; NEAA= non-essential amino acids

Amino acids	*Aphanizomenon flos-aquae*	*Caulerpa lentillifera*	*Codium sp.*	*Dunaliella salina*	*Athrospira platensis*	*Chlorella vulgaris*	*Tetraselmis chui*	*Auxenochlorella pyrenoidosa*	*Nanochloropsis sp.*	*Ulva lactuca*
His	1.18 ± 0.77	1.40 ± 0.27	1.00 ± 0.06	0.47 ± 0.03	1.55 ± 0.19	3.00 ± 0.22	2.07 ± 0.19	2.56 ± 0.18	0.59 ± 0.06	1.13 ± 0.15
Thr	5.08 ± 0.66	1.62 ± 0.06	0.77 ± 0.17	1.59 ± 0.13	1.57 ± 0.13	4.55 ± 0.83	2.43 ± 1.22	5.08 ± 1.15	3.02 ± 0.79	2.94 ± 0.77
Lys	5.85 ± 0.48	0.31 ± 0.02	1.78 ± 0.03	2.62 ± 0.77	5.57 ± 0.98	10.3 ± 1.02	3.48 ± 0.83	7.55 ± 1.06	2.30 ± 0.84	3.47 ± 1.08
Met	1.00 ± 0.11	1.15 ± 0.16	0.68 ± 0.10	0.03 ± 0.00	2.48 ± 0.15	1.26 ± 0.25	0.60 ± 0.15	0.56 ± 0.14	0.13 ± 0.01	1.84 ± 0.08
Val	1.52 ± 0.14	6.33 ± 0.87	2.35 ± 0.45	1.73 ± 0.14	5.24 ± 0.96	2.80 ± 0.18	4.31 ± 1.23	4.30 ± 1.11	4.62 ± 1.09	2.40 ± 1.15
Ileu	5.90 ± 0.01	3.42 ± 0.53	1.16 ± 0.06	7.65 ± 0.82	4.27 ± 0.80	4.44 ± 0.93	1.86 ± 1.20	3.13 ± 0.09	11.9 ± 1.21	2.16 ± 0.99
Leu	7.28 ± 1.65	5.79 ± 0.72	1.92 ± 0.55	6.34 ± 0.84	7.36 ± 1.00	6.94 ± 0.82	3.51 ± 0.73	5.08 ± 1.02	3.86 ± 0.75	4.02 ± 1.17
Phe	5.60 ± 0.79	3.30 ± 0.87	1.35 ± 0.33	1.45 ± 0.80	2.21 ± 0.18	5.38 ± 0.18	0.01 ± 0.00	4.22 ± 0.81	0.32 ± 0.04	2.46 ± 0.04
∑EAA	33.4	23.3	11.0	21.9	30.3	38.8	18.3	32.5	26.8	20.4
Hyptau	nd	nd	nd	nd	nd	nd	nd	0.01 ± 0.01	nd	nd
Hyp	0.02 ± 0.00	0.08 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	1.07 ± 0.13	0.36 ± 0.01	0.04 ± 0.00	0.37 ± 0.05	0.02 ± 0.01	0.82 ± 0.01
Tau	0.24 ± 0.01	0.39 ± 0.02	0.38 ± 0.01	0.35 ± 0.00	0.35 ± 0.03	0.31 ± 0.00	0.25 ± 0.05	0.27 ± 0.00	0.15 ± 0.06	0.03 ± 0.00
Htau	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Arg	5.35 ± 0.42	2.15 ± 0.21	1.03 ± 0.12	0.37 ± 0.03	11.6 ± 0.76	10.3 ± 1.04	1.75 ± 0.06	6.16 ± 0.98	1.50 ± 0.92	2.65 ± 0.99
Ser	4.72 ± 0.35	3.52 ± 0.92	1.04 ± 0.03	1.62 ± 0.19	5.81 ± 1.06	5.47 ± 1.05	3.20 ± 0.99	3.41 ± 0.07	2.75 ± 0.18	4.55 ± 1.20
Gly	5.35 ± 0.34	3.87 ± 0.42	1.78 ± 0.16	3.28 ± 0.04	7.45 ± 1.06	8.97 ± 1.17	2.67 ± 1.18	8.97 ± 0.82	7.62 ± 0.79	1.75 ± 0.03
Asp	7.47 ± 1.76	13.0 ± 1.65	1.35 ± 0.18	4.21 ± 0.73	13.1 ± 0.99	12.1 ± 0.77	5.48 ± 1.25	10.7 ± 1.01	8.43 ± 1.13	2.97 ± 0.94
Glu	11.3 ± 0.45	11.6 ± 0.67	4.35± 0.39	5.78 ± 1.04	19.9 ± 0.99	14.5 ± 1.09	6.05 ± 1.09	12.5 ± 0.85	11.5 ± 0.91	2.35 ± 0.88
Cys	0.02 ± 0.00	0.10 ± 0.06	0.17 ± 0.07	1.10 ±0.01	1.75 ± 0.11	1.75 ± 0.24	0.31 ± 0.02	0.29 ± 0.04	0.01 ± 0.00	1.26 ± 0.12
Pro	5.00 ± 1.64	5.71 ± 1.16	1.39± 0 .35	3.78 ± 0.74	3.18 ± 0.93	3.61 ± 1 .14	1.58 ± 0.31	5.36 ± 1.09	4.91 ± 0.79	1.87 ± 0.07
Ala	6.95 ± 0.34	8.03 ± 0.01	1.68 ± 0.47	7.03 ± 0.55	10.7 ± 0.82	9.85 ± 1.04	5.37 ± 0.87	10.0 ± 0.73	9.59 ± 0.75	2.18 ± 0.15
GABA	1.05 ± 0.08	1.28 ± 0.09	1.27 ± 0.05	0.91± 0 .03	0.83 ± 0.05	0.94 ± 0.05	0.63 ± 0.20	0.72 ± 0.12	0.49 ± 0.05	0.37 ± 0.03
Tyr	0.42 ± 0.03	2.97 ± 0.20	1.00 ± 0.12	0.48 ± 0.16	1.88 ± 0.12	2.09 ± 0.06	1.23 ± 0.11	2.86 ± 0.05	1.40 ± 0.19	1.40 ± 0.22
∑NEAA	47.9	52.8	15.5	28.9	77.7	70.4	28.6	61.7	48.4	22.2
Total Amino acids	81.4	76.1	26.5	50.9	108	109	46.9	94.3	75.2	42.6

Table S1. Amino acid profiles of 36 algal protein hydrolysates (continued). Data are expressed as g / 100 g protein. Each value is the mean of n = 3 independent determinations ± standard deviation.

* EAA= essential amino acid; NEAA= non-essential amino acids

Amino acids

	Codium	*Ascophyllu*	*Sargassum*	*Eisenia*	*Laminaria*	*Himanthali*	*Undaria*	*Odonella*	*Fucus*	*Bifurcaria*
His	0.55 ± 0.15	0.53 ± 0.13	0.21 ± 0.08	0.50 ± 0.01	3.12 ± 0.48	5.66 ± 0.69	0.90 ± 0.01	0.74 ± 0.11	1.83 ± 0.50	1.91 ± 0.20
Thr	0.91 ± 0.03	1.08 ± 0.11	0.70 ± 0.05	0.86 ± 0.14	1.61 ± 0.22	2.29 ± 0.36	9.04 ± 0.57	2.73 ± 0.21	3.84 ± 0.63	5.36 ± 0.35
Lys	1.67 ± 0.21	2.06 ± 0.86	0.86± 0.01	0.58 ± 0.08	3.10 ± 0.44	5.05 ± 0.54	7.48 ± 0.66	12.1 ± 0.32	9.53 ± 0.40	5.60 ± 0.21
Met	1.21 ± 0.22	0.06 ± 0.01	0.69 ± 0.09	1.22 ± 0.06	0.12 ± 0.02	0.35 ± 0.06	0.16 ± 0.02	0.16 ± 0.06	0.14 ± 0.01	0.30 ± 0.02
Val	3.92 ± 1.20	1.29 ± 0.12	0.52 ± 0.02	1.01 ± 0.15	5.97 ± 0.33	3.53 ± 0.21	3.07 ± 0.52	2.20 ± 0.37	3.86 ± 0.59	5.38 ± 0.23
Ileu	1.88 ± 0.19	0.85 ± 0.02	0.38 ± 0.12	0.80 ± 0.13	3.65 ± 0.69	0.09 ± 0.01	5.07 ± 0.60	8.74 ± 0.25	1.36 ± 0.24	4.34 ± 0.63
Leu	2.47 ± 0.24	1.09 ± 0.06	1.83 ± 0.21	1.84 ± 0.08	6.58 ± 0.23	2.30 ± 0.52	0.88 ± 0.02	3.38 ± 0.70	3.41 ± 0.22	7.31 ± 0.30
Phe	1.88 ± 0.24	0.48 ± 0.18	0.77 ± 018	0.77 ± 0.06	0.78 ± 0.06	0.04 ± 0.01	3.51 ± 0.68	2.86 ± 0.44	1.22 ± 0.34	5.10 ± 0.27
∑EAA	14.5	7.44	5.96	7.58	24.9	19.3	30.1	32.9	25.2	35.3
Hyptau	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Hyp	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.39 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.10 ± 0.01	0.14 ± 0.00
Tau	0.009 ± 0.000	0.19 ± 0.00	0.25 ± 0.00	0.23 ± 0.00	0.25 ± 0.01	0.22 ± 0.01	0.19 ± 0.05	0.19 ± 0.01	0.21 ± 0.01	0.001 ± 0.000
Htau	0.007 ± 0.00	0.045 ± 0.00	0.035 ± 0.00	0.033 ± 0.00	0.045 ± 0.00	0.045 ± 0.00	0.038 ± 0.00	0.038 ± 0.00	0.031 ± 0.00	0.008 ± 0.00
Arg	1.35 ± 0.02	0.28 ± 0.01	1.25 ± 0.08	0.98 ± 0.01	1.78 ± 0.01	0.88 ± 0.11	6.93 ± 0.13	0.04 ± 0.00	3.00 ± 0.22	5.10 ± 0.42
Ser	1.62 ± 0.08	0.88 ± 0.07	0.61 ± 0.03	0.95 ± 0.08	7.45 ± 0.68	5.66 ± 0.40	9.11 ± 0.48	1.46 ± 0.20	4.04 ± 0.23	4.91 ± 0.71
Gly	1.93 ± 0.23	0.60 ± 0.05	0.84 ± 0.09	1.22 ± 0.16	11.1 ± 0.63	1.36 ± 012	5.37 ± 0.60	11.4 ± 0.60	9.53 ± 0.53	5.38 ± 0.45
Asp	1.44 ± 0.18	1.55 ± 0.15	2.35 ± 0.11	2.22 ± 0.31	3.26 ± 0.21	11.2 ± 0.51	7.56 ± 0.28	2.20 ± 0.19	11.7 ± 0.36	17.6 ± 0.51
Glu	4.00 ± 1.1	2.55 ± 0.84	3.49 ± 1.20	4.64 ± 1.20	3.38 ± 0.71	6.87 ± 0.38	8.37 ± 0.28	6.75 ± 0.33	11.3 ± 0.47	23.3 ± 0.48
Cys	0.21 ± 0.06	0.03 ± 0.00	0.48 ± 0.03	1.50 ± 0.18	3.36 ± 0.29	2.12 ± 0.05	0.16 ± 0.06	0.01 ± 0.00	0.05 ± 0.01	0.02 ± 0.00
Pro	3.15 ± 0.51	0.63 ± 0.05	0.60 ± 0.01	0.60 ± 0.01	9.48 ± 0.64	1.21 ± 0.21	1.64 ± 0.25	8.08 ± 0.55	6.74 ± 0.10	4.91 ± 0.21
Ala	1.62 ± 0.08	0.75 ± 0.11	1.54 ± 0.17	1.16 ± 0.22	9.48 ± 0.71	10.4 ± 0157	2.47 ± 0.33	0.65 ± 0.09	9.53 ± 0.71	12.3 ± 0.41
GABA	0.18 ± 001	0.68 ± 0.02	0.72 ± 0.01	0.74 ± 0.00	0.85 ± 0.01	0.65 ± 0.06	0.64 ± 0.08	1.12 ± 0.13	0.51 ± 0.03	0.008 ± 0.000
Tyr	2.39 ± 0.03	0.25 ± 0.09	0.16 ± 0.02	0.34 ± 0.08	0.64 ± 0.09	1.43 ± 0.12	0.04 ± 0.06	1.7 5 ±0.23	1.73 ± 0.52	2.16 ± 0.31
∑NEAA	24.4	8.45	12.3	14.6	51.1	42.5	42.6	33.8	58.4	75.8
Total Amino acids	38.9	15.8	18.3	22.2	76.0	61.8	72.7	66.8	83.6	111

