UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
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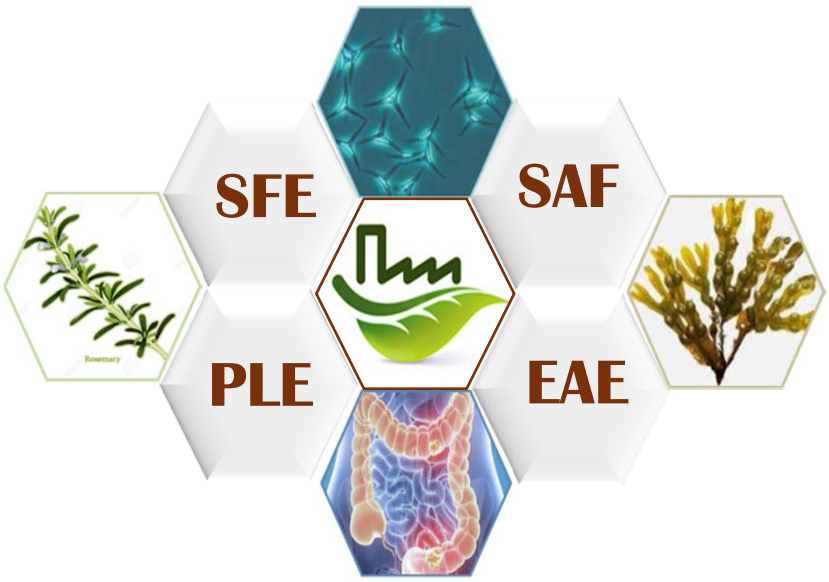
DESARROLLO DE NUEVAS ESTRATEGIAS DE EXTRACCIÓN PARA LA OBTENCIÓN DE COMPUESTOS BIOACTIVOS



TESIS DOCTORAL

ANDREA DEL PILAR SÁNCHEZ CAMARGO

MADRID, 2017

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ANDREA DEL PILAR SÁNCHEZ CAMARGO

Tesis doctoral

INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC)

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Memoria presentada por:
ANDREA DEL PILAR SÁNCHEZ CAMARGO

Para optar al grado de
DOCTOR EN BIOLOGÍA Y CIENCIAS DE LA ALIMENTACIÓN

Trabajo realizado bajo la dirección de:
Dra. Elena Ibáñez Ezequiel
Dr. Miguel Herrero Calleja
Dra. Virginia García Cañas
Instituto de Investigación en Ciencias de la Alimentación
(CIAL-CSIC)

Tutor académico:
Dr. Guillermo Reglero Rada
Universidad Autónoma de Madrid (UAM)

Dª. Elena Ibáñez Ezequiel, Dra. en Ciencias Químicas, Profesora de Investigación en el Instituto de Investigación en Ciencias de la Alimentación del CSIC.

D. Miguel Herrero Calleja, Dr. en Ciencia y Tecnología de los Alimentos, Científico Titular en el Instituto de Investigación en Ciencias de la Alimentación del CSIC.

y

Dª. Virginia García Cañas, Dra. en Ciencia y Tecnología de los Alimentos, Científico Titular en el Instituto de Investigación en Ciencias de la Alimentación del CSIC.

CERTIFICAN:

Que la presente Memoria titulada **"DESARROLLO DE NUEVAS ESTRATEGIAS DE EXTRACCIÓN PARA LA OBTENCIÓN DE COMPUESTOS BIOACTIVOS",** que presenta **Andrea del Pilar Sánchez Camargo,** M.Sc. Ingeniería de Alimentos por la Universidade Estadual de Campinas (Brasil), ha sido realizada bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación. Y que para que así conste, firman el presente certificado en Madrid a 24 de abril de 2017.

Dª. Elena Ibáñez Ezequiel **Dr. Miguel Herrero Calleja**

Dª. Virginia García Cañas,

A mis padres....

"Viajar es marcharse de casa,
es dejar los amigos
es intentar volar
volar conociendo otras ramas
recorriendo caminos
es intentar cambiar (...)"

Gabriel García Márquez

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2. Novel extraction techniques for bioactive compounds from herbs and spices.
3. Bioactives obtained from plants, seaweeds, microalgae and food by-products using preessurized liquid extraction and supercritical fluid extraction.

ABREVIATURAS

ABTS	3-ethylbenzothiazoline-6-sulphonic acid.
APCI	*(Atmospheric-pressure chemical ionization)*: Ionización química a presión atmosférica.
DAD	*(Diode-array detection)*: Detector de haz de diodos.
DMBA	2,4-dimetoxy benzaldehide.
DNA	(Deoxyribonucleic acid): ácido desoxirribonucleico.
DPPH	2,2-diphenyl-1-picrylhydrazyl.
EAE	*(Enzyme-assisted extraction)*: Extracción asistida por enzimas.
EFSA	*(European Food Safety Authority): Autoridad europea de seguridad alimentaria.*
ESI	(*Electrospray ionization*): ionización por electrospray.
GAE	(Galic acid Equivalents): Equivalentes de ácido gálico.
GC	*(Gas Chromatography)*: Cromatografía de gases.
GI50	(50% growth inhibition): concentración inhibitoria del 50% del crecimiento.
GRAS	*(Generally recognized as safe)*: Generalmente reconocido como seguro.
HSP	*(Hansen solubility parameter)*: Parámetro de solubilidad de Hansen.
LC	*(Liquid Chromatography)*: Cromatografia de líquidos.
LC×LC	*(Comprehensive Two-Dimensional Liquid Chromatography)*: Cromatografía de líquidos bidimensional completa.
LC50	*(50% lethal concentration)*: concentración letal del 50% de las células.
MS	*(Mass spectrometry)*: Espectrometría de masas.
MS/MS	*(Tandem mass spectrometry)*: Espectrometría de masas en tandem.
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide).
PGE	(Phloroglucinol Equivalents): Equivalentes de floroglucinol.
PLE	*(Pressurized Liquid Extraction)*: Extracción con líquidos presurizados.
Q	*(quadrupole)*: cuadrupolo.
Ra	Distancia entre parametros de solubilidad de Hansen.
RED	*(Relative energy distance)*: Distancia de energía relativa.

NMR	*(Nuclear magnetic resonance)*: resonancia magnética nuclear.
Ro	(Interaction radius): Radio de interacción de la esfera de Hansen.
SAF	*(Supercritical Antisolvent Fractionation)*: Fraccionamiento supercrítico antisolvente.
SC-CO_2	*(Supercritical carbon dioxide)*: Dióxido de carbono supercrítico.
SLE	*(Solid-liquid extraction)*: Extracción sólido-líquido.
SFE	*(Supercritical fluid extraction)*: Extracción con fluidos supercríticos.
SWE	*(Subcritical water extraction)*: Extracción con agua subcrítica.
TOF	*(Time-of-flight)*: Tiempo de vuelo.
TPC	*(Total phenols content)*: Contenido de fenoles totales.
UHPLC	*(Ultra high performance liquid chromatography)*: cromatografía de líquidos de muy alta resolución.
UV/Vis	Ultravioleta/visible
% p/p	Porcentaje en peso seco.
% v/v	Porcentaje en volumen.

RESUMEN/SUMMARY

RESUMEN

En la última década, la investigación sobre compuestos bioactivos procedentes de fuentes naturales ha suscitado una gran atención debido al creciente interés de la población en el consumo de alimentos funcionales que puedan promover efectos beneficiosos para la salud. Este interés también se ha visto potenciado por el progresivo aumento en la incidencia de ciertas enfermedades crónicas, como el cáncer de colon que, según diversos estudios, posee una relación directa con la dieta. Entre las diferentes fuentes naturales para la obtención de compuestos bioactivos, las plantas y, específicamente, las hierbas y especias, han sido las fuentes naturales más estudiadas debido a sus usos en medicina tradicional. No obstante, las algas marinas también han demostrado un gran potencial. Para la extracción de estos compuestos, el uso de bio-disolventes como CO_2, etanol y agua en estado sub- y supercrítico se ha estudiado ampliamente en los últimos años, demostrando su viabilidad tecnológica en un gran número de aplicaciones. No obstante, recientemente, se ha propuesto un enfoque más holístico acerca de los principios básicos para la extracción "verde" de productos naturales. Estos principios incluyen el uso de recursos vegetales renovables, el uso de disolventes alternativos, la recuperación de energía y el empleo de tecnologías innovadoras, reduciendo las operaciones unitarias y favoreciendo el diseño de procesos seguros y la generación de co-productos en lugar de residuos, donde el concepto de agro- y biorrefinerías cobra un papel importante (Capítulo 1). Así, teniendo en cuenta este enfoque, el objetivo de la presente Tesis Doctoral ha sido **el desarrollo de nuevas estrategias de extracción para la obtención de compuestos bioactivos empleando como fuentes naturales, romero (*Rosmarinus officinalis*), dos tipos de algas marrones, *Sargassum muticum y Cystoseira abies-marina*, y una microalga, *Phaeodactylum tricornutum*.**

Inicialmente, se estudió la integración e intensificación de diferentes procesos de extracción empleando CO_2 supercrítico (SC-CO_2) para la obtención y fraccionamiento selectivo de extractos de romero, con potencial actividad inhibitoria de la proliferación celular *in vitro* en dos modelos de células cancerígenas de colon, HT-29 y HCT116 (Capítulo 4,

sección 4.1). De los dos procesos desarrollados en la presente Tesis Doctoral (extracción secuencial con fluidos supercríticos en 2 etapas y extracción con líquidos presurizados combinada con fraccionamiento supercrítico antisolvente (PLE+SAF)), el fraccionamiento supercrítico antisolvente de un extracto hidroalcohólico obtenido mediante PLE, proporcionó los extractos de romero más activos en ambas líneas celulares. Éstos se caracterizaron por poseer concentraciones de ácido carnósico y carnosol en el extracto superiores al 26% y 3% (p/p), respectivamente.

Posteriormente, se llevó a cabo el estudio de diferentes técnicas de extracción de compuestos bioactivos de algas marinas, como la extracción asistida por enzimas (EAE), la extracción con líquidos presurizados (PLE) y la extracción con SC-CO_2 (SFE) empleando modificadores alternativos. Además, se utilizaron herramientas teóricas de predicción de solubilidad para la selección del bio-disolvente que mejorase la selectividad del proceso (Capítulo 4, sección 4.2). El uso de técnicas analíticas avanzadas como la cromatografía de líquidos bidimensional completa acoplada a espectrometría de masas (LC × LC-MS/MS) fue clave para la caracterización de los extractos obtenidos a partir de algas marrones. En relación al alga *Sargassum muticum*, la PLE demostró ser la técnica de extracción más eficiente para la extracción de florotaninos, entre los cuales se pudieron identificar como más abundantes diferentes fuhaloles, hidroxifuhaloles y floretoles con grados de polimerización entre 3 y 11 unidades. Tras el estudio de muestras de este alga recolectadas en diferentes localizaciones geográficas a lo largo de las costa Altántica, las procedentes de Noruega se revelaron como las más activas en células de cáncer de colon HT-29. Por otra parte, la identificación de los florotaninos más abundantes en los extractos de PLE de *Cystoseira abies-marina* mostró la predominancia de heptafucoles o heptafloretoles; la estimación teórica de sus parámetros de solubilidad permitió confirmar que el etanol era el disolvente más selectivo para su extracción. En relación a la microalga *Phaeodactylum tricornutum,* la estimación teórica de los parámetros de solubilidad de fucoxantina permitió determinar que el disolvente más selectivo para su extracción era el d-limoneno. Asimismo, el empleo de PLE en un ciclo utilizando este bio-disolvente, se mostró como la más apropiada para obtener extractos ricos en el carotenoide fucoxantina.

Los resultados derivados de estos trabajos han sido publicados en revistas científicas internacionales del área de Química, Ingeniería Química y Ciencia y Tecnología de Alimentos, sumando un total de 7 artículos de investigación originales (6 publicados y uno actualmente en proceso de revisión), y 2 artículos de revisión (aceptados) en revistas incluidas en el SCI, además de 3 capítulos de libros (aceptados) y un artículo de revisión en una revista no-SCI.

SUMMARY

In the last decade, there has been a growing interest in the research on bioactive compounds from natural sources that can be employed as functional food ingredients to help in the prevention of certain diseases, such as colon cancer, which occurrence has a strong relationship with diet. Among the different natural sources to obtain bioactive compounds, plants and, more specifically, herbs and spices have been the most-widely studied due to their traditional medicinal use; however, marine algae have also demonstrated a huge potential. To extract these bioactive compounds, bio-based solvents such as CO_2, ethanol and water at sub- and supercritical conditions have been widely studied and have demonstrated their technological ability in an important number of applications. Nevertheless, more recently, a holistic approach on the principles of "green extraction" of natural products has been proposed, including the use of renewable vegetable sources, the employment of alternative solvents, the maximization of energy recovery and the use of innovative technologies. The main goal of this strategy is to reduce unit operations, to foster the development of safer processes and to generate co-products instead of by-products, approaching the concept of agro- and biorefinery (Chapter 1).

Therefore, considering this perspective, the objective of the present PhD Thesis has been **the development of new extraction strategies to obtain bioactive compounds from natural sources such as rosemary (*Rosmarinus officinalis*), seaweeds (*Sargassum muticum* and *Cystoseira abies-marina*) and microalgae (*Phaeodactylum tricornutum*).**

Initially, the integration and intensification of different extraction processes based on the use of supercritical CO_2 (SC-CO_2) was studied to extract and selectively fractionate rosemary extracts with potential inhibitory activity against the proliferation of human colon cancer cell lines, HT-29 and HCT116 (Chapter 4, section 4.1). Among the different processes developed in the present work (two-step sequential supercritical fluid extraction and pressurized liquid extraction-supercritical antisolvent fractionation (PLE+SAF)), those obtained using supercritical antisolvent fractionation (SAF) provided with the most active rosemary extracts against both

colon cancer cell lines. Those extracts possessed carnosic acid and carnosol concentrations higher than 26% and 3% (w/w), respectively.

Later on, the study of different extraction processes (such as enzyme-assisted extraction (EAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) using SC-CO_2 and alternative co-solvents) was carried out to extract bioactive compounds from algae. Moreover, theoretical tools to predict the solubility of the target compounds on different bio-based solvents were employed to improve the selectivity of the extraction processes (Chapter 4, section 4.2). The use of advanced analytical techniques such as comprehensive two-dimensional liquid chromatography coupled to mass spectrometry (LC × LC-MS/MS) was critical to chemically characterize the extracts from the studied brown seaweeds. For *Sargassum muticum*, PLE was the most efficient extraction technique for the recovery of phlorotannins; fuhaols, hydroxyfuhaols and phloretols (with degrees of polymerization ranging between 3-11 units) were the most abundant among the identified phlorotannins. Among the different *Sargassum muticum* samples studied collected along the Atlantic coast, those grown in Norway were the most active against HT-29 colon cancer cells. On the other hand, heptafucols and heptaphloretols were the most abundant phlorotannins found in *Cystoseira abies-marina* PLE extracts; the theoretical estimation of their solubility parameters demonstrated that ethanol was the best solvent in terms of selectivity towards the extraction of these compounds. As for *Phaeodactylum tricornutum* microalgae, the theoretical prediction of the solubility parameters of fucoxanthin (the most active compound) demonstrated that d-limonene was the most selective solvent towards this carotenoid. Moreover, one-cycle PLE using d-limonene as extracting solvent was the most appropriate extraction process to obtain fucoxanthin-rich extracts from this organism.

The results obtained during the development of this PhD have resulted in the publication of 7 original research articles (6 published and one submitted for publication) and 2 reviews in scientific journals included in the SCI (within Chemistry, Chemical Engineering and Food Science and Technology categories). Besides, 3 international book chapters (2 published and one accepted) and one review paper in a No-SCI Journal have also been published.

ESTRUCTURA DE LA MEMORIA

La presente Memoria se estructura en ocho secciones, como se detalla a continuación:

- **Introducción,** donde se describen los fundamentos, antecedentes y estado del arte de los métodos de extracción y fuentes naturales objeto de estudio (Capítulo 1).
- **Justificación y objetivos,** donde se presenta el problema, se describe la motivación e importancia de su estudio, y se formulan los objetivos generales y específicos de esta Tesis Doctoral (Capítulo 2).
- **Plan de trabajo,** donde se describe la metodología seguida, procedimientos aplicados y las tareas realizadas para alcanzar los objetivos propuestos (Capítulo 3).
- **Resultados y discusión,** donde exponen los resultados obtenidos divididos en dos subsecciones: una relacionada con los procesos desarrollados empleando como fuente natural el romero, y la otra donde se incluyen todos los estudios llevados a cabo con algas (macro y micro). Cada subsección viene precedida por un prefacio, donde se resume el contenido de la misma, y a continuación se incluyen las publicaciones derivadas de los estudios realizados (en inglés y en el formato original de cada revista) (Capítulo 4).
- **Discusión general**, donde se presenta una discusión integradora de los trabajos presentados en esta Memoria (Capítulo 5).
- **Conclusiones,** donde se recogen las conclusiones más relevantes obtenidas en la presente Tesis Doctoral (Capítulo 6).
- **Bibliografía,** que incluye la relación de las publicaciones más relevantes que se han empleado para la escritura de esta Memoria (Capítulo 7).
- **Anexos,** incluyendo un extracto del *Curriculum vitae* de la doctoranda, la lista de publicaciones a las que ha dado lugar la presente Tesis Doctoral y las publicaciones no recogidas en la misma, pero que se consideran de gran interés por su relación directa con el trabajo presentado en esta Memoria.

CAPÍTULO 1.
INTRODUCCIÓN

1. INTRODUCCIÓN

En la actualidad, existe un enorme interés en dar respuesta a uno de los grandes retos de la sociedad, **la sostenibilidad**, entendida como una forma racional de mejorar procesos con el objetivo de aumentar la producción y a la vez minimizar el impacto medioambiental. Sin embargo, para el desarrollo de procesos sostenibles es necesario tener en cuenta los 12 principios de la Química Verde considerando que es posible, desde esta perspectiva, diseñar y mejorar productos, materiales, procesos y sistemas manteniendo como uno de los objetivos primordiales la preservación del medioambiente. Desde este punto de vista, son especialmente importantes los procesos de extracción que permitan alcanzar este desarrollo sostenible y que cumplan con los principios de la Química Verde, y a su vez, con los criterios necesarios de rapidez, selectividad, eficacia, elevada producción y bajo coste. Algunos de estos procesos se basan en el empleo de fluidos comprimidos, como la extracción con fluidos supercríticos (SFE), con líquidos presurizados (PLE) o con agua subcrítica (SWE, PHWE). En este campo, cobra especial importancia la obtención de productos de alto valor añadido (con actividad biológica) a partir de fuentes naturales como plantas y algas, puesto que su producción puede contribuir notablemente a consolidar la idea de los procesos sostenibles.

En las Áreas de la Ciencia y Tecnología de Alimentos y la Nutrición la búsqueda de nuevos productos o ingredientes alimentarios capaces de mejorar la salud del consumidor, o incluso prevenir la aparición de ciertas enfermedades, es uno de los campos de investigación más activos. Un aspecto de especial relevancia es la confirmación, con evidencias científicas probadas, de la actividad biológica de los productos e ingredientes alimentarios que justificarán su posterior producción a gran escala. Aunque existen evidencias sobre la actividad biológica de, por ejemplo, algunos compuestos fenólicos con actividad antioxidante asociados a la reducción de riesgos de padecer enfermedades cardiovasculares o cáncer, es necesario realizar esfuerzos importantes para probar su eficacia real en seres humanos. En este sentido, el desarrollo de la Alimentómica (García-Cañas et al., 2012) puede proporcionar importantes avances en este campo.

La presente Tesis Doctoral se centra en el **desarrollo de nuevos procesos sostenibles**, basados en el empleo de tecnologías limpias de extracción y herramientas teóricas de predicción de solubilidad, **para la obtención de extractos con actividad antiproliferativa en líneas celulares de cáncer de colon a partir de fuentes naturales como el romero y distintos tipos de algas**.

A continuación, se revisan con detalle los distintos aspectos implicados en la investigación que se ha desarrollado durante el período de realización de la presente Tesis Doctoral en el Laboratorio de Alimentómica del Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM).

1.1 ALIMENTOS E INGREDIENTES FUNCIONALES.

La extracción de compuestos bioactivos de fuentes naturales es un tema de investigación de gran interés para las industrias alimentarias y afines, debido al creciente desarrollo del sector de los alimentos funcionales (Gul et al., 2016). Un *alimento funcional* se define como el producto que, además de cubrir las necesidades energéticas y nutricionales que todo alimento debe aportar, es capaz de proporcionar un beneficio fisiológico adicional (Goldberg, 1994). Por lo general, este beneficio adicional para la salud se obtiene a través de la adición o presencia de un *ingrediente funcional*, que es el responsable de la bioactividad pretendida. Existen actualmente en el mercado varios ejemplos de ingredientes funcionales incluidos en productos comerciales, tales como ácidos grasos poliinsaturados (Lopez-Huertas, 2010), esteroles y estanoles vegetales (Smet et al., 2012), péptidos (Patil et al., 2015) o polifenoles (Dueñas et al., 2015), por nombrar solo algunos. A modo de ejemplo, se ha descrito que algunos polifenoles poseen efectos positivos contra enfermedades cardiovasculares, neurodegenerativas y cáncer, gracias a sus actividades antioxidantes, antiinflamatorias y antiproliferativas (Del Rio et al., 2013). A pesar de que los mecanismos de acción de los efectos beneficiosos de estos componentes aún no han sido completamente elucidados, la búsqueda de nuevos componentes naturales bioactivos es un tema importante en el que se están realizando notables esfuerzos por parte de la comunidad científica (Manach et al., 2009; Monteiro et al., 2015). Asimismo, el desarrollo de la Alimentómica (Foodomics) como una nueva disciplina dentro de la Ciencia de

los Alimentos, está permitiendo obtener una visión holística de los mecanismos moleculares responsables de la actividad biológica de algunos compuestos alimentarios bioactivos (Cifuentes, 2013, 2009; Valdés et al., 2014).

1.2 FUENTES POTENCIALES DE COMPUESTOS BIOACTIVOS.

Generalmente, los compuestos bioactivos de origen natural son sintetizados como metabolitos secundarios; éstos son un grupo de compuestos que, si bien no tienen un rol directo en el desarrollo y crecimiento del organismo, se encargan de aumentar su capacidad general para sobrevivir y superar los desafíos de su medio, permitiéndole interactuar con su entorno (Azmir et al., 2013). Los metabolitos secundarios de origen natural de mayor interés pueden agruparse en compuestos fenólicos, terpenos y derivados, alcaloides, esteroides, lípidos y ácidos grasos, policétidos y glicósidos (Azmir et al., 2013; Hamed et al., 2015).

Entre los diferentes tipos de fuentes naturales de compuestos bioactivos, las plantas son, con toda seguridad, las más estudiadas, principalmente debido a su inmediata disponibilidad y diversidad (Azmir et al., 2013; Herrero et al., 2015; Sasidharan et al., 2010). Se han sugerido una amplia gama de productos naturales derivados de plantas como fuente de compuestos bioactivos (Brusotti et al., 2014), aunque, sin embargo, este campo investigador también se ha abierto a otras fuentes naturales interesantes, como las algas y microalgas (Hayes, 2012; Holdt and Kraan, 2011; Vaz et al., 2016). El medio marino se considera una fuente subexplotada de compuestos bioactivos (Hamed et al., 2015). Las algas y microalgas son probablemente los dos grupos de organismos marinos que han atraído la mayor atención por su potencial para el aprovechamiento industrial (de Jesus Raposo et al., 2013; Holdt and Kraan, 2011). Además de las fuentes naturales mencionadas, el uso de subproductos procedentes de la industria agroalimentaria también está ganando interés, ya que algunos de los residuos generados durante la fabricación de alimentos han demostrado poseer aun cantidades significativas de compuestos bioactivos (Castro-Vargas et al., 2016, 2013; Wijngaard et al., 2012). De hecho, el uso de subproductos se está promoviendo ampliamente, dado que permite una reducción de residuos alimentarios, otorgándoles un valor añadido y agregando importantes beneficios medioambientales (Viganó et al., 2015). A continuación, se presentarán en detalle las fuentes

naturales empleadas en la presente Tesis Doctoral para la obtención de compuestos bioactivos: romero (*Rosmarinus officinalis* L.) y algas marinas (micro y macroalgas).

1.2.1 ROMERO

El romero (*Rosmarinus officinalis* L.) es una planta aromática perteneciente a la familia de las lamiaceas (*Lamiaceae*) que se consume ampliamente como ingrediente alimentario, especialmente como especia culinaria. Esta planta endémica de regiones Mediterráneas, es capaz de contribuir significativamente a las características organolépticas de los alimentos en los que se incluye. Sin embargo, el romero también se ha utilizado tradicionalmente en la medicina popular para tratar varios trastornos de salud, despertando un enorme interés en cuanto a los compuestos mayoritarios que podrían ser responsables de las propiedades beneficiosas que le son atribuidas. Las principales bioactividades relacionadas con el romero son: antioxidante (Yesil-Celiktas et al., 2007), antiinflamatoria (Arranz et al., 2015), quimioprotectora (González-Vallinas et al., 2015), antiproliferativa (Đilas et al., 2012), antimicrobiana (Wang et al., 2012), protectora frente a trastornos neurológicos (de Oliveira, 2016), así como de disminución de los riesgos relacionados con la obesidad, diabetes y síndromes metabólicos (Hassani et al., 2016; Sedighi et al., 2015), entre otros (Bahri et al., 2016).

Dentro de los compuestos presentes en esta planta, los más importantes son los compuestos fenólicos y los compuestos volátiles, ambos responsables de sus características funcionales (Kontogianni et al., 2013). Entre los compuestos fenólicos es posible encontrar dos familias con polaridades diferentes: i) ácidos fenólicos y ii) diterpenos fenólicos. En el primer grupo (de naturaleza más polar), destaca el ácido rosmarínico como uno de los más abundantes, mientras que en el segundo grupo el ácido carnósico y el carnosol, son predominantes (Herrero et al., 2010). En cualquier caso, la composición química del romero es compleja y puede variar en función de la especie, la variedad, las condiciones de crecimiento, el momento de la recolección, las propiedades del suelo, el clima y la localización geográfica. También se han identificado otros compuestos fenólicos tales como galocatequina y genkwanina (flavonoides), rosmanol, rosmadial, carnosato de metilo, ácido betulínico, ácido

oleanólico y ácido ursólico (di- y triterpenoides fenólicos), entre otros (Borrás-Linares et al., 2014; Kontogianni et al., 2013). Por su parte, los compuestos volátiles conforman el aceite esencial de romero, que puede representar entre el 1.0 - 2.5% de la composición de la hoja (Fornari et al., 2012). En el aceite esencial se han identificado compuestos volátiles pertenecientes a diferentes familias tales como derivados de terpenos oxigenados (1,8-cineol, alcanfor, borneol, α-terpineol, verberona y acetato de borneol), terpenos (α-pineno, canfeno, limoneno) y sesquiterpenos (β-cariofileno, α-humuleno), entre otros (Fornari et al., 2012).

Las propiedades bioactivas del romero se han atribuido principalmente a la presencia de ácido carnósico y carnosol (Figura 1.1), que pueden representar hasta el 5% en peso fresco de la hoja de romero (Wellwood and Cole, 2004). Sin embargo, se ha observado incluso cómo los extractos de romero enriquecidos en ácido carnósico y carnosol pueden ser más activos que la cantidad equivalente de estos compuestos estudiados por separado, lo que sugiere un efecto sinérgico o aditivo con otros componentes presentes en dichos extractos en menor concentración (Valdés et al., 2014). Los efectos anticancerígenos del extracto de romero y sus principales compuestos fenólicos se han estudiado en los últimos años empleando modelos *in-vitro* de líneas celulares de cáncer, incluyendo leucemia (Ishida et al., 2014; Valdés et al., 2012), mama (Cheung and Tai, 2007; González-Vallinas et al., 2014a), pulmón (Yesil-Celiktas et al., 2010), hígado (Vicente et al., 2013; Yesil-Celiktas et al., 2010), páncreas (González-Vallinas et al., 2014b; Kontogianni et al., 2013), próstata (Kar et al., 2012; Petiwala et al., 2014; Yesil-Celiktas et al., 2010), colon (Đilas et al., 2012; González-Vallinas et al., 2013b; Ibáñez et al., 2012; Valdés et al., 2013), cervix (Berrington and Lall, 2012) y ovario (Tai et al., 2012).

El ácido carnósico y el carnosol son diterpenos orto-difenólicos (Figura 1.1.) y representan más del 90% de la actividad antioxidante de los extractos de romero (de Oliveira, 2016; Wellwood and Cole, 2004).

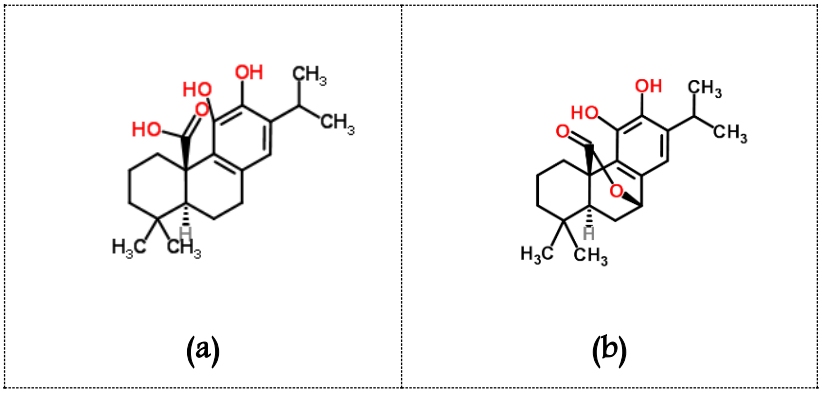


Figura 1.1 Estructura química de los compuestos fenólicos mayoritarios presentes en las hojas de romero, a) Ácido carnósico y b) Carnosol.