Table S1. Amino acid profiles of 36 algal protein hydrolysates (continued). Data are expressed as g / 100 g protein. Each value is the mean of n = 3 independent determinations ± standard deviation.
* EAA= essential amino acid; NEAA= non-essential amino acids

Amino acids	*Fucus guiryi*	*Pelvetia canaliculata*	*Halopteris scoparia*	*Gongolaria baccata*	*Cladostephus spongiosus*	*Ericaria selaginoides*
His	0.49 ± 0.04	0.02 ± 0.00	0.28 ± 0.01	0.54 ± 0.06	0.24 ± 0.02	0.54 ± 0.01
Thr	0.99 ± 0.02	0.84 ± 0.02	0.54 ± 0.03	0.39 ± 0.01	0.27 ± 0.06	0.60 ± 0.07
Lys	2.40 ± 0.06	0.84 ± 0.03	0.58 ± 0.07	1.81 ± 0.03	0.76 ± 0.04	2.43 ± 0.15
Met	2.05 ± 0.00	0.03 ± 0.01	0.35 ± 0.04	0.14 ± 0.00	0.09 ± 0.02	0.21 ± 0.01
Val	0.69 ± 0.04	0.94 ± 0.05	0.65 ± 0.06	0.55 ± 0.08	0.74 ± 0.09	0.87 ± 0.13
Ileu	0.26 ± 0.08	0.73 ± 0.03	0.54 ± 0.07	0.45 ± 0.04	0.48 ± 0.02	0.50 ± 0.04
Leu	0.93 ± 0.07	1.22 ± 0.16	1.11 ± 0.14	1.29 ± 0.17	0.79 ± 0.15	1.40 ± 0.16
Phe	0.26 ± 0.12	0.67 ± 0.11	0.22 ± 0.04	0.38 ± 0.05	6.06 ± 0.69	0.87 ± 0.13
∑EAA	8.07	5.29	4.27	5.55	9.40	7.40
Hyptau	nd	0.01 ± 0.00	nd	nd	nd	nd
Hyp	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.04 ± 0.01
Tau	0.12 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.01
Htau	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Arg	0.84 ± 0.15	0.74 ± 0.01	0.55 ± 0.08	0.64 ± 0.01	0.38 ± 0.02	0.60 ± 0.07
Ser	0.99 ± 0.04	0.90 ± 0.03	0.94 ± 0.04	0.39 ± 0.02	0.70 ± 0.12	0.67 ± 0.11
Gly	3.84 ± 0.17	0.96 ± 0.03	2.22 ± 0.18	0.81 ± 0.12	1.76 ± 0.46	1.32 ± 0.20
Asp	3.86 ± 0.58	1.46 ± 0.26	2.85 ± 0.36	3.80 ± 0.25	2.00 ± 0.32	3.48 ± 0.27
Glu	4.24 ± 0.36	3.74 ± 0.54	3.13 ± 0.52	2.60 ± 0.12	2.80 ± 0.40	3.86 ± 0.11
Cys	0.02 ± 0.00	3.63 ± 0.01	1.34 ± 0.32	1.97 ± 0.13	1.81 ± 0.16	0.98 ± 0.00
Pro	1.87 ± 0.41	1.34 ± 0.29	1.60 ± 0.13	1.66 ± 0.30	0.76 ± 0.09	1.27 ± 0.11
Ala	2.78 ± 0.17	2.57 ± 0.29	3.49 ± 0.18	3.90 ± 0.35	1.49 ± 0.10	1.25 ± 0.12
GABA	0.27 ± 0.02	0.09 ± 0.01	0.25 ± 0.12	0.11 ± 0.10	0.11 ± 0.01	0.39 ± 0.01
Tyr	0.55 ± 0.02	0.29 ± 0.10	0.32 ± 0.01	0.15 ± 0.02	0.49 ± 0.02	0.32 ± 0.09
∑NEAA	19.4	15.8	16.7	16.1	12.1	14.3
Total Amino acids	27.5	21.1	21.0	22.7	21.6	21.7

Antioxidant activities. TEAC and DPPH assays. Strong antioxidant (TEAC) and antiradical (DPPH) activities (both higher than 70%) were observed in EPHs from 10 algae species (*Porphyra sp*., *Gigartina pistillata*, *Plocammium cartilagineum*, *Caulerpa lentillifera*, *Spirulina plantensis*, *Chlorella vulgaris*, *Tetraselmis chui*, *Ulva lactuca*, *Enteromorpha intestinalis*, and *Nannocloropsis sp*.).

EPHs from green and red species showed significantly higher median antioxidant (TEAC) and free radical scavenging (DPPH) activities than those of the brown species (**Table 3**). The highest TEAC and DPPH activities in the different groups were found in the EPHs of *Porphyra sp*., *Caulerpa lentillifera*, *Enteromorpha intestinalis*, and *Nannochloropsis sp*.

These results are in good agreement with the studies of Je at al. [15] and Norzagaray-Valenzuela et al. [48], who observed high and comparable values of the TEAC and DPPH assays in algal protein hydrolysates from *Nannochloropsis sp*. and *Dunaliella tertiolecta* and relatively low free radical scavenging activities in Alcalase® extracts from *Undaria pinnatifida*. To the best of our knowledge, the antioxidant properties of EPHs from almost all the algae species included in the study (except *Undaria pinnatifida* [15], *Ascophyllum nodosum* [27], *Nannochloropsis sp*. [48], and *Fucus vesiculosus* [49]) have never been evaluated.

Besides, our results are in line with the DPPH/TEAC activities observed in Alcalase® protein hydrolysates from other animal and plant sources, such insects [50], scallop [51], cuttlefish [52], and black beans [53], indicating that EPHs from algae show interesting antioxidative properties with potential applications in the food industry.

Table 3. Antioxidant (TEAC, DPPH) activities in enzymatic protein hydrolysates (EPHs) from the algae samples (mean of n=3 independent determinations ± std. dev.). Different small letters in the same column indicate significant differences between medians of the algae groups ($p < 0.05$).

	TEAC	DPPH
Red Algae	%	%
Porphyra sp.	71.2 ± 1.65	70.4 ± 0.15
Gigartina pistillata	54.1 ± 0.32	51.4 ± 0.36
Chondrus crispus	56.5 ± 0.11	50.2 ± 0.42
Mastocarpus stellatus	54.3 ± 0.12	53.2 ± 0.14
Palmaria palmata	54.6 ± 0.25	56.6 ± 1.65
Gelidium corneum	69.2 ± 0.36	67.4 ± 2.54
Plocammium Cartilagineum	68.8 ± 0.41	67.9 ± 2.14
Centroceras clavulatum	69.4 ± 0.09	68.8 ± 1.54
Halopithys incurva	68.5 ± 0.11	65.7 ± 0.85
Median	**68.5** [a]	**65.7** [a]
Green Algae		
Aphanizomenon flos-aquae	65.4 ± 0.07	63.5 ± 2.10
Caulerpa lentillifera	72.1 ± 0.05	70.0 ± 2.52
Codium sp.	52.3 ± 0.15	55.8 ± 2.77
Dunaliella salina	60.2 ± 0.23	58.8 ± 1.41
Athrospira platensis	70.2 ± 1.25	68.6 ± 3.65
Chlorella vulgaris	70.5 ± 2.36	68.8 ± 0.54
Tetraselmis chui	48.9 ± 1.87	50.1 ± 1.44
Auxenoclorella pyrenoidosa	65.5 ± 2.47	63.4 ± 2.58
Ulva lactuca	51.1 ± 2.87	52.3 ± 0.88
Enteromorpha intestinalis	71.2 ± 0.08	73.6 ± 0.65
Codium decorticatum	60.5 ± 3.11	58.9 ± 0.96
Median	**60.5** [a]	**58.9** [a]
Brown Algae		
Ascophyllum nodosum	40.7 ± 1.54	42.6 ± 2.22
Sagassum fusiforme	41.2 ± 3.36	45.2 ± 2.74
Eisenia bicyclis	47.8 ± 2.87	49.3 ± 0.98
Laminaria ochroleuca	59.1 ±2.54	58.6 ± 1.11
Himanthalia elongata	47.7 ± 3.47	45.5 ± 2.41
Undaria pinnatifida	48.8 ± 1.74	50.1 ± 3.55
Odonella aurita	60.7 ± 1.23	58.5 ± 2.22
Fucus vesiculosus	54.3 ± 1.69	52.4 ± 2.64
Bifurcaria bifurcata	61.2 ± 3.44	60.3 ± 2.27
Fucus guiryi	46.4 ± 1.27	44.5 ± 1.55
Pelvetia canaliculata	49.7 ± 2.27	47.7 ± 1.48
Halopteris scoparia	39.5 ± 1.13	37.8 ± 2.28
Gongolaria baccata	45.5 ± 1.54	42.8 ± 3.24
Cladostephus spongiosus	50.2 ± 1.74	47.5 ± 1.05
Ericaria selaginoides	43.5 ± 2.28	41.9 ± 1.49
Nanochloropsis sp.	58.9 ± 2.14	60.1 ± 1.74
Median	**49.7** [b]	**47.5** [b]

SIRT1 activity. **Figure 1** summarizes the results expressed as median fold activation or inhibition for the EPHs of each algae group. Significant differences ($p < 0.05$) were found between the algae groups, indicating that EPHs´ from the red and green algae groups were the most potent SIRT1 activators.

EPHs from *Porphyra sp*., *Caulerpa lentillifera*, *Codium sp*., and *Odontella aurita* were the most effective SIRT1 activating agents with values higher than 80 %. Surprisingly, all the EPH from seaweed collected on the Atlantic coast of northern Spain but only four commercial species showed SIRT1 inhibition (**Table S2**, *Supplementary material*).

These results could be associated with the harvesting, drying, and storage conditions applied to "experimental" and commercial samples, which, even if no specific information was available on the labels of the commercial products, are likely to be quite different.

This hypothesis appears to be supported by studies indicating that different drying methods used on algal biomass have a significant impact on the nutritional and/or functional components of the final product (e.g., protein content and total antioxidant capacity) [54,55].

In the case of samples inducing SIRT1 activation, all the three algae groups induced a median fold activation around 1.50 or higher (**Figure 1**). Dutot et al. [33] reported similar results working with an extract rich in phlorotannins from a commercial sample of *Ascophyllum nodosum*, while Fitton et al. [56] found that a fucoidan-rich extract from *Undaria pinnatifida* increased SIRT1 expression of around 28.8%. SIRT1 fold activation results of around 1.30 have been previously described for natural compounds such as quercetin, ferulic acid, tyrosol, etc. at concentrations between 0.5-2.0 mg/mL [57].

Algal EPHs that inhibited SIRT1 showed a median fold inhibition of around 1.65, corresponding to median % inhibition between 32 and 57 % (**Figure 1** and **Table S2**,

Supplementary material). Selisistat (EX – 527), a synthetic compound identified as the most potent SIRT1 inhibitor to date, can reach extremely high SIRT1 inhibition values up to 83.6 % at 0.0125 mg/mL [58]. Besides, in a recent study [59], two phenolic compounds, rhuschalcone I and rhuschalcone IV, previously isolated from the twigs and root bark of the medicinal plant Rhus pyroides Burch (Anacardiaceae), showed inhibitory effects against SIRT1 at 0.02 mg/ml (rhuschalcone I = 53.5%; rhuschalcone IV = 61.2%). In this study EPHs from algae reached similar effects, even if at higher concentrations (5 mg/mL).

Our results seem to indicate that EPHs from microalgae and seaweed included in this study had a substantial capacity to modulate SIRT1 “in vitro”, especially considering they were not purified fractions.

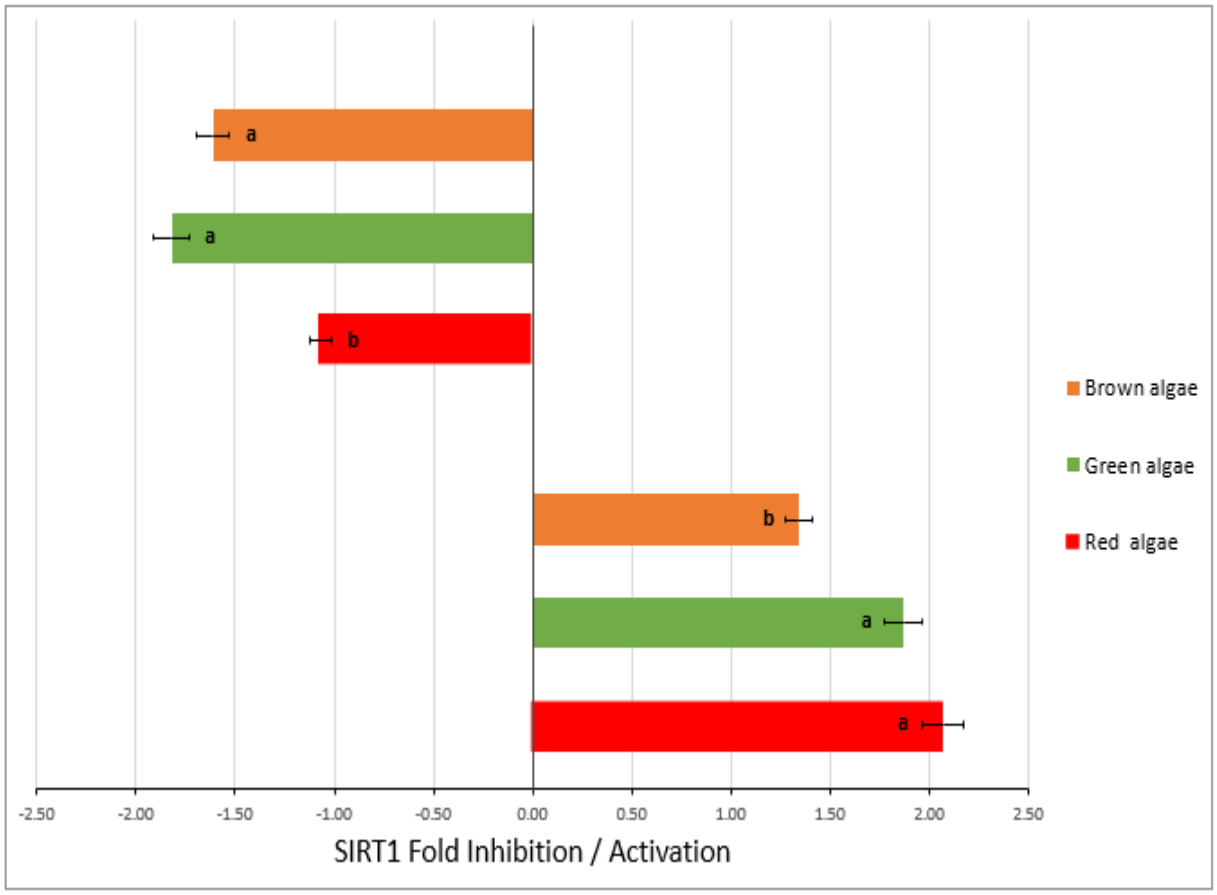


Figure 1. SIRT1 median fold activation / inhibition induced by EPHs from brown, red, and green algae. SIRT1 enzyme initial activity was used as a control. Different small letters indicate significant differences between algae groups ($p < 0.05$) with EPHs providing Fold inhibition. Different capital letters indicate significant differences between algae groups ($p < 0.05$) with EPHs providing Fold activation.

Table S2. SIRT1 activation / inhibition percentages of the enzymatic protein hydrolysates from algae species. (Mean of n=3 independent determinations ± std. dev.).

	Alage specie	% Inhibition	%Activation
Red Algae	*Porphyra sp*		83.3 ± 1.23
	Gigartina pistillata		68.8 ± 0.98
	Chondrus crispus		56.5 ± 2.32
	Mastocarpus stellatus		32.6 ± 2.47
	Palmaria palmata	16.0 ± 0.85	
	Gelidium corneum	46.9 ± 1.49	
	Plocammium Cartilagineum	32.1 ± 2.38	
	Centroceras clavulatum	57.7 ± 2.85	
	Halopithys incurva	23.4 ± 0.65	
	Median	**32.1[b]**	**62.7[a]**
Green Algae	*Afanizomenon flos-aquae*		62.8 ± 2.85
	Caulerpa lentillifera		98.2 ± 3.44
	Codium sp		88.6 ± 3.87
	Dunaliella salina		63.2 ± 2.14
	Spirulina platensis		66.7 ± 1.45
	Chlorella vulgaris		60.9 ± 0.54
	Tetraselmis chui		39.1 ± 0.85
	Auxenochlorella pyrenoidosa		54.5 ± 1.58
	Ulva lactuca	41.4 ± 1.14	
	Enteromorpha intestinalis		60.3 ± 1.45
	Codium decorticatum	73.5 ± 2.85	
	Median	**57.5[a]**	**62.8[a]**
Brown Algae	*Ascophyllum nodosum*		30.5 ± 2.55
	Sargassum fusiforme		33.6 ± 1.74
	Eisenia byciclis		26.7 ± 0.41
	Laminaria ochroleuca		49.2 ± 0.18
	Himanthalia elongata		41.9 ± 2.22
	Undaria pinnatifida		25.6 ± 2.96
	Odonella aurita		105 ± 3.87
	Fucus vesiculosus	2.30 ± 0.11	
	Bifurcaria bifurcata	80.5 ± 1.85	
	Fucus guiryi	46.8 ± 1.37	
	Pelvetia canaliculata	55.8 ± 2.49	
	Stypocaulon scoparium	63.9 ± 2.97	
	Gongolaria baccata	57.7 ± 1.75	
	Cladostephus spongiosum	70.2 ± 1.69	
	Cystoseira tamariscifolia	34.4 ± 0.65	
	Nanochloropsis sp.	4.80 ± 0.17	
	Median	**55.8[a]**	**37.8[b]**

Correlation analysis and Principal Component Analysis

Pearson's Correlation Coefficients (PCCs, **Figure 2**) showed highly significant correlations between peptide content with both TEAC and DPPH activities, in good agreement with previous findings reported in wheat germ and in the microalgae *Dunaliella salina* [60,61]. Additionally, a significant correlation between EPH hydrophobic amino acid content and TEAC and DPPH was observed in algae EPHs, as already described in other biological extracts; it was suggested that hydrophobic amino acids probably act as antioxidants by increasing the solubility of peptides in lipids and, therefore, facilitating their better interaction with free radicals that cause oxidative damage [62].

Peptide and hydrophobic amino acids content showed a PCC of around 0.76, ($p < 0.01$), indicating that peptides in the protein hydrolysates contain a great proportion of these amino acids. Hence, our results are in line with other studies underlying hydrophobic amino acids originated by the hydrolysis of jumbo squid skin gelatin and giant squid muscle, or conger eel (*Conger myriaster*) contributed to the antioxidant activities of the peptide fractions [11-13].

Results of TEAC and DPPH assays were highly correlated, in line with results observed in other studies carried out in methanolic extracts from several fruits, vegetables, and beverages [63].

Significant, positive correlations were also observed between Tau, Htau and GABA concentrations in algae EPHs and SIRT1 modulation activity (**Figure 2**).

This correlation has been previously observed in the study of KP et al. [30], where Tau was established as a positive regulator of SIRT1 activity in hepatic cells "in vitro". Our results also agreed with previous studies in human cultured cells where homotaurine and GABA significantly increased the expression of SIRT1 [31,32].

A principal component analysis (PCA) was performed in order to detect structure in the relationship between the independent analytical variables and EPHs from the different algae samples. According to the Kaiser criterion the first two principal components (PCs) were the only significant ones and accounted for 43.8% and 25.4% of the total variance of the data, respectively. EPHs configuration and analytical attributes loadings (plotted as vectors) are presented for the first two PCs in **figure 3**. The combination of the two PCs discriminates three groups (G1–G3) of EPHs. G1 gathers EPHs with the highest content of hydrophobic amino acids, peptides and antioxidant activities. The EPHs grouped within G2 were characterized by high content of Tau, Htau, GABA, and SIRT1 activation capacity. On the other hand, EPHs grouped in G3 showed inhibition of the SIRT1 activity, the lowest antioxidants capacities and low contents of Tau, Htau, GABA and hydrophobic amino acids.