El ácido carnósico ($C_{20}H_{28}O_4$, MW 332.434 g/mol) presenta, además, propiedades antiinflamatorias y quimioprotectoras en varios modelos celulares (Birtić et al., 2015), así como actividad antiproliferativa en células de cáncer de colon (Valdés et al., 2013) y de carcinoma hepático (Vicente et al., 2013; Yesil-Celiktas et al., 2010), entre otros. Por su parte, el carnosol ($C_{20}H_{26}O_4$, MW 330.4 g/mol) posee una importante actividad antioxidante y quimioprotectora (Chun et al., 2014); este diterpeno presenta también actividades antiinflamatorias y anticancerígenas, por ejemplo, en células de cáncer de próstata, piel, mama y colon (Johnson, 2011).

Se han documentado una amplia variedad de métodos para el análisis de compuestos fenólicos y compuestos volátiles en extractos vegetales, y concretamente en extractos de romero. Para la caracterización cuantitativa y cualitativa de la fracción volátil, se emplea comúnmente la cromatografía de gases acoplada a espectrometría de masas (GC-MS) (Jalali-Heravi et al., 2011; Jiang et al., 2011; Napoli et al., 2010). Recientemente, también se ha utilizado esta técnica para determinar la composición enantiomérica de sus compuestos volátiles y estudiar así su posible incidencia en los efectos fisiológicos en seres humanos (Tomi et al., 2016).

Por su parte, la cromatografía de líquidos (LC) es la técnica de separación por excelencia para el análisis de compuestos fenólicos presentes en el extracto de romero, debido a su versatilidad, reproducibilidad, selectividad y eficacia. Esta técnica de separación se suele

acoplar a diferentes sistemas de detección, fundamentalmente, a sistemas de absorción UV-Vis y a espectrometría de masas (MS) (Borrás-Linares et al., 2014; Herrero et al., 2010; Kontogianni et al., 2013). En MS, el sistema de ionización más comúnmente usado para la detección de este tipo de compuestos es la ionización por electrospray (ESI), ya que es muy eficaz en el análisis de compuestos polares, lábiles o con bajo peso molecular. En cuanto a los analizadores de masas, el analizador híbrido cuadruplo-tiempo de vuelo (QTOF) ha permitido la detección de estos compuestos con un elevado grado de sensibilidad, resolución y selectividad, proporcionando con mayor exactitud información acerca de la masa molecular de los analitos, facilitando así su identificación (Borrás-Linares et al., 2015, 2014; Romo Vaquero et al., 2013)

1.2.2 ALGAS MARINAS

En los últimos años, las algas marinas se han considerado como "el alimento *vegetal* del futuro" (Cardoso et al., 2015), ya que su composición comprende una amplia e interesante gama de nutrientes y micronutrientes, entre los que se cuentan lípidos y ácidos grasos poliinsaturados (PUFA), pigmentos como carotenoides y clorofilas, proteínas, polisacáridos y compuestos fenólicos (Herrero et al., 2015; Stengel et al., 2011). No obstante, su naturaleza es totalmente diferente a la de las plantas terrestres. Si bien son organismos autótrofos fotosintéticos, poseen características comunes diferentes a las plantas, como son la ausencia de tallos, raíces y hojas. En cualquier caso, la variedad morfológica es enorme, pudiendo abarcar desde organismos unicelulares microscópicos (microalgas) a grandes organismos multicelulares (macroalgas). Los países asiáticos cuentan con una larga tradición en el uso de algas marinas como alimento (Holdt and Kraan, 2011; Liu et al., 2012). Los datos muestran que el consumo típico diario de algas por persona en estos países alcanza entre 8,5 y 10 g/día en Corea y Japón, respectivamente (Gomez-Gutierrez et al., 2011). Hoy en día, la creciente información sobre los beneficios de las algas, así como los movimientos de población entre países y culturas, han sido los responsables del creciente interés en las algas también en los países occidentales.

Aunque los organismos clasificados históricamente dentro del término algas pueden pertenecer a tres de los seis grandes reinos taxonómicos más frecuentemente empleados hoy día para agrupar sistemáticamente a todos los seres vivos, *Bacteria*, *Protista* y *Plantae*, de manera general, las algas se clasifican en tres categorías: algas verdes *(Chlorophyceae),* algas rojas *(Rhodophyceae)* y algas marrones *(Phaeophyceae),* de acuerdo a la composición de los pigmentos responsables de su color (Suganya et al., 2016).

Dentro de las algas marrones, *Cystoseira abies-marina* y *Sargassum muticum* (Figura 1.2 a, b) se cuentan entre las especies más importantes que se pueden encontrar en los ecosistemas del océano Atlántico y del mar Mediterráneo (Amico, 1995; Guiry and Guiry, 2015; Tanniou et al., 2014). Se ha descrito que estas macroalgas producen una amplia variedad de metabolitos secundarios, los cuales incluyen terpenoides (Balboa et al., 2013; Gouveia et al., 2013), florotaninos (Montero et al., 2016, 2014; Stiger-Pouvreau et al., 2014), carotenoides (como fucoxantina) (Balboa et al., 2015; Moraes et al., 2011) y algunos ácidos grasos, especialmente, poliinsaturados (PUFAs) (Plaza et al., 2008; Vizetto-Duarte et al., 2015). De igual manera, también se ha descrito que presentan un buen potencial farmacológico, ya que sus extractos pueden exhibir apreciables actividades antioxidantes (Balboa et al., 2013; Yende et al., 2014), antiproliferativas (Montero et al., 2016; Namvar et al., 2013; Zubia et al., 2009) así como antiinflamatorias (Liu et al., 2012). Estas dos especies de macroalgas se han empleado durante el desarrollo de la presente Tesis Doctoral.

Figura 1.2. Fotografías de las macroalgas a) *Cystoseira abies-marina* y b) *Sargassum muticum* y, c) de la microalga *Phaeodactylum tricornutum,* empleadas en esta Tesis Doctoral.

Por su parte, la industria biotecnológica relacionada con las microalgas ha crecido y se ha diversificado significativamente desde los años setenta. En la actualidad, algunas microalgas se cultivan a gran escala como fuente de nutrientes para seres humanos y animales (*Chlorella y Arthrospira*, por ejemplo) o para la obtención de pigmentos (*Dunaliella salina* y *Haematococcus pluvialis* como fuentes de β-caroteno y astaxantina, respectivamente) (Spolaore et al., 2006). Otra microalga de gran interés comercial es *Phaeodactylum tricornutum* (Figura 1.2c). Esta diatomea marina es rica en PUFAs, como el EPA (ácido eicosapentaenoico), y posee también un alto contenido de fucoxantina, un carotenoide al que se le ha atribuido una elevada capacidad para proteger el organismo contra algunas enfermedades (Kim et al., 2012). Teniendo en cuenta esta prometedora composición química, esta microalga también se ha incluido para su estudio en el presente trabajo.

A continuación, se describen las principales características de las moléculas "diana" encontradas en las algas objeto de estudio y que pueden ser responsables de las bioactividades relacionadas.

1.2.2.1 Florotaninos

Aunque generalmente los compuestos fenólicos se han asociado a las plantas, las algas son también una fuente rica de polifenoles. Entre las diferentes clases de algas, las algas marrones son las que poseen una mayor cantidad de estos compuestos (Heffernan et al., 2015). De hecho, hay una variedad de compuestos fenólicos, denominados florotaninos, que son exclusivos de este tipo de algas (Isaza Martinez and Torres Castaneda, 2013; Steevensz et al., 2012; Wijesekara et al., 2011). Los florotaninos poseen una función estructural en las algas debido a su contribución en la formación y fortificación de la pared celular. Además, como metabolitos secundarios, se les han atribuido otras funciones celulares como, por ejemplo, la capacidad de unir iones metálicos y la absorción de la radiación UV. También pueden actuar como agentes antibacterianos, inhibidores de incrustación y antiherbívoros (Amsler and Fairhead, 2006). Desde el punto de vista químico y estructural, los florotaninos son compuestos fenólicos poliméricos complejos formados por unidades de floroglucinol unidas entre sí a través de diferentes tipos de enlaces. El peso molecular de los florotaninos varía de 0.126 a 650

kDa, aunque el intervalo observado más común es de 10 a 100 kDa (Wijesekara et al., 2011). Según Martínez y Castañeda (2013), dependiendo de los enlaces de las unidades de floroglucinol (Figura 1.3a), los florotaninos pueden clasificarse en: fucoles (Figura 1.3b, enlaces fenilo), floretoles (Figura 1.3c, enlaces éter), fuhaloles (Figura 1.3d, enlaces éter y grupos hidroxilo adicionales), fucofloretoles (Figura 1.3e, con enlaces éter y fenilo) y eckoles (Figura 1.3f, enlaces dibenzodioxina) (Isaza Martinez and Torres Castaneda, 2013).

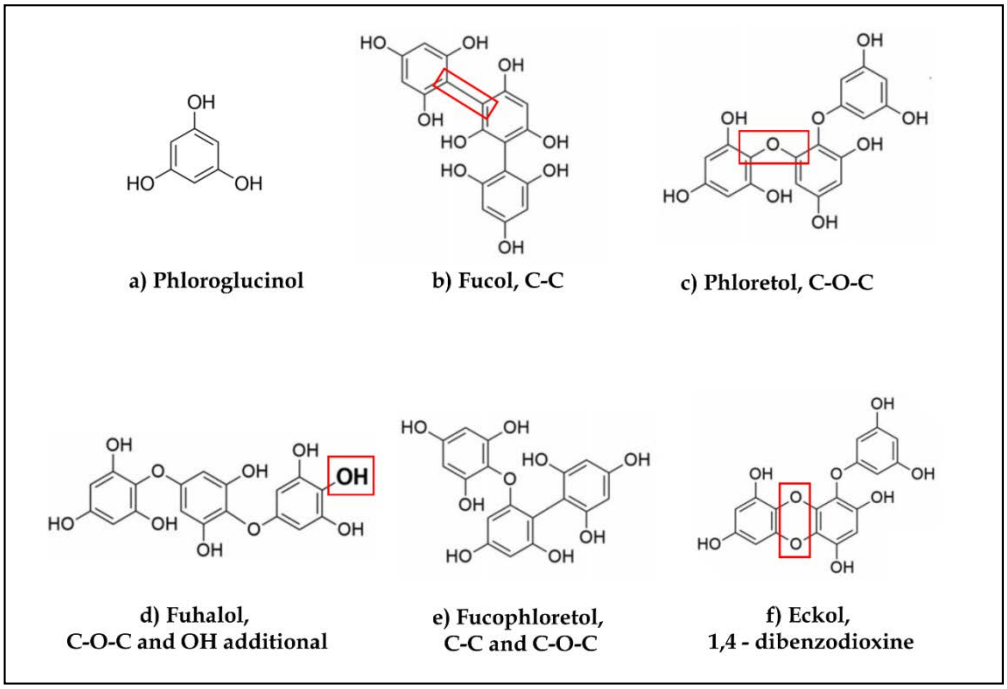


Figura 1.3. Estructura química del floroglucinol (a) y las principales clases de florotaninos derivados de algas marrones: b) fucoles, c) floretoles, d), fuhaloles, e) fucofloretoles, y f) eckoles (Adaptado de Martínez y Castañeda, 2013).

A medida que el número de unidades de floroglucinol aumenta, la complejidad de la estructura y diversidad de los florotaninos se incrementa significativamente (Heffernan et al., 2015). Este nivel de complejidad hace que la identificación y caracterización química de dichos compuestos sea un desafío interesante. Se han empleado diferentes técnicas analíticas para separar e identificar los florotaninos de diferentes algas marrones, tales como HPLC-DAD-MS (Ferreres et al., 2012; Steevensz et al., 2012; Wang et al., 2012), UHPLC-DAD-MS (Heffernan et al., 2015) o incluso la cromatografía de líquidos bidimensional completa acoplada a MS (LC

× LC-MS/MS). Recientemente, nuestro grupo de investigación ha utilizado con éxito esta técnica para separar florotaninos con diferentes estructuras químicas y grado de polimerización en extractos de *Cystoseira abies marina* y *Sargassum muticum* por primera vez (Montero et al., 2016, 2014). Otras técnicas, como NMR ^{1}H y ^{13}C, también se han usado para la elucidación de las estructuras de florotaninos presentes en algas marrones (Stiger-Pouvreau et al., 2014).

1.2.2.2 Fucoxantina

Como se ha mencionado previamente, la fucoxantina ($C_{42}H_{58}O_6$, MW 658.92 g/mol) es un importante carotenoide oxigenado que se produce abundantemente en las algas marrones así como en algunas microalgas y contribuye a más del 10% de la producción total estimada de carotenoides en la naturaleza (Kim et al., 2012). Este pigmento está unido a varias proteínas y, junto con la clorofila *a*, forma complejos del tipo fucoxantina-clorofila *a*-proteína en los tilacoides, donde actúa como un carotenoide primario para captar luz y transferir energía (Kim et al., 2012). A diferencia de otros carotenoides, la fucoxantina tiene una estructura única (Figura 1.4), en la que están presentes un enlace alénico inusual, 9 enlaces conjugados dobles, un enlace 5,6-monoepóxido y algunos grupos funcionales oxigenados incluyendo hidroxilo, epoxi, carbonilo y carboxilo, lo que le confiere propiedades biológicas especiales (Zhang et al., 2015).

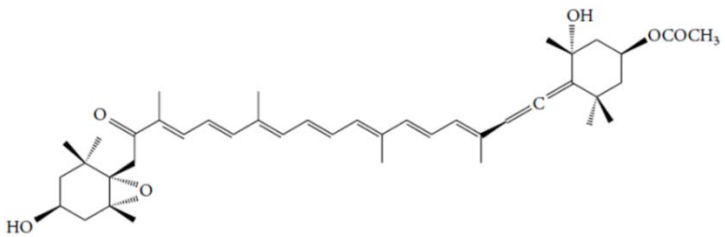


Figura 1.4. Estructura química de la fucoxantina. Adaptado de Zhang et al. (2015).

Este carotenoide ha demostrado ser muy efectivo inhibiendo el crecimiento celular e induciendo apoptosis en células cancerígenas de colon y próstata (Hosokawa et al., 2004; Kotake-Nara et al., 2005). También posee propiedades antiinflamatorias (Kim et al., 2010),

antioxidantes (Ha et al., 2013; Kawee-ai et al., 2013), antidiabéticas y de prevención de la obesidad (Maeda et al., 2009, 2007; Park et al., 2011).

1.3 CÁNCER DE COLON

Según datos extraídos del informe World Cancer Report 2014 elaborado por la Agencia Internacional para la Investigación del Cáncer de la Organización Mundial de la Salud (OMS), en el año 2012, el cáncer figuró entre las principales causas de morbilidad y mortalidad en todo el mundo, con aproximadamente 14 millones de nuevos casos y 8,2 millones de muertes relacionadas. No obstante, la preocupación es todavía mayor si se tiene en cuenta que se espera que el número de nuevos casos aumente en un 70% en los próximos dos decenios. Entre los hombres, los 5 tipos de cáncer más diagnosticados en 2012 fueron cáncer de pulmón, próstata, colorrectal, estómago e hígado. Por otro lado, entre las mujeres lo fueron los cánceres de mama, colorrectal, pulmón, cuello uterino y estómago, por ese orden (Stewart and Wild, 2014). En Europa, el cáncer de colon es el segundo cáncer con mayor incidencia, diagnosticándose en 2012, 447.000 nuevos casos y alcanzando las 215.000 muertes relacionadas. En España, el cáncer de colon ocupa la primera posición, y se ha estimado que el número de nuevos casos en 2014 fue de 39.500 hombres y 16.000 mujeres (Stewart and Wild, 2014).

El cáncer de colon surge como una consecuencia de la inestabilidad genómica de una acumulación de errores genéticos que son generados en la desregulación de las vías moleculares que controlan la migración celular, la diferenciación, la apoptosis y la proliferación (Watson and Collins, 2011). El cáncer colorrectal se origina en el colon o el recto y en un 95% de los casos comienza como un crecimiento en el revestimiento interno del colon o del recto llamado pólipo (Watson and Collins, 2011). Dentro de los factores de riesgo modificables que aumentan las probabilidades de que una persona presente pólipos ademotosos o cáncer de colon, el estilo de vida y, en particular, la dieta, se han señalado por tener una gran importancia. De hecho, el consumo de grasa, alcohol y carne roja se asocia con un mayor riesgo a padecer esta enfermedad (Stewart and Wild, 2014; Watson and Collins, 2011). Debido a ello, se está prestando atención a los fitoquímicos o metabolitos secundarios, que se caracterizan por ser sustancias químicas no-nutritivas obtenidas a partir de fuentes

naturales, de modo que puedan contribuir como nuevos moduladores de las principales vías de señalización celular ejerciendo un potencial efecto anticancerígeno comprobado (González-Vallinas et al., 2013a). Actualmente, más del 50% de los agentes antitumorales utilizados en el entorno clínico derivan de productos naturales, ya sea obtenidos directamente de plantas u otras fuentes naturales, o químicamente modificados de ellos (González-Vallinas et al., 2015). En general, la actividad anticancerígena de los extractos ricos en compuestos bioactivos puede dividirse en función de su capacidad para proteger o inhibir frente al desarrollo de las diferentes etapas del cáncer: la iniciación (o actividad quimioprotectora), la promoción (o actividad antiproliferativa), la progresión del cáncer (o actividad antiinvasiva o antimetastásica) (Valdés, 2016).

La actividad quimioprotectora de los extractos de romero se ha relacionado con sus propiedades antioxidantes, en particular, con su capacidad para secuestrar radicales libres, lo que podría proteger frente el daño oxidativo inducido por especies reactivas del oxígeno en lípidos, proteínas y DNA (Bahri et al., 2016; González-Vallinas et al., 2015; Sánchez-Camargo and Herrero, 2017; Xiang et al., 2013). Además de los efectos protectores frente al estrés oxidativo, también hay varios estudios que sugieren que el ácido carnósico y el carnosol tienen actividad antiproliferativa. Por ejemplo, Valdés et al. (2013) analizaron el efecto inhibidor de la proliferación celular de 5 extractos de romero, 3 de ellos obtenidos empleando líquidos presurizados (usando etanol y agua como disolventes a diferentes temperaturas) y 2 obtenidos mediante CO_2 supercrítico, en dos líneas celulares de adenocarcinoma de colon humano, SW840 y HT-29 (Valdés et al., 2013). Los resultados mostraron que aquellos extractos que contenían una alta concentración de ácido carnósico y carnosol presentaban una actividad antiproliferativa superior, la cual se asociaba con alteraciones en el ciclo celular (Ibáñez et al., 2012; Valdés et al., 2013). Además, los extractos supercríticos de romero también indujeron la muerte en células tumorales de colon debido, al menos en parte, a un proceso apoptótico (González-Vallinas et al., 2013b; Ibáñez et al., 2012). También se analizó el efecto antitumoral de un extracto supercrítico de romero *in vivo* empleando xenoinjertos de cáncer de colon en ratones, demostrando la disminución del volumen del tumor después de la administración oral del extracto (González-Vallinas et al., 2013b). Recientemente, en un estudio basado en el

fraccionamiento de un extracto de romero obtenido por SFE, el ácido carnósico se destacó como el principal responsable de la actividad antiproliferativa del extracto, seguido por el carnosol y también el ácido betulínico. En este caso, la concentración de ácido betulínico en el extracto fue de 2,1 μM, y la incubación de células HT-29 con una fracción purificada del mismo extracto que contenía 81% de ácido betulínico y 19% de hinokiona mostró la misma citotoxicidad que el extracto completo (Borrás-Linares et al., 2015).

Por otra parte, los extractos ricos en florotaninos, también han demostrado actividad inhibitoria de la proliferación celular (Thomas and Kim, 2011). Por ejemplo, la fracción fenólica cruda de un extracto del hidrolizado enzimático (CphF) de *Ecklonia cava* mostró los mejores resultados de actividad antiproliferativa en la línea celular de cáncer de colon murino (CT26) con un valor GI50[1] de 5,1 μg/mL. En este estudio, se sugirió que la actividad antiproliferativa del extracto podía ser debida a su elevado contenido de polifenoles (Athukorala et al., 2006). Aunque los derivados de los floretoles y fucofloretoles han demostrado efectos interesantes en algunas líneas de leucemia, adenocarcinoma hepático y mama (Kong et al., 2009; Li et al., 2011), en la actualidad, existen muy pocos trabajos publicados que tengan por objeto el estudio de la actividad antiproliferativa de extractos ricos en florotaninos en modelos celulares de cáncer de colon humano.

1.4 TÉCNICAS AVANZADAS DE EXTRACCIÓN: GENERALIDADES.

En la actualidad, existen una serie de técnicas basadas en el uso de fluidos comprimidos que tienen en común una mayor eficiencia en comparación con los procesos convencionales realizados a presión atmosférica, y que implican el empleo de condiciones operativas que permitan a los disolventes adquirir propiedades físico-químicas no alcanzables de otro modo. Además, en ciertas condiciones, estas técnicas cumplen con los principios de la Química Verde (Ibáñez and Cifuentes, 2015). La extracción con líquidos presurizados y la extracción con fluidos supercríticos son las dos técnicas más empleadas basadas en el uso de fluidos

[1] GI50 (50% Growth Inhibition) se define como la concentración del compuesto que causa la inhibición del 50% en el crecimiento *in vitro* de las células con respecto al control no tratado.

comprimidos, para obtener compuestos bioactivos de fuentes naturales; sus principales características se describen a continuación.

1.4.1 Extracción con líquidos presurizados (PLE)

La extracción con líquidos presurizados se basa en el uso de disolventes a elevadas presiones y temperaturas (siempre por debajo de sus puntos críticos), en condiciones en las que los disolventes se mantienen en estado líquido durante el proceso de extracción. En dichas condiciones, estos disolventes adquieren propiedades diferentes en comparación con los utilizados en condiciones ambientales (de presión y temperatura). Esta técnica también se conoce comúnmente como extracción con fluidos presurizados (PFE, de sus siglas en inglés), extracción presurizada con disolventes calentados (PHSE) o extracción acelerada con disolventes (ASE) (Herrero et al., 2013; Plaza and Turner, 2015). Es posible emplear una gran variedad de disolventes para llevar a cabo este tipo de extracciones; sin embargo hoy en día se busca el uso de disolventes lo menos perjudiciales para el medio ambiente. Entre ellos, el agua llama especialmente la atención debido a la modificación de sus propiedades físico-químicas que se produce cuando ésta se somete a altas temperaturas y presiones (Plaza and Turner, 2015). En este caso, la técnica es incluso renombrada como extracción con agua subcrítica (SWE), extracción con agua sobrecalentada (SHWE) o extracción con agua calentada presurizada (PHWE). En cualquier caso, los principios y los requisitos instrumentales son los mismos independientemente del disolvente empleado. Cuando se utiliza agua como disolvente, el parámetro principal que más influye en la extracción es su constante dieléctrica (ε). Este valor se considera generalmente como una medida de la polaridad de un disolvente; cuando el agua se calienta bajo suficiente presión de manera que se mantiene su estado líquido, su constante dieléctrica sufre una disminución drástica. De hecho, los valores de ε van desde 80 a temperatura ambiente hasta aproximadamente 27 a 250 °C, valores muy similares a las de algunos disolventes orgánicos como etanol o metanol (Figura 1.5A). Por lo tanto, se puede deducir que el agua sometida a las condiciones típicas empleadas en PLE podría ser capaz de extraer compuestos de polaridad media-baja.

Los requisitos instrumentales necesarios para llevar a cabo un proceso de extracción con líquidos presurizados en principio son simples (Figura 1.5B): i) una bomba introduce el

disolvente en el sistema y ayuda a desalojar el extracto en el vial de recolección una vez ha terminado el proceso (además, la bomba es la encargada de mantener la presión seleccionada, normalmente entre 5-10 MPa); ii) una celda de extracción, donde se coloca la muestra; la celda debe soportar altas presiones y debe estar equipada con al menos dos válvulas on/off para poder mantener las condiciones de extracción estables; iii) un horno, donde se sitúa la celda de extracción de modo que se pueda calentar hasta el valor de temperatura deseado. La temperatura máxima de trabajo en la mayoría de los instrumentos es de unos 200 °C; iv) por último, se necesita un vial de recolección.

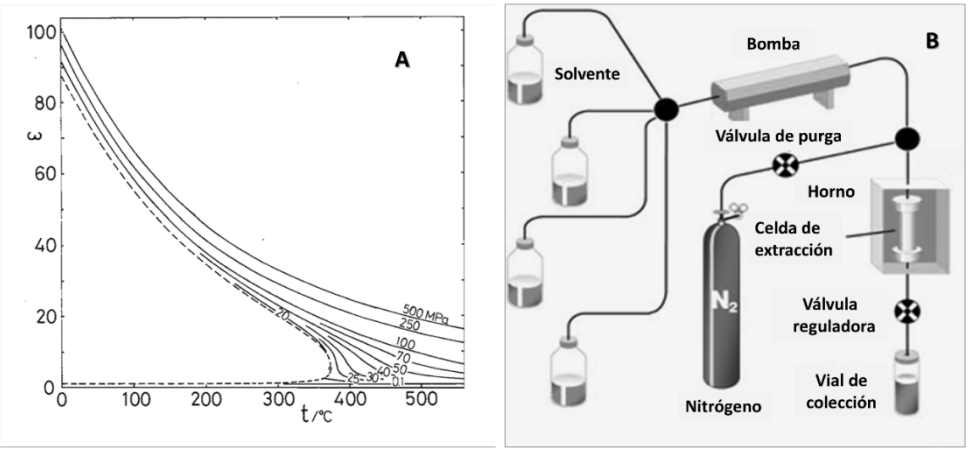


Figura 1.5. Constante dieléctrica (ε) del agua en función de la temperatura (A) y diagrama esquemático de la instrumentación básica necesaria para operar un proceso de extracción con líquidos presurizados (B). Figura 1.5A adaptada de Uematsu y Frank, 1980. Figura 1.5B adaptada de Dionex Thermo Scientific website.

Sin embargo, la instrumentación empleada puede ser más o menos sofisticada, dependiendo de los requisitos del proceso. Por ejemplo, puede incluir un circuito de nitrógeno que ayuda a desalojar todo el disolvente de las líneas después de la extracción. Existen varios instrumentos comerciales disponibles en el mercado, aunque también se han presentado diferentes aplicaciones con instrumentos diseñados y construidos a escala de laboratorio. En ambos casos deben utilizarse materiales resistentes a la corrosión, habida cuenta de las presiones de funcionamiento y las temperaturas habitualmente empleadas (Saldaña and Valdivieso-Ramírez, 2015).

En PLE, el tipo de disolvente empleado es el factor más determinante ya que influirá directamente en el tipo de compuestos extraídos. La selección del disolvente depende del objetivo particular del proceso de extracción; por tanto, el disolvente más adecuado debe elegirse teniendo en cuenta la naturaleza y características de los analitos objetivo (Mendiola et al., 2007). Una vez seleccionado el disolvente o la mezcla de disolventes, la temperatura es el parámetro que más influye en cualquier proceso de PLE. Como regla general, cuando se extraen compuestos a partir de matrices naturales, un incremento en la temperatura de extracción proporcionará un mayor rendimiento de extracción. Se ha observado repetidamente cómo el uso de temperaturas muy altas no siempre es perjudicial para la recuperación de compuestos bioactivos a partir de matrices naturales. Sin embargo, debido a la naturaleza de estos componentes y a su labilidad, se debe considerar que temperaturas de extracción demasiado altas pueden conducir potencialmente a la degradación de dichos compuestos (Mustafa and Turner, 2011). Por esta razón, se recomienda el uso de diseños experimentales para optimizar la temperatura de extracción, monitorizando como variable de respuesta la presencia de compuestos bioactivos en los extractos (Saravana et al., 2016). La presión de extracción también puede tener un impacto importante en el proceso. Sin embargo, diferentes investigaciones han demostrado que, una vez que la presión es suficiente para mantener el disolvente en estado líquido, su efecto es bastante limitado (Herrero et al., 2013; Mustafa and Turner, 2011).

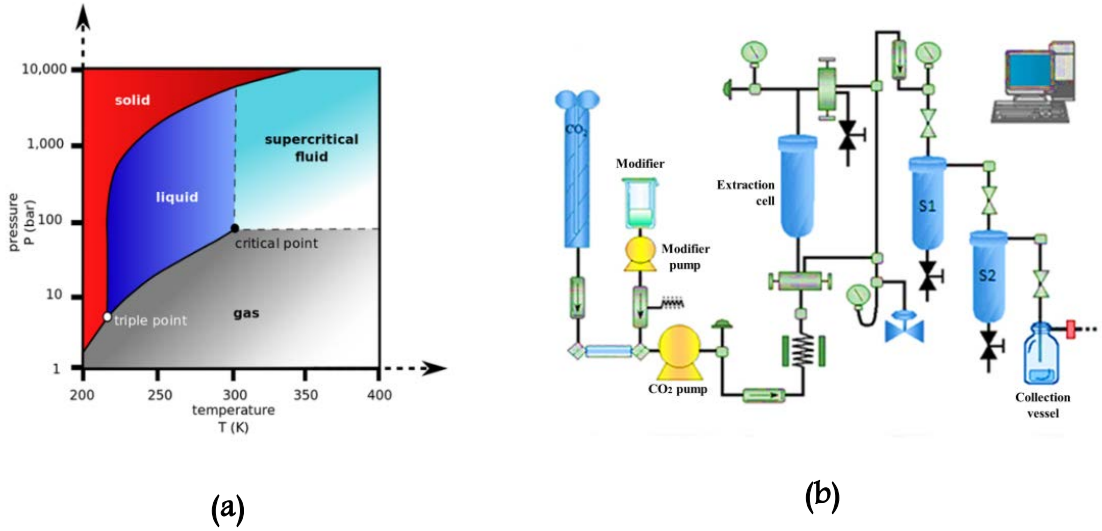
En cuanto al tiempo de extracción, esta variable, aunque importante, tiene menor influencia en el proceso resultante. El tiempo de extracción se refiere al tiempo en el que el disolvente está en contacto directo con la muestra que se extrae a la temperatura y presión fijadas. También se llama tiempo de extracción estática. Este último término se refiere a extracciones estáticas en las que un volumen dado de disolvente se deja en contacto con la muestra en las condiciones de extracción deseadas durante un periodo de tiempo específico. En general, se aplican tiempos de extracción estática relativamente cortos (5–20 min) para la extracción de bioactivos a partir de matrices naturales. Los tiempos más largos generalmente no producen mejores resultados cuando se alcanza un equilibrio entre los componentes de la muestra y el disolvente (Mendiola et al., 2007). Otro modo de operación implica el uso de extracciones dinámicas. En este caso, el disolvente calentado y presurizado fluirá

continuamente a través de la muestra. Aunque este modo podría ser más favorable para la extracción completa de la muestra, dado que se evita la formación de equilibrio, pueden ser necesarios mayores volúmenes de disolvente. Además, en este último caso, el caudal de disolvente también debe optimizarse ya que influirá directamente en el tiempo de extracción. Por otra parte, un enfoque más frecuentemente descrito en la bibliografía es el uso de ciclos cortos consecutivos de extracción estática, los cuales pueden ayudar eficazmente a agotar la matriz de la muestra que se está extrayendo (Herrero et al., 2013).