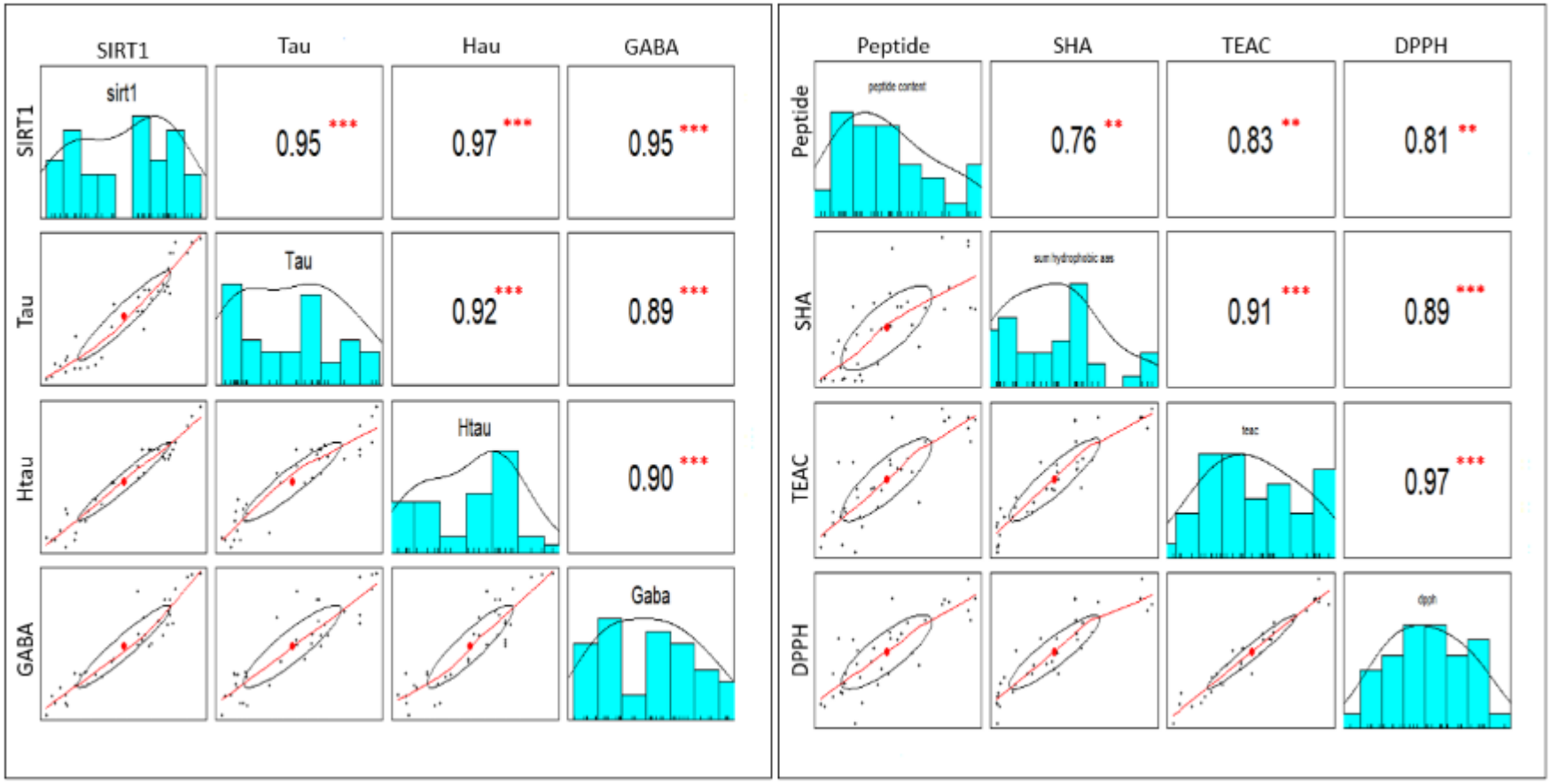


Figure 2. Pairwise scatter plot matrix, distribution, and Pearson correlation coefficients (R) for comparisons among specific amino acids, peptide content, antioxidant and SIRT1 activities in enzymatic protein hydrolysates from 36 algae species. On the bottom of the diagonal, the bivariate scatter plots with a fitted line, and on the top of the diagonal, the value of the correlation (R) plus the significance level as asterisks (**p<0.01, ***p<0.001). Peptide= Peptide concentration; SHA= Sum of hydrophobic amino acids; DPPH and ABTS= antioxidant activities; SIRT1= EPH modulators of Sirtuin 1; GABA= Gamma amino butyric acid; Tau: taurine; Htau: Homotaurine.

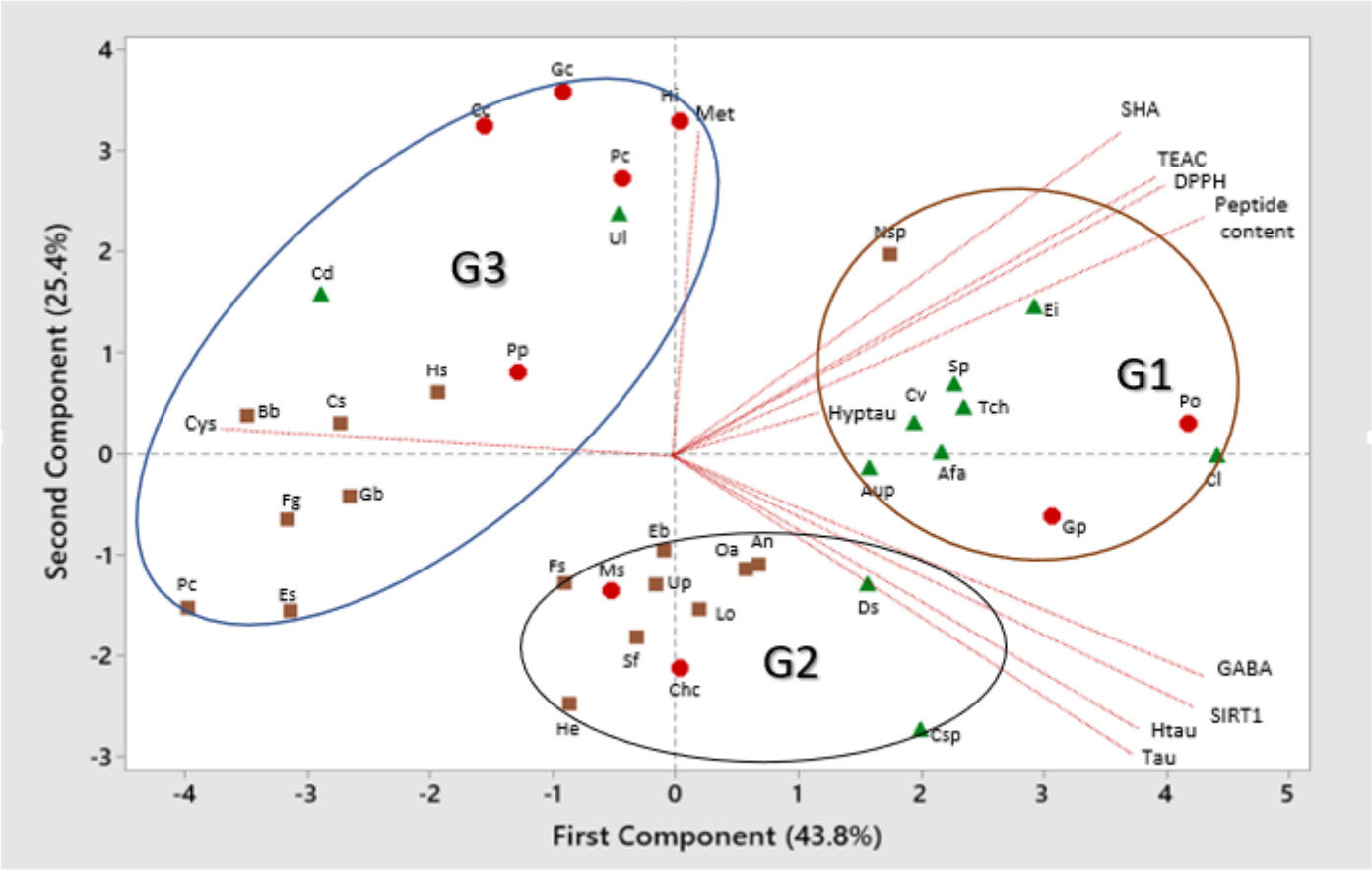


Figure 3. Principal Components Analysis of EPHs from 36 algae samples. Met= Methionine; SHaas= Sum of hydrophobic amino acids; DPPH and ABTS= antioxidant activities; SIRT1= Sirtuin 1 inhibition/activation activity; GABA= Gamma amino butyric acid; Tau: taurine; Htau: Homotaurine; Hyptau: Hypotauine; Cys: Cysteine. Circles, squares, and triangles represent red, brown, and green algae species, respectively. Abbreviations of algae species as listed in Table 1.

Conclusions

Food ingredients and extracts from algae are an interesting field of research due to their richness in different classes of bioactive compounds. However, there is a substantial lack of knowledge regarding the capacity of algae EPHs to modulate sirtuins activities, which has been pointed out for their influence on cell aging and metabolism.

To the best of our knowledge, it is the first time that EPHs antioxidant and SIRT1 modulation activities have been evaluated in most of the algae species included in this study.

Our results indicated that EPHs from a broad range of algae species provided significant antioxidant and SIRT1 modulation activities. SIRT1 activation was significantly correlated to the content of Tau, Htau and GABA in EPHs, while antioxidant activities (TEAC and DPPH) were significantly correlated to the total hydrophobic amino acids in algal EPH.

The main conclusion of this study is that enzymatic hydrolysates from water-soluble proteins of different algae species are a potential source of ingredients with interesting functional properties that could find suitable applications in both the food and pharmaceutical industries.

Hence, further studies are needed in order to purify and further characterize target compounds in EPHs responsible for the observed antioxidant and SIRT1 modulation activities, investigate their effects "in vivo" as well as to clarify the influence of algae biomass storage and processing on the EPHs composition and their functional activities.

Materials and Methods

Chemicals and reagents

All the chemicals used in the experiments were of analytical grade. Acetonitrile (ACN) and methanol (MeOH) were HPLC gradient-grade (Merck KGaA (Darmstadt, Germany). Per-chloric (60%) and hydrochloric acid (37%) were from J.T. Baker (NJ, United States). Sigma-Aldrich Chemie (Sant Quentin Fallavier, France) provided Alcalase 2.4 L FG (CAS no. 9014-01-1), ortho-phthaldialdehyde reagent (OPA), l-glutathione, borate buffer, formic acid (for LC-MS LiChropur™), ammonium acetate, ammonium formate, phenyl isothiocyanate (PITC), triethylamine (TEA), pure standards for 19 amino acids, taurine, hypotaurine and homotaurine, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), α-diphenyl-β-picrylhydrazyl (DPPH), potassium persulfate, Folin-Ciocalteu's reagent, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO). The SIRT1 Direct Fluorescent Activity Assay Kit was from ABNOVA (Cat # KA1366, Taiwan).

Algae material

The currently accepted scientific names of algae species used in this study are given according to Algaebase [64] and following the rules of the International Code of Nomenclature for algae, fungi, and plants (ICN) [65].

To make easier the discussion of the results, the species were divided based on their size and morphology into two typical algal groups, microalgae and macroalgae, which were classified as follows: green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Ochrophyta). The cyanobacteria were also included in the green algae group as commonly considered for commercial purposes (**Table 4**). Dried samples of twenty-four commercially available algae species (**Table 4**) were purchased in local stores. Three packs, from different production batches, were acquired for each specie.

The product of each pack was homogenized with a ball mill (Retsch GmbH & Co., KG, Germany), and the powdered samples were stored in sealed plastic vacuum bags at ambient temperature under dry and dark conditions and analyzed within one month. Additionally, fresh samples of twelve macroalgal species were collected during the 23rd–25th August 2017 (summer season) on the Atlantic coast of northern Spain (Comillas - Cantabria, 43° 23 N and 4° 17 W) (**Table 5**). Sample collections were done at the same rocky shore site in the intertidal and subtidal (1 m depth) zones during low water. The samples of adult fresh macroalgae were selected and carefully washed with fresh seawater to remove sand or remaining debris, and any epiphytes or animals attached to the algal surface. They were then wrapped in sterile cloths moistened with seawater and kept dark and cool with ice packs (<15 ºC) to preserve the algae alive and healthy until transport to the laboratory. Then they were dried in a cabinet laboratory dryer with air circulation at a constant temperature of 40 °C for 72 hours approximately (until a constant weight was achieved). The dried samples were milled as previously described, and the powdered samples were stored in sealed plastic vacuum bags at ambient temperature under dry and dark conditions and analyzed within one month.

Table 4. Product information retrieved from the label in commercial microalgae and macroalgae (or seaweeds). The algae species included in the novel food catalogue, the union list of authorized novel foods, and official member states' lists of food and food supplements in Europe are highlighted in bold.

Abbreviation	Name	Currently accepted scientific names	Classification	Sample type	Origin
Po	Nori	*Porphyra* sensu lato	Rhodophyta (Macroalgae)	Dried	Spain
Gp	Fresh Gigartina	*Gigartina pistillata*	Rhodophyta (Macroalgae)	Fresh	Spain
Chc	Irish moss	*Chondrus crispus*	Rhodophyta (Macroalgae)	Dried	Spain
Ei	Green Aonori (*Enteromorpha*	***Ulva intestinalis***	Chlorophyta (Macroalgae)	Fresh	Japan
Ms	Irish Moss	*Mastocarpus stellatus*	Rhodophyta (Macroalgae)	Dried	Spain
Pp	Dulse	***Palmaria palmata***	Rhodophyta (Macroalgae)	Dried	Spain
Afa	Klamath eco	***Aphanizomenon flos-aquae***	Cyanobacteria (Microalgae)	Dried	Spain
Cl	Green Caviar	*Caulerpa lentillifera*	Chlorophyta (Macroalgae)	Dried	Unknown
Csp	Barnacle seaweed	*Codium* sp	Chlorophyta (Macroalgae)	Dried	Spain
Ds	Dunaliella	***Dunaliella salina***	Chlorophyta (Microalgae)	Dried	Spain
Sp	Spìrulina (*Spirulina platensis*)	***Arthrospira platensis***	Cyanobacteria (Microalgae)	Dried	Spain
Cv	Chlorella	***Chlorella vulgaris***	Chlorophyta (Microalgae)	Dried	Spain
Tch	Holofit TetraSod capsules	***Tetraselmis chui***	Chlorophyta (Microalgae)	Dried	Spain
Aup	Holofit Chlorella (*Chlorella*	***Auxenochlorella pyrenoidosa***	Chlorophyta (Microalgae)	Dried	Spain
Nsp	Nannochloropsis	***Nannochloropsis sp.***	Ochrophyta (Microalgae)	Dried	Spain
Ul	Sea Lettuce	***Ulva lactuca***	Chlorophyta (Macroalgae)	Dried	Spain
An	*Ascophyllum nodosum*	***Ascophyllum nodosum***	Ochrophyta, *Phaeophyceae*	Dried	Spain
Sf	Iziki seaweed (*Hizikia fusiformis*)	***Sargassum fusiforme***	Ochrophyta, *Phaeophyceae* (Macroalgae)	Dried	Spain
Eb	Arame	***Eisenia bicyclis***	Ochrophyta, *Phaeophyceae*	Dried	Unknown
Lo	Kombu bio	***Laminaria ochroleuca***	Ochrophyta, *Phaeophyceae*	Dried	Spain
He	Sea Spaguetti bio	***Himanthalia elongata***	Ochrophyta, *Phaeophyceae*	Dried	Spain
Up	Wakame	***Undaria pinnatifida***	Ochrophyta, *Phaeophyceae*	Dried	Spain
Oa	Odontella capsules	***Odontella aurita***	Bacillariophyta (Microalga)	Dried	France
Fv	Fucus capsules	***Fucus vesiculosus***	Ochrophyta, *Phaeophyceae*	Dried	Spain

Table 5. Sampling location of macroalgae collected from wild populations on Spain's north-western coast

Abbreviation	Currently accepted scientific names	Class	Location
Gc	*Gelidium corneum*	*Rhodophyceae*	Trasvia, Comillas (Cantabria)
Pc	*Plocammium cartilagineum*	*Rhodophyceae*	Trasvia, Comillas (Cantabria)
Cc	*Centroceras clavulatum*	*Rhodophyceae*	Comillas' beach (Cantabria)
Hi	*Halopithys incurva*	*Rhodophyceae*	Trasvia, Comillas (Cantabria)
Cd	*Codium decorticatum*	*Chlorophyceae*	Trasvia, Comillas (Cantabria)
Bb	*Bifurcaria bifurcata*	*Phaeophyceae*	Trasvia, Comillas (Cantabria)
Fg	*Fucus guiryi*	*Phaeophyceae*	Comillas' beach (Cantabria)
Pc	*Pelvetia canaliculata*	*Phaeophyceae*	Comillas' beach (Cantabria)
Hs	*Halopteris scoparia*	*Phaeophyceae*	Trasvia, Comillas (Cantabria)
Gb	*Gongolaria baccata*	*Phaeophyceae*	Trasvia, Comillas (Cantabria)
Cs	*Cladostephus spongiosus*	*Phaeophyceae*	Comillas' beach (Cantabria)
Es	*Ericaria selaginoides*	*Phaeophyceae*	Trasvia, Comillas (Cantabria)

Preparation of the enzymatic protein hydrolysates (EPH)

With minor modifications, the procedure used to obtain the EPH fractions was based on a previously described method [66]. In brief, 0.5 g of dried milled algae powder was suspended in 10 mL of milli-Q water, stirred gently with a Reax 2 shaker (Heidolph Instruments GmbH & CO. KG, Schwabach-Germany) for 3 h at 4 °C and then centrifuged at 4000 g for 15 min at 4 °C (mod. 5430R, Eppendorf AG, Hamburg - Germany). Water-soluble proteins were precipitated by adjusting the pH of the supernatant fraction to 3.5 with HCl 0.1 N and keeping the sample at 4 °C for 30 min. After centrifuging the sample at 4000 g for 15 min at 4 °C, the pellets were collected and freeze-dried with a LyoMicron (Cool Vacuum Technologies SL, Barcelona, Spain). Three independent extracts were obtained for each algae specie.

EPH fractions were obtained by enzymatic hydrolysis of the dried pellets by using the enzyme Alcalase® (Merck KGaA, Darmstadt, Germany) following Pérez-Míguez et al. [66]. Pellets (50 mg) were dissolved, with the help of an ultrasonic bath (J.P. Selecta, BCN, Spain), in 10 mL of 5 mM borate buffer, pH = 8.5 (final concentration of the EPH fractions = 5 mg/mL). Then, Alcalase® was added at an enzyme/substrate ratio of 0.15 AU per gram of dried sample. The digestion was performed at 50 °C for 4 h with agitation (700 rpm) in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany). The digestion/reaction was stopped by heating (100 °C for 10 min) and the solution was centrifuged for 10 min at 6000 g (mod. 5430R; Eppendorf AG, Hamburg Germany). Finally, the supernatants (EPH) were collected and stored at -80 ºC until analysis.

Determination of protein content and protein recovery

The protein content of the dried algae samples and enzymatic protein hydrolysates (EPH) was determined following a previously published method [67], with minor modifications. In brief, 5 mg of dried sample or five microliters of EPH were re-suspended by vortexing in 200 L of 24% (w/v) TCA, incubated in a water bath for 15 min at 95 C, and then allowed to cool at room temperature. After the addition of 600 L of MilliQ water and mixing, the samples were centrifuged at 15,000 g for 20 min at 4 C (Microcentrifuge 5415 R, Eppendorf AG, Hamburg, Germany) and their supernatants discarded. The pellets were resuspended in 0.5 mL of Lowry Reagent D and incubated for 10 min at 55 C. Samples were then cooled at room temperature, centrifuged at 15,000 g for 20 min, and the supernatant retained. For protein quantification, a stock of Lowry Reagent D was prepared daily in a 48:1:1 ratio (v/v/v) of Lowry Reagents A, B, and C. A suitable volume (up to 50 µL) of the protein extract was added into a 1.5 mL microfuge tube, together with 950 L of Lowry Reagent D, followed by immediate mixing. After incubation for 10 min at room temperature, 0.1 mL of 0.2 N Folin-Ciocalteu phenol reagent was added to each tube and mixed immediately. After 30 min at room temperature, absorbance was read at 600 nm (1800 UV–Vis spectrophotometer, Shimadzu Co., Madison, WI) using UVProbe™ software (Shimadzu Co., Madison, WI). Calibration curves were prepared for each assay with a bovine serum albumin (BSA) stock solution (200 mg/mL) and using a polynomial line of best fit generated in Microsoft Excel 365. Analysis was performed in triplicate and expressed in g / 100 g d.w.

The recovery of the protein fraction (RP) was determined using the equation [1]:

$$RP = \frac{P_1}{P_2} \times 100 \qquad [1]$$

Where P1 is the protein content in algae protein hydrolysates and P2 is the algae protein in dried algal samples.

Amino acid and peptide quantification in algae hydrolysates

Total amino acids were quantified as previously described [68]. Briefly, 10 mg of EPH fractions were weighted and added with 1 mL of 8 M per chloric acid and maintained 24 h at 110 °C (Precisiterm, J.P. Selecta, BCN, Spain). After cooling at room temperature, the samples were filtered through 0.2 µm membrane syringe filters (GMP filter membranes, Merck KGaA, Darmstadt, Germany), derivatized with a methanol: water: TEA: PITC solution (7:1:1:1, v:v:v:v) and evaporated under nitrogen. Then, 24 µL of mobile phase B and 226 µL of mobile phase A to the derivatized samples centrifuged at 11000 × g for 5 min and filtered through a Single Step Standard Filter Vials (Thomson Instrument Company, CA, USA). Four µL of the sample were injected into the chromatographic system, consisting of an Acquity UPLC® equipped with a PDA detector, an electrospray (ESI) as a source of ionization operated in the positive mode, and a TQD triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The data were acquired with MassLynx v.4.1 software (Waters, Milford, MA, USA). Chromatographic separation was carried out on a BEH-C18, 1.7 µm, 100 mm x 2.1 mm i.d. column (Waters, Milford, MA, USA). Peak identity was confirmed by comparing their retention times, UV spectra, MS and MS/MS spectra with the corresponding data obtained from pure standards.

The total hydrophobic amino acid content was determined as the sum of phenylalanine, leucine, isoleucine, tyrosine, valine, methionine, and proline concentrations as previously described [69].

Peptide content was estimated by using the O-phthaldialdehyde method (OPA) [70] with minor modifications. Every day, a fresh OPA solution was prepared by combining: i) solution A (7.62 g sodium tetrahydroborate and 200 mg sodium dodecyl sulfate dissolved in 150 mL of deionized water), ii) solution B (160 mg of OPA

dissolved in 4 mL of ethanol (96%), and iii) solution C (400 L of -mercaptoethanol, adjusted to a final volume of μL of the EPH fraction was mixed with 270 μL of OPA reagent in a 96-well plate. The mixture was incubated at room temperature for 2 min, and then the absorbance was measured at 340 nm using a microplate reader system (Varioskan, Thermo Scientific, MA, USA). The analysis was carried out in triplicate for each of the 3 extracts from each algae specie (n=9). The peptide content, expressed as mg of glutathione (GSH) per mL of EPH, was calculated by creating a calibration curve with glutathione solutions in the range of 0.1–5.0 mg/mL.

Trolox equivalent antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) assay was carried out according to Li et al. [71]. In the TEAC assay, antioxidant action by hydrogen atom transfer (HAT) as well as single electron transfer (SET) is measured. For the assay, an ABTS●+ radical cation was generated by preparing a solution of 7 mM ABTS and 2.45 mM potassium persulfate in milliQ-water. The reaction mixture was kept in the dark for 16 hours at room temperature and used within two days. The ABTS●+ solution was diluted with deionized water to give an absorbance of 0.700 ± 0.050 at 734 nm. 50 μL of EPH fraction was mixed with 1.9 mL of diluted ABTS●+ solution. After incubating for 10 minutes at room temperature, the absorbance was measured with a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan) at 734 nm. The antioxidant capacity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the equation [5]:

$$\%TEAC = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} x100 \qquad [5]$$

Where $Abs_{control}$ and Abs_{sample} are the absorbances of the $ABTS^{\bullet+}$ and the tested samples, respectively.

DPPH Free Radical Scavenging Activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was determined according to the method of Zhang et al. [72], with slight modifications. Briefly, 100 µL of EPH fraction were mixed with 100 µL of 0.16 mM DPPH methanolic solution. This mixture was vortexed for 1 min, kept for 30 min in the dark, and then the absorbance was read at 517 nm in an automated microplate reader (Sunrise-Elisa Reader, Tecan, Swiss). The radical scavenging activity was calculated using the equation [6]:

$$\%DPPH = \frac{[(Abs_{control}) - (Abs_{sample} - Abs_{blank})]}{Abs_{control}} x100 \qquad [6]$$

Where the $Abs_{control}$ is the absorbance of the control (DPPH without sample), the Abs_{sample} is the absorbance of the sample (sample plus DPPH solution), and the Abs_{blank} is the absorbance of the sample blank (Sample without the DPPH solution).