1.4.2 Extracción con fluidos supercríticos (SFE)

El empleo de fluidos supercríticos para la extracción de compuestos bioactivos implica el uso de un fluido a presión y temperatura por encima de sus valores críticos (Figura 1.6a). Una vez se sobrepasa el punto crítico, el fluido adquiere propiedades significativamente diferentes como disolvente, como consecuencia de los diferentes cambios físicos que se producen. En estas condiciones, el fluido supercrítico posee propiedades intermedias entre las de los gases y las de los líquidos. Su viscosidad es similar a la de un gas (50-100 μPa s), mientras que la difusividad es intermedia entre la de los líquidos y de los gases (0.01-0.1 mm^2/s). Por su parte, la densidad es cercana a los valores encontrados para líquidos (200-900 kg/m^3), lo que modifica significativamente su capacidad como disolvente y permite conseguir altos grados de selectividad en el proceso de extracción (Brunner, 2005; Sánchez-Camargo et al., 2014). En la actualidad, la mayoría de las aplicaciones de extracción de compuestos bioactivos a partir de fuentes naturales empleando fluidos supercríticos, se basan en las ventajas que presenta el dióxido de carbono gracias a unos valores de temperatura y presión críticos fácilmente alcanzables (Tc= 31.2 °C, Pc= 7.38 MPa). Además, el CO_2 posee una serie de propiedades que lo convierten en un disolvente supercrítico ideal, incluyendo entre otras: a) es considerado como GRAS (*Generally recognized as safe*) para la industria alimentaria, b) es barato y fácilmente disponible, c) permite la reutilización del CO_2 generado en otros procesos industriales, cumpliendo así con algunos de los principios de la Química Verde, y d) es gas a temperatura ambiente. Esta última propiedad es muy importante ya que permite obtener un extracto libre de disolvente una vez finaliza el proceso de extracción y se produce la despresurización del

sistema. Dada su baja temperatura crítica, el CO_2 supercrítico se puede utilizar a temperaturas de extracción suaves, lo cual es muy apropiado para la recuperación de sustancias termolábiles (da Silva et al., 2016).



(a) (b)

Figura 1.6. (a) Diagrama de fases presión-temperatura del dióxido de carbono, (b) Diagrama esquemático de la instrumentación básica necesaria para operar un proceso de extracción con fluidos supercríticos. S1, separador 1; S2, separador 2.

Entre los parámetros que afectan a la extracción por SFE, la presión y la temperatura son los más influyentes. Estos dos parámetros combinados determinarán la solubilidad del compuesto "diana u objetivo" en el fluido supercrítico (Brunner, 2005). La selección de los valores de temperatura y presión a emplear en un proceso debe realizarse cuidadosamente de acuerdo con el objetivo del mismo, considerando que se obtendrá una mayor densidad del fluido supercrítico a través de un aumento de presión, lo que puede conducir a una mayor solubilidad de los componentes de la muestra. Sin embargo, un aumento isobárico de la temperatura de extracción producirá una disminución en la densidad del disolvente, aunque, por otro lado, producirá un incremento en la presión de vapor de los analitos presentes en la matriz, promoviendo a través de este mecanismo, la transferencia de materia. A pesar de las ventajas comentadas anteriormente del CO_2 supercrítico, la principal limitación en su aplicación es su baja polaridad. Los extractos de SFE obtenidos de matrices naturales son típicamente mezclas de familias de compuestos tales como triglicéridos (Lopes et al., 2012), ácidos grasos (Sánchez-Camargo et al., 2011), terpenoides (Herrero et al., 2010), fitoesteroles

(Sajfrtová et al., 2010), tocoferoles (Ibáñez et al., 2002), carotenoides (Durante et al., 2014), y algunos fenólicos de baja polaridad (Marques et al., 2016), entre otros. En la Figura 1.7, se presentan ejemplos de cada familia.

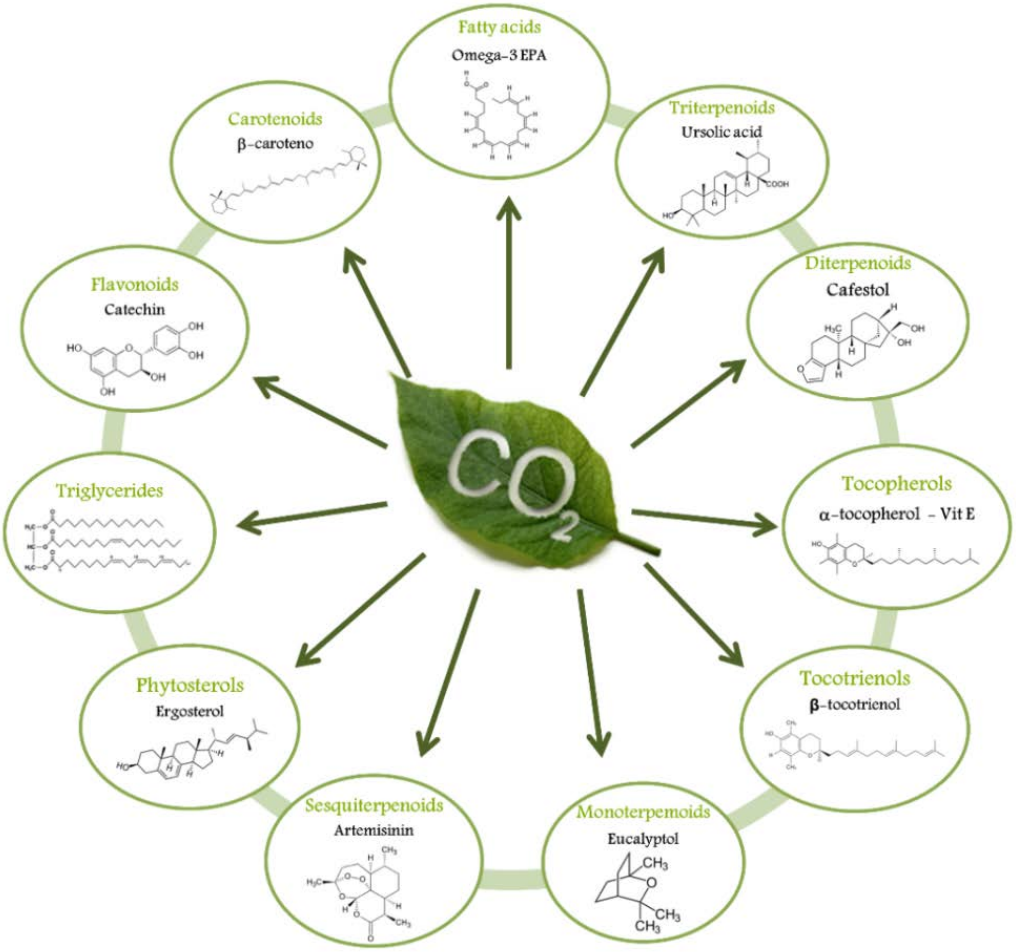


Figura 1.7. Ejemplos de las principales familias de compuestos encontrados en los extractos obtenidos empleando SFE de fuentes naturales.

Para ampliar el campo de aplicación de esta técnica es necesario el uso de modificadores (disolventes orgánicos de carácter polar, generalmente) que se emplean en una determinada relación (5-10%) junto con el CO_2 supercrítico, dando como resultado un aumento de la polaridad global del disolvente de extracción. De esta manera, se pueden desarrollar otras aplicaciones que implican la extracción de componentes más polares, frecuentes en el campo de los compuestos bioactivos naturales. Por esta razón, la selección del modificador así como la proporción en la que se utiliza debe ser cuidadosamente optimizada para producir una

extracción eficiente (Herrero et al., 2015; Mendiola et al., 2013; Sánchez-Camargo et al., 2014).

En cuanto a la instrumentación necesaria, al igual que en PLE, ésta debe ser capaz de soportar altas presiones (hasta 50 MPa o incluso más). En la Figura 1.6b se presenta un esquema típico de instrumentación requerida para procesos de extracción por SFE para matrices sólidas. El primer requisito es un tanque para el disolvente de extracción, usualmente CO_2, una bomba para presurizar el gas a la presión de extracción deseada, y un limitador o válvula para mantener la alta presión dentro del sistema. Se necesita una bomba adicional cuando se van a emplear modificadores orgánicos. En el caso de muestras sólidas, se necesita una celda de extracción de alta presión, mientras que para la extracción de líquidos se utiliza una columna en la que la entrada de muestra y de fluido supercrítico se lleva a cabo a contracorriente. Finalmente, es necesario un recipiente de recolección del extracto (o celdas de separación, también llamadas celdas de fraccionamiento) para la recuperación del mismo. Habitualmente, se pueden emplear diferentes métodos de recolección, incluyendo el atrapamiento sólido, el atrapamiento de líquidos o el atrapamiento en frío, entre otros (Turner et al., 2002). En sistemas a escala piloto e industrial, la recolección de los solutos extraídos se realiza reduciendo rápidamente la presión, aumentando la temperatura, o ambas cosas. La despresurización después de la extracción también se puede realizar en cascada usando varias celdas de fraccionamiento o separación, considerando que cada recipiente de separación podría tener una temperatura y presión particulares para que algunos de los compuestos extraídos precipiten y se separen selectivamente (Herrero et al., 2013).

1.4.3 Obtención de extractos de romero

Las propiedades antioxidantes del extracto y el aceite esencial de romero han sido ampliamente estudiadas desde los años 50, siendo catalogada desde entonces como una de las especias con mayor bioactividad (Chipault et al., 1952). Es por esto que los extractos de hojas de romero obtenidos por diversas técnicas de extracción y el aislamiento de los compuestos mayoritarios han recibido especial atención. Para la obtención de la fracción de aceite esencial se emplea, comúnmente, la hidrodestilación o la destilación por arrastre de vapor (Fornari et

al., 2012), y más recientemente, la hidrodestilación asistida por microondas (González-Rivera et al., 2016; Karakaya et al., 2014).

En cuanto a los extractos de romero, la extracción sólido-líquido (SLE) empleando disolventes como metanol, etanol, agua, acetona y sus mezclas, ha sido el procedimiento más comúnmente empleado debido a que es simple, se obtienen altos rendimientos de extracción y resulta fácil su escalado (Azmir et al., 2013; Oliveira et al., 2016). No obstante, otras técnicas de extracción como la extracción asistida por ultrasonidos o extracción asistida por microondas (Bellumori et al., 2016; Borrás-Linares et al., 2014), así como la extracción con fluidos presurizados (Carvalho et al., 2005; García-Risco et al., 2011; Herrero et al., 2010) también se han empleado, principalmente, para mejorar la eficiencia del proceso de extracción, la calidad del extracto y su concentración en compuestos con actividad biológica (Valdés et al., 2013; Zabot et al., 2015). En la Tabla 1.1, se presentan los trabajos más relevantes sobre la obtención de extractos de romero empleando fluidos presurizados publicados en los últimos años.

Yesil-Celiktas et al. (2007), demostraron que los extractos obtenidos empleando extracción supercrítica (35 MPa, 100 °C, 5% (p/p) metanol como modificador) de hojas recolectadas en diferentes lugares y época de cosecha en Turquía presentaban diferencias en su composición, con contenidos de ácido carnósico entre 0,5 y 11,6% (p/p) (Yesil-Celiktas et al., 2007). Por su parte, Carvalho et al. (2005) estudiaron la extracción supercrítica del romero, encontrando que los extractos obtenidos a 30 MPa y 40°C poseían la concentración más alta de ácido carnósico (hasta 21,5% en peso) con un rendimiento de extracción global alrededor del 5,0%. La extracción con líquidos presurizados también se ha empleado para la obtención de extractos de romero, aunque en este caso la selectividad del proceso ha demostrado ser menor que la extracción supercrítica para la obtención de ácido carnósico y carnosol.

Con el creciente aumento en las restricciones del uso de antioxidantes de origen sintético, debido a sus potenciales efectos cancerígenos (Branen, 1975; Ito et al., 1985), se han llevado a cabo estudios que han demostrado que es posible obtener extractos de romero (y sus compuestos mayoritarios) con una mayor actividad antioxidante que el butilhidroxianisol (BHA) y el butilhidroxitolueno (BHT), antioxidantes sintéticos ampliamente utilizados en la industria de alimentos (Guitard et al., 2016; Raadt et al., 2015). Este hecho motivó a la

Asociación Europea de Productores de Extracto de Romero (EREMG, por sus siglas en inglés) a solicitar la autorización de la EFSA (Autoridad Europea de Seguridad Alimentaria) para la utilización del extracto de romero como aditivo alimentario. En la directiva 2008/84/CE, la EFSA aprobó su utilización como aditivo alimentario seguro (a dosis especificas), el cual se puede añadir directamente al alimento o incorporarse durante el envasado, actuando como agente antimicrobiano y antioxidante (Aguilar et al., 2008). Asimismo, la directiva 2008/84/CE también describe 4 diferentes procesos permitidos para producir el extracto de romero: i) SLE empleando acetona, ii) extracción con CO_2 supercrítico, iii) SLE empleando etanol como disolvente y desodorizante y iv) extracción en dos etapas: la primera etapa como la descrita en el proceso (iii), seguida por una etapa de decoloración del extracto empleando hexano. Dependiendo del tipo de proceso de extracción utilizado, la normativa establece una reglamentación diferente (Aguilar et al., 2008). Recientemente, se ha llevado a cabo una extensión del uso de extractos de romero en grasas para untar (contenido en grasa inferior al 80%) bajo la directiva 2016/56/CE, ampliando así sus aplicaciones de uso en la industria alimentaria (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2015).

Tabla 1.1. Condiciones de extracción empleadas para la extracción de romero utilizando fluidos comprimidos y cantidades de ácido carnósico y carnosol recuperadas.

Origen Hojas de Romero	Tecnología empleada, disolvente	Presión (MPa)	Temp. (ºC)	Tiempo (min)	Modificador (% p/p)	CA (% p/p)	CS (% p/p)	Referencia
Turquía	SFE, SC-CO_2	35	100	40	Metanol, 5	11,6	1,5	(Yesil-Celiktas et al., 2007)
Brasil	SFE, SC-CO_2	30	40	180	NE	21,5	NR	(Carvalho et al., 2005)
España	SFE, SC-CO_2	30	40	480	NE	28,0	NR	(García-Risco et al., 2011)
España	SFE, SC-CO_2	30	40	360	NE	10,9	1,05	(Vicente et al., 2012)
España	SFE, SC-CO_2	15	40	180	Etanol, 5	25,7	-	(Vicente et al., 2013)
España	SFE, SC-CO_2	15	40	180	Etanol, 10	14,2	-	(Vicente et al., 2012)
España	SFE, SC-CO_2	30	40	360	NE	18,0	1,6	(Arranz et al., 2015)
España	SFE, SC-CO_2	15	40	300	Etanol, 7	25,6	3,7	(Valdés et al., 2014)
España	PLE, Etanol	10	50	20	-	7,6	13,7	(Herrero et al., 2010)
España	PLE, H_2O	10	50	20	-	0,003	0,8	(Herrero et al., 2010)

NE: No empleado; NR: No descrito: CA: Ácido carnósico; CS: Carnosol

1.4.4. Obtención de extractos a partir de algas marinas

Dada la creciente importancia de los compuestos fenólicos y los carotenoides y su posible efecto beneficioso sobre la salud humana, nuestro grupo de investigación se ha centrado en la última década en el desarrollo de procedimientos de extracción medioambientalmente limpios que permitan el aislamiento a gran escala de estos metabolitos a partir de macro- y microalgas (Herrero et al., 2006, 2005; Mendiola et al., 2008; Plaza et al., 2012; Reyes et al., 2014).

Para la extracción de florotaninos se ha empleado comúnmente la extracción sólido-líquido convencional con disolventes polares puros o mezclas hidroalcohólicas a baja temperatura durante varias horas en agitación y con luz reducida. Posteriormente, las muestras se centrifugan y el disolvente orgánico se evapora a vacío, y se elimina el agua remanente por medio de liofilización, si es necesario (Breton et al., 2011; Lann et al., 2012; Tanniou et al., 2014). Este procedimiento convencional consume mucho tiempo y a menudo grandes volúmenes de disolventes orgánicos tóxicos. Con el fin de corregir estas desventajas, nuestro grupo de investigación ha propuesto varios procesos innovadores en los últimos años (Golmakani et al., 2012; Ibañez et al., 2012; Plaza et al., 2012; Tanniou et al., 2014). En un trabajo realizado en colaboración con el Laboratoire des Sciences de l'Environnement Marin (Université de Bretagne Occidentale, Francia), se comparó la extracción convencional sólido líquido con otros métodos tales como (1) SFE empleando etanol como modificador, (2) extracción por partición centrífuga y (3) PLE, con el fin de identificar el proceso más eficiente para extraer los florotaninos a partir del alga marrón *S. muticum*. De todas las técnicas empleadas, la extracción con líquidos presurizados resultó ser la alternativa más eficaz. Sin embargo, los extractos de florotaninos también contenían compuestos lipídicos así como carbohidratos (Tanniou et al., 2013). Entre los métodos más usados para la posterior purificación de los florotaninos extraidos se encuentran la diálisis, la extracción líquido-líquido, y la extracción en fase sólida (SPE) (Stiger-Pouvreau et al., 2014).

Por su parte, para la extracción de fucoxantina a partir de microalgas se han descrito en la bibliografía diferentes métodos utilizando una variedad de disolventes de extracción. Como es frecuente, la extracción convencional con acetona, etanol y acetato de etilo (entre muchos otros disolventes orgánicos) se ha estudiado ampliamente (Kim, 2014; Kim et al., 2012; Mäki-

Arvela et al., 2014). Sin embargo, debido a los inconvenientes anteriormente mencionados, recientemente, se han evaluado procesos verdes como la extracción asistida por ultrasonidos (Kim et al., 2012; Pasquet et al., 2011), la extracción asistida por microondas (Gilbert-López et al., 2016), SFE (Quitain et al., 2013; Roh et al., 2008) y PLE (Gilbert-López et al., 2016; Kim et al., 2012). En estos trabajos se han estudiado, entre otros factores, el tipo de disolvente, el tiempo de extracción y la temperatura empleada (Gilbert-López et al., 2016; Mäki-Arvela et al., 2014).

Además del estudio individual de cada uno de estos procesos, la combinación de algunos de ellos se ha convertido en objeto de estudio en nuestro laboratorio, para la obtención de diferentes compuestos con valor añadido, incluyendo carotenoides. Recientemente, se ha establecido una metodología de extracción secuencial de compuestos bioactivos de la microalga *Isochrysis galbana* empleando varios disolventes presurizados. La metodología incluía: 1) la aplicación de SFE con CO_2 supercrítico puro, 2) seguido de una extracción intermedia con etanol expandido con CO_2, y finalizando con 3) una extracción PLE empleando etanol y agua. En los dos primeros pasos se recuperaron componentes de polaridad baja a media, principalmente fucoxantina, mientras que los compuestos más polares se extrajeron mediante PLE (Gilbert-López et al., 2015) en la tercera etapa.

1.5 INTEGRACIÓN E INTENSIFICACIÓN DE PROCESOS

Actualmente, existe una gran cantidad de información sobre las condiciones de extracción necesarias para obtener compuestos bioactivos químicamente diferentes a partir de una amplia variedad de muestras. Sin embargo, es importante reconocer que todavía hay un largo camino por recorrer en la aplicación de estas técnicas de acuerdo con las últimas tendencias en este campo. El desarrollo de nuevos procesos de extracción está relacionado con el uso y estudio de nuevos disolventes y la integración de procesos. Si bien aún no se prevé la sustitución del CO_2 supercrítico como el disolvente preferido en los procesos SFE, en el caso de PLE, la situación es totalmente diferente. De hecho, la tendencia actual, muestra un incremento en el uso de nuevos disolventes respetuosos con el medio ambiente (y de grado alimentario) en

dichos procesos de PLE. Ya se han desarrollado algunas aplicaciones que han mostrado la posibilidad de obtener bioactivos de diferentes fuentes naturales utilizando nuevos disolventes como el lactato de etilo (Lores et al., 2015) o el d-limoneno (Golmakani et al., 2014). Asimismo, la evolución de los procesos basados en PLE y SFE está relacionada con la aplicación de la idea de integración e intensificación de procesos encaminada a un mayor desarrollo del concepto de biorrefinería (Zabot et al., 2015).

En la intensificación de procesos se utiliza el mismo equipo de manera polivalente para llevar a cabo diferentes operaciones unitarias, mientras que en un proceso integrado se busca el mejor proceso para obtener cada producto, aunque se empleen equipos diferentes (Zabot et al., 2015). El concepto de intensificación de procesos utilizando fluidos supercríticos se ha aplicado en varias áreas como una opción para la producción de sustitutos de derivados petroquímicos a partir de biomasa, principalmente como fuente de energía y biocombustibles (Lee et al., 2014; Sanders et al., 2012). Sin embargo, recientemente, también se han desarrollado estrategias basadas en la intensificación de procesos aplicadas en el campo de la extracción de compuestos bioactivos (Carvalho et al., 2015; Osorio-Tobón et al., 2014; Vardanega et al., 2017; Zabot et al., 2015).

Algunos procesos combinados pueden integrar otras técnicas asistidas con PLE y/o SFE, produciendo procesos mejorados en términos de eficiencia, como es el caso de la extracción asistida por enzimas (EAE) (Puri et al., 2012; Wijesinghe and Jeon, 2012). La aplicación de estas técnicas puede ayudar significativamente a alterar las matrices naturales que van a ser extraídas, lo que provoca un aumento en los rendimientos de extracción y puede facilitar la recuperación de los compuestos bioactivos de interés. Debido a las ventajas intrínsecas que estos procesos acoplados pueden proporcionar, el desarrollo de este tipo de enfoques puede ser altamente favorable para aplicaciones comerciales.

1.5.1 Extracción supercrítica en etapas secuenciales.

La obtención de extractos naturales empleando fluidos supercríticos en una sola etapa se ha estudiado ampliamente (de Melo et al., 2014; Herrero et al., 2015). Sin embargo, a

menudo, las matrices naturales empleadas contienen numerosas familias de compuestos que poseen diferente naturaleza y bioactividades interesantes, y su extracción en una sola etapa se ve limitada. Asimismo, en algunos casos son necesarias etapas posteriores de purificación debido a la co-extracción de compuestos con menor bioactividad y/o que no son de interés. Es por esto que las nuevas perspectivas para las cadenas productivas se centran en mejorar el uso de las materias primas utilizando tecnologías respetuosas con el medio ambiente que aumenten la productividad sin afectar la calidad del producto (Vardanega et al., 2017). Este enfoque responde al concepto de intensificación de procesos, que incluye iniciativas que aumentan la capacidad de producción dentro de un determinado volumen de equipo, disminuyendo el consumo de energía por tonelada de producto y reduciendo la formación de residuos. En este contexto, los procesos de extracción que dan lugar a varios compuestos de valor añadido de una matriz vegetal utilizando el mismo equipo, o mejoras de proceso que aumentan su eficiencia, se consideran procesos intensificados (Shirsath et al., 2012). A modo de ejemplo, Osorio-Tobón et al. (2014) desarrollaron un proceso intensificado para obtener aceite de cúrcuma (*Curcuma longa L.*) y un extracto rico en curcuminoides a partir de esta matriz. El aceite de cúrcuma se obtuvo usando SFE y el extracto se consiguió empleando PLE. Ambos procesos se realizaron secuencialmente con el mismo equipo (Osorio-Tobón et al., 2014). Vardanega et al. (2017), desarrollaron un proceso intensificado similar para la obtención de β-ecdisona, saponinas y fructo-oligosacaridos a partir de raíces de ginseng brasileño (*Pfaffia glomerata*) (Vardanega et al., 2017).

Siguiendo este mismo enfoque, el fraccionamiento supercrítico puede ser un procedimiento eficaz para concentrar selectivamente compuestos bioactivos de interés que se han extraido previamente. Por ejemplo, es posible concentrar compuestos fenólicos en una fracción (y así obtener un producto con mayor capacidad antioxidante) y obtener compuestos volátiles en otra fracción. Dicho proceso puede llevarse a cabo de dos modos diferentes: mediante una extracción en etapas secuenciales, aplicando diferentes condiciones de presión y temperatura de extracción en cada etapa, o mediante un fraccionamiento en línea a través de una despresurización en cascada.

Ibáñez et al. (1999), describieron por primera vez el fraccionamiento de extractos de romero, donde dos etapas sucesivas de extracción dieron como resultado una fracción con baja actividad antioxidante en la primera etapa (10 MPa y 40 °C) y una fracción con alta actividad antioxidante en la segunda etapa (40 MPa y 60 °C), obteniendo un extracto desodorizado rico en compuestos fenólicos. De la misma manera, se ha estudiado el fraccionamiento en línea del extracto en un sistema de despresurización (compuesto de dos separadores) para producir una separación selectiva de las sustancias antioxidantes (Cavero et al., 2005), confirmando la existencia de una relación directa entre el contenido de ácido carnósico y la actividad antioxidante de las 16 muestras recogidas empleando diferentes condiciones de extracción y fraccionamiento. Por su parte, Vicente et al. (2012) emplearon un enfoque similar (tanto etapas secuenciales como fraccionamiento en línea), probando diferentes condiciones de temperatura, presión y tiempo para la obtención de extractos supercríticos de romero. Los resultados obtenidos mostraron que la adición de etanol en la segunda etapa secuencial (1ª etapa: 30 MPa, 40°C; 2ª etapa: 15 MPa, 10% (p/p) etanol), permitía obtener un enriquecimiento de ácido carnósico en el extracto hasta valores de 30% en peso.

1.5.2 Fraccionamiento supercrítico antisolvente (SAF)

A pesar de las ventajas y la flexibilidad de los sistemas que emplean fluidos sub- y supercríticos para la extracción de fuentes naturales, resulta conveniente, y en muchos casos necesario, llevar a cabo operaciones de procesamiento adicionales antes, durante o después del empleo del fluido supercrítico (Catchpole et al., 2012). Esta integración de procesos incluye la operación previa de un proceso unitario (fermentación, extracción, pretratamiento enzimático, fraccionamiento físico o reducción de tamaño) seguida de procesos de extracción o fraccionamiento sub- o supercríticos (cromatografía supercrítica, precipitación y recubrimiento de solutos, entre otros) (Catchpole et al., 2012). En relación a este enfoque, se ha sugerido el uso combinado de procesos de extracción, como el CO_2 supercrítico seguido de extracción con líquidos presurizados (PLE, empleando etanol y agua) para mejorar la recuperación de compuestos con diferentes polaridades y bioactividades (Garcia-Mendoza et al., 2015; Osorio-Tobón et al., 2016; Zabot et al., 2015). Otro proceso excepcionalmente

versátil que se ha utilizado para fraccionar extractos de naturaleza compleja es el fraccionamiento supercrítico antisolvente (SAF). Este proceso se beneficia de las propiedades antisolventes del SC-CO_2 y permite la precipitación de compuestos insolubles de una solución de una mezcla orgánica de naturaleza polar y SC-CO_2 (Catchpole et al., 2012; Gonzalez-Coloma et al., 2012). La mezcla orgánica polar es un extracto previamente obtenido a partir de una matriz natural usando mezclas etanol-agua. De esta forma, el contacto continuo con el SC-CO_2 permite la precipitación de los componentes más polares insolubles en SC-CO_2 (incluyendo el agua y los compuestos hidrófilos, fracción denominada *refinado*) en una primera cámara de separación, mientras que otros componentes, así como la otra parte de mezcla orgánica soluble (componentes menos polares y etanol, fracción denominada *extracto*) permanecen en disolución y se recuperan posteriormente mediante la reducción de la presión en una segunda cámara de separación. Recientemente, se ha demostrado que el fraccionamiento supercrítico antisolvente es útil para separar compuestos bioactivos de diferentes polaridades a partir de extractos hidroalcohólicos de plantas como *Persea indica* (Gonzalez-Coloma et al., 2012), romero y salvia (Catchpole et al., 2012; Durling et al., 2007a; Visentín et al., 2011), así como de propóleo (Catchpole et al., 2004).

El estudio del equilibrio de fase del sistema SC-CO_2-Etanol-H_2O es crucial para comprender los factores que afectan al comportamiento de los componentes de la mezcla. En algunos trabajos previos se ha demostrado que la selectividad del disolvente y los coeficientes de partición del etanol están altamente influenciados por la presión, el porcentaje de masa de agua en la alimentación y la relación másica de flujo de extracto hidroalcohólico/SC-CO_2 (Durling et al., 2007b). Estos parámetros se deben optimizar de acuerdo con la naturaleza de los compuestos a extraer (Catchpole et al., 2012).

Por otra parte, los extractos hidroalcohólicos de partida se pueden obtener empleando métodos convencionales como la extracción líquido-sólido. Sin embargo, con el empleo de la PLE se alcanzan mayores rendimientos de extracción, debido principalmente a la mejora en la transferencia de materia y el aumento de la solubilidad de los analitos (Herrero et al., 2015). Además, éste es un proceso que requiere menos tiempo y menores relaciones de

disolvente/sólido en comparación con las técnicas tradicionales de extracción a presión atmosférica (Herrero et al., 2010).

1.5.3 Extracción asistida con enzimas combinada con extracción con líquidos presurizados (EAE + PLE).

La extracción asistida por enzimas también se considera una técnica verde que aprovecha los procesos de hidrólisis enzimática para ayudar a la liberación de compuestos interesantes al disolvente de extracción (Wijesinghe and Jeon, 2012). En el caso de material vegetal y, específicamente, de las algas, esta técnica podría presentar la ventaja adicional de producir la hidrólisis de componentes bioactivos que están unidos a componentes de la pared celular, aumentando así su eficiencia de extracción (Puri et al., 2012). La pared celular de las algas marrones está compuesta principalmente por polisacáridos, incluyendo ácido algínico, alginatos (polisacáridos carboxilados, sales de ácido algínico) y fucoidanos (polisacáridos sulfatados) (Balboa et al., 2013). La presencia de estos compuestos en la pared celular limita la accesibilidad de los disolventes a los compuestos bioactivos, reduciendo la eficiencia de extracción durante la aplicación de métodos de extracción tradicionales. De este modo, la degradación de los polisacáridos de la pared celular puede ser un paso importante en la liberación de componentes activos como florotaninos (Jeon et al., 2011). Por otra parte, se ha descrito previamente que los florotaninos se pueden encontrar naturalmente formando complejos fuertes con proteínas, ya sea por enlace de hidrógeno reversible o por condensación covalente irreversible (Stern et al., 1996). Para llevar a cabo estos procesos, es interesante conocer la estructura y composición de la pared celular, puesto que ésta permitirá la selección precisa de las enzimas más adecuadas para promover su debilitamiento. Así, en la mayoría de las aplicaciones que se han llevado a cabo empleando EAE de algas marrones, se han utilizado enzimas del tipo carbohidrasas (incluyendo arabanasas, celulasas, β-glucanasas, hemicelulasas, y xylanasas) o proteasas (endo/exo peptidasas) (Balboa et al., 2013). En la Tabla 1.2 se muestran algunos ejemplos de estudios realizados empleando enzimas para la obtención de fracciones hidrolizadas con actividad antioxidante a partir de algas marrones. Una vez seleccionada la enzima, o mezcla enzimática, se deben considerar y optimizar diferentes parámetros,

incluyendo la concentración de la enzima, la temperatura de incubación, el tiempo de reacción, el pH del medio de reacción, así como otras consideraciones relacionadas con el estado físico de la muestra (Balboa et al., 2013). En general, un tamaño de partícula reducido ayudará a aumentar la eficacia del tratamiento enzimático, mejorando el contacto superficial. Respecto al resto de parámetros, éstos estarán fuertemente relacionados con la enzima o enzimas a utilizar, ya que cada enzima posee generalmente rangos de trabajo limitados en términos de temperatura y pH. Por lo tanto, si se va a utilizar una mezcla de enzimas, se deben seleccionar valores apropiados para que todas las reacciones de hidrólisis puedan tener lugar.

Tabla 1.2. Ejemplos de enzimas empleadas en EAE para la obtención de extractos bioactivos de algas marrones.

Alga marrón	Enzimas empleadas	Referencia
Sargassum horneri	Carbohidrasas (Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L, Ultraflo L) y proteasas (Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG, Alcalase 2.4L FG) comerciales.	(Park et al., 2004)
Scytosiphon lomentaria	Carbohidrasas (AMG, Celluclast, Termamyl, Ultraflo, Viscozyme) y proteasas (Alcalase, Flavourzyme, Kojizyme, Neutrase, Protamex) comerciales.	(Ahn et al., 2004)
Ecklonia cava	Carbohidrasas comerciales (Viscozyme L, Celluclast 1.5L FG, AMG 300L, Ultraflo L)	(Heo et al., 2005)
	Carbohidrasas comerciales (AMG 300L)	(Athukorala et al., 2006)
Hizikia fusiformis	Carbohidrasas (Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L, Ultraflo L) y proteasas (Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG, Alcalase 2.4L FG) comerciales.	(Siriwardhana et al., 2004)

El tiempo de reacción también es un parámetro importante, ya que no todas las enzimas poseen velocidades de hidrólisis rápidas. Una vez terminada la reacción, la materia sólida se separa de la solución por centrifugación. Otros procesos pueden requerir inactivación enzimática, generalmente aumentando la temperatura. Aunque hay aplicaciones sobre el uso de EAE por sí sola para la extracción de compuestos bioactivos (Jeon et al., 2011; Wijesinghe and Jeon, 2012), el empleo de esta técnica es más conveniente si se acopla a otras técnicas avanzadas de extracción, como PLE o SFE, ya que el daño causado a las células ayudará eficazmente a mejorar la penetración de los disolventes en la matriz, aumentando así la velocidad de transferencia de materia y favoreciendo la recuperación de los compuestos objetivo.

Las aplicaciones de la extracción asistida con enzimas en plantas se ha estudiado ampliamente (Puri et al., 2012), si bien los estudios acerca del uso de este proceso integrado para la obtención de compuestos bioactivos a partir de algas, y más específicamente, para la extracción de sus compuestos fenólicos, son escasos. Billakanti et al. (2013) demostraron la eficacia en la extracción de compuestos bioactivos lipídicos del alga marron *Undaria pinnatifida*, utilizando la extracción asistida por enzimas (alginato liasas) y una mezcla de éter dimetílico y etanol. Los resultados de este estudio indicaron que el pretratamiento enzimático mejoraba el rendimiento de extracción de fucoxantina en más del 50% y el de lípidos totales en más del 10%, en comparación con las algas control (no pretratadas). La digestión enzimática de los polisacáridos de la pared celular contribuyó a la liberación de compuestos liposolubles del alga marrón. Por su parte, Zuorro et al. (2016), han optimizado recientemente la extracción asistida por enzimas de lípidos de la microalga *Nannochloropsis sp.* Los resultados de este estudio indicaron que un tratamiento enzimático optimizado basado en el uso combinado de celulasa y β-mananasa mejoraba significativamente la recuperación de los lípidos; en particular, se obtuvieron rendimientos de extracción cercanos al 90% mediante un tratamiento enzimático de 3,5 h a 53 °C, seguido por una extracción convencional con disolvente (hexano/isopropanol (3:2, v/v)) durante 4 h.

1.6 NUEVOS ENFOQUES PARA LA INCREMENTO DE SELECTIVIDAD DE PROCESOS EMPLEANDO LOS PARÁMETROS DE SOLUBILIDAD DE HANSEN.

La teoría de los parámetros de solubilidad se ha empleado desde la primera mitad del siglo pasado. Sin embargo, recientemente, ha recibido una atención especial por su utilidad como herramienta predictiva de la disolución de un soluto en un disolvente (Al-Hamimi et al., 2016; Li et al., 2014, 2016; Pereira et al., 2013; Srinivas and King, 2010). El enfoque de la teoría de los parámetros de solubilidad se basa en el principio de "semejante disuelve a semejante" y puede ser muy favorable para obtener una primera aproximación a los procesos de extracción, indicando el disolvente más adecuado para una aplicación determinada, evitando así la selección de condiciones experimentales poco prácticas. En un esfuerzo por mejorar la aplicabilidad del parámetro de solubilidad total, Hansen (Hansen, 2000) dividió el parámetro de Hildebrand en componentes tridimensionales: E_D, energía de dispersión (relacionada con las fuerzas de Van der Waals), E_P, energía de polaridad (relacionada con el momento dipolar) y E_H, energía de enlace de hidrógeno. Dividiendo estas energías por el volumen molar se obtiene el cuadrado del parámetro de solubilidad total (δ_T^2) como la suma de los cuadrados de los componentes individuales (D, P y H) definidos por las Ecuaciones 1 y 2, como sigue (Hansen, 2000; King, 2014):

$$\frac{E_T}{V} = \frac{E_D}{V} + \frac{E_P}{V} + \frac{E_H}{V} \qquad \text{Ec. (1)}$$

$$\delta_T(MPa^{1/2}) = \sqrt{\delta_D^2 + \delta_P^2 + \delta_H^2} \qquad \text{Ec. (2)}$$

Los parámetros de solubilidad de Hansen (HSP) se han empleado como una estimación numérica para predecir la solubilidad de muchos productos industriales tales como polímeros, biopolímeros, fármacos, pigmentos, y materiales de origen biológico, entre otros. Es importante tener en cuenta que Hansen se basa en modelos puramente termodinámicos y no en modelos cinéticos (Hansen, 2000).

Los tres parámetros individuales se pueden visualizar en un espacio tridimensional, como se muestra en la Figura 1.8, mientras que la interacción entre un disolvente y un soluto se puede representar como una esfera. De esta manera, para saber si un disolvente es bueno o malo para una extracción selectiva, es necesario calcular el valor de diferencia de energía relativa (RED), que es la relación de dos parámetros R_a y R_O (Ecuación 3).

$$RED = R_a/R_0 \qquad \text{Ec. (3)}$$

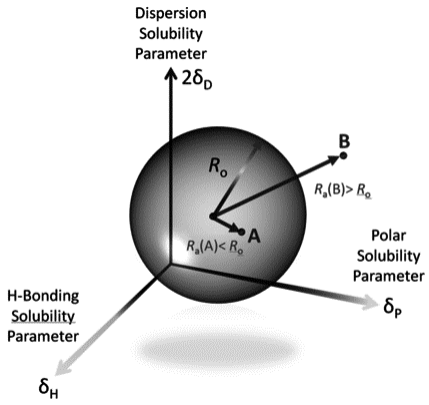


Figura 1.8. Esquema de visualización tridimensional de los parámetros de solubilidad de Hansen (HSP). Adaptado de Hansen (2000).

El modelo de Hansen considera a "R_a" como la distancia teórica entre un disolvente *j* y un soluto *i* (el centro de la esfera) en términos de sus parámetros de solubilidad y se puede calcular tal y como se describe en la ecuación 4. El criterio empleado para evaluar el parámetro R_a se basa en que cuanto menor sea su valor más apropiado será el disolvente para solubilizar el soluto. Por otra parte, R_O o "radio de la esfera" se calcula mediante observaciones experimentales, probando diferentes disolventes que crearan el espacio de la "esfera de solubilidad". Este espacio se refiere a la región particular donde las combinaciones disolvente-soluto ocurren como una solución, en cuyo radio se incluyen todos los disolventes "buenos" ($R_a < R_O$) y se excluye los disolventes "malos" fuera de la esfera ($R_a > R_O$) (Figura 1.8).

$$R_a = \sqrt{4\left(\delta_{Di}-\delta_{Dj}\right)^2 + \left(\delta_{Pi}-\delta_{Pj}\right)^2 + \left(\delta_{Hi}-\delta_{Hj}\right)^2} \qquad \text{Ec. (4)}$$

De esta forma, si RED = 0, no existen diferencias de energía, lo que indica un "disolvente perfecto"; Si RED <1,0 indica afinidad alta; si RED = 1,0 indica la condición límite; y si RED> 1,0 indica una afinidad baja entre el soluto y el disolvente.

La estimación de la solubilidad de una sustancia depende en gran medida de la disponibilidad de datos de los parámetros de solubilidad de la molécula objeto de estudio y del disolvente a emplear. Actualmente, existen herramientas computacionales que cuentan con bases de datos donde se encuentran disponibles los parámetros de solubilidad de un gran número de disolventes (Li et al., 2014). Cuando no existe suficiente información en las bases de datos sobre una molécula, más específicamente sobre el soluto, los métodos de contribución de grupo (GCM) son la forma más común de predecir las propiedades y los parámetros de solubilidad a partir de estructuras moleculares, utilizando reglas aditivas (Grulke, 1999). Para la estimación de propiedades de compuestos puros (Kolská et al., 2012), se han empleado, con algunas limitaciones, algunos GMC como los dados por Lydersen (1955), Klincewicz y Reid (1984) y Joback y Reid (1987), entre otros. Sin embargo, algunos métodos más recientes han introducido incluso grupos de segundo y tercer orden para mejorar la capacidad predictiva para representar moléculas complejas (Gani and Constantinou, 1996; Marrero and Gani, 2001). Por otra parte, siguiendo el mismo enfoque descrito por Hansen, Small (1953), Hoy (1970), Fedors (1974) y Van Krevelen y Hoftyzer, (1976) desarrollaron algunas técnicas de contribución de grupos para estimar los parámetros de solubilidad de una amplia variedad de compuestos (Grulke, 1999; Hansen, 2000). Aunque algunas de estas predicciones solo son válidas en condiciones normales, se han desarrollado algunos métodos para corregir el efecto de la presión y la temperatura en condiciones supercríticas y subcríticas, debido a la creciente importancia de este tipo de procesos de extracción (Machida et al., 2011; Marcus, 2012, 2006; Williams et al., 2004).

Existen algunos ejemplos en la bibliografía reciente en los que la estimación de los HSP se indica como una herramienta útil para optimizar la extracción presurizada de metabolitos bioactivos a partir de fuentes naturales (Al-Hamimi et al., 2016; Filly et al., 2014; Kagliwal et al., 2011; Sajilata et al., 2010; Srinivas et al., 2009; Srinivas and King, 2010). Srinivas y King

(2010) estimaron los HSP de algunos carotenoides presentes en especias (tales como pimienta negra, canela, ajo, jengibre) en SC-CO_2 a diferentes temperaturas y presiones. A su vez, recientemente, también se han empleado los parámetros de solubilidad para evaluar la eficacia de varios disolventes alternativos para extraer compuestos aromáticos, grasas y aceites, y carotenoides, a partir de diferentes matrices alimenticias, con el fin de sustituir disolventes petroquímicos (tales como hexano y diclorometano) (Bundeesomchok et al., 2016; Filly et al., 2014; Li et al., 2014; Sicaire et al., 2015; Yara-Varón et al., 2016). De esta forma, el enfoque teórico desarrollado por Hansen, puede ser ventajoso en el desarrollo de procesos selectivos de extracción de compuestos con valor añadido a partir de fuentes naturales. Otra de las ventajas de utilizar este enfoque para elegir el disolvente más apropiado para la extracción es que permite reducir el número de experimentos a llevar a cabo. Además, dado que las extracciones son más selectivas, se reducen las etapas de purificación, promoviendo una reducción en la generación de residuos, lo que es muy importante si se desea lograr procesos medioambientalmente limpios desde un enfoque sostenible.

1.7 EMPLEO DE NUEVOS DISOLVENTES ALTERNATIVOS: BIO-DISOLVENTES

Otro aspecto que se debe tener en cuenta para el desarrollo de procesos respetuosos con el medio ambiente es la selección del tipo de disolventes de extracción a emplear. Diferentes organizaciones han establecido clasificaciones de "*greenness*" de los disolventes atendiendo a diferentes parámetros, por ejemplo: impacto ambiental, salud, seguridad, evaluación del ciclo de vida (LCA), residuos, etc. (Byrne et al., 2016; Capello et al., 2007). En este sentido, se ha comenzado a explorar el potencial uso de bio-disolventes para el desarrollo de procesos de extracción sostenibles, siendo este uno de los retos más difíciles a los que las industrias químicas y farmacéuticas se enfrentan hoy en día, ya que, además, deben contar con características tecnológicas y de proceso similares o mejoradas (Li et al., 2016).

Los bio-disolventes son sustancias de origen vegetal totalmente biodegradables y que se han propuesto como alternativa a los disolventes petroquímicos (Byrne et al., 2016). Pueden obtenerse mediante procesos de fermentación, esterificación o mediante procesos enzimáticos (Clark et al., 2015). Además del CO_2, otros bio-disolventes poseen importantes características y

ventajas como disolventes de extracción, ya que son comparables con los disolventes orgánicos volátiles convencionales en términos de rendimiento y selectividad en la extracción de compuestos a partir de matrices naturales (Li et al., 2016).

Por ejemplo, el terpeno d-limoneno (Figura 1.9a), es el principal componente de los aceites esenciales extraídos de cáscaras de cítricos y se ha sugerido como una valiosa alternativa ecológica a disolventes como el hexano y los hidrocarburos halogenados (Aissou et al., 2016). El d-limoneno posee una constante dieléctrica muy próxima a la del hexano (Yara-Varón et al., 2016), y se ha empleado para la extracción de aceite de salvado de arroz, aceite de los residuos de oliva, carotenoides a partir de tomate o algas y, recientemente, para la extracción de lípidos de algas (Aissou et al., 2016; Golmakani et al., 2014).

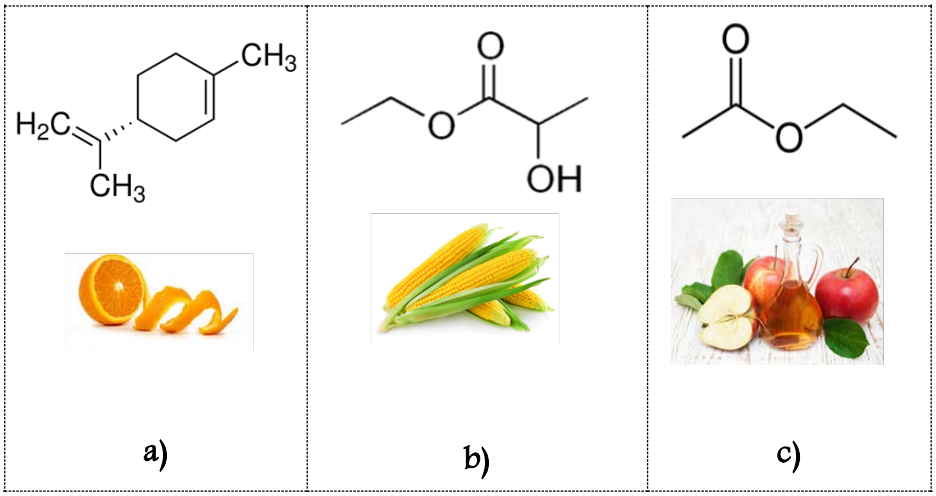


Figura 1.9. Estructuras moleculares de algunos bio-disolventes empleados en la presente Tesis Doctoral. a) d-limoneno, b) lactato de etilo y c) acetato de etilo

.

Por su parte, el lactato de etilo (2-hidroxipropanoato de etilo) (Figura 9b) es una alternativa agroquímica y económicamente viable a los disolventes líquidos tradicionales, ya que es totalmente biodegradable, no corrosivo, no cancerígeno y no destruye la capa de ozono (Pereira et al., 2011). Proviene de la esterificación de ácido láctico con etanol, que se lleva a cabo en biorrefinerías a partir de subproductos agrícolas ricos en carbohidratos, como por ejemplo, residuos de maíz o residuos de la industria azucarera (Pereira et al., 2011). El lactato

de etilo es reconocido como una sustancia tipo GRAS y debido a su baja toxicidad se aprobó como aditivo farmacéutico y alimentario por parte de la Administración de Alimentos y Medicamentos de los Estados Unidos (FDA) (Bermejo et al., 2015). Estas características han despertado el interés de su empleo como un disolvente verde para la industria alimentaria. Su constante dieléctrica de 15,7 a 25 °C, hace que se le pueda considerar un disolvente moderadamente polar (Aparicio and Alcalde, 2009). En la bibliografía se pueden encontrar varias aplicaciones ya publicadas relacionadas con la extracción de carotenoides a partir de residuos de tomate (Strati and Oreopoulou, 2011), la extracción de ácido linolénico a partir de la microalga *Spirulina* (Golmakani et al., 2012), el fraccionamiento de compuestos de aceite comestible (escualeno y tocoferol) (Hernández et al., 2011) y la extracción de cafeína a partir de hojas de té verde (Bermejo et al., 2015), entre otros.

Por su parte, el acetato de etilo (Figura 1.9c) es un bio-disolvente orgánico importante y ampliamente utilizado en la producción de barnices, tinta, resinas sintéticas y agentes adhesivos. Se emplea comercialmente en la industria de alimentos para el proceso de descafeinado de café y hojas de té, ya que presenta una toxicidad mucho menor que los disolventes clorados (Bermejo et al., 2015). Comúnmente, se produce por reacción de esterificación de ácido acético con etanol. En el año 2012 fue catalogado como un bio-disolvente "recomendado" de acuerdo a una guía de selección de disolventes, analizada y recopilada por seis importantes compañías farmacéuticas pertenecientes a la Fundación Europea de Industrias y Asociaciones Farmacéuticas (EFPIA), diez universidades y cinco pequeñas y medianas empresas; esta selección se publicó en la revista *Green Chemistry* (Prat et al., 2014). Esta categoría considera criterios como la seguridad, la salud ocupacional, el medio ambiente, la calidad (riesgo de impurezas en la sustancia farmacéutica), las limitaciones industriales (por ejemplo, punto de ebullición, temperatura de congelación, densidad, reciclabilidad) y el coste.

CAPÍTULO 2.
JUSTIFICACIÓN Y OBJETIVOS

2. JUSTIFICACIÓN Y OBJETIVOS

En los últimos años, el desarrollo de nuevos procesos para la extracción de compuestos bioactivos de algas y plantas ha recibido gran atención. Las metodologías tradicionales que emplean disolventes orgánicos para la extracción de dichos compuestos (tales como cloroformo, acetona, hexano, etc.) presentan importantes desventajas ya que es necesario usar grandes cantidades de disolventes así como varias etapas de extracción para obtener rendimientos considerables. Además, la creciente preocupación medioambiental por el uso de este tipo de disolventes ha redireccionado la investigación e innovación tecnológica hacia el desarrollo de procesos alternativos de producción, que vayan de la mano del concepto de desarrollo sostenible. En la última década, la extracción de compuestos bioactivos empleando fluidos presurizados (PLE y SFE) se ha aplicado ampliamente a múltiples fuentes naturales, utilizando varios tipos de bio-disolventes y condiciones de proceso, demostrando así su gran potencial tecnológico. Sin embargo, es necesario emplear un enfoque más amplio que proporcione una mayor eficiencia de extracción y un mejor aprovechamiento de las fuentes naturales de partida, ya que éstas cuentan en su composición química con diferentes tipos de compuestos bioactivos, que no pueden obtenerse aplicando solo una operación unitaria de proceso. De esta manera, la integración e intensificación de procesos permiten incorporar diferentes etapas (pretratamiento de la muestra, reacción, extracción selectiva y purificación) de manera combinada en un mismo protocolo de procesamiento, y como en el caso de la intensificación, en un mismo equipamiento. Teniendo en cuenta este enfoque, el concepto de selectividad adquiere gran importancia y la selección del bio-disolvente juega un papel crucial. Así, el uso de herramientas de predicción, como la estimación de los parámetros de solubilidad de Hansen, permite acotar y enfocar la selección del disolvente de extracción hacia los compuestos de interés.

Una fuente importante de polifenoles que se ha estudiado extensamente en los últimos años empleando esta metodología de extracción es el romero (*Rosmarinus officinalis*). Existen varias razones para la selección de esta fuente natural de polifenoles en la presente Tesis Doctoral, entre ellas, su potente actividad antioxidante (con variadas aplicaciones como

conservante en la industria de alimentos) y, principalmente, la existencia de evidencias científicas de su capacidad para inhibir la proliferación celular en varios modelos de cáncer, incluyendo el cáncer de colon. Este tipo particular de cáncer, constituye un importante problema de salud pública en España y a nivel mundial, debido a su elevada incidencia. Por su parte, el mar y su biodiversidad constituyen una fuente casi inagotable, y en gran medida inexplorada, de compuestos bioactivos, siendo las algas marinas uno de los recursos más prometedores como fuente de nuevas estructuras químicas, con actividad biológica. Estos organismos son capaces de sintetizar y acumular en determinadas condiciones diferentes metabolitos secundarios, tales como florotaninos y carotenoides (como fucoxantina) que ya se han descrito como inhibidores de la proliferación celular *in vitro* en modelos de cáncer de colon.

Estas consideraciones han motivado los estudios llevados a cabo en la presente Tesis Doctoral, en la que se ha buscado **desarrollar nuevas estrategias de extracción para la obtención de compuestos bioactivos empleando, como fuentes naturales, romero (*Rosmarinus officinalis*), dos tipos de algas marrones, *Sargassum muticum* y *Cystoseira abies-marina*, y una microalga, *Phaeodactylum tricornutum*.** El desarrollo de los mencionados procesos alternativos permitiría el aprovechamiento de recursos naturales cercanos, empleando tecnologías limpias de obtención y generando nuevos conocimientos sobre la bioactividad de los extractos naturales generados en células de adenocarcinoma de colon humano.

Teniendo en cuenta lo mencionado anteriormente, el proyecto de Tesis Doctoral se llevó a cabo en dos fases, cuyos objetivos se describen a continuación:

Fase I. Uso de fluidos presurizados para la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol

a. Objetivo General

Desarrollar nuevas metodologías de extracción basadas en la integración e intensificación de procesos a escala piloto que conduzcan a la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol, principalmente, a fin de llevar a cabo

ensayos de confirmación de su actividad antiproliferativa *in vitro* en células de cáncer de colon humano.

b. Objetivos Específicos

1. Evaluar el uso de dos etapas secuenciales de extracción empleando CO_2 supercrítico para la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol y estudiar su actividad antiproliferativa en células de cáncer de colon, comparándolos con los resultados alcanzables empleando un extracto supercrítico obtenido en una sola etapa.

2. Optimizar el proceso integrado, a escala de laboratorio, para el fraccionamiento supercrítico antisolvente (SAF) de extractos hidro-etanólicos de romero obtenidos por extracción con líquidos presurizados (PLE), con el fin de obtener dos fracciones bioactivas, una enriquecida en ácido carnósico y carnosol y otra en ácido rosmarínico, así como proceder a la evaluación de su actividad biológica en células de cáncer de colon.

3. Realizar un estudio comparativo de los cuatro tipos de procesos desarrollados para la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol: a) PLE (escala laboratorio); b) SFE en una sola etapa; c) SFE en dos etapas secuenciales, d) PLE + SAF (escala piloto) y evaluar su potencial para obtener extractos de romero con actividad antiproliferativa *in vitro* en dos líneas celulares de cáncer de colon.

Fase II. Obtención de extractos ricos en polifenoles y carotenoides a partir de algas marinas empleando fluidos presurizados

a. Objetivo General

Desarrollar nuevas estrategias de extracción para la obtención de extractos enriquecidos en compuestos bioactivos a partir de macro- y microalgas, empleando para ello procesos combinados, disolventes alternativos y herramientas teóricas de predicción de solubilidad con el fin de proponer procesos más eficientes y selectivos.

Objetivos Específicos

1. Evaluar el potencial aumento de la eficiencia de extracción para el aislamiento de florotaninos a partir del alga marrón *Sargassum muticum,* empleando extracción asistida con enzimas seguida de extracción con líquidos presurizados.

2. De acuerdo a los resultados obtenidos en el objetivo anterior, estudiar la influencia de la ubicación geográfica en el contenido y tipo de florotaninos extraídos empleando líquidos presurizados a partir de muestras de *Sargassum muticum* recogidas en 13 lugares diferentes a lo largo de las costas del Atlántico Norte, así como corroborar su potencial actividad antiproliferativa en células de cáncer de colon.

3. Estimar los parámetros de solubilidad de Hansen para los florotaninos más abundantes encontrados en el alga marrón *Cystoseira abies-marina* en bio-disolventes subcríticos y supercríticos con el fin de diseñar un nuevo protocolo de extracción para su aislamiento selectivo.

4. Desarrollar un método de extracción eficiente y selectivo de fucoxantina a partir de la microalga *Phaeodactylum tricornutum* mediante la predicción teórica de los parámetros de solubilidad de Hansen de 5 bio-disolventes presurizados (CO_2, etanol, acetato de etilo, lactato de etilo y d-limoneno), evaluando dichas predicciones experimentalmente.

CAPÍTULO 3.
PLAN DE TRABAJO

3. PLAN DE TRABAJO

Teniendo en cuenta los antecedentes expuestos y con el fin de alcanzar los objetivos planteados en la presente Tesis Doctoral, se siguió el plan de trabajo que se describe a continuación, y que se muestra de forma esquemática en las Figuras 3.1 y 3.2. Los planes de trabajo se ciñen a las fases de ejecución mencionadas en los objetivos.

En la primera parte de esta Tesis Doctoral (Fase I), se ha estudiado el desarrollo de nuevas estrategias de extracción de compuestos bioactivos a partir de romero y su actividad en células de cáncer de colon (Figura 3.1).

A continuación, se detalla el plan de trabajo seguido para el desarrollo de esta primera fase:

1. Obtención de extractos supercríticos de romero en una única etapa a escala piloto, empleando parámetros de extracción previamente optimizados en el grupo de investigación.
2. Selección y ensayo de diferentes condiciones de extracción para la obtención de extractos supercríticos de romero a escala piloto en dos etapas secuenciales.
3. Caracterización química y funcional de los extractos y fracciones obtenidas en los apartados 1 y 2.

    3.1 Identificación del perfil de compuestos fenólicos y cuantificación de ácido carnósico y carnosol mediante cromatografía líquida de alta eficacia acoplada a espectrometría de masas (UHPLC-DAD-MS).

    3.2 Identificación del perfil de compuestos volátiles mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS)

    3.3 Cuantificación de fenoles totales (TPC) (método de Folin-Ciocalteu) y determinación de la actividad antioxidante utilizando los métodos de captación de radicales libres: 2,2-diphenyl-1-picrylhydrazyl (DPPH) y 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).

3.4 Determinación del efecto antiproliferativo de los extractos obtenidos en la línea celular de cáncer de colon HT-29 mediante el método colorimétrico MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide).

4. Selección de las mejores condiciones de extracción del proceso en dos etapas secuenciales.
5. Obtención de un extracto de romero usando una mezcla de etanol y agua en condiciones subcríticas (PLE) previamente optimizadas, para su uso como extracto de partida del posterior proceso de fraccionamiento supercrítico antisolvente (SAF) a escala de laboratorio.
6. Optimización del proceso SAF a escala de laboratorio empleando un diseño experimental de tres factores y tres niveles: (i) presión de CO_2, (ii) porcentaje de agua en el extracto de PLE y (iii) relación másica de flujo de extracto de PLE/SC-CO_2.
   6.1 Estudio teórico de la selectividad del sistema de equilibrio ternario de fases SC-CO_2–Etanol–H_2O
7. Caracterización química y funcional tanto del extracto PLE de partida como de las fracciones resultantes del proceso SAF (*refinado* y *extracto,* para cada condición experimental), bajo los parámetros descritos a continuación:
   7.1 Cuantificación de ácido rosmarínico, ácido carnósico y carnosol mediante UHPLC-DAD-MS.
   7.2 Cuantificación de fenoles totales y determinación de la actividad antioxidante utilizando los métodos de radicales libres DPPH y ABTS.
   7.3 Determinación del efecto antiproliferativo en la línea celular de cáncer de colon HT-29 mediante el método colorimétrico MTT
8. Selección de las mejores condiciones de extracción obtenidas del diseño experimental del proceso SAF a escala laboratorio, para ser ensayadas a escala piloto.
9. Adecuación y puesta en marcha del equipo de extracción supercrítica para llevar a cabo el proceso SAF a escala piloto.
10. Obtención de *extractos* empleando el proceso SAF a escala piloto.
11. Caracterización funcional de los *extractos* obtenidos, empleando los parámetros descritos en el apartado 7.1 y 7.2.