SIRT1 direct fluorescence assay

SIRT1 activity modulation was assessed by using a Direct Fluorescent Screening Assay Kit (SIRT1 Direct Fluorescent Screening Assay Kit, Cat # KA1366, ABNOVA, Taiwan) following the instructions of the provider (**Figures S1** and **S2**, *Supplementary material*). The formation of the final fluorescent product was detected using a Varioskan (Varioskan, Thermo Scientific, MA, USA) with an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

For each EPH fraction, the % inhibition/activation was calculated following the equation [7], and the fold activation was calculated following the instructions of the provider by using the equation [8]:

$$\%\frac{Inhibition}{Activation} = \frac{Initial\ activity\ fluorescence_{control} - Sample\ fluorescence}{Initial\ activity\ fluorescence_{control}} x100$$

[7]

$$Fold\ Activation = \frac{Sample\ fluorescence}{Initial\ Activity\ fluorescence_{control}}$$

[8]

where:

“Initial Activity fluorescence control” is the fluorescence obtained in wells with SIRT1 dissolved in assay buffer and solvent;

“Sample fluorescence” is the fluorescence from wells with SIRT1 dissolved in buffer assay and solvent plus samples.

To perform the Pearson´s correlation test and the principal component analysis, negative values of SIRT1 activity were scaled and normalized into a range of 0 and 1, according to Teknomo, K. [73].

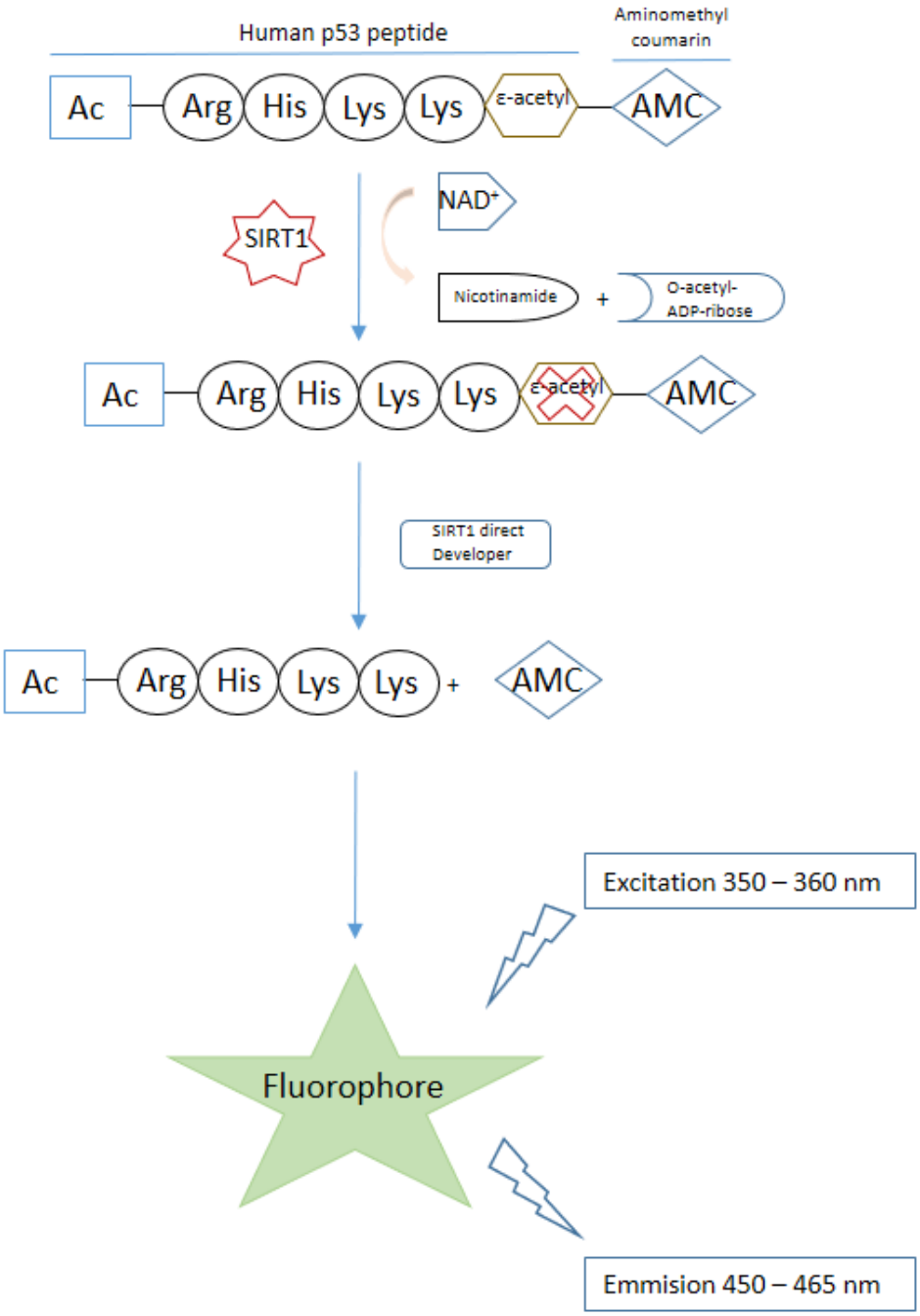


Figure S1. Principle of the SIRT1 Fluorescence screening assay used in this study.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Background Wells	Background Wells	Background Wells	Sample x	Sample x	Sample x						
B	100% Initial Activity Wells	100% Initial Activity Wells	100% Initial Activity Wells									
C	Sample 1	Sample 1	Sample 1									

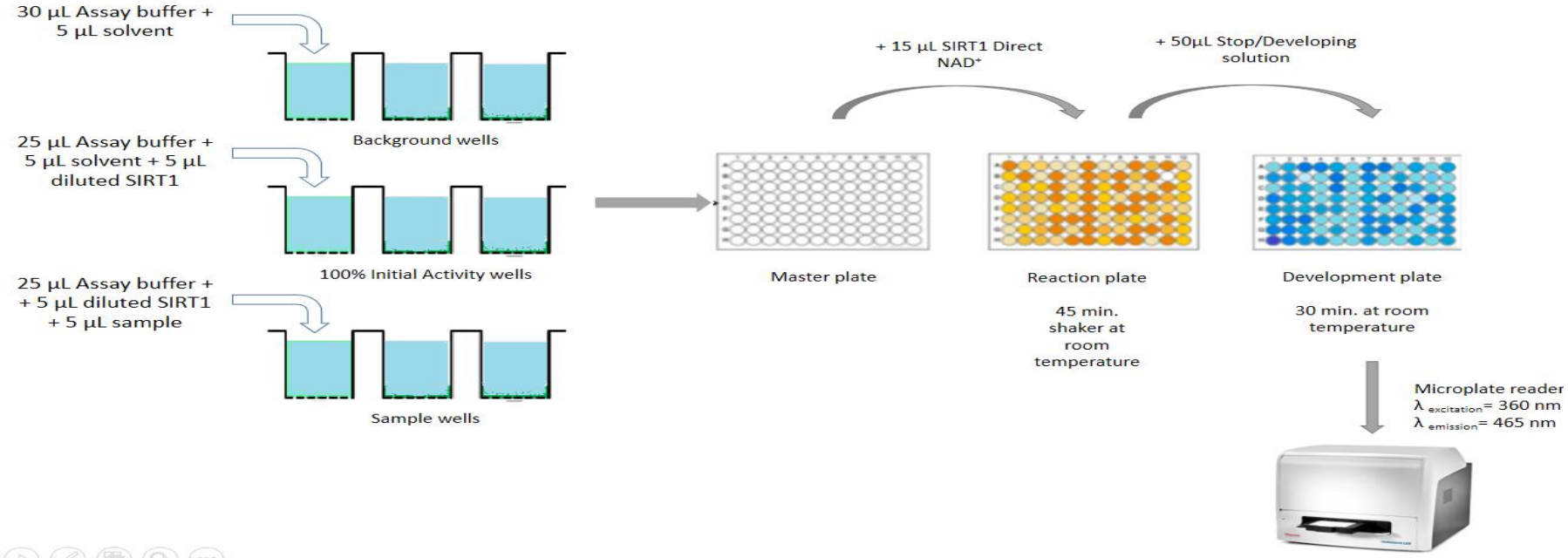


Figure S2. Scheme of the Assay protocol of ABNOVA SIRT1 Fluorescence kit.

Statistical analysis

All results were expressed as the mean ± standard deviation.

A Kruskal-Wallis non-parametric test and Mann-Whitney pairwise comparisons of median values were carried out to evidence significant differences between groups ($p < 0.05$). Principal component analysis (PCA) and Pearson's correlation coefficients (PCC) were used. Statistical analyses were performed with the software Minitab® version 19.2, 2019 (Minitab Inc., State College, PA, USA), and RStudio version 2022.02.3, Build 492 (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA).

References

1. Wang, X., Yu, H., Xing, R. & Li, P. Characterization, Preparation, and Purification of Marine Bioactive Peptides. *Biomed Res. Int.* **2017**, (2017). https://doi.org/10.1155/2017/9746720.

2. Lafarga, T., Acién-Fernández, F. G. & Garcia-Vaquero, M. Bioactive peptides and carbohydrates from seaweed for food applications: Natural occurrence, isolation, purification, and identification. *Algal Res.* **48**, (2020). https://doi.org/10.1016/j.algal.2020.101909.

3. Verdugo-González, L., Acosta-Cárdenas, A., Zapata-Zapata, A. D., Cadena-Chamorro, E. M. & Pulido-Muñoz, S. A. Antimicrobial potential of a hydrolyzed protein extract of the microalgae nannochloropsis sp.•. *DYNA* **86**, 192–198 (2019). https://doi.org/10.15446/dyna.v86n211.78865.

4. El-Baz, F. K., El-Senousy, W. M., El-Sayed, A. B. & Kamel, M. M. In vitro antiviral and antimicrobial activities of Spirulina platensis extract. *J. Appl. Pharm. Sci.* **3**, 52–56 (2013). https://doi.org/10.7324/JAPS.2013.31209.

5. Sadeghi, S., Jalili, H., Ranaei Siadat, S. O. & Sedighi, M. Anticancer and antibacterial properties in peptide fractions from hydrolyzed spirulina protein. *J. Agric. Sci. Technol.* **20**, 673–683 (2018). http://jast.modares.ac.ir/article-23-19830-en.html.

6. Sheih, I. C., Wu, T. K. & Fang, T. J. Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. *Bioresour. Technol.* **100**, 3419–3425 (2009). https://doi.org/10.1016/j.biortech.2009.02.014.

7. Heo, S. J., Park, E. J., Lee, K. W. & Jeon, Y. J. Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresour. Technol.* **96**, 1613–1623 (2005). https://doi.org/10.1016/j.biortech.2004.07.013.

8. Wang, T. *et al.* Enzyme-enhanced extraction of antioxidant ingredients from red algae Palmaria palmata. *LWT - Food Sci. Technol.* **43**, 1387–1393 (2010). https://doi.org/10.1016/j.lwt.2010.05.010.

9. Hu, X. *et al.* Antioxidant Properties of Microalgae Protein Hydrolysates Prepared by Neutral Protease Digestion. *Appl. Mech. Mater.* **707**, 149–153 (2014). https://doi.org/10.4028/www.scientific.net/AMM.707.149.

10. Morris, H. J. *et al.* Immunostimulant activity of an enzymatic protein hydrolysate from green microalga Chlorella vulgaris on undernourished mice. *Enzyme Microb. Technol.* **40**, 456–460 (2007). https://doi.org/10.1016/j.enzmictec.2006.07.021.