12. Caracterización química de los mejores extractos obtenidos de los tres procesos de extracción estudiados a escala piloto: i) SFE en una única etapa, ii) SFE en dos etapas secuenciales y iii) SAF (así como del extracto PLE de partida) por medio de cromatografía de líquidos acoplada a espectrometría de masas con analizador cuadrupolo-tiempo de vuelo (LC-QTOF-MS).
13. Determinación del efecto antiproliferativo de los extractos mencionados en el punto anterior en las líneas de cáncer de colon HT-29 y HCT116 mediante el método colorimétrico MTT.
14. Análisis global de la relación del perfil y contenido de compuestos fenólicos de cada tipo de extracción estudiada en respuesta a la actividad antiproliferativa en cada línea celular ensayada.

En la segunda parte de esta Tesis Doctoral (Fase II), se ha investigado el desarrollo de estrategias de extracción para la obtención de extractos enriquecidos en compuestos bioactivos a partir de macro y microalgas. En la Figura 4.2 se presenta un esquema general de las etapas llevadas a cabo.

A continuación, se detalla el plan de trabajo seguido para el desarrollo de esta fase y se divide de acuerdo al tipo de alga empleada:

Sargassum muticum (Francia)

15. Obtención de extractos hidrolizados a partir de *Sargassum muticum* empleando dos tipos de enzimas (una carbohidrasa y una peptidasa) y estudio de diferentes tiempos de hidrólisis.
16. Caracterización funcional de los hidrolizados obtenidos, empleando los métodos de cuantificación de fenoles totales y determinación de la actividad antioxidante utilizando el método del radical libre ABTS.

17. Estudio del proceso integrado de extracción asistida por enzimas (EAE) seguido de extracción con líquidos presurizados (PLE), empleando como material de partida el residuo de alga posterior a la hidrólisis.
18. Caracterización química y funcional de los extractos obtenidos por medio de HPLC-DAD-MS y tal y como se ha descrito en el ítem 16, respectivamente.
19. Optimización de las condiciones de PLE empleando un diseño experimental de dos factores a tres niveles: i) temperatura de extracción y ii) contenido de etanol en la mezcla disolvente.
20. Caracterización química y funcional de los extractos obtenidos para cada condición de diseño, bajo los parámetros descritos a continuación:
    20.1 Cuantificación de fenoles totales, determinación de la actividad antioxidante utilizando el método del radical libre ABTS y cuantificación de los florotaninos totales, mediante el método colorimétrico DMBA (2,4-dimetoxy benzaldehide).

Sargassum muticum (13 localizaciones, costa Atlántica Europea)

21. De acuerdo a las condiciones optimizadas de extracción de PLE (ítem 19), obtención de extractos a partir de 13 muestras de *Sargassum muticum*, recogidas en diferentes localizaciones a lo largo de las costas del Atlántico Norte.
22. De cada extracto, obtención de una fracción enriquecida en florotaninos empleando protocolos de purificación líquido-líquido.
23. Caracterización química y funcional de las fracciones purificadas, por medio de cromatografía de líquidos bidimensional completa acoplada a espectrometría de masas (LC × LC-MS/MS) y tal y como se ha descrito en el ítem 20.1, respectivamente.
24. Selección de los extractos con mayor contenido en florotaninos y estudio de su efecto antiproliferativo en las células de cáncer de colon HT-29, mediante el método colorimétrico MTT.

Cystoseira abies-marina (Banco Español de Algas, Las Palmas de Gran Canaria, España):

25. Obtención de extractos a partir de *Cystosiera abies-marina* empleando PLE y extracción sólido-líquido, en condiciones previamente optimizadas.
26. Obtención de una fracción enriquecida en florotaninos, empleando el protocolo de purificación previamente descrito en ítem 22.
27. Identificación tentativa del tipo y grado de polimerización de los florotaninos presentes en los extractos purificados de *C. abies-marina,* por medio de LC × LC-MS/MS
28. Estimación teórica de los parámetros de solubilidad de Hansen para los florotaninos más abundantes, en cuatro bio-disolventes presurizados
29. Selección de las mejores condiciones teóricas de extracción, que proporcionen la mayor selectividad para el aislamiento de florotaninos.
30. Ensayo experimental de las condiciones seleccionadas y comparación con el análisis teórico.

Phaeodactylum tricornutum (Fitoplacton marino S.A., Cádiz, España)

31. Estimación teórica de los parámetros de solubilidad de Hansen de fucoxantina en cinco bio-disolventes seleccionados en condiciones sub-y supercríticas.
32. Determinación experimental del radio de interacción de la esfera de Hansen (R_0) para la fucoxantina estándar.
33. Evaluación experimental de los diferentes bio-disolventes empleando PLE y SFE.
34. Determinación de la selectividad de los bio-disolventes en la extracción de fucoxantina, sometiendo a los extractos obtenidos a las siguientes técnicas de análisis:
    - 34.1 Contenido de carotenoides y clorofilas totales, por medio de un método espectrofotométrico.
    - 34.2 Cuantificación de E-fucoxantina por medio de HPLC-DAD-APCI-MS/MS.
35. Comparación del análisis teórico frente a los datos experimentales y selección del disolvente más selectivo.
36. Optimización del proceso de extracción de SFE y PLE empleando los disolventes más selectivos.

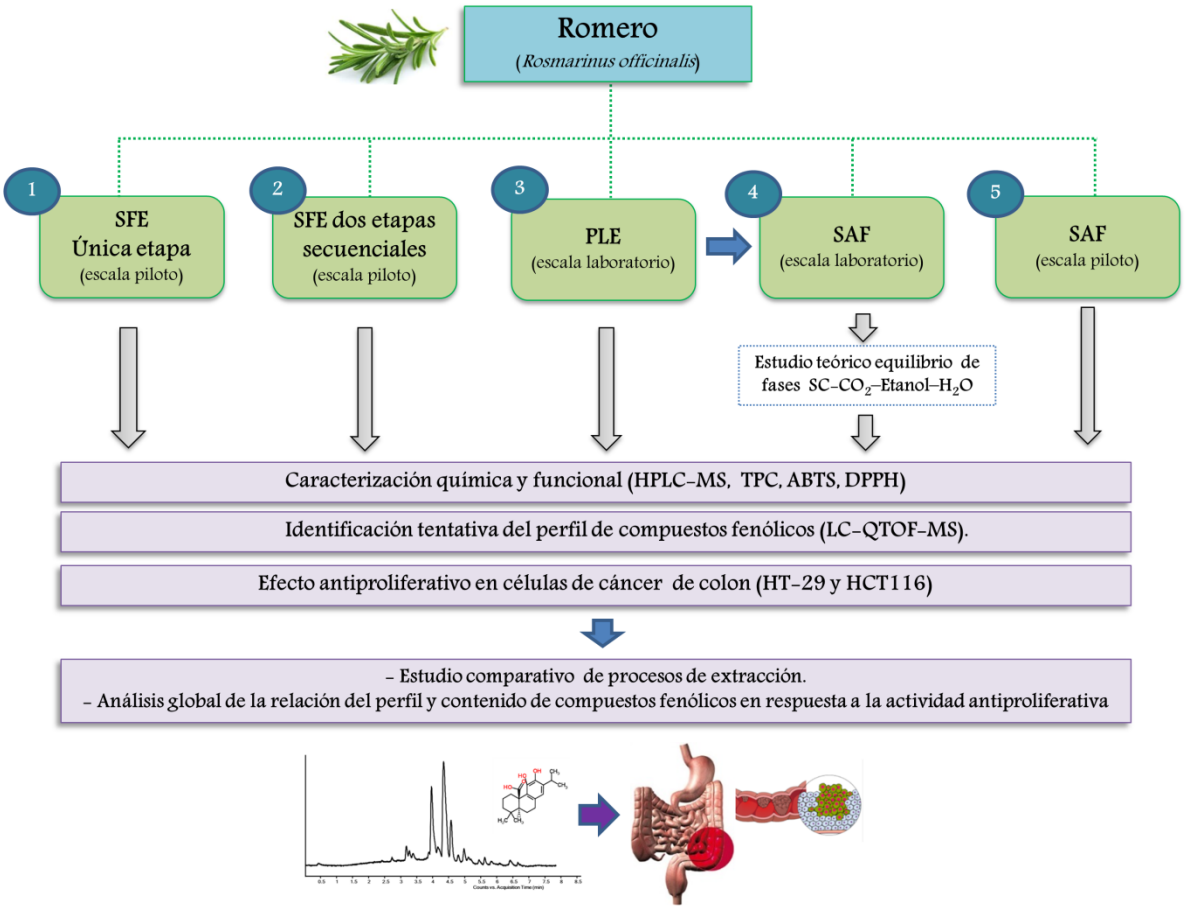


Figura 3.1. Esquema general de la primera fase del plan de trabajo llevado a cabo en la presente Tesis Doctoral.

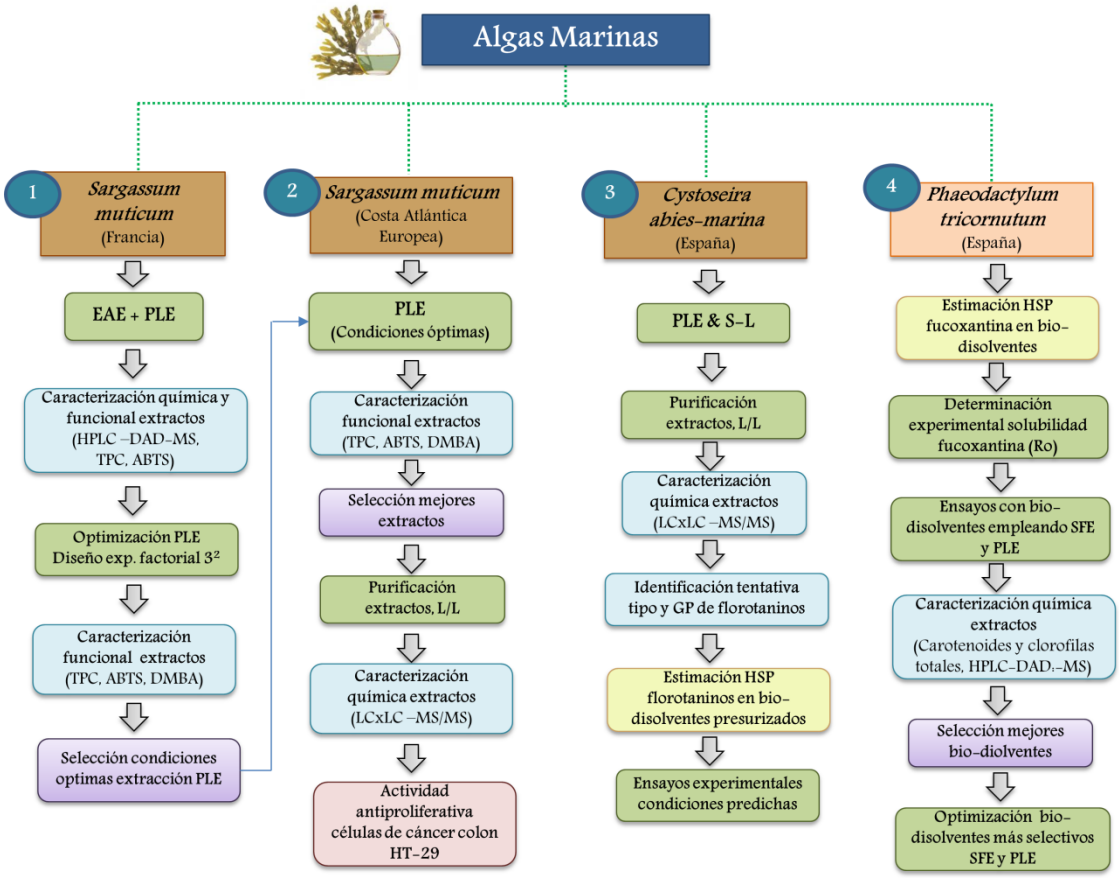


Figura 3.2. Esquema general de la segunda fase del plan de trabajo llevado a cabo en la presente Tesis Doctoral. GP: Grado de polimerización.

CAPÍTULO 4.
RESULTADOS Y DISCUSIÓN

4. RESULTADOS Y DISCUSIÓN

4.1 Uso de fluidos presurizados para la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol

4.1.1 Prefacio

Hoy en día, la extracción con fluidos supercríticos empleando CO_2 es un proceso industrial establecido para la obtención de productos de alto valor añadido. Los datos del año 2014 indicaban que había más de 150 plantas industriales de SFE en el mundo, con un volumen total de extracción de más de 500 L. Muchas de estas plantas de producción se dedican generalmente a la SFE de compuestos bioactivos o "moléculas diana" procedentes de fuentes naturales (King, 2014) con fines nutracéuticos y funcionales. Como se ha descrito en la introducción de esta Memoria, la recuperación de compuestos bioactivos de romero empleando fluidos sub- y supercríticos se ha intensificado en los últimos 10 años, debido principalmente a la variedad de propiedades biológicas que se le atribuyen, entre ellas su actividad antiproliferativa en células de cáncer de colon. En el grupo de investigación de Alimentómica, el desarrollo de procesos de extracción verdes aplicados a romero se ha centrado principalmente en el diseño de nuevas estrategias para aumentar la concentración de ácido carnósico y carnosol en extractos de romero, ya que estos compuestos se han descrito como los principales responsables de la actividad antiproliferativa en líneas celulares de cáncer de colon. En trabajos previos realizados en el laboratorio, se han estudiado distintos procesos de extracción tales como extracción con agua subcrítica y formación de partículas on-line (WEPO, Water Extraction and Particle formation On-line) y PLE empleando agua y etanol a diferentes temperaturas (50-200 °C). Sin embargo, la extracción en una única etapa empleando SC-CO_2 a 40 °C, 15.0 MPa, durante 300 min de extracción y 7% (p/p) de etanol como modificador, se ha destacado como el proceso más adecuado para el enriquecimiento en ácido carnósico y carnosol en el extracto resultante (Herrero et al., 2010). Teniendo en cuenta estos antecedentes, en la presente Tesis Doctoral se propuso una nueva estrategia de intensificación del proceso de extracción supercrítica en dos etapas secuenciales (Figura 4.1):

una primera etapa (15,0 o 30,0 MPa, 40°C, CO_2 puro, 60 min) donde se eliminan los compuestos menos activos, principalmente compuestos volátiles, ácidos grasos y ceras; y una segunda etapa (15.0 MPa, 40°C, CO_2 + 7% (p/p) etanol, 120 min) donde se obtiene un extracto enriquecido en compuestos fenólicos, principalmente ácido carnósico y carnosol.

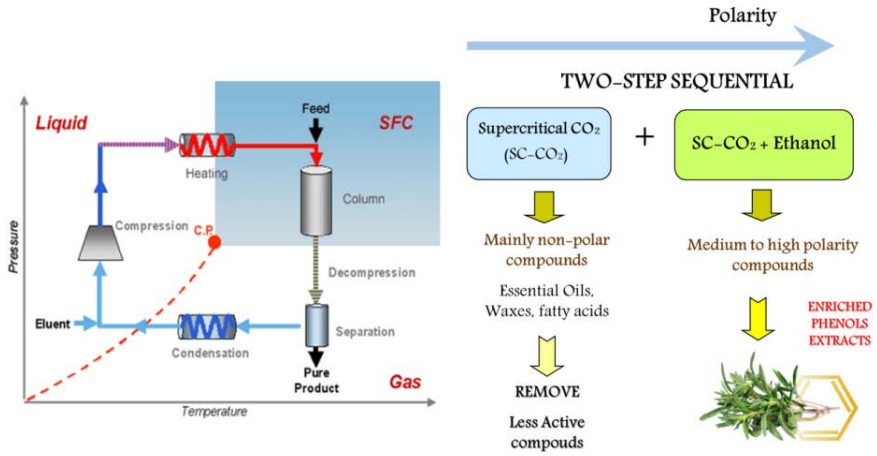


Figura 4.1. Proceso intensificado en dos etapas secuenciales empleando extracción de fluidos supercríticos.

A continuación, en la sección 4.1.2 se incluyen los resultados de este estudio titulado *"Two-step sequential supercritical fluid extracts from rosemary with enhanced antiproliferative activity"* de Sánchez-Camargo et al. publicado en la revista Journal of Functional Foods 2014, 11, 293–303.

Una vez obtenidos los extractos empleando el enfoque de i) una única etapa y ii) dos etapas secuenciales, bajo las condiciones descritas previamente a escala piloto, los extractos se caracterizaron química y funcionalmente. Con el fin de poder correlacionar el contenido de fenoles totales (ensayo que emplea el reactivo de Folin-Ciocalteu) y la actividad antioxidante de los extractos con el efecto antiproliferativo de los mismos, se realizaron análisis *in vitro* de actividad antioxidante, los cuales se basaron en la medición de la capacidad que tienen los compuestos antioxidantes presentes en los extractos para reaccionar con un radical libre determinado (ABTS y DPPH). En este trabajo, el empleo de herramientas de análisis como UHPLC-DAD-MS permitió la identificación tentativa del perfil de compuestos fenólicos presentes en la primera y segunda etapa del proceso de extracción secuencial, así como el del proceso en una única etapa. Por otra parte, el perfil de compuestos volátiles presentes en las

fracciones obtenidas del proceso secuencial se analizó por medio de GC-MS. Posteriormente, se llevó a cabo la determinación de la actividad antiproliferativa *in vitro* tanto de las fracciones enriquecidas en compuestos volátiles como de los extractos de romero concentrados en compuestos fenólicos, en la línea celular de cáncer de colon HT-29 y se observó la respuesta a tres concentraciones diferentes de los extractos durante tiempos de incubación de 24, 48 y 72 h.

Teniendo en cuenta que el enriquecimiento en ácido carnósico y carnosol en los extractos estudiados resultó en una disminución de la proliferación celular, la siguiente etapa del trabajo consistió en proponer otra estrategia de extracción integrando dos procesos, PLE y SAF, con el fin de producir una fracción aún más enriquecida en ácido carnósico y carnosol, que fuese más activa en las células de cáncer de colon HT-29. Este estudio se presenta en la sección 4.1.3, y corresponde al trabajo titulado "*Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity*" de Sánchez-Camargo et al., publicado en la revista Journal of Supercritical Fluids (2016), 107, 581–589. En este trabajo, previo al desarrollo experimental, se realizó un análisis teórico de balance de materia de los proceso PLE (*"upstream process"*) y SAF (*"downstream process"*), y de los principales factores que influyen en la selectividad del sistema de equilibrio de fases ternario SC-CO_2–Etanol-H_2O (Figura 4.2b). Una vez seleccionados dichos factores, es decir, presión de CO_2, porcentaje de agua en el extracto de PLE y relación másica de flujo de extracto de PLE/SC-CO_2, se formuló un diseño experimental factorial para su optimización a escala de laboratorio. En la fase experimental, inicialmente se obtuvieron extractos hidroalcohólicos de romero empleando PLE. Este extracto fue sometido a fraccionamiento antisolvente empleando una corriente de SC-CO_2, obteniéndose dos fracciones enriquecidas en diferentes familias de compuestos presentes en el romero: la fracción *refinado* (enriquecida en ácidos fenólicos, principalmente ácido rosmarínico) y la fracción *extracto* (enriquecida en diterpenos fenólicos, principalmente ácido carnosico y carnosol) (Figura 4.2a). Para evaluar la efectividad del proceso se estudiaron como variables respuesta entre otros, el porcentaje de recuperación, el contenido de ácido carnósico, carnosol y ácido rosmarínico, el contenido de fenoles totales y la actividad antioxidante, empleando los métodos anteriormente mencionados. También se realizó

un análisis comparativo entre la selectividad teórica y la selectividad experimental. A su vez, se estudió la actividad antiproliferativa de los compuestos presentes tanto en la fracción *extracto* como en la fracción *refinado*, en células de cáncer de colon HT-29 a una concentración seleccionada durante 24 h, para cada una de las condiciones propuestas en el diseño experimental.

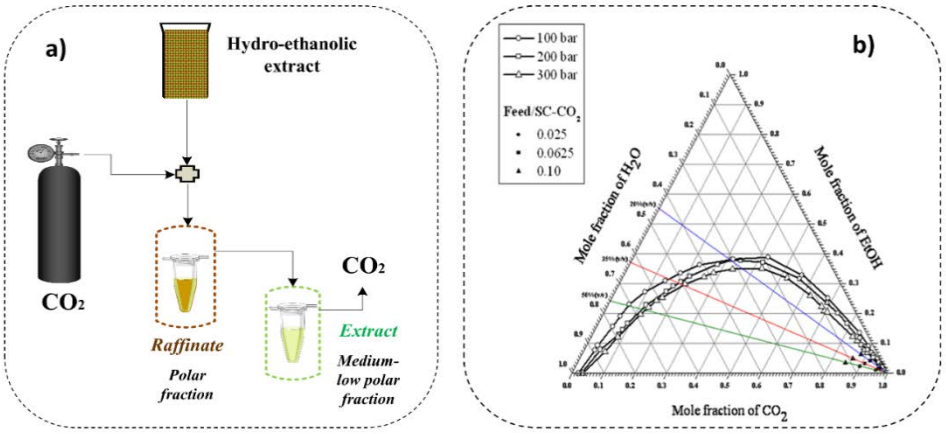


Figura 4.2. a) Esquema básico de un sistema de fraccionamiento supercrítico antisolvente, b) Diagrama de equilibrio de fases para el sistema de CO_2–etanol–agua a 40 °C a 10,0, 20,0 y 30,0 MPa. Porcentaje de agua en la alimentación: 20% (v/v) (línea azul), 35% (v/v) (línea roja) y 50% (v/v) (línea verde). Los datos de equilibrio de fase se han adaptado de Durling et al., 2007b.

Una vez optimizadas las condiciones del proceso integrado PLE + SAF a escala de laboratorio, se implementó y adecuó un equipo de extracción supercrítica para la etapa SAF a escala piloto. Se seleccionaron tres condiciones de extracción de acuerdo a los resultados de la optimización. Posteriormente, se llevó a cabo un estudio comparativo de los procesos de extracción previamente estudiados y la actividad biológica de los extractos obtenidos en dos líneas celulares de cáncer de colon. Los resultados de este este estudio se presentan en la sección 4.1.4 y están recogidos en artículo titulado *"Comparative study of green sub- and supercritical processes to obtain carnosic acid and carnosol-enriched rosemary extracts with in vitro antiproliferative activity on colon cancer cells"* de Sánchez-Camargo et al., publicado en la revista International Journal of Molecular Sciences 2016, 17, 2046. La comparación de los extractos resultantes se realizó en términos de rendimiento de extracción, composición química, actividad antioxidante y actividad antiproliferativa. Se evaluaron las posibles

correlaciones entre el contenido de compuestos fenólicos, la actividad antioxidante y el tipo de proceso de extracción empleado. Las actividades citoestáticas y citotóxicas de los extractos en las líneas celulares de cáncer HT-29 y HCT116, se estimaron mediante la incubación de cada tipo celular con cinco concentraciones de extracto diferentes, durante 24 y 72h. Todos los extractos de romero se caracterizaron químicamente utilizando diferentes técnicas analíticas basadas en espectrometría de masas tales como LC-DAD-MS, LC-QTOF-MS y GC-MS en un intento de correlacionar la presencia de componentes específicos de romero (compuestos volátiles y fenólicos) con las bioact ividades observadas.

4.1.2 Two-step sequential supercritical fluid extracts from rosemary with enhanced antiproliferative activity

A.P. Sánchez-Camargo, A. Valdés, G. Sullini, V. García-Cañas, A. Cifuentes, E. Ibáñez & M. Herrero

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Two-step sequential supercritical fluid extracts from rosemary with enhanced anti-proliferative activity

Andrea del Pilar Sánchez-Camargo [a], *Alberto Valdés* [a], *Giuseppe Sullini* [a,b], *Virginia García-Cañas* [a], *Alejandro Cifuentes* [a], *Elena Ibáñez* [a,*], *Miguel Herrero* [a]

[a] *Laboratory of Foodomics, Institute of Food Science Research (CIAL, CSIC), Calle Nicolas Cabrera 9, Madrid 28049, Spain*

[b] *Dipart. di Scienze del Farmaco e dei Prodotti per la Salute (S.C.I.F.A.R.), University of Messina, Viale Annunziata, Messina 98168, Italy*

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ABSTRACT

Previously, carnosic acid and carnosol have demonstrated anti-proliferative activity against different types of cancer. To obtain extracts enriched in these two key phenolic compounds, two different processes have been developed in the present work based on the use of two-step sequential supercritical fluid extraction (SFE). By removing the interfering, less active fractions in a first step (150 or 300 bar, 40°C, neat CO_2, 60 min), suitable enrichment is achieved in the second step (150 bar, 40°C, CO_2 + 7% ethanol, 120 min), and this leads to carnosic acid concentrations in the extract as high as 40% of total dry weight, which are among the highest concentrations that have been described with this type of process. The enriched extracts were tested against the HT-29 human adenocarcinoma cell line, showing enhancement of their antiproliferative activity by approximately 3-fold compared to previously reported SFE rosemary extracts and higher inhibitory effects at lower concentrations (30 μg mL^{-1} of extract). Thus, the proposed two-step SFE process effectively improves the carnosic acid and carnosol recovery in shorter processing times (180 min vs. 300 min). Moreover, the obtained extracts possess higher anti-proliferative activity and consume less solvent.

1. Introduction

The influence of dietary polyphenols on human health is currently widely accepted. In fact, these compounds have been suggested to positively influence the prevention of several different chronic diseases, including obesity (Wang et al., 2014), diabetes (Xiao & Högger, 2014), cardiovascular disease (Wang, Chun, & Song, 2013), Alzheimer's disease (Hu et al., 2013), and cancer (Henning, Wang, Carpenter, & Heber, 2013), among others. Moreover, a positive impact on gut microbiota has been proposed (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013), which could also provide additional benefits for human health. However, the particular mechanisms of action of polyphenols on these diseases have not yet been completely elucidated (Del Rio et al., 2013).

* *Corresponding author.* Institute of Food Science Research (CIAL, CSIC), Laboratory of Foodomics, Calle Nicolas Cabrera 9, 28049 Madrid, Spain. Tel.: +34 910017956; fax: +34 910017905.
E-mail address: elena.ibanez@csic.es (E. Ibáñez).
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1756-4646/

One of the dietary sources of polyphenols that has garnered attention is rosemary (*Rosmarinus officinalis*). This Mediterranean species belongs to the *Lamiaceae* family and has traditionally been used as a food ingredient to increase food flavor. However, this plant has also been described as an attractive source of phenolic compounds with important bioactivities (Ben Jemia et al., 2013; Borrás Linares et al., 2011).

The anti-proliferative activity of dietary polyphenols is one of the most-studied bioactivities, and rosemary phenolic compounds are not an exception. Several studies have demonstrated the positive influence of rosemary extracts against different types of cancer (Kontogianni et al., 2013). This activity has been associated with their antioxidant capacity, mainly due to the presence of several phenolic compounds, such as rosmarinic and carnosic acids, carnosol, rosmanol, epirosmanol and methyl carnosate (Herrero, Plaza, Cifuentes, & Ibañez, 2010b), among others. In previous studies, we reported that different advanced, environmentally-friendly extraction techniques, such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE), are able to selectively produce bioactive rosemary extracts with anti-proliferative effects on different human cancer cell lines, specifically colon cancer (Valdés et al., 2013) and leukemia (Valdés et al., 2012) cell lines. Through different foodomics-based approaches, it has been possible to explain some of the molecular mechanisms of action of these phenolic compounds (Ibáñez et al., 2012a and Ibáñez et al., 2012b). SFE is a valuable tool to extract bioactive compounds from natural sources (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013). The use of supercritical CO_2 has many advantages in these applications, allowing the extraction of labile or easily oxidizable compounds. However, because of its very low polarity, CO_2 is unable to extract some of the most interesting natural bioactive components, which possess higher polarity. For this reason, the use of a co-solvent during extraction, often ethanol, is widely employed (Herrero, Mendiola, Cifuentes, & Ibáñez, 2010a). By using a co-solvent, the properties of the CO_2 as a solvent are modified and more polar compounds can be extracted. Among the different extraction techniques previously studied in our laboratory to obtain bioactive rosemary extracts, SFE has shown the most promise, because it was able to produce active extracts when ethanol was employed as cosolvent together with supercritical CO_2 (Valdés et al., 2013). These rosemary extracts were comparatively richer in medium to low polarity phenolic compounds (i.e., carnosic acid and carnosol), whereas other techniques such as PLE were more suited for the enrichment of the more polar phenolics, mainly rosmarinic acid, which were less active (Valdés et al., 2013). Consequently, these less polar phenolic compounds present in rosemary are more interesting as functional ingredients to prevent cell proliferation. In fact, carnosic acid has been the focus of research that has concluded that this compound may have not only anti-proliferative activities (Visanji, Thompson, & Padfield, 2006) but also anti-inflammatory properties (Kuo et al., 2011; Xiang et al., 2013) and neuroprotective activities (Satoh et al., 2008) in addition to its well-known antioxidant capacity (Jordan, Lax, Rota, Loran, & Sotomayor, 2012). The other related phenolic diterpene, carnosol, has also possesses anti-proliferative effects (Lopez-Jimenez, Garcia-Caballero, Medina, & Quesada, 2013 and Johnson, 2011). Thus, strategies directed at the enrichment of carnosic acid and carnosol in rosemary extracts are important for the functional food industry to achieve more active natural fractions.

Therefore, the main goal of the present work was to develop new strategies based on the use of sequential SFE processes to produce supercritical rosemary extracts enriched in carnosic acid and carnosol. Additionally, their anti-proliferative activities against human colon cancer cells were compared to a previously reported SFE extract (Herrero et al., 2010b and Valdés et al., 2013). Moreover, a complete chemical characterization together with *in vitro* activity measurements (total phenol content and antioxidant activity) were performed to create as broad of a picture as possible of their observed bioactivity.

2. Materials and methods

2.1. Samples and chemicals

The rosemary (*Rosmarinus officinalis*) samples consisted of dried rosemary leaves obtained from Herboristeria Murciana (Murcia, Spain). The dried rosemary leaves were ground using a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at low temperature for 30 s. The ground samples were then vacuum-packed and stored at 4 °C until further use.

The solvents used in the supercritical extraction process were 99% pure carbon dioxide purchased from Carburos Metálicos (X50S, Barcelona, Spain) and ethanol (99.5%) provided by VWR Chemicals (Fontenay-sous-Bois, France).

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 99%), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, ≥99%), carnosic acid (≥97%) and carnosol (≥ 98%) were purchased from Sigma-Aldrich. The Folin-Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany). For the inhibition of cell proliferation experiments, the dry extracts were dissolved in DMSO (Sigma-Aldrich) at the appropriate concentrations and stored as aliquots at –80 °C until future use.

2.2. Supercritical fluid extraction (SFE)

The extractions were carried out using a pilot scale SFE instrument (Thar Technologies, model SF2000, Pittsburg, PA) equipped with a 2 L extraction cell and two 0.5 L separators with independent pressure and temperature controls. For each extraction, 500 g of rosemary was used with a CO_2 flow rate of 60 g min^{-1}. For each extraction condition studied, a two-step extraction protocol was employed, utilizing a 60 min extraction with neat supercritical CO_2 first and then a second extraction with supercritical CO_2 and 7% ethanol as the cosolvent at 150 bar. Duplicate extraction procedures were carried out, and two different extraction pressures for the first step (150 and 300 bar) were used.

After extraction, the extracts were collected in vials, the residual ethanol was evaporated under vacuum and the dried extracts were stored at –20 °C and protected from light until analysis.

2.3. Total phenol content (Folin-Ciocalteu method)

The total phenol contents of the rosemary extracts were measured using the Folin–Ciocalteu assay with some modifications,

as previously described (Montero, Herrero, Ibáñez, & Cifuentes, 2013). Briefly, 600 μL of water and 10 μL of each extract (1 mg mL^{-1} of rosemary extract in methanol) were mixed, and 50 μL of undiluted Folin–Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 20% (w/v) Na_2CO_3 were added and the volume was brought to 1.0 mL with ultrapure water. After incubation for 2 h at 25 °C, 300 μL of the mixture were transferred into one well of a 96-well microplate. The absorbance was measured at 760 nm using a microplate spectrophotometer reader (Synergy HT, Bio Tek Instruments, Winooski, VT), and this value was compared to a gallic acid calibration curve (0.032–2 mg mL^{-1}) constructed in the same manner. Data were presented as the average of triplicate analyses.

2.4. Antioxidant activity determination

2.4.1. TEAC assay

The antioxidant activity of the SFE rosemary extracts was determined using the Trolox Equivalents Antioxidant Capacity (TEAC) assay. TEAC was determined using the method described by Re et al. (1999) with some modifications. The $ABTS^{\bullet+}$ radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature for 16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7 (±0.02) at 734 nm. The samples (10 μL; 5 different concentrations) and 1 mL of $ABTS^{\bullet+}$ solution were mixed in an Eppendorf vial and 300 μL of the mixture were transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min for 45 min in a microplate spectrophotometer reader (Synergy HT, BioTek). Trolox was used as the reference standard and the results were expressed as TEAC values (mM trolox g^{-1} extract). These values were obtained from five different concentrations of each extract that were tested in the assay and gave a linear response between 20 and 80% of the blank absorbance. All analyses were performed in triplicate.

2.4.2. DPPH radical scavenging assay

The DPPH radical scavenging method was based on a procedure described by Brand-Williams, Cuvelier, and Berset (1995). Briefly, a solution was prepared by dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts were tested (from 0.125 to 1.0 mg mL^{-1}), and these extract solutions (25 μL) were added to 975 μL of a dilute DPPH solution to complete the final reaction solution (1 mL). After 4 h in the dark at room temperature, 300 μL of the mixture were transferred into a 96-well microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (Synergy HT, BioTek). A DPPH–methanol solution was used as the reference sample. The DPPH concentration remaining in the reaction medium at the end of the reaction was calculated using a calibration curve. The percentage of remaining DPPH was then plotted against the extract concentration to obtain the EC_{50}, the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. Therefore, the lower the EC_{50} value, the higher the antioxidant capacity. Measurements were done in at least triplicate.

2.5. Anti-proliferative activity against human colon cancer cells

Colon adenocarcinoma HT-29 cells obtained from the ATCC (American Type Culture Collection, LGC Promochem, UK) were grown in McCoy's 5A media supplemented with 10% heat-inactivated fetal calf serum, 50 U mL^{-1} penicillin G, and 50 U mL^{-1} streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. When the cells reached ~50% confluence, they were trypsinized, neutralized with culture medium, plated in different culture plates and allowed to adhere overnight at 37 °C. To study the effects of rosemary extracts on the proliferation of the HT-29 cell line, the cells were seeded onto 96-well culture plates at 10,000 cells cm^{-2}, permitted to adhere overnight at 37 °C, and exposed to different treatments with different concentrations of rosemary extracts (from 0 to 60 μg ml^{-1}) for 24–72 h. After incubation with the bioactive extracts for the indicated time, cell proliferation was estimated by the MTT assay as follows: 0.5 mg mL^{-1} of MTT reagent (Sigma-Aldrich) was added and incubated for 3 h at 37 °C in a humidified 5% CO_2/air atmosphere. After the incubation, the media was aspirated and 100 μL of DMSO were added to each well to dissolve the formazan (the metabolic product of MTT). Then, the absorbance was measured at 570 nm in a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Shanghai, China). The results were provided as the mean ± 95% confidence interval of at least three independent experiments, each performed in triplicate. The cell viability at the beginning of the treatment (time zero) was used to calculate the percent of growth (PG). The PG was obtained using the formula $PG = 100 \times (T - T0)/(C - T0)$, where T = mean cell number after a given treatment time; T0= mean cell number at time 0; and C = mean cell number at a given time of exposure to 0.2% DMSO (vehicle). If T is less than $T0$, cell death has occurred and can be calculated by $100\times[(T-T0)/T0]$. The PG values emphasize the correction for cell count at time 0 (i.e., cell number at the beginning of the treatment). To assess the inhibition of cell proliferation, curves were generated where the PG values were determined and plotted vs. treatment time.

2.6. Chemical characterization of the rosemary extracts

2.6.1. Liquid chromatography-diode array detection-mass spectrometry analysis of rosemary extracts

The rosemary extracts were analyzed using an Agilent 1100 series liquid chromatograph (Agilent, Santa Clara, CA) equipped with a diode array detector (DAD) and an autosampler, which was connected to an Agilent 6320 ion trap mass spectrometer via an electrospray interface. A Hypersil C18-AR (150 mm × 4.6 mm, d.p. 3 μm, Thermo Scientific, San Jose, CA) column was used. The mobile phases used were (A) 0.1% formic acid in acetonitrile and (B) 0.1% formic acid in water. The extracts were eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 48 min, 40% B; 55 min, 5% B; 60 min, 5% B; and 65 min, 95% B. The flow rate was 0.7 mL min^{-1} and the injection volume was 10 μL. The DAD recorded the spectra from 190 to 550 nm. The MS detection conditions (negative ionization mode) were as follows: dry temperature, 350 °C; mass range, m/z 90–800 Da; dry gas flow rate, 12 L min^{-1}; and nebulization pressure, 40 psi.

Quantitative amounts of carnosic acid and carnosol were determined using the same protocol and the external standard calibration method.

2.6.2. *Gas chromatography-mass spectrometry analysis of rosemary extracts*

GC-MS was employed to characterize the volatile compound profiles of the different rosemary extracts. A GCMS-QP2010 plus system (Shimadzu, Kyoto, Japan) equipped with a DB-5ms column (30 m × 0.25 mm I.D. × 0.25 μm df, Quadrex Corporation, Woodbridge, CT) was used. The separation was performed according to the following oven temperature program: initial temperature was 60 °C and held for 4 min, then it was raised to 100 °C at 3°C min^{-1}, followed by an increase to 110 °C at 1°C min^{-1} and then to 150 °C at 5°C min^{-1}. Finally, the temperature was raised to 300 °C at 15°C min^{-1} and this value was maintained for 25 min. The injection volume was 1 μL in split mode (split ratio 1:25) with the injector temperature at 250 °C. The carrier gas was He at 36.5 cm s^{-1}. The MS detection parameters were: interface temperature, 280 °C; source temperature, 230 °C; mass range, m/z 40–500; scan speed, 2500 amu s^{-1}; and event time, 0.20 s. The data collection and handling were performed using the GCMS solution (ver. 2.50 SU3, Shimadzu) software. The system was equipped with a commercial mass spectral database (Wiley) that was employed to identify the separated compounds as well as their respective linear retention indices (LRIs). For determination of the LRIs, a hydrocarbon mixture ranging from C_8 to C_{30} was employed, and it was analyzed under the same experimental conditions as the samples.

2.7. *Statistical analysis*

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95%. One-way analysis of variance (ANOVA), together with the Student–Newman–Keuls test, was employed to group the extracts based on statistically significant differences. These differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. *Enrichment of carnosic acid and carnosol by SFE*

We have previously shown that a supercritical CO_2 extract of rosemary obtained using 7% ethanol as the co-solvent at 150 bar and 40 °C provided significant activity against human cancer cell proliferation in colon adenocarcinoma HT-29 and SW480 cell models (Valdés et al., 2013) as well as leukemia cells (Valdés et al., 2012). Furthermore, we proved that the antiproliferative action was chiefly related to the presence of polyphenols in the extracts, particularly carnosic acid and carnosol, which affected cell cytostaticity through multiple pathways (Valdés et al., 2013). Studies of spice and plant extractions using CO_2 plus alcohol indicate that, although the overall yield obtained using alcohol as the co-solvent is higher, the percentage of the desired active compound in these extracts is lower (Mukhopadhyay, 2008). This is mainly because other undesirable components that provide a more complex chemical pattern are also co-extracted (Herrero et al., 2010a).

Taking these facts into consideration, in the present work, we developed two different sequential SFE-based extraction processes to remove interfering (and less active) compounds in the first step while allowing a good enrichment of the phenolic compounds in the second extraction step. It is worth mentioning that these experiments were carried out using a pilot scale SFE system to further demonstrate the applicability of this technology to produce relevant amounts of bioactive extracts that could be later used in the food industry. **Table 1** shows the tested extraction conditions along with the extraction yield, the functional characterization and the total content of target bioactive compounds (carnosic acid and carnosol). There were two extraction processes selected. Process 1 consisted of a 60 min extraction at 150 bar using neat supercritical CO_2 as the solvent to remove the essential oils that are preferentially extracted at relatively low pressures, followed by a second 120 min extraction step at 150 bar using 7% ethanol as the co-solvent. The conditions selected for the second step were chosen based on previous works (Valdés et al., 2012 and Valdés et al., 2013). Process 2 consisted of a 60 min extraction at 300 bar using neat supercritical CO_2 as the solvent, which was selected to remove the waxes and miscellaneous compounds belonging to the less polar oleoresin fraction, followed by a second 120 min extraction step at 150 bar using 7% ethanol as the co-solvent. Moreover, these two-step processes were compared to the aforementioned one-step SFE process (Valdés et al., 2012 and Valdés et al., 2013) that was carried out for 300 min at 150 bar using 7% ethanol as the co-solvent, which is equal to the extraction conditions used in the second step of the alternative processes.

As seen in **Table 1**, significantly higher extraction yields were obtained in the second extraction step, resulting from the use of ethanol. This behavior is very well documented and is in agreement to the increase on solvent extractability when ethanol is added . However, the extraction yield obtained using the single process was the highest ($p < 0.05$). Nevertheless, higher combined yields were attained when a 300 bar step was employed first, followed by extraction with a co-solvent (total extraction yield of 7.4%). In terms of composition, total phenols content determinations (Folin-Ciocalteu method) allowed a preliminary idea of the chemical composition present in the generated extracts. As shown in **Table 1**, the extracts produced in the second step of both processes were significantly ($p < 0.05$) enriched in phenolic compounds, not only compared to the first step extracts but also compared to the single extraction procedure. The quantity of total phenols obtained in Process 2 after the first 300 bar step was slightly higher, although not statistically significant ($p > 0.05$). The antioxidant activity presented by the extracts followed a similar pattern; both TEAC and DPPH assays revealed a significantly higher antioxidant activity in the second step of the extraction than in the first step, whereas a similar antioxidant activity was obtained for the single step process ($p > 0.05$). Once this preliminary functional characterization was performed, the chemical characterization was evaluated to reveal the composition of the extracts, quantifying the carnosic acid and carnosol contents and determining their antiproliferative activity against HT-29 human colon cancer cells.

Table 1 – Conditions employed for the SFE rosemary extraction, as well as extraction yields (%), total phenols content, antioxidant activities (TEAC and DPPH assays) and carnosic acid and carnosol amounts determined in the extracts.

Process	Step	P (bar)	T (°C)	% Ethanol	Ext. time (min)	Ext. yield (%)	Functional characterization				
							Total phenols (mg GAE g^{-1})[a]	TEAC (mM trolox g^{-1})[b]	EC_{50} (μg ml^{-1})[c]	Carnosic acid (mg g^{-1})[d]	Carnosol (mg g^{-1})[d]
1	1	150	40	0	60	1.61 ± 0.15	34.31 ± 3.22	0.38 ± 0.10	42.95 ± 2.39	36.70 ± 2.66	6.81 ± 0.31*
	2	150	40	7	120	4.30 ± 0.25*	198.38 ± 9.78*	2.21 ± 0.23*	4.48 ± 0.57*	384.37 ± 13.17	32.75 ± 0.92
2	1	300	40	0	60	2.73 ± 0.58	53.77 ± 2.63	0.55 ± 0.10	31.85 ± 3.31	49.84 ± 4.23	6.17 ± 0.25*
	2	150	40	7	120	4.67 ± 0.01*	203.92 ± 9.02*	2.19 ± 0.11*	5.02 ± 0.48*	403.04 ± 6.92	44.56 ± 6.58**
Single Step		150	40	7	300	6.50 ± 0.01	177.61 ± 8.94	2.30 ± 0.04*	6.76 ± 0.11*	363.32 ± 9.74	45.61 ± 1.08**

[a] mg Gallic acid equivalents (GAE) g^{-1} extract.
[b] mM Trolox g^{-1} extract.
[c] EC_{50}, Efficient concentration, μg extract ml^{-1}.
[d] amounts in (mg g^{-1}) with respect to dried extract.
*, **Not statistically different ($p > 0.05$) for that assay.

3.2. *Characterization of extracts by LC-MS and GC-MS*

The extracts were chemically characterized using an HPLC-DAD-MS method previously developed in our laboratory (Herrero et al., 2010b). By using DAD and MS detectors connected in series, it was possible to collect the UV-Vis and MS spectra of the separated compounds, thus allowing the tentative identification of the most relevant components (see **Table 2**).

Figure 1 shows representative chromatograms of the extracts obtained during the first (Fig. 1C) and second (Fig. 1A) steps, as well as for the single step extraction procedure (Fig. 1B). The chemical composition among them is clearly varied, not only qualitatively but also quantitatively. In fact, even though the chromatographic profiles were quite similar among the samples, the extracts generated during the first step were significantly poorer in phenolic compounds. Carnosic acid (peak 17) and carnosol (peak 13) were among the most relevant compounds and were obtained at notably lower concentrations in the first extraction step.

Following these analyses, the concentrations of carnosic acid and carnosol present in the extracts were quantified. As summarized in **Table 1**, the quantity of carnosic acid recovered in the second step was clearly higher than the first; in fact, it was nearly 10-times more (all values significantly different, $p < 0.05$). Similarly, the amount of recovered carnosol also increased in the second step, although not as markedly. The two-step sequential SFE processes studied afforded a significant enrichment of carnosic acid and carnosol in the second step, which came after the removal of either the essential oils (150 bar) or the waxes and lower polarity oleoresin compounds (300 bar). Thus, the extraction strategy developed is a good choice for the enrichment of carnosic acid and carnosol (preferentially carnosic acid, which is the most active compound in rosemary extracts) in the resulting extracts using shorter extraction times compared to a single step extraction. Moreover, the entire extraction process consumes less ethanol, which is important for the total energy consumption needed to remove the organic solvent and considering the global cost of the process. Other extraction approaches have been previously developed to produce an enrichment in the carnosic acid in a rosemary supercritical fluid extract (Vicente, Martín, García-Risco, Fornari, & Reglero, 2012), although the percentage of carnosic acid in the final extract was significantly lower than in our approach.

To gain further insight regarding the chemical differences among the produced extracts, they were also analyzed by GC-MS to determine the most important volatiles that may be responsible for the bioactivity observed in the natural extracts. **Table 3** summarizes the compounds identified in addition to their relative contributions (normalized by the total area of the chromatogram), their retention times and the LRIs employed to confirm their identity. Both extracts from the first SFE step as well as the single extraction extract possessed higher but similar amounts of volatile compounds, whereas the two extracts from the second SFE steps contained lower amounts of volatiles. This observed difference implied an approximate reduction of 30 to 40% of the volatiles compared to the single extraction process. The volatile compounds identified can be classified according to their nature: oxygenated terpene derivatives (1,8-cineole, camphor, borneol, α-terpineol, verberone

Table 2 – Identification of phenolic compounds in the rosemary extracts including their UV-Vis and MS spectra characteristics.

ID	Rt (min)	Proposed Identification	UV-Vis maxima (nm)	$[M-H]^-$	Main fragments detected
1	16.9	*NI*	260, 292	529.2[a]	[a] 511.1
2	20.7	*NI*	310, 300s	498.6	451.4
3	27.1	*NI*	257	207.1[a]	[a] 176.9
4	30.2	*NI*[1]	283	149.5	
5	32.5	*NI*[2]	257	151.1[a]	[a] 123.0, 109.1
6	38.6	Rosmanol	285	345.2	301.2
7	39.0	Cirsimaritin	334, 275	315.1[a]	[a] 300.1, 282.1
8	39.9	Epiisorosmanol	340, 280	345.4	283.2, 301.1
9	41.2	Epirosmanol	288	345.6	283.2, 301.0
10	43.1	Scutellarein	334, 267	285.2	267.9
11	43.3	Genkwanin/Acacetin	335, 267	283.1	267.9
12	48.1	Salvigenin	331, 276	329[a]	[a] 296.0, 314.1, 268.0
13	50.1	Carnosol	285	329.3	285.3
14	51.3	Rosmadial / Rosmanol quinone	286	343.3	315.3, 299.9
15	51.7	Epirosmanol ethyl ether	288	373.5	284.3, 329.9
16	52.1	*NI*	329, 268	318.6	287.1
17	52.6	Carnosic acid	285	331.2	287.3
18	54.1	Methyl carnosate	284	345.3	301.3

NI, Not identified; [a] Detected in positive ESI ionization mode as $[M+H]^+$; s, spectral shoulder.
[1] MS and UV-Vis spectral characteristics match with compound NI3 of reference (Señoráns, Ibañez, Cavero, Tabera, & Reglero, 2000).
[2] MS and UV-Vis spectral characteristics match with compound NI1 of reference (Señoráns et al., 2000).

and isobornyl acetate), terpene hydrocarbons (α-pinene, camphene, myrcene, α-terpinene and limonene), sesquiterpene hydrocarbons (γ-cadinene, β-caryophyllene, α-humulene and caryophyllene-oxide), sesquiterpene alcohols (Z,E)-farnesol and phenolic diterpenes (totarol acetate). The relative composition of the oxygenated terpene derivatives, which are associated with the quality of the essential oils present in the extracts, ranged from 70 to 89%. Additionally, some of these components have been previously described as possessing anti-proliferative activity as well (Bayala et al., 2014; Murata et al., 2013). With the increase of extraction pressure in the first SFE step from 150 to 300 bar (Process 2), the relative percentage of terpene hydrocarbons was favored, increasing from 4.56% to 25.85%.

Thus, after chemical characterization, it could be concluded that it was possible to obtain a volatile-rich extract by performing a first extraction step with neat CO_2, and furthermore, that the use of ethanol as a modifier allowed the enrichment of phenolic compounds in a second SFE step. Once these results were obtained, the anti-proliferative activity of the produced extracts was studied and compared to the extract obtained from a single SFE extraction.

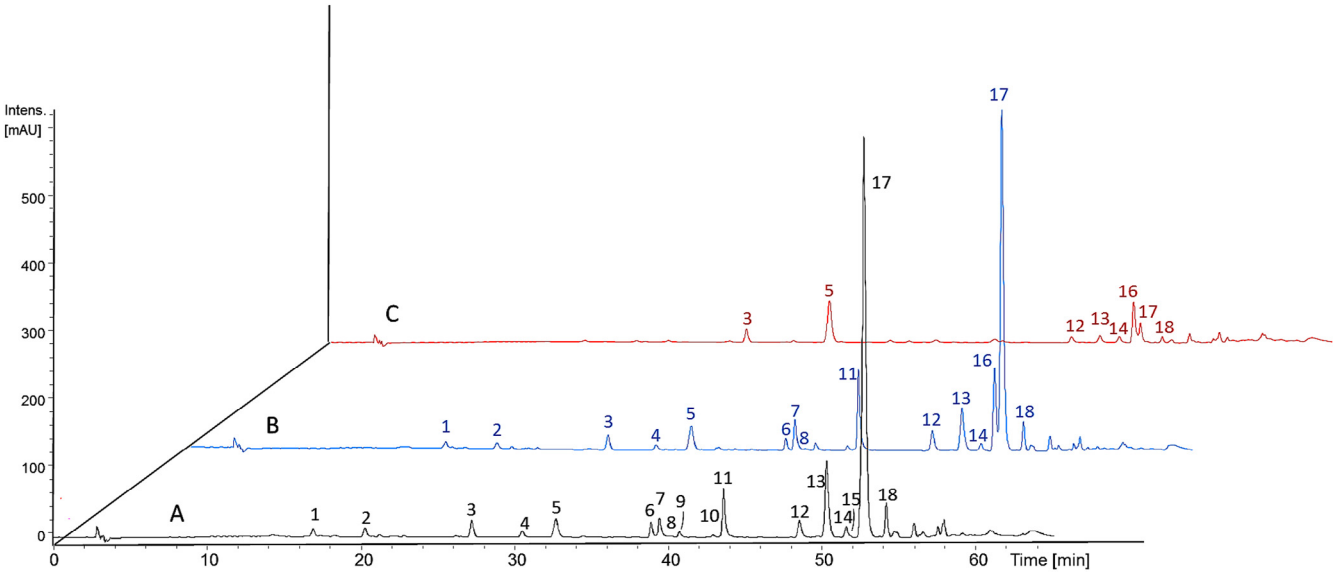

Fig. 1 – Chromatograms (280 nm) corresponding to the first (C) and second (A) extraction step (Process 1) as well as to the single extraction protocol (B). For peak identification, see Table 2.

Table 3 – Identified compounds by GC-MS and total peak areas in the different SFE extracts. Percentage from total peak area between parentheses. For more detailed extraction conditions, see Table 1.

ID	Compound	R_t (min)	Calculated LRI[a]	Referenced LRI	Relative peak areas (% total peak area)				
					Process 1		Process 2		Single step
					Step 1	Step 2	Step 1	Step 2	
1	α-Pinene	8.13	937	939	227046 (0.70)	46197 (0.52)	6735313 (14.17)	27724 (0.36)	69560 (0.56)
2	Camphene	8.98	957	953	115999 (0.36)	32538 (0.37)	2046808 (4.31)	19770 (0.26)	25808 (0.21)
3	Myricene	10.66	998	991	218913 (0.67)	37535 (0.43)	966458 (2.03)	45123 (0.59)	31844 (0.26)
4	α-Terpinene	12.10	1027	1018	79503 (0.25)	33227 (0.38)	205423 (0.43)	41540 (0.54)	58417 (0.47)
5	Limonene	12.68	1039	1031	836314 (2.58)	163215 (1.85)	2330841 (4.90)	151554 (1.98)	158526 (1.27)
6	1,8-Cineole	13.00	1045	1033	13473514 (41.60)	4433782 (50.26)	20148556 (42.39)	3940260 (51.44)	4988023 (40.05)
7	Linalool	16.45	1114	1098	510490 (1.58)	103092 (1.17)	421251 (0.89)	90200 (1.18)	189953 (1.52)
8	Camphor	19.73	1173	1143	7895333 (24.38)	1840242 (20.86)	6751107 (14.20)	1562490 (20.40)	3176089 (25.51)
9	Borneol	20.92	1195	1165	2338490 (7.22)	523226 (5.93)	2204850 (4.64)	423046 (5.52)	901171 (7.24)
10	Terpineol-4	21.25	1201	1177	504086 (1.56)	104599 (1.18)	451903 (0.95)	89894 (1.17)	199088 (1.60)
11	α-Terpineol	22.40	1219	1189	2020162 (6.24)	407053 (4.61)	1887276 (3.97)	342083 (4.47)	837310 (6.73)
12	Verbenone	23.69	1240	1204	1599299 (4.94)	397324 (4.50)	1181526 (2.49)	328078 (4.28)	613593 (4.93)
13	Isobornyl-acetate	28.41	1314	1285	250887 (0.77)	45548 (0.52)	174017 (0.36)	50589 (0.66)	101715 (0.82)
14	β-Caryophyllene	34.93	1434	1418	1098149 (3.39)	221657 (2.51)	851816 (1.79)	161136 (2.10)	467713 (3.76)
15	α-Humulene	36.31	1475	1454	355308 (1.10)	66529 (0.75)	266138 (0.56)	51778 (0.68)	127746 (1.03)
16	δ-Cadiene	37.80	1535	1513	173762 (0.54)	40461 (0.46)	138791 (0.29)	28796 (0.38)	77383 (0.62)
17	Caryophyllene-oxide	39.19	1613	1581	89541 (0.28)	25096 (0.28)	59877 (0.13)	33562 (0.44)	89844 (0.72)
18	(Z,E)-Farnesol	40.25	1692	1697	397547 (1.23)	91360 (1.04)	444951 (0.94)	73957 (0.96)	139667 (1.12)
19	Totarol acetate	46.09	2419	2417	197855 (0.61)	209792 (2.38)	266357 (0.56)	199003 (2.59)	197212 (1.58)
	Total peak areas				32382204	8822478	47533264	7660589	12450669

[a] Linear Retention indices.

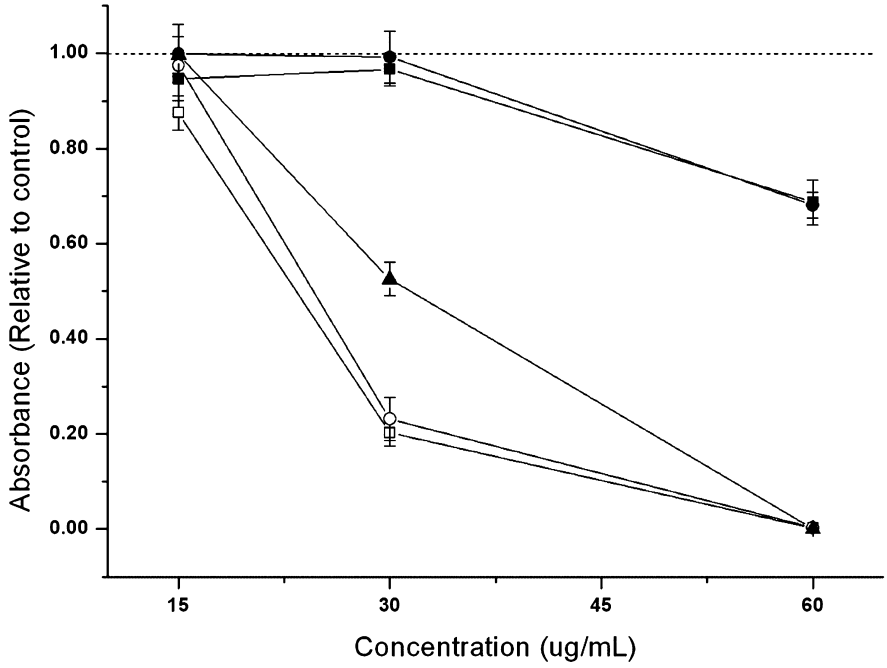

Fig. 2 – HT-29 colon cancer cell viability upon treatment for 48 h with different concentrations of extracts generated in extraction procedure 1, step 1 (■) and step 2 (□); extraction procedure 2, step 1 (●) and step 2 (○); and single step extraction procedure (▲). Error bars are given as 95% confidence interval.

3.3. *Anti-proliferative activity*

To determine the anti-proliferative effects of the extracts, HT-29 cells were incubated with increasing concentrations (from 0 to 60 μg mL^{-1}) of the different extracts for 24, 48 and 72 h, and cell proliferation was analyzed by the MTT assay. As illustrated in **Fig. 2**, for the 48 h incubation period, all extracts exerted a dose-dependent reduction in cell proliferation after treatment (after the longest incubation time). The three extracts most enriched in carnosic acid exhibited stronger inhibitory effects on cell proliferation than those obtained in the first step of both extraction processes, which was provided by the lower values obtained at 30 and 60 μg mL^{-1}. Moreover, at intermediate extract concentrations (30 μg mL^{-1}), significantly higher inhibitory effects (p-values < 0.05; $p = 2.2 \times 10^{-6}$ and 3.1×10^{-7} for step 2 of process 1 and step 2 of process 2, respectively) were observed for the extracts most enriched in carnosic acid (both obtained in the second step of the extraction processes) compared to the extract obtained in the single step process. Interestingly, the extracts generated in the first step of both extraction processes provided some inhibitory effects, which was unexpected due to their low carnosic acid and carnosol contents. These results suggest that the activity of other rosemary constituents, in addition to carnosic acid and carnosol, against colon cancer cells cannot be dismissed. Indeed, as mentioned previously, these extracts were enriched in monoterpenes such as 1,8-cineole, alpha-pinene, camphor and terpineol, which have reported anti-proliferative activity on different cancer cell models (Murata et al., 2013 and Bayala et al., 2014). For example, Murata et al. (2013) recently showed that 1,8-cineole induces specific apoptosis in human colon cancer cell lines HCT116 and RKO. Additionally, alpha-pinene has inhibited the proliferation of several cancer cell lines (Bayala et al., 2014). In some studies, monoterpenes such as camphor and terpineol have also been reported to act synergistically against colon cancer cell proliferation (Itani et al., 2008). Thus, the presence of monoterpenes, together with minor amounts of carnosic acid and carnosol, may explain the anti-proliferative activity observed in the fractions obtained in the first step of the extraction processes. However, further investigations would be required to determine the identity and potency of the bioactive constituents in these extracts against the HT-29 cell line.