11. Mendis, E., Rajapakse, N., Byun, H. G. & Kim, S. K. Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their in vitro antioxidant effects. *Life Sci.* **77**, 2166–2178 (2005). https://doi.org/10.1016/j.lfs.2005.03.016

12. Rajapakse, N., Mendis, E., Byun, H. G. & Kim, S. K. Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J. Nutr. Biochem.* **16**, 562–569 (2005). https://doi.org/10.1016/j.jnutbio.2005.02.005.

13. Ranathunga, S., Rajapakse, N. & Kim, S. K. Purification and characterization of antioxidative peptide derived from muscle of conger eel (Conger myriaster). *Eur. Food Res. Technol.* **222**, 310–315 (2006). https://doi.org/10.1007/s00217-005-0079-x.

14. Wattanasiritham, L., Theerakulkait, C., Wickramasekara, S., Maier, C. S. & Stevens, J. F. Isolation and identification of antioxidant peptides from enzymatically hydrolyzed rice bran protein. *Food Chem.* **192**, 156–162 (2016). https://doi.org/10.1016/j.foodchem.2015.06.057.

15. Je, J. Y. *et al.* Antioxidant activity of enzymatic extracts from the brown seaweed Undaria pinnatifida by electron spin resonance spectroscopy. *LWT - Food Sci. Technol.* **42**, 874–878 (2009). https://doi.org/10.1016/j.lwt.2008.10.012.

16. Imai, S. ichiro & Guarente, L. NAD+ and sirtuins in aging and disease. *Trends Cell Biol.* **24**, 464–471 (2014). https://doi.org/10.1016/j.tcb.2014.04.002.

17. Lin, S. J., Defossez, P. A. & Guarente, L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in saccharomyces cerevisiae. *Science (80-).* **289**, 2126–2128 (2000). https://doi.org/10.1126/science.289.5487.2126.

18. Meng, F. *et al.* Synergy between SIRT1 and SIRT6 helps recognize DNA breaks and potentiates the DNA damage response and repair in humans and mice. *Elife* **9**, 1–22 (2020). https://doi.org/10.7554/eLife.55828.

19. Fan, W. *et al.* SIRT1 regulates sphingolipid metabolism and neural differentiation of mouse embryonic stem cells through c-Myc- SMPDL3B. *Elife* **10**, 1–33 (2021). https://doi.org/10.7554/eLife.67452.

20. Young, S. A., Mina, J. G., Denny, P. W. & Smith, T. K. Sphingolipid and ceramide homeostasis: Potential therapeutic targets. *Biochem. Res. Int.* **2012**, (2012). https://doi.org/10.1155/2012/248135.
21. D'Onofrio, N., Servillo, L. & Balestrieri, M. L. SIRT1 and SIRT6 Signaling Pathways in Cardiovascular Disease Protection. *Antioxidants Redox Signal.* **28**, 711–732 (2018). https://doi.org/10.1089/ars.2017.7178.
22. Herskovits, A. Z. & Guarente, L. SIRT1 in Neurodevelopment and Brain Senescence. *Neuron* **81**, 471–483 (2014). https://doi.org/10.1016/j.neuron.2014.01.028.
23. Ng, F., Wijaya, L. & Tang, B. L. SIRT1 in the brain—connections with aging-associated disorders and lifespan. *Front. Cell. Neurosci.* **9**, 1–13 (2015). https://doi.org/10.3389/fncel.2015.00064.
24. Wang, Y., Liang, X., Chen, Y. & Zhao, X. Evaluación SIRT1 Activators from Medicinal Plants as Bioactive Compounds against Oxidative Damage in Mitochondrial Function. *Oxid. Med. Cell. Longev.* **2016**, 1–9 (2016). https://doi.org/10.1155/2016/4206392.
25. Hegedus, C. *et al.* SIRT1 activation by *Equisetum arvense* L. (Horsetail) modulates insulin sensitivity in streptozotocin induced diabetic rats. *Molecules* **25**, 1–19 (2020). https://doi.org/10.3390/molecules25112541.
26. Huang, Q. *et al.* A SIRT1 Activator, Ginsenoside Rc, Promotes Energy Metabolism in Cardiomyocytes and Neurons. *J. Am. Chem. Soc.* **143**, 1416–1427 (2021). https://doi.org/10.1021/jacs.0c10836.
27. Salekeen, R. *et al.* In-silico Evaluación of bioactive phytopeptides for novel anti-Ageing therapeutics. *J. Biomol. Struct. Dyn.* **0**, 1–13 (2020). https://doi.org/10.1080/07391102.2020.1859411.
28. Dai, H., Sinclair, D. A., Ellis, J. L. & Steegborn, C. Sirtuin activators and inhibitors: Promises, achievements, and challenges. *Pharmacol. Ther.* **188**, 140–154 (2018). https://doi.org/10.1016/j.pharmthera.2018.03.004.
29. Stacchiotti, A., Favero, G. & Rezzani, R. Resveratrol and SIRT1 Activators for the Treatment of Aging and Age-Related Diseases. *Resveratrol - Adding Life to Years, Not Adding Years to Life* (2019) doi:10.5772/intechopen.78977. https://doi.org/10.5772/intechopen.78977.
30. KP, A. D., Shimoga Janakirama, A. R. & Martin, A. SIRT1 activation by Taurine: in vitro evaluation, molecular docking and molecular dynamics simulation studies. *J. Nutr. Biochem.* **102**, 108948 (2022). https://doi.org/10.1016/j.jnutbio.2022.108948.
31. Lakshmi, S. *et al.* Marine derived bioactive compounds for treatment of Alzheimer's disease. *Front. Biosci. - Elit.* **10**, 537–548 (2018). https://doi.org/10.2741/E840.
32. Prud'Homme, G. J. *et al.* GABA protects pancreatic beta cells against apoptosis by increasing SIRT1 expression and activity. *Biochem. Biophys.*

Res. Commun. **452**, 649–654 (2014). https://doi.org/10.1016/j.bbrc.2014.08.135.

33. Dutot, M., Fagon, R., Hemon, M. & Rat, P. Antioxidant, anti-inflammatory, and anti-senescence activities of a phlorotannin-rich natural extract from Brown Seaweed Ascophyllum nodosum. *Appl. Biochem. Biotechnol.* **167**, 2234–2240 (2012). https://doi.org/10.1007/s12010-012-9761-1.
34. Eo, H., Jeon, Y. J., Lee, M. & Lim, Y. Brown alga Ecklonia cava polyphenol extract ameliorates hepatic lipogenesis, oxidative stress, and inflammation by activation of AMPK and SIRT1 in high-fat diet-induced obese mice. *J. Agric. Food Chem.* **63**, 349–359 (2015). https://doi.org/10.1021/jf502830b.
35. Hu, J., Jing, H. & Lin, H. Sirtuin inhibitors as anticancer agents. *Future Med. Chem.* **6**, 945–966 (2014). https://doi.org/10.4155/fmc.14.44.
36. Kozako, T. *et al.* Novel small-molecule SIRT1 inhibitors induce cell death in adult T-cell leukaemia cells. *Sci. Rep.* **5**, 1–14 (2015). https://doi.org/10.1038/srep11345.
37. Bhullar, K. S. & Wu, J. Dietary peptides in aging: Evidence and prospects. *Food Sci. Hum. Wellness* **9**, 1–7 (2020). https://doi.org/10.1016/j.fshw.2020.01.001.
38. Ramis, M. R., Esteban, S., Miralles, A., Tan, D. X. & Reiter, R. J. Caloric restriction, resveratrol and melatonin: Role of SIRT1 and implications for aging and related-diseases. *Mech. Ageing Dev.* **146**–**148**, 28–41 (2015). https://doi.org/10.1016/j.mad.2015.03.008.
39. Yuan, X. Y. *et al.* Replacing fish meal with cottonseed meal protein hydrolysate affects amino acid metabolism via AMPK/SIRT1 and TOR signaling pathway of *Megalobrama amblycephala*. *Aquaculture* **510**, 225–233 (2019). https://doi.org/10.1016/j.aquaculture.2019.05.056.
40. Hatab, H. M. *et al.* A combined treatment of curcumin, piperine, and taurine alters the circulating levels of IL-10 and miR-21 in hepatocellular carcinoma patients: A pilot study. *J. Gastrointest. Oncol.* **10**, 766–776 (2019). doi: 10.21037/jgo.2019.03.07.
41. Schaffer, S. & Kim, H. W. Effects and mechanisms of taurine as a therapeutic agent. *Biomol. Ther.* **26**, 225–241 (2018). https://doi.org/10.4062/biomolther.2017.251.
42. Gossai, D. & Lau-Cam, C. A. The effects of taurine, taurine homologs and hypotaurine on cell and membrane antioxidative system alterations caused by type 2 diabetes in rat erythrocytes. *Adv. Exp. Med. Biol.* 643, 359–368 (2009). https://doi.org/10.1007/978-0-387-75681-3_37.
43. Surai, P. F., Earle-payne, K. & Kidd, M. T. Taurine as a natural antioxidant: From direct antioxidant effects to protective action in various toxicological models. *Antioxidants* vol. 10 (2021). https://doi.org/10.3390/antiox10121876.

44. Manzano, S., Agüera, L., Aguilar, M. & Olazarán, J. A review on tramiprosate (homotaurine) in Alzheimer's disease and other neurocognitive disorders. Front. Neurol. 11, 1–10 (2020).
45. Diez-Gutiérrez, L., San Vicente, L., Luis, L. J., del Carmen Villarán, M. & Chávarri, M. Gamma-aminobutyric acid and probiotics: Multiple health benefts and their future in the global functional food and nutraceuticals market. J. Funct. Foods 64, 103669 (2020).
46. Wang, Y., Tibbetts, S. & McGinn, P. Microalgae as Sources of High-Quality Protein for Human Food and Protein Supplements. *Foods* **10**, 3002 (2021). https://doi.org/10.3390/foods10123002.
47. Liaset, B. & Espe, M. Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish-raw materials. *Process Biochem.* **43**, 42–48 (2008). https://doi.org/10.1016/j.procbio.2007.10.007.
48. Lassoued, I. *et al.* Characterization, antioxidative and ACE inhibitory properties of hydrolysates obtained from thornback ray (Raja clavata) muscle. *J. Proteomics* **128**, 458–468 (2015). https://doi.org/10.1016/j.jprot.2015.05.007.
49. Echave, J. *et al.* Seaweed protein hydrolysates and bioactive peptides: Extraction, purification, and applications. *Mar. Drugs* **19**, 1–22 (2021). https://doi.org/10.3390/md19090500.
50. Norzagaray-Valenzuela, C. D. *et al.* Residual biomasses and protein hydrolysates of three green microalgae species exhibit antioxidant and anti-aging activity. *J. Appl. Phycol.* **29**, 189–198 (2017). https://doi.org/10.1007/s10811-016-0938-9.
51. Catarino, M. D., Silva, A. M. S. & Cardoso, S. M. Phycochemical constituents and biological activities of Fucus spp. *Mar. Drugs* **16**, (2018). https://doi.org/10.3390/md16080249.
52. Firmansyah, M. & Abduh, M. Y. Production of protein hydrolysate containing antioxidant activity from *Hermetia illucens*. *Heliyon* **5**, e02005 (2019). https://doi.org/10.1016/j.heliyon.2019.e02005.
53. Wang, Z. *et al.* Antioxidant activity and functional properties of Alcalase-hydrolyzed scallop protein hydrolysate and its role in the inhibition of cytotoxicity in vitro. *Food Chem.* **344**, 128566 (2021). https://doi.org/10.1016/j.foodchem.2020.128566.
54. Balti, R. *et al.* Comparative Study on Biochemical Properties and Antioxidative Activity of Cuttlefish (*Sepia officinalis*) Protein Hydrolysates Produced by Alcalase and Bacillus licheniformis NH1 Proteases. *J. Amino Acids* **2011**, 1–11 (2011). https://doi.org/10.4061/2011/107179.
55. do Evangelho, J. A. *et al.* Antioxidant activity of black bean (Phaseolus vulgaris L.) protein hydrolysates. *Food Sci. Technol.* **36**, 23–27 (2016). https://doi.org/10.1590/1678-457X.0047.