To determine the mechanisms that can explain the inhibitory activity of these extracts on HT-29 cell proliferation, the percentage of growth (PG) values were calculated for each extract. **Figure 3** shows the PG values calculated for each day of treatment with 30 μg mL^{-1} of each extract. PG values above zero are indicative of cytostatic activity, because they represent the percentage of growth relative to the control cell number at the beginning of the treatment. Conversely, PG values lower than zero indicate cytotoxicity (i.e., lower cell number than at the beginning of treatment). As observed in **Fig. 3**, the extracts obtained from the first step yielded PG values between 50 and 100, indicating moderate cytostatic effects. Differences in the effect of both extracts on the percentage of growth of the cells were only significant at the longest incubation time (72 h), showing higher inhibitory activity than the extract obtained in the first step from Process 1. In contrast, using 30 μg mL^{-1} of the extract obtained in the second step provided PG values that fell below the initial cell density at the beginning of the treatment ($PG < 0$), thus indicating cytotoxic activity. Interestingly, according to the calculated PG values, the extract obtained in the single step process exerted a cytostatic effect,

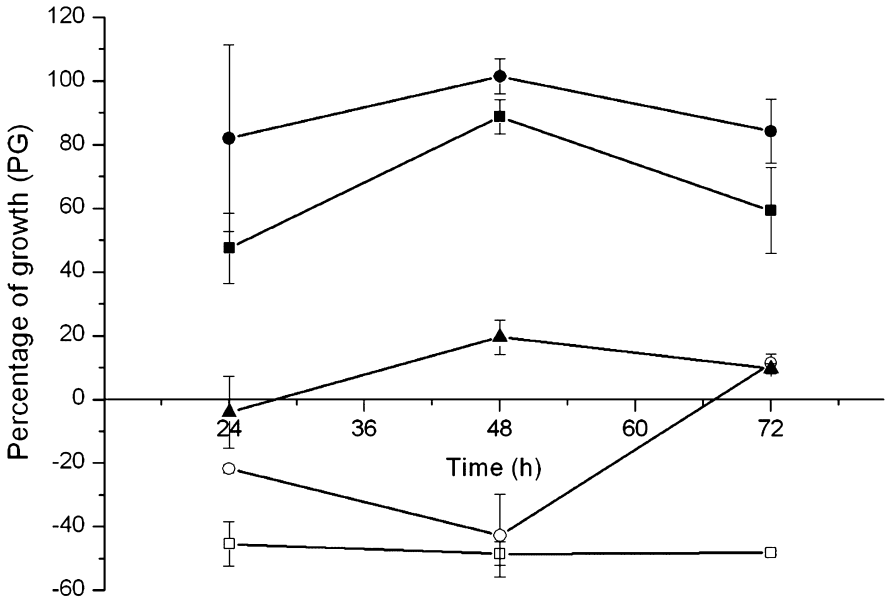

Fig. 3 – Percentage of growth (PG) of HT-29 colon cancer cells exposed to the different extracts (30 μg mL^{-1}) for 24, 48 and 72 h: Extraction procedure 1, step 1 (■) and step 2 (□); extraction procedure 2, step 1 (●) and step 2 (○); and single step extraction procedure (▲). Error bars are given as the mean standard error.

showing an intermediate effect on cell proliferation, which fell between the effects exhibited by extracts from the first and second steps. Furthermore, the dependence of the cytotoxic effect on time was negligible for the extract obtained in the second step of process 1, whereas for the extract obtained in step 2 of process 2, the percentage of cell growth recovered at 72 h after reaching its minimum value at 48 h (Fig. 3). According to literature data, extracts enriched in carnosic acid and carnosic acid alone have demonstrated different *in vitro* cytotoxic and cytostatic effects, depending on the cell type, concentration and time of exposure. The cytostatic and cytotoxic effects observed in the present study for the carnosic acid-enriched extracts (10.90 – 12.09 μg carnosic acid mL^{-1} of culture medium) are more pronounced than those observed in our laboratory for pure carnosic acid solutions on the same cell model (unpublished data, 9.5 ug mL^{-1} GI_{50} and 21.1 ug mL^{-1} LC_{50}) and those reported *in vitro* for other cancer cell models (Barni, Carlini, Cafferata, Puricelli, & Moreno, 2012; Gigante et al., 2003; Visanji et al., 2006 and Yesil-Celiktas, Sevimli, Bedir, & Vardar-Sukan, 2010). For example, Gigante et al. (2003) reported GI_{50} values ranging from 21.6 to 47.2 μM carnosic acid (~7.2 μg mL^{-1}-15.7 μg mL^{-1}) for five cancer cell lines. Our results suggest that although carnosic acid and carnosol are likely the most active compounds, other extract constituents may also be contributing to the observed anti-proliferative activity of the enriched extracts on HT-29 cells. These results corroborate and complement other recent research conducted in our laboratory that has demonstrated the additive effect of carnosic acid and carnosol on cell proliferation inhibition (Valdés et al., 2014). Moreover, according to our studies on rosemary polyphenols, carnosic acid contributes to the observed cytostatic effect that rosemary extracts exert on HT-29 cells at similar concentrations as those assayed in the present work. In addition, recently published data indicate that carnosic acid exerts a differential effect on the cell cycle as a function of the assayed concentration and the cellular model selected for the study (Einbond et al., 2012), thus providing an explanation for the divergences observed on the type and severity of the effect exerted by the different enriched rosemary extracts.

According to the literature, the favorable results obtained for the extracts generated using the new optimized two-step process increase the chance for a successful further scale up of this rosemary extraction methodology (De Melo, Silvestre, & Silva, 2014; Pereira & Meireles, 2010). Several studies concerning natural products have demonstrated the viability of SFE technology (Danielski, Zetzl, Hense, & Brunner, 2005, Leal, Alexandre, Kfuri, & Meireles, 2006; Leal et al., 2008; de Melo, Barbosa, Passos, & Silva, 2014; Pereira & Meireles, 2007; Prado, Assis, Maróstica, & Meireles, 2007; Rosa & Meireles, 2005). In most of this research, the cost-of-manufacturing (COM) for extracts and essential oils was lower than those produced by traditional methods and were also lower when compared to the market price of the extracts. For instance, Pereira and Meireles (2007) developed an economic analysis of their SFE scale up work with essential oils and rosemary extracts in terms of COM. Their results showed that the COM of extracts and essential oils produced by SFE were 1.8-2.5 times lower than those produced by steam distillation, indicating the viability of SFE to recover bioactive fractions from natural plants. Meanwhile, Prado et al. (2007) verified that the COM of Buriti oil depends on the composition of the oil; however, the cost of the raw material (Buriti Palm, *Mauritia flexuosa*) represented at least 62.25% of the COM. Therefore, despite the relatively high cost of SFE equipment, it is not always the predominant cost, as it is often mistakenly considered.

The economic analyses described above on SFE have addressed how to obtain bulk extracts and/or essential oils. To this end, it is worth noting that in our case, the purpose of the

SFE technology was to produce extracts with fractions enriched in high-value compounds. For this scenario, any further economic analysis and scale up studies should carefully take into consideration different factors, from raw material market price to unit working hours, human resources needed, energy costs, pressure needed, potential extract market price, extract drying costs, waste treatment costs, and carbon dioxide recycling possibility, among many others.

4. Conclusions

Taken together, these findings support the suitability of using a two-step extraction process based on the application of supercritical fluids to obtain natural extracts that are more active against colon cancer cell proliferation. The use of a first extraction step that employs neat supercritical CO_2 followed by a second extraction step using CO_2 with 7% ethanol as modifier led to rosemary extracts that were highly enriched in carnosic acid and carnosol, two of the more active phenolic compounds found in this plant. This type of two-step process permitted the attainment of more active extracts using shorter extraction times with a reduced amount of co-solvent (ethanol), providing better selectivity towards the extraction of key phenolic compounds.

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4.1.3 Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity

A.P. Sánchez-Camargo, J.A. Mendiola, A. Valdés, M. Castro-Puyana, V. García-Cañas, A. Cifuentes, M. Herrero & E. Ibañez

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Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity

A.P. Sánchez-Camargo [a], J.A. Mendiola [a], A. Valdés [a], M. Castro-Puyana [b], V. García-Cañas [a], A. Cifuentes [a], M. Herrero [a], E. Ibáñez [a,*]

[a] Laboratory of Foodomics, Institute of Food Science Research (CIAL, CSIC-UAM), Nicolas Cabrera 9, Campus de Cantoblanco, 28049 Madrid, Spain
[b] Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering, Faculty of Biology, Environmental Science and Chemistry, University of Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Community of Madrid, Spain

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ABSTRACT

Rosemary bioactivity is correlated with the presence of phenolic diterpenes (carnosic acid, carnosol) and phenolic acids (rosmarinic acid). In the present work, two processes, pressurized liquid extraction (PLE) and supercritical antisolvent fractionation (SAF) were integrated to separate and enrich these families of compounds. The optimization of the SAF process was carried out using a response surface methodology (RSM) with three factors: (i) CO_2 pressure, (ii) percentage of water in the PLE extract and (iii) PLE extract/SC-CO_2 flow ratio. The selected responses were the relative amount of each compound (rosmarinic acid (RA) and carnosic acid + carnosol (CA + CS)), total phenolic content, antioxidant activity and antiproliferative activity against HT-29 colon cancer cells. The higher CA + CS content was obtained at 100 bar, 50% (v/v) of water in the feed solution and 0.025 feed/SC-CO_2 mass flow ratio, achieving values of 478.1 mg/g extract and 17% of cell survival after 24 h of treatment.

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) is a Mediterranean plant well known for its antioxidant properties that are mainly related to the presence of two families of compounds with different polarities: phenolic diterpenes, such as carnosic acid and carnosol [1,2] and phenolic acids (mainly, rosmarinic acid) [3,4]. Carnosic acid (CA) has proven anti-inflammatory and chemoprotective properties in several *in-vitro* cell models [5,6], and antiproliferative activity in human colon cancer [7] and liver carcinoma cells [8]. Carnosol (CS) possesses strong antioxidant and chemopreventive activities; this diterpene has demonstrated anti-inflammatory and anti-cancer activities, for instance, on prostate, skin, breast, or colon cancer cells [9]. On the other hand, rosmarinic acid (RA) can have synergistic antiproliferative effect with some synthetic drugs such as cisplatin on ovarian cancer cell lines [10].

The chemical structures of carnosic acid, carnosol and rosmarinic acid are shown in the Supplementary material (Fig. S1). Due to their structure, CA and CS have poor solubility in water, while RA is completely soluble in aqueous media [3,11]. Hydro-alcoholic mixtures are usually employed to obtain extracts enriched on these three antioxidant compounds. Durling et al. [11] described that RA from sage (*Salvia officinalis*) was preferentially recovered with a solvent mixture of 30–70% ethanol, while carnosic acid-type compounds were better extracted within the range of 70 to 100% of ethanol. As a compromise, the optimal solvent composition to achieve good yields of antioxidants belonging to the carnosic and rosmarinic acids families ranged between 60 and 80% ethanol. Thus, it seems clear that different ethanol–water proportions cause different behavior in the extraction of these compounds of interest. On the other hand, the solubility of CA and CS in supercritical neat CO_2 is quite high and is even improved when ethanol is used as entrainer [3,12]. However, other more polar phenolic compounds are not effectively extracted using supercritical fluid extraction (SFE) [3].

Nowadays, the manufacturing process of food-grade commercial rosemary extract is mainly carried out by extraction from rosemary leaves using food-grade acetone, ethanol, hexane, or a combination of hexane and ethanol (in a two-step process) as extraction solvents. Also, food-grade extracts are obtained using supercritical carbon dioxide fractionation, achieving the highest

* Corresponding author. Tel.: +34 910 017 956; fax: +34 910 017 905.
E-mail address: elena.ibanez@csic.es (E. Ibáñez).

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concentration of phenolic diterpenes (*ca.* 30% w/w of CA + CS) among the aforementioned processes [13,14]. Since the approval in 2008 of the use of rosemary extracts as food additives (natural antioxidant) by the European Food Safety Authority (EFSA), rosemary extracts have been mainly used for that purpose within the food industry [13]. Thus, the development of more environmentally friendly extraction methods to obtain more active extracts in the most efficient possible way is constantly sought. In this regard, in previous studies, we reported that different advanced environmentally-friendly extraction techniques, such as SFE and pressurized liquid extraction (PLE), were able to selectively produce bioactive rosemary extracts with antiproliferative effects on different human cancer cell lines, such as leukemia [15] and colon cancer [16]. A strategy recently developed in our laboratory, consisted of using a two-step supercritical fluid extraction process. By removing the interfering less active fractions in a first step (150 or 300 bar, 40 °C, neat CO_2, 60 min), a suitable enrichment is achieved in the second step (150 bar, 40 °C, CO_2 + 7% ethanol, 120 min), that allows the attainment of CA + CS-enriched rosemary extracts (*ca.* 40–45% w/w). The enriched extracts showed an enhancement of their antiproliferative activity by approximately 3-fold (tested against HT-29 human colon cancer cells) compared to previously reported SFE rosemary extracts [17].

Also, PLE has been employed previously in our laboratory as part of the green process extraction explorations. PLE is based on the use of solvents at high pressures and temperatures of extraction, so that the solvent in the liquid state is guaranteed throughout the extraction process. Under PLE conditions, higher extraction yields are achieved mainly because of the enhancement of mass transfer rate and the increase in analytes' solubility [18,19]. Besides, it is less time-consuming process and requires smaller ratios of solvent to solids when compared to traditional atmospheric pressure extraction techniques [3,20]. PLE using ethanol at high temperature (150–200 °C) has proven to be one of the most effective techniques to extract both families of compounds present in the rosemary leaves, due its relatively low selectivity [3].

Another alternative approach to obtain CA + CS-enriched extracts could be the fractionation from a complex mixture into two differentiated fractions, a highly polar (containing phenolic acids, such as RA) and other less polar fraction where CA + CS may be concentrated and separated from other constituents. Among the different technologies to fractionate a complex extract, supercritical antisolvent fractionation (SAF) has been shown useful to fractionate phenolic compounds from plant extracts, such as *Persea indica* [21], propolis [22] or rosemary ethanolic oleoresins [23,24]. SAF is achieved by the continuous contact between a supercritical carbon dioxide (SC-CO_2) current and a polar liquid mixture flow in a pressurized separation chamber. The polar liquid mixture is an extract previously obtained from a natural matrix using ethanol + water mixtures. This contact allows the precipitation of the more polar components from the liquid mixture (including water and hydrophilic compounds) insoluble in SC-CO_2, whereas other components as well as another part of the solvent mixture (less polar components and ethanol) remain dissolved in the SC-CO_2 and are recovered by downstream pressure reduction [22,25]. Studying the phase equilibria of the SC-CO_2–EtOH–H_2O system is crucial to understand the factors that affect the behavior of components in the mixture. Previous works have shown that the solvent selectivity and the ethanol partition coefficients are highly influenced by the pressure, the mass percentage of water in feed and the feed/SC-CO_2 mass ratio [26]. These parameters must be optimized according to the nature of target compounds to be extracted [24].

Therefore, the objective of the present work was to optimize the single-step supercritical antisolvent fractionation of a rosemary PLE extract (obtained using a mixture of ethanol and water as extracting solvent), in order to produce a CA + CS-enriched fraction which is expected to be more active against human colon cancer cells. The optimization was performed using an experimental design and selecting the key parameters controlling the SAF process such as solvent composition, feed/SC-CO_2 flow rate ratio and pressure. Besides the content of CA and CS in the fractions, other response variables considered were RA content, total phenols content (TPC), antioxidant activity (measured by using TEAC and DPPH assays) and the antiproliferative activity against HT-29 human colon cancer cells (measured as % cell viability).

2. Materials and methods

2.1. Samples and reagents

Dry rosemary leaves (*R. officinalis*) obtained from Herboristeria Murciana (Murcia, Spain) were ground using a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at low temperature (10 °C) employing small rocks of dry ice for this purpose. The particle size was determined by sieving the ground material to appropriate size (between 999 and 500 μm). Then, the whole sample was vacuum-packed and stored at 4 °C until its use.

Ultrapure water obtained from a Millipore system (Billerica, MA, USA) and ethanol (99.5%) provided by VWR Chemicals (Fontenay-sous-Bois, France) were used for pressurized liquid extraction (PLE). For the antisolvent fractionation process, carbon dioxide (99% purity) purchased from Carburos Metálicos (X50S, Barcelona, Spain) was used. Rosmarinic acid (RA, ≥98%), carnosic acid (CA, ≥97%), carnosol (CS, ≥98%), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS, ≥99%), 2,2-diphenil-1-pycril hydrazyl hydrate (DPPH, 99%) were purchased from Sigma-Aldrich (Madrid, Spain). Folin–Ciocalteu phenol reagent (2 N) was provided by Merck (Darmstadt, Germany). For the antiproliferative activity assays, dry extracts were dissolved in dimethyl sulfoxide (DMSO, 99.9%, Sigma-Aldrich) and stored as aliquots at −80 °C until their use.

2.2. Pressurized liquid extraction (PLE)

Firstly, extractions of dried and ground rosemary samples were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. For each extraction, 2.0 g of rosemary dry leaves were loaded into an 11 mL stainless steel extraction cell after being mixed with the same quantity of sea sand, employed as dispersive agent. The conditions of the extraction were 150 °C, 100 bar, using a mixture of ethanol + water (80:20, v/v or 76:24 w/w) as extraction solvent for 20 min. The procedure and the extraction conditions were selected based on a previous work, to maximize the contents of RA, CA and CS [3]. For each PLE extraction between 26 and 28 mL of extraction solvent were employed and successive extractions were performed at the same conditions to obtain 500 mL of extract. After the PLE process, the liquid extract solution was diluted properly to obtain different percentages of water needed according to the planned experimental design (20, 35 and 50% v/v or 24, 40.5 and 55.8% w/w, respectively). Once diluted, the PLE extracts were filtered through Whatman cellulose filter paper and kept stored in the dark at −20 °C until their use for the antisolvent fractionation experiments.

2.3. Antisolvent fractionation

Because commercial systems are not available for antisolvent fractionation, the equipment used in this work (Fig. 1) was designed and built in-lab based on the one previously described by Catchpole

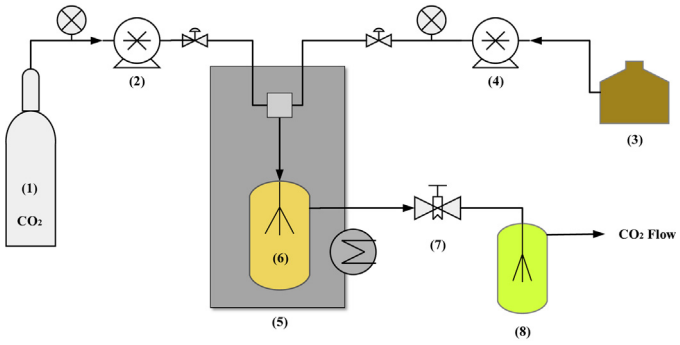

Fig. 1. Scheme of the antisolvent fractionation equipment: (1) CO_2 cylinder, (2) high-pressure CO_2 pump, (3) PLE extract reservoir flask, (4) high-pressure liquid pump, (5) oven with temperature control, (6) separator 1 (*raffinate* collection cell), (7) back-pressure regulator, (8) separator 2 (*extract* collection cell).

et al. [22]. A semi-continuous fractionation process was accomplished as follows: CO_2 was provided from a pressurized cylinder and subcooled in a heat exchanger placed within the high pressure pump (Jasco, PU-2080 CO_2Plus, Tokyo, Japan). Immediately, it was compressed and continuously pumped at a constant flow rate (2.061 mL/min ± 2 μL/min or 2 g/min) (2), and just before reaching the separation chamber 1 (6), the CO_2 was co-currently mixed in a T-tube device with the feed (rosemary PLE extract), which was supplied by another high pressure pump (Jasco, PU-2080Plus, Tokyo, Japan; 0.5 to 5.0 mL/min ± 2 μL/min) (4) from the PLE extract reservoir (3). After mixing, the rosemary extract components that were not soluble in SC–CO_2 + EtOH mixture precipitate and were collected at the bottom of the separator 1 (the non-soluble fraction is called *raffinate*) (6). At the mixing point as well as in the separation chamber 1, the temperature was controlled by the oven and was maintained constant at 40 °C throughout the experiment. The fractionation pressure was also kept constant in the equipment by means of the regulation of an upstream back pressure valve (7) (LF40, Pressure Tech, Houston, TX, USA). The fraction soluble in SC–CO_2 + EtOH goes through the tubing to the next vessel where reduced pressure turns CO_2 in a gas, precipitating into the second separation chamber (the soluble fraction is called *extract*) (8), which is kept at room temperature (25 °C). The antisolvent fractionation time was kept to 120 min for each experiment. After obtaining the fractions (raffinate and extract in separator 1 and 2, respectively) and in order to obtain dry extracts, the ethanol evaporation was completed using a vacuum-concentrator (SpeedVac Concentrator SC200, Savant Instrument, Inc., Farmingdale, NY, USA) and the water was eliminated by freeze-drying (Labconco Corporation, Missouri, USA). These dry extracts were kept under freezing at −20 °C until analysis. The recovery was determined by a gravimetric method, as the ratio of the mass of dry extract recovered in the separators and the mass of dry PLE extract fed, and expressed as percentage. In the same way, the CA + CS recovery (%) was calculated as the ratio of the mass of CA + CS recovered in the separator 2 and the mass of CA + CS in the start PLE extract.

2.4. Experimental design

The antisolvent fractionation was optimized using a 3-level factorial experimental design 2^3 (including three central points) based on three factors: pressure (100–300 bar), PLE rosemary extract to SC-CO_2 flow rate ratio in terms of w/w (0.025–0.1) and percentage of water in the PLE extract (20–50% v/v). The effect of the factors on different responses, including the relative amount of each compound (RA and CA + CS), the total phenolic content (TPC), the antioxidant activity (TEAC and DPPH assays) and the antiproliferative activity (% cell viability) was studied. Measurements were carried out on the *raffinate*, the *extract* and on the original PLE extract employed to perform the experimental design. A total of 11 experiments were conducted in a randomized order (as shown in Table 1). The experimental design and data analysis were carried out using response surface methodology (RSM) with the software Statgraphics Centurion XVI® (StatPoint Technologies, Inc., Warrenton, VA, USA). The effects of the independent variables on the response variables in the separation process were assessed using the pure error, considering a level of confidence of 95% for all the variables. The linear regression model having three predictor variables proposed for each response variable (Y_i) was

$$Y_i = \beta_0 + \beta_1 P + \beta_2 R + \beta_3 W + \beta_{1,2} P \times R + \beta_{1,3} P \times W + \beta_{2,3} R \times W + \beta_{1,2,3} P \times R \times W + \text{error} \quad (1)$$

where P is the pressure, R is the PLE rosemary extract/SC-CO_2 flow rate ratio, W is the volume percentage of water in the mixture, β_0 the intercept, β_1, β_2, β_3 are the linear effects, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ are the two factor interaction effects, $\beta_{1,2,3}$ is the three factor interaction effect and error is the error variable. Linear models (Eq. (1)) were evaluated considering the percent variation explained by the determination coefficient (R^2), the residual standard deviation (RSD), and the lack-of-fit test for the model from the analysis of variance table, as the significance criteria. The effect of each factor and its statistical significance, for each of the response variables, was analyzed from the standardized Pareto chart. The response surfaces of the respective mathematical models were also obtained, and the significances were accepted at $p \leq 0.05$. A multiple response optimization was carried out by the combination of experimental factors, looking for maximizing the desirability function for the responses in the extract fraction, relative amount of CA + CS, total phenolic content and antioxidant activity (TEAC), and minimizing the desirability function for the EC_{50} value (DPPH) and % cell viability.

2.5. Chemical characterization of rosemary fractions by liquid chromatography–diode array detection–mass spectrometry

An ACCELA UHPLC system (Thermo Scientific, San Jose, CA, USA) was used to analyze the fractions recovered in the *raffinate*, the *extract* and the PLE original extract (80:20 v/v ethanol + water as extracting solvent). The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyzer *via* an electrospray interface. The analytical method was previously developed in our laboratory [3]. Briefly, the analytical conditions employed consisted of a Hypersil Gold column (50 mm × 2.1 mm, d.p. 1.9 μm) (Thermo Scientific) using as mobile phases acetonitrile (+0.1% formic acid, A) and water (+0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 0.35 min, 95% B; 3.5 min, 40% B; 6.2 min, 5% B; 6.5 min; 5% B; 7 min, 95% B; 9 min, 95% B. The flow rate was 0.4 mL min^{-1} while the injection volume was 5 μL. The diode array detector recorded the spectra from 200 to 450 nm. To quantify CA, CS and RA, the mass spectrometer was operated in the negative ESI mode using multiple reaction monitoring (MRM) with a Q1 and Q3 resolution of 0.7 Da FWHM, scan width 0.010 Da and scan time of 0.240 s. The values corresponding to the tube lens voltage and collision energy for each ion transition were optimized for each quantified compound: carnosic acid m/z 331.4 ($[M-H]^-$) and m/z 287.1 ($[M-H]^-$, product ion using 80 V and 25 V as TLV and CE, respectively), for carnosol m/z 329.3 ($[M-H]^-$) and 285.1 m/z ($[M-H]^-$, product ion using 129 V and 19 V as TLV and CE, respectively) and for rosmarinic acid m/z 359.07 ($[M-H]^-$) and m/z 161.22 ($[M-H]^-$, product ion using 180 V and 20 V as TLV and CE, respectively). Calibration curves were constructed using external calibration method for each compound (0.078–4.0 μg mL^{-1} for CA; 0.05–12.5 μg mL^{-1} for CS and 0.10–10.0 μg mL^{-1} for RA).

Table 1
Factor levels of the experimental design selected to optimize the SAF of PLE rosemary extract, mole fraction phase equilibrium data and calculated selectivity for the experimental design conditions for the SC-CO_2–EtOH–H_2O system at 40 °C. Phase equilibria data adopted from Durling et al. [25].

				Mole fraction								
				Raffinate (liquid phase)			Extract (gaseous phase)					
Experiment	Pressure (bar)	Feed/SC-CO_2	Water in feed (% v/v)	CO_2	EtOH	Water	CO_2	EtOH	Water	K_{EtOH}	K_{H_2O}	Selectivity
1	100	0.0250	50	0.0330	0.0490	0.9180	0.9870	0.0090	0.0040	0.184	0.004	46.00
2	100	0.1000	20	0.0952	0.2728	0.6320	0.9415	0.0481	0.0104	0.176	0.016	11.00
3	300	0.1000	20	0.1100	0.1810	0.7090	0.9140	0.0600	0.0260	0.331	0.037	8.95
4	300	0.1000	50	0.0820	0.1190	0.7990	0.9590	0.0240	0.0170	0.202	0.021	9.62
5	100	0.1000	50	0.0450	0.1320	0.8230	0.9720	0.0220	0.0060	0.167	0.007	23.86
6	300	0.0250	50	0.0620	0.0480	0.8900	0.9810	0.0090	0.0100	0.188	0.011	17.09
7, 9, 11 (CP*)	200	0.0625	35	0.0840	0.1340	0.7820	0.9670	0.0250	0.0080	0.187	0.010	18.70
8	100	0.0250	20	0.0360	0.1030	0.8610	0.9760	0.0170	0.0070	0.165	0.008	20.63
10	300	0.0250	20	0.0770	0.0970	0.8260	0.9690	0.0180	0.0130	0.186	0.016	11.63

* CP, experimental design center point.

2.6. In-vitro *assays*

2.6.1. *Total phenols content (Folin method)*

The total phenols content of the rosemary fractions and the PLE original extract were measured using the Folin–Ciocalteu assay with some modifications [27]. Briefly, 10 μL of each extract (2.5–5 mg mL^{-1} of rosemary fraction extract in ethanol or ethanol + water mixtures) and 600 μL of water were mixed, to which 50 μL of undiluted Folin–Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 20% (w/v) Na_2CO_3 solution were added and the volume was made up to 1.0 mL with water. After 2 h of incubation at 25 °C, 300 μL of the mixture was transferred into a well of a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader (Synergy HT, Bio Tek Instruments, Winooski, VT, USA). A gallic acid calibration curve (0.032–2.00 mg mL^{-1}) was established in the same way and the phenolic content (TPC) was expressed as mg of gallic acid equivalents (GAE) per g of extract. All analyses were done in triplicate.

2.6.2. *Antioxidant activity determination: TEAC assay*

The antioxidant activity of the different rosemary extracts and fractions was determined using the Trolox Equivalents Antioxidant Capacity (TEAC) methodology. The analyses were carried out following the method described by Re et al. [28] with some modifications. $ABTS^{\bullet+}$ radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature (25 °C) during 16 h before its use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) until an absorbance of 0.7 (±0.02) at 734 nm was achieved. One mL of $ABTS^{\bullet+}$ solution was mixed with 10 μL of sample (5 different concentrations) in an eppendorf vial and 300 μL of the mixture were transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min during 45 min in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). Trolox was used as reference standard and results were expressed as TEAC values (mmol of Trolox/g extract). These values were obtained from five different concentrations of each extract tested (between 0.0625 and 1 mg mL^{-1}) in the assay giving a linear response between 20% and 80% blank absorbance. All analyses were done in triplicate.