56. Nakagawa, K., Ritcharoen, W., Sri-Uam, P., Pavasant, P. & Adachi, S. Antioxidant properties of convective-air-dried *Spirulina maxima*: Evaluation of phycocyanin retention by a simple mathematical model of air-drying. *Food Bioprod. Process.* **100**, 292–302 (2016). https://doi.org/10.1016/j.fbp.2016.07.014.
57. Zepka, L. Q., Jacob-Lopes, E., Goldbeck, R. & Queiroz, M. I. Production and biochemical profile of the microalgae *Aphanothece microscopica Nägeli* submitted to different drying conditions. *Chem. Eng. Process. Process Intensif.* **47**, 1305–1310 (2008). https://doi.org/10.1016/j.cep.2007.04.013.
58. Fitton, J. H. *et al.* Topical benefits of two fucoidan-rich extracts from marine macroalgae. *Cosmetics* **2**, 66–81 (2015).
59. Fusi, J. *et al.* An in vitro comparative study of the antioxidant activity and SIRT1 modulation of natural compounds. *Biomed. Pharmacother.* **101**, 805–819 (2018). https://doi.org/10.1016/j.biopha.2018.03.006.
60. Mellini, P. *et al.* Carprofen Analogues as Sirtuin Inhibitors: Enzyme and Cellular Studies. *ChemMedChem* **7**, 1905–1908 (2012). https://doi.org/10.1002/cmdc.201200318.
61. Karaman, B. *et al.* Identification of bichalcones as sirtuin inhibitors by virtual Evaluación and in vitro testing. *Molecules* **23**, 1–10 (2018). https://doi.org/10.3390/molecules23020416.
62. Liu, F., Chen, Z., Shao, J., Wang, C. & Zhan, C. Effect of fermentation on the peptide content, phenolics and antioxidant activity of defatted wheat germ. *Food Biosci.* **20**, 141–148 (2017). https://doi.org/10.1016/j.fbio.2017.10.002.
63. Xia, E. *et al.* Optimization and identification of antioxidant peptide from underutilized *Dunaliella salina* protein: Extraction, in vitro gastrointestinal digestion, and fractionation. *Biomed Res. Int.* **2019**, (2019). https://doi.org/10.1155/2019/6424651.
64. Intiquilla, A., Jiménez-Aliaga, K., Zavaleta, A. I. & Hernández-Ledesma, B. Production of antioxidant hydrolyzates from a *Lupinus mutabilis* (Tarwi) protein concentrate with alcalase: Optimization by response surface methodology. *Nat. Prod. Commun.* **13**, 751–756 (2018). https://doi.org/10.1177/1934578x1801300626.
65. Floegel, A., Kim, D. O., Chung, S. J., Koo, S. I. & Chun, O. K. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *J. Food Compos. Anal.* **24**, 1043–1048 (2011).
66. Guiry, M.D. & Guiry, G.M. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. https://www.algaebase.org; searched on 19 de abril de 2022. (2022).
67. Turland, N. J., Wiersema, J. H., Barrie, F. R., Greuter, W., Hawksworth, D. L., Herendeen, P. S., Knapp, S., Kusber, W.-H., Li, D.-Z., Marhold, K., May, T. W., McNeill, J., Monro, A. M., Prado, J., Price, M. J. & Smith, G. F. (eds.):

International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017. Regnum Vegetabile 159. Glashütten: Koeltz Botanical Books. DOI https://doi.org/10.12705/Code.2018. (2018).

68. Pérez-Míguez, R., Plaza, M., Castro-Puyana, M. & Marina, M. L. Separation and identification of peptides in hydrolysed protein extracts from edible macroalgae by HPLC-ESI-QTOF/MS. *Algal Res.* **39**, 101465 (2019). https://doi.org/10.1016/j.algal.2019.101465.
69. Slocombe, S. P., Ross, M., Thomas, N., McNeill, S. & Stanley, M. S. A rapid and general method for measurement of protein in micro-algal biomass. *Bioresour. Technol.* **129**, 51–57 (2013). https://doi.org/10.1016/j.biortech.2012.10.163.
70. Terriente-Palacios, C., Diaz, I. & Castellari, M. A validated ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry method to simultaneously quantify taurine, homotaurine, hypotaurine and amino acids in macro- and mic. *J. Chromatogr. A* **1589**, (2019). https://doi.org/10.1016/j.chroma.2018.12.058.
71. Widyarani, Sari, Y. W., Ratnaningsih, E., Sanders, J. P. M. & Bruins, M. E. Production of hydrophobic amino acids from biobased resources: wheat gluten and rubber seed proteins. *Appl. Microbiol. Biotechnol.* **100**, 7909–7920 (2016). https://doi.org/10.1007/s00253-016-7441-8.
72. Ghanbari, R. *et al.* Angiotensin-I converting enzyme (ACE) inhibitory and anti-oxidant activities of sea cucumber (*Actinopyga lecanora*) hydrolysates. *Int. J. Mol. Sci.* **16**, 28870–28885 (2015). https://doi.org/10.3390/ijms161226140.
73. Li, H. Bin *et al.* Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chem.* **102**, 771–776 (2007). https://doi.org/10.1016/j.foodchem.2006.06.022.
74. Zhang, W. W., Duan, X. J., Huang, H. L., Zhang, Y. & Wang, B. G. Evaluation of 28 marine algae from the Qingdao coast for antioxidative capacity and determination of antioxidant efficiency and total phenolic content of fractions and subfractions derived from *Symphyocladia latiuscula* (Rhodomelaceae). *J. Appl. Phycol.* **19**, 97–108 (2007). https://doi.org/10.1007/s10811-006-9115-x.
75. 2015 (Teknomo, Kardi. Similarity Measurement. 2015. [Online]. (http://people.revoledu.com/kardi/tutorial/Similarity/).

Acknowledgements

This work was supported by ProFuture project (2019-2023 "*Microalgae protein-rich ingredients for the food and feed of the future*"- EU H2020 GA nº 862980) and CERCA Programme (Generalitat de Catalunya).

Author Contributions

CTP: Conceptualization, Writing – original draft, Data curation, Formal Analysis, Methodology. SRC & MH: Resources, Supervision. CP: Resources, Supervision. MC: Conceptualization, Writing – review & editing, Supervision.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-25130-4.

Correspondence and requests for materials should be addressed to M.C.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

CONCLUSIONES

1. Se ha llevado a cabo la **optimización** de un nuevo método analítico para la cuantificación de 19 aminoácidos y de tres derivados del ácido sulfónico (Taurina, Hipotaurina y Homotaurina) en muestras de algas mediante la instrumentación UPLC-PDA-ESI-Triple Quad MS/MS. Para llevar a cabo esta optimización, se han estudiado diferentes parámetros de la separación cromatográfica, como el uso de diferentes columnas cromatográficas (en relación a la naturaleza química de su empaquetamiento), diferente composición de las fases móviles y su pH, flujos de caudal, programas de elución en gradiente, y temperatura de columna. Los resultados fueron satisfactorios, en términos de resolución en la separación cromatográfica de los 22 analitos objeto de estudio. Asimismo, se llevaron a cabo experimentos de optimización en espectrometría de masas, para la confirmación de cada uno de los analitos. El resultado final fue un método analítico novedoso, dado que es la primera vez que se ha desarrollado un método para el análisis simultáneo de 19 aminoácidos y de los tres derivados del ácido sulfónico (Taurina, Hipotaurina y Homotaurina), con un tiempo total de análisis de 15 minutos.
   Asimismo, se procedió a la **validación** del método analítico previamente optimizado, incluyendo los parámetros expuestos en normas internacionales de calidad como AOAC, IUPAC, etc., con resultados que cumplen con las normas de calidad antes mencionadas.

2. El método optimizado y validado ha sido utilizado para llevar a cabo una evaluación del contenido de 19 aminoácidos y de tres derivados del ácido sulfónico (Taurina, Hipotaurina y Homotaurina), en 26 especies diferentes de algas comestibles disponibles en el mercado para consumo humano. Este estudio ha proporcionado, por primera vez, datos sobre el contenido de estas moléculas cuyas implicaciones para la salud humana han sido investigadas en estudios publicados previamente. Igualmente, el protocolo analítico se ha validado también en dos tipos de alimentos enriquecidos con algas (pasta y crackers), permitiendo cuantificar los 22 analitos en estas matrices.

3. Finalmente, se han evaluado las actividades “in vitro” de hidrolizados proteicos enzimáticos (EPH) obtenidos de 36 especies de algas tanto comerciales como recolectadas en zonas de la costa atlántica española. Estas actividades biológicas fueron:

    i. La actividad antioxidante mediante 2 tipos de ensayos (ABTS y DPPH),
    ii. La capacidad de modulación de una importante enzima humana, la Sirtuina 1 (SIRT1).

    Los EPH de una amplia gama de especies de algas presentaron interesantes propiedades antioxidantes y actividad de modulación de la SIRT1. Asimismo, también se han hallado correlaciones significativas entre diferentes parámetros:

    iii. Contenido total de péptidos en los EPH y las actividades antioxidantes, ABTS, y DPPH. Esto parece indicar que una proporción significativa de péptidos, tienen propiedades antioxidantes.
    iv. Proporción de aminoácidos hidrofóbicos que forman parte de péptidos, proteínas o en estado libre en los EPH y las actividades antioxidantes.
    v. Contenido en Taurina, Homotaurina y GABA en los EPH y capacidad de activación de la SRT1.

Es la primera vez que estas correlaciones han sido halladas en muestras de algas.

En resumen, las algas y los extractos de algas son un interesante campo de investigación por su riqueza en una amplia diversidad de compuestos bioactivos. Sin embargo, existe una falta significativa de estudios validados o debidamente validados para la correcta evaluación y cuantificación de estos compuestos bioactivos. Asimismo, las capacidades que tienen ciertos alimentos y / o compuestos específicos procedentes de estos, para activar o inhibir las sirtuinas y, en concreto, la sirtuina 1,

explican por qué hay cada vez más investigaciones sobre la búsqueda de estos moduladores naturales de sirtuinas, y todas las implicaciones en la salud humana que estos poseen.

Las algas pueden ser consideradas como fuentes ricas en moléculas interesantes tanto a nivel nutritivo, como de ingrediente para su incorporación en alimentos para hacerlas “funcionales”. Se trata de organismos muy abundantes en la naturaleza, pero no solo eso, la capacidad que tienen de crecer in vitro es excepcional, lo que nos lleva a pensar en una fuente de alimentos, proteína, compuestos bioactivos, energía, casi inagotables. En un futuro cercano, con las tecnologías más avanzadas de análisis químico, tanto en el desarrollo de nuevas partículas de empaquetamiento cromatográfico, como en los nuevos sistemas de detección por masa exacta, se podrá llegar a determinar la identidad de un gran número de estos compuestos bioactivos, así como sus implicaciones para la salud.

INDICE DE TABLAS

INDICE DE FIGURAS

Compuestos bioactivos en algas y en alimentos enriquecidos con algas: Caracterización analítica y ensayos de actividad biológica

Carlos Gerónimo Terriente Palacios

Agosto 2023

UNED
Escuela Internacional de Doctorado
EIDUNED

GOBIERNO DE ESPAÑA
MINISTERIO DE CIENCIA E INNOVACIÓN

IRTA
Institut de Recerca i Tecnologia Agroalimentàries