2.6.3. *DPPH radical scavenging assay*

The DPPH radical scavenging method was carried out adapting the procedure described by Brand-Williams et al. [29]. A stock solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol which was further diluted 1:10 with methanol to give the working solution. Both stock and working solutions were stored at 4 °C until use. Different concentrations of extracts were tested (from 0.0625 to 0.5 mg mL^{-1}). Nine hundred and seventy-five microliters of DPPH diluted solution were added to 25 μL of each extract concentration solution and the reaction was kept in darkness for 4 h at room temperature. Once the reaction was finished, 300 μL of this mixture was transferred into a well of a microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). DPPH–methanol solution was used as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary (expressed in μg mL^{-1}) to decrease the initial DPPH concentration by 50% or EC_{50}. Therefore, the lower the EC_{50} value, the higher the antioxidant capacity. Measurements were done in triplicate.

2.7. *Antiproliferative activity assay*

Colon adenocarcinoma HT-29 cells (American Type Culture Collection, LGC Promochem, Middlesex, UK) were cultured in McCoy's 5A supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL penicillin G, and 50 U/mL streptomycin, at 37 °C in humidified atmosphere and 5% CO_2. Cell viability was determined using MTT assay. Briefly, HT-29 cells were seeded onto 96-well culture plates at 10,000 cells cm^{-2}, and permitted to adhere overnight at 37 °C. Cells were treated with the vehicle (medium with 0.2% DMSO) or rosemary fractions (*raffinate* and *extract*) at a concentration of 30 μg mL^{-1} for 24 h. After incubation, the medium was aspirated and 0.5 mg mL^{-1} of MTT reagent (Sigma-Aldrich) was added to the cells and incubated for 3 h at 37 °C in humidified 5% CO_2/air atmosphere. The medium was then removed, and the purple formazan crystals were dissolved in 100 μL of DMSO. The absorbance at 570 nm was measured in a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland). Results are provided as the mean of the relative cell viability (%; relative to control) ±95% confidence interval (CI) of at least three independent experiments each performed in triplicate.

3. Results and discussion

3.1. *PLE rosemary extraction: Upstream process*

As mentioned, a PLE process has been studied in the present contribution to obtain enriched extracts in phenolic antioxidants from rosemary. The extraction yield obtained for the PLE process was 39.86% (w/w dry weight basis (d.w.b)), with a total concentration of solids of 28.42 g L^{-1} of extract solution. The chemical characterization of the PLE rosemary extract provided values of 25.1, 109.0 and 20.5 mg g^{-1} extract d.w.b. of RA, CA and CS, respectively.

The *in-vitro* assays showed that the total phenolic content was 208.32 mg GAE g^{-1} extract d.w.b., whereas the antioxidant activity determined by TEAC and DPPH methods was 2.33 mM TE g^{-1} extract d.w.b., and EC_{50} = 8.51 μg mL^{-1}, respectively.

3.2. Theoretical selectivity for SC-CO_2–EtOH system

In this section, the influence of the factors to be studied on the SC-CO_2–EtOH–H_2O phase equilibria and on the theoretical selectivity of the antisolvent fractionation is discussed. Mole fraction equilibrium data for this ternary system at 40 °C and pressures of 100–300 bar was already measured by Durling et al. [26] and is represented in Fig. S2 (Supplementary material). As can be observed, the size of the two-phase region (below the curve) of the ternary diagram decreases with increasing pressure (from 100 to 300 bar) at constant temperature, conditions that influence the miscibility of the binary mixture of ethanol + water in SC-CO_2. Also, the portion of the solvent mixture that is dissolved in the SC-CO_2 acts as a co-solvent (ethanol), increasing the solvent power of the near-critical fluid and modifying the equilibrium. In the composition of the feed, the mass fraction of water can range from zero to near 100% and must be optimized according to the range of compounds to be extracted. Besides, according to the experimental data [26], solvent selectivity of ethanol reaches high values when decreasing the mole fraction of ethanol (CO_2 free basis) in the liquid phase (*raffinate*). This condition is achieved when small feed solution-to-supercritical fluid mass ratios are employed (≤0.125), being in agreement with the results obtained for sage and propolis hydro-alcoholic extract fractionations in other researches [22,24]. However, the ratio cannot be decreased indefinitely, because the profitability of the process can worsen due to the low throughput of extract obtained.

Taking into account as process separation input streams: SC-CO_2 and PLE extract feed, the composition of the mixing points (*M*) for each feed/SC-CO_2 mass flow ratio (0.025, 0.0625 and 0.1) and the mass fraction of water in the feed selected (24, 40.5 and 55.8% w/w) were obtained by solving the mass balance for the antisolvent separation process (data not shown). In Fig. S2, mixing points (indicated with full circles, triangles and squares) were located on the straight lines of water mole fraction (blue line (24% w/w or 0.45), red line (40.5% w/w or 0.63) and green line (55.8% w/w or 0.76)) in feed, under the binodal curve. The mole compositions of *raffinate* and *extract* were obtained by the end of tie lines passing across them, following the Sherwood's method [30].

Once the compositions were known, the ethanol and water partition coefficients (K_i), between CO_2-rich phase (*extract*) and water-rich phase (*raffinate*) were calculated according to Eq. (2), where *y* and *x* are the mole fractions of ethanol (or water) in the gaseous and liquid phases, respectively.

$$K_i = \frac{y_i}{x_i} \tag{2}$$

The partition coefficients are necessary to assess the theoretical selectivity, which was calculated as ratio of the partition coefficients between ethanol and water. Both theoretical selectivity and partition coefficients values are shown in Table 1 together with the corresponding levels of the factors (in real units) selected in the experimental design. The results showed that, at given water content in the feed and feed/SC-CO_2 flow mass rate ratio, selectivity values increase with decreasing pressure. Theoretical selectivity also increases with the increase of the mass fraction of water in the feed, when pressure and flow rate ratios are kept constant. Moreover, when feed/SC-CO_2 flow rate ratio is decreased, while maintaining pressure and percentage of water constant, the selectivity also improves. Thus, among the conditions proposed, the theoretical selectivity suggests that low values of pressure, feed/SC-CO_2 flow rate ratio and high values of % water in the feed could be used to achieve the maximum separation of ethanol in the extract and water in the *raffinate* phase on a SC-CO_2–EtOH–H_2O system. These results could give an idea about the behavior of the pure ternary system. However, the real (experimental) selectivity and the recovery of the compounds of interest present in the complex PLE extract must be studied (next sections) in order to assess if there is a correlation among them.

3.3. Optimization of the supercritical antisolvent fractionation process: Downstream process

The levels of the factors (in coded and real units) as well as the outcomes for the response variables studied are summarized in Tables 2 and 3, for *raffinate* and *extract*, respectively. The analysis of the effects of each factor on the different responses for each fraction will be discussed separately below.

3.3.1. Raffinate

As it can be seen in Table 2, the recovery of *raffinate* fraction was high (72.1–96.7%) for all of the conditions proposed, which can be interpreted as a high concentration of compounds in the PLE extract lacking solubility in SC-CO_2 + EtOH mixture. The fraction obtained in the *raffinate* was effectively enriched (6.76 wt.%, 2.7-fold compared to the PLE original extract) in RA (the most polar of the studied compounds) when the fractionation was performed at 300 bar, 20% (v/v) of water in the mixture and 0.025 PLE extract/SC-CO_2 flow rate ratio. Moreover, under those fractionation conditions, the highest phenolic content and antioxidant activity were obtained. The individual effects of pressure and % water in the feed showed a positive significant effect, while the PLE extract/SC-CO_2 ratio showed a negative effect on RA recovery, as shown in Fig. 2a. As expected, as the PLE extract/SC-CO_2 flow rate ratio decreases, the level of ethanol in the *raffinate* also decreases and the amount of water increases, and thus, the separation of solutes that are not soluble in CO_2 + EtOH favors the *raffinate* phase. On the other hand, the highest values of CA and CS in the *raffinate* matched the conditions obtained for the lowest RA content (experiment 2, Table 2). For the CA + CS, the individual effects of each factor exhibited the opposite behavior than RA content, being pressure the most influencing factor, followed by water content in PLE extract (Fig. 2b). Regarding the total phenolic content, it was possible to recover between 66 and 85% more phenolic compounds in the *raffinate* than in the *extract* fraction. However, considering the inhibitory activity of the *raffinate* on HT-29 cells proliferation, results showed a maximum reduction of 35% on cell viability, which can again be related to the amount of CA + CS present on that fraction, as reported previously [7,31,32]. Indeed, some recent results have suggested that no significant inhibition of cell proliferation was observed on different cancer cell models treated with RA alone [32–34].

3.3.2. Extract

The results of the experimental design for the *extract* fraction (see Table 3) showed that the less polar compounds (CA and CS) were obtained, as expected, preferentially in that fraction. Rosmarinic acid determined by HPLC–MS was below the limits of quantification for all the experiments. The recovery of the *extract* reached values between 3.3 and 23.5% (wt.). The highest enrichment in CA + CS was equal to 47.81% (wt.), achieved at 100 bar, 50% (v/v) of water in the feed and 0.025 PLE extract/SC-CO_2 mass flow ratio. To our knowledge, this is the highest mass fraction of CA + CS obtained so far from rosemary applying green extraction-fractionation processes such as PLE, SFE, SFE + EtOH, SWE, or two-step sequential SFE [3,13,17,35]. Also under these conditions, the highest percentage of recovery of carnosic acid + carnosol (78.5 wt.%) was obtained. All of the above results seem to be in

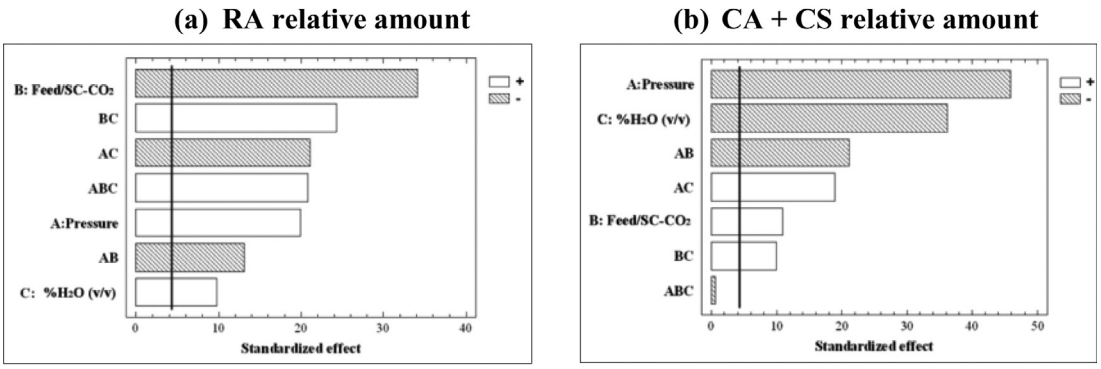

Fig. 2. Standardized Pareto charts for the rosmarinic acid and carnosic acid + carnosol relative amount in the experimental design for *raffinate* phase.

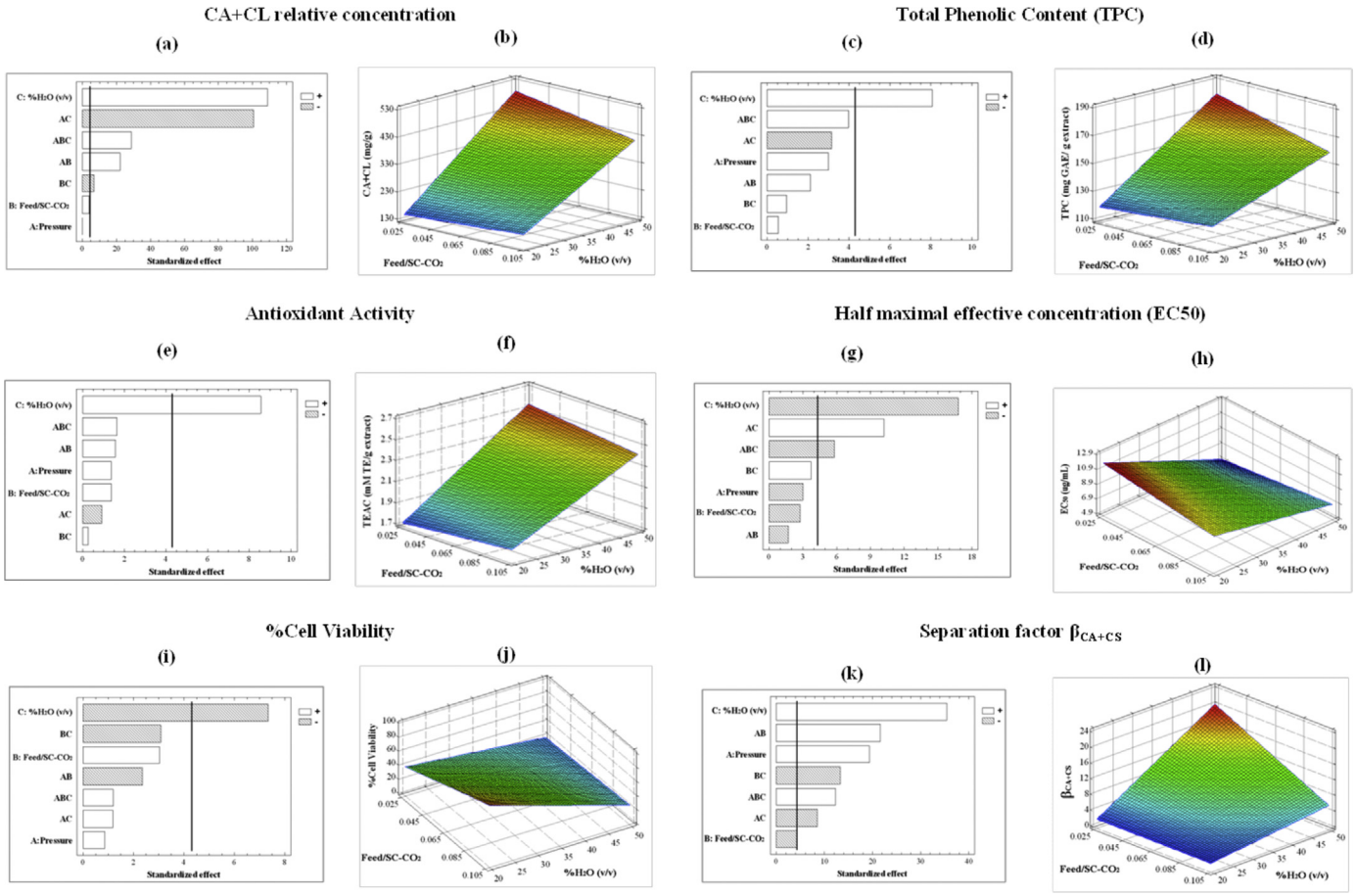

Fig. 3. Standardized Pareto charts for the six response variables of phase equilibrium studied in the experimental design for *extract* phase and the separation factor β_{CL+CA} (striped and white bars show negative and positive effects, respectively), and their corresponding response surfaces, keeping constant the pressure (100 bar).

agreement with the theoretical selectivity assessed in Section 3.2. As can be seen in Table 3, all the response variables studied for this fraction reached the highest values at those conditions, except total phenolic content. It is also worth to note that the CA + CS concentration in the extract obtained at the conditions of experiment 1 (Table 3) was concentrated 3.7-fold compared to the corresponding mass fraction in the original PLE extract. Table S1 (Supplementary material) shows the regression coefficients obtained from the mathematical model provided by Statgraphics software for the effects of the independent factors on the response variables in the SAF process, considering a level of confidence of 95% for all the variables. The *p*-value showed that the regression was highly significant and valid to describe the experimental data; however, the *F*-test for the lack-of-fit showed that the model for the combined CA + CS content was not predictive (the response surface shown in Fig. 3b was generated only for qualitative purposes). For the other responses, the *p*-value for lack-of-fit in the ANOVA was higher than 0.05 and the models seemed to be adequate to describe the observed data. The determination coefficient (R^2) of the model for the total phenolic content was 0.97, while for the TEAC, EC_{50} and % cell viability were 0.92, 0.98 and 0.93, respectively. In Fig. 3, the standardized Pareto charts for the six response variables studied are shown together with their corresponding response surfaces. Different bar shadings indicate positive and negative effects of the factors in the response variables and the vertical line demonstrates the significance of the effects at the 95% confidence level. As it can

Table 2
Experimental design in real and coded (between brackets) values of the factors and values of the response variables studied for the *raffinate*.

Experim.	*P* (bar)	Feed/SC-CO_2	Water in feed (% v/v)	Recovery (wt.%)	RA (mg g^{-1})	CS (mg g^{-1})	CA (mg g^{-1})	TPC (mg GAE g^{-1} extract)	TEAC (mM TE g^{-1} extract)	EC_{50} (μg mL^{-1})	Cell viability % (±95%CI)
1	100 (−1)	0.0250 (−1)	50 (1)	78.7	40.03	8.94	14.83	272.46	3.06	6.29	74.2 ± 2.2
2	100 (−1)	0.1000 (1)	20 (−1)	93.8	9.98	17.23	87.07	224.27	2.52	8.52	65.0 ± 1.9
3	300 (1)	0.1000 (1)	20 (−1)	76.5	14.06	5.97	19.43	239.83	2.58	9.01	72.6 ± 2.6
4	300 (1)	0.1000 (1)	50 (1)	87.7	33.67	4.40	13.74	234.59	2.71	7.95	79.2 ± 1.6
5	100 (−1)	0.1000 (1)	50 (1)	96.7	29.90	13.21	49.99	245.20	2.75	8.21	86.0 ± 1.5
6	300 (1)	0.0250 (−1)	50 (1)	96.0	34.92	4.58	14.34	239.14	2.64	5.89	88.4 ± 2.7
7 (CP)	200 (0)	0.0625 (0)	35 (0)	84.0	31.95	10.38	22.22	237.46	2.72	8.35	92.7 ± 2.4
8	100 (−1)	0.0250 (−1)	20 (−1)	95.7	24.15	15.61	68.84	195.77	2.27	6.04	77.4 ± 2.7
9 (CP)	200 (0)	0.0625 (0)	35 (0)	83.1	32.82	11.39	23.80	234.11	2.68	7.86	106.0 ± 3.2
10	300 (1)	0.0250 (−1)	20 (−1)	84.7	67.66	8.64	34.80	340.67	4.16	9.32	82.1 ± 3.5
11(CP)	200 (0)	0.0625 (0)	35 (0)	85.6	31.18	11.83	22.38	243.34	2.93	7.87	94.5 ± 2.5

CP: experimental design center point.

Table 3
Experimental design in real and coded (between brackets) values of the factors and values of the response variables studied for the *extract*.

Experiment	*P* (bar)	Feed/SC-CO_2	Water in feed (% v/v)	Recovery (wt.%)	RA (mg g^{-1})	CS (mg g^{-1})	CA (mg g^{-1})	TPC (mg GAE g^{-1} extract)	TEAC (mM TE g^{-1} extract)	EC50 (μg mL^{-1})	Cell viability % (±95%CI)	Recovery %CA + CS (wt.)	β_{CA+CS}
1	100 (−1)	0.0250 (−1)	50 (1)	21.3	<LOQ	132.30	345.80	178.82	2.58	4.95	16.9 ± 2.3	78.5	20.1
2	100 (−1)	0.1000 (1)	20 (−1)	6.2	<LOQ	36.24	120.63	123.90	1.84	9.71	80.6 ± 3.7	7.6	1.5
3	300 (1)	0.1000 (1)	20 (−1)	23.5	<LOQ	66.28	223.06	142.18	2.01	8.04	62.9 ± 1.9	52.5	11.4
4	300 (1)	0.1000 (1)	50 (1)	12.3	<LOQ	84.59	247.71	184.47	2.74	6.45	26.0 ± 1.6	31.5	18.3
5	100 (−1)	0.1000 (1)	50 (1)	3.3	<LOQ	60.52	341.95	158.97	2.46	6.51	22.0 ± 1.9	10.3	6.4
6	300 (1)	0.0250 (−1)	50 (1)	4.0	<LOQ	80.84	183.71	151.91	2.37	7.65	31.6 ± 1.9	16.5	14.0
7 (CP)	200 (0)	0.0625 (0)	35 (0)	16.0	<LOQ	144.15	197.30	148.09	2.17	8.49	45.6 ± 4.1	22.8	10.5
8	100 (−1)	0.0250 (−1)	20 (−1)	4.3	<LOQ	24.33	108.14	117.60	1.75	11.65	36.4 ± 1.5	4.2	1.6
9 (CP)	200 (0)	0.0625 (0)	35 (0)	16.9	<LOQ	152.51	189.54	157.74	1.96	8.56	57.1 ± 3.9	24.0	9.7
10	300 (1)	0.0250 (−1)	20 (−1)	15.3	<LOQ	95.21	188.48	152.02	1.93	8.48	51.1 ± 1.4	31.8	6.5
11(CP)	200 (0)	0.0625 (0)	35 (0)	14.4	<LOQ	151.29	193.88	159.32	2.02	8.08	46.3 ± 2.4	20.7	10.1

LOQ: Limit of quantification.
CP:experimental design center point.

be observed, percentage of water in the PLE extract was always the most important factor, although for some responses, two and three factor interaction effects also had an important influence. Response surface for variables related to the combined CA + CS content, total phenolic content and antioxidant activity were similar, increasing the values according to an increase of water content in the feed and also, to a lesser extent, with the decrease in the PLE extract/SC-CO_2 flow rate ratio. Minimum values of EC_{50} and percentage of cell viability were also obtained following the same trend. In order to consider the six response variables at the same time, a multiple response optimization was calculated maximizing the combined CA + CS content, TPC and the TEAC value and minimizing EC_{50} and cell viability, all of them given the same weight. The optimum fractionation conditions provided by the statistical model are those of experiment 1 (pressure, 100 bar; 0.025 PLE extract/SC-CO_2 flow rate ratio; and 50% water in the feed). Under these conditions, predicted values of 491.85 mg g^{-1}, 179.86 mg GAE g^{-1}, 5.07 mM TE g^{-1}, 2.54 μg mL^{-1} and 19.33% were obtained for CA + CS content, TPC, TEAC, EC_{50} and % cell viability respectively. Moreover, an overall desirability value of 0.931 was attained. Experimental values obtained in experiment 1 (corresponding to the optimum process parameters) presented an average of the Relative Standard Deviation (%RSD) of 2.88, 2.50, 7.43, 2.46 and 14.06%, for the CA + CS content, TPC, TEAC, EC_{50} and % cell viability respectively, thus, demonstrating the usefulness of the proposed model to assess the SAF process. Considering the important antiproliferative activity observed for the *extract* fraction, this will be analyzed in detail and discussed separately in Section 3.4.

3.3.3. Separation factor β_{CA+CS}

One way of evaluating the actual selectivity of the SC-CO_2–EtOH–H_2O ternary system to fractionate the phenolic diterpenoids is comparing their content in the *extract* and in the *raffinate*, or calculating the separation factor β_{CA+CS} which indicates how many times the light phase (*extract*) is more concentrated (or diluted) than the heavy phase (*raffinate*) in terms of CA and CS content. A $\beta_{CA+CS} = 1$ indicates no fractionation and, therefore, the solvent is not selective; whereas $\beta_{CA+CS} < 1$ or $\beta_{CA+CS} > 1$ indicates selectivity, in which CA and CS will be enriched in the *raffinate* or in the *extract*, respectively. In Table 3, the calculated experimental separation factors are shown; values of enrichment between 1.5 and 20.1 were achieved. Considering those values as an additional response variable to be analyzed by RSM, the standardized Pareto chart and its response surface were obtained. As can be observed in Fig. 3k, the composition of the PLE extract has the highest positive influence on the separation process, followed by the pressure. The optimization of β_{CA+CS} provided a mathematical model (see Eq. (3)) with a $R^2 = 0.991$ (99.7% of the experimental variability explained by the model at the 95% confidence level, $p < 0.5$).

$$\beta_{CA+CL} = -20.59 + 0.068 \times P + 146.34 \times R + 1.03 \times W - 0.26 \times P \times R - 0.0026 \times P \times W - 9.00 \times R \times W + 0.03 \times P \times R \times W \quad (3)$$

The optimum fractionation was obtained using the lowest flow rate (0.025 mL min^{-1}), the lowest process pressure (100 bar) and highest water content in the feed, being in concordance again with the results obtained for the theoretical selectivity and optimum conditions to fractionate PLE rosemary extracts. As can be seen, the highest theoretical selectivity matched with the highest separation factor $\beta_{CA+CS.}$ Regarding other conditions studied however, while the theoretical selectivity decreases with a pressure increase, the separation factor (β_{CA+CS}) increases under these conditions, probably due to an improvement of the solubility of CA in SC-CO_2 + ethanol as reported by Chafer et al. [12].

3.4. Antiproliferative activity of SAF fractions

According to the literature, CA and CS exert different antiproliferative effects depending on their concentration, the cancer cell type and duration of the treatment. For instance, medium μM concentrations (*ca.* 25–33 μM) of CA exert cytostatic effects on human colon adenocarcinoma cells [9], whereas, a lower CA concentration (10 μM) has shown to induce cell death on human hepatocellular carcinoma [36]. Reported data also suggest that CA and CS exhibit additive cytostatic effects when they are combined in solution (GI50 value of 15.2 μg CA + CS mL^{-1}) [9]. In addition, the antiproliferative activities of CA- and CS-enriched rosemary extracts on HT-29 colon cancer cells generally surpass those observed for pure solutions with similar CA and CS content [17].

In the present work, the viability screening performed on the *raffinate* and *extract* fractions at a concentration of 30 μg mL^{-1} also indicated that the most active extracts were those with higher CA + CS content. Moreover, the inhibitory effects observed for the three most enriched *extracts* in CA + CS (10.3–14.3 μg of total CA + CS mL^{-1} of culture medium) were superior (% cell viability < 50) to the reported activity of pure solutions with similar total CA + CS concentrations on the same cell model (GI50 15.2 μg CA + CS mL^{-1}, Valdés et al. [9]). Furthermore, the antiproliferative activities of the *extracts* obtained at the conditions of 50% (v/v) of water in the feed (experiments 1, 4, 5, and 6) were strikingly higher than those observed for other rosemary extracts with comparable CA + CS content. Among those extracts, the most active one reduced cell viability down to 17%. To note, 30 μg mL^{-1} of the *extract* obtained in the present work at the conditions of experiment 5, containing a total CA + CS concentration of 12.1 μg mL^{-1}, reduced HT-29 cell viability up to 22.0% after incubation for 24 h, whereas the reported viability value for an extract (30 μg mL^{-1}; 12.3 μg CA + CS mL^{-1}) obtained by a single step SFE procedure was 64.5% [17]. These results suggest that conditions corresponding to 50% (v/v) of water in the feed enhance the cytotoxic potency of the *extracts*. This finding was also corroborated by the comparison of the antiproliferative effect observed with 30 μg mL^{-1} of e*xtracts* obtained at the conditions of experiment 4 and the central point experiments (7, 9 and 11), containing similar total CA + CS concentrations (~10 μg mL^{-1}), but showing a difference of approximately 20% on cell viability (Table 3). No direct dependence between the molar CA/CS ratio of the extracts and their potency could be found; therefore, it seems unlikely that differences on molar CA/CS ratio, ranging from 1.3 to 5.7, will account for the observed variation in the cell viability between extracts. Indeed, the viability differences between extracts with similar total CA + CS concentration supports the hypothesis that although CA and CS account for most of the observed effect, other constituents may also positively or negatively influence the antiproliferative activity of the extracts against HT-29 colon cancer cells. With this idea in mind, besides the HPLC–DAD–MS analysis of the fractions, we carried out the GC–MS analysis of the volatile compounds in these fractions; however, not correlation could be established based on the GC–MS results (data not shown). A detailed study is now being carried out in our laboratory to identify other compounds, besides CA and CS, in the rosemary fractions responsible of the antiproliferative activity observed. Taken together, these data demonstrate the good potential of the strategy adopted in this work for the optimization of SAF conditions to provide rosemary extracts with potent antiproliferative activity.

4. Conclusions

In the present work, an integrated process based on the use of pressurized liquid extraction and supercritical antisolvent

fractionation has been optimized to obtain rosemary extracts with improved antiproliferative activity against HT-29 colon cancer cells. By employing a RSM it has been possible to optimize the most important factors involved in the SAF process (CO_2 pressure, percentage of water in the PLE extract and PLE extract/SC-CO_2 flow ratio). The extract obtained at 100 bar, 50% (v/v) of water in the feeding solution and a 0.025 feed/SC-CO_2 flow mass ratio of presented the highest anti-proliferative activity with 17% of cell survival after 24 h of treatment. This extract presented also the highest CA + CS content, 478.1 mg/g extract, although results suggested that other compounds are also involved in the important antiproliferative activity observed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.supflu.2015.07.019

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SUPPLEMENTARY MATERIAL

Figure S1. Structures of phenolic compounds of interest in this study.

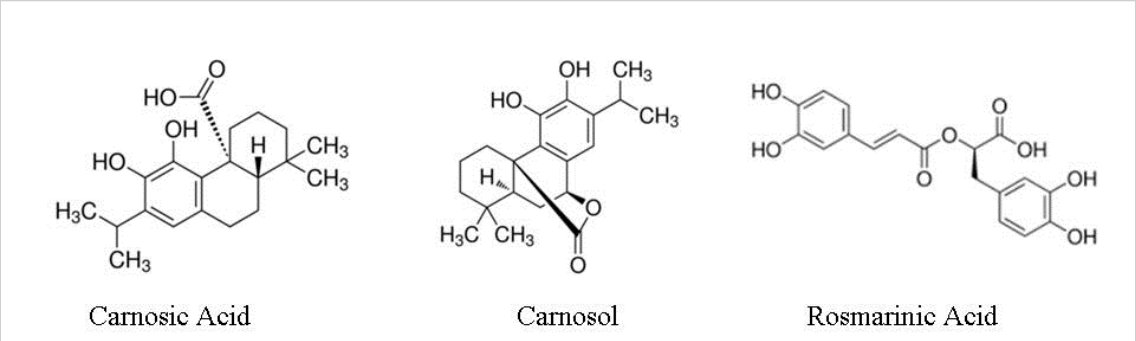

Figure S2. Phase equilibrium for CO_2-ethanol-water system at 40°C at 100, 200 and 300 bar. Percentage of water in the feed: 20% (v/v) (blue line), 35% (v/v) (red line) and 50% (v/v) (green line). Phase equilibria data adopted from Durling et al. [26]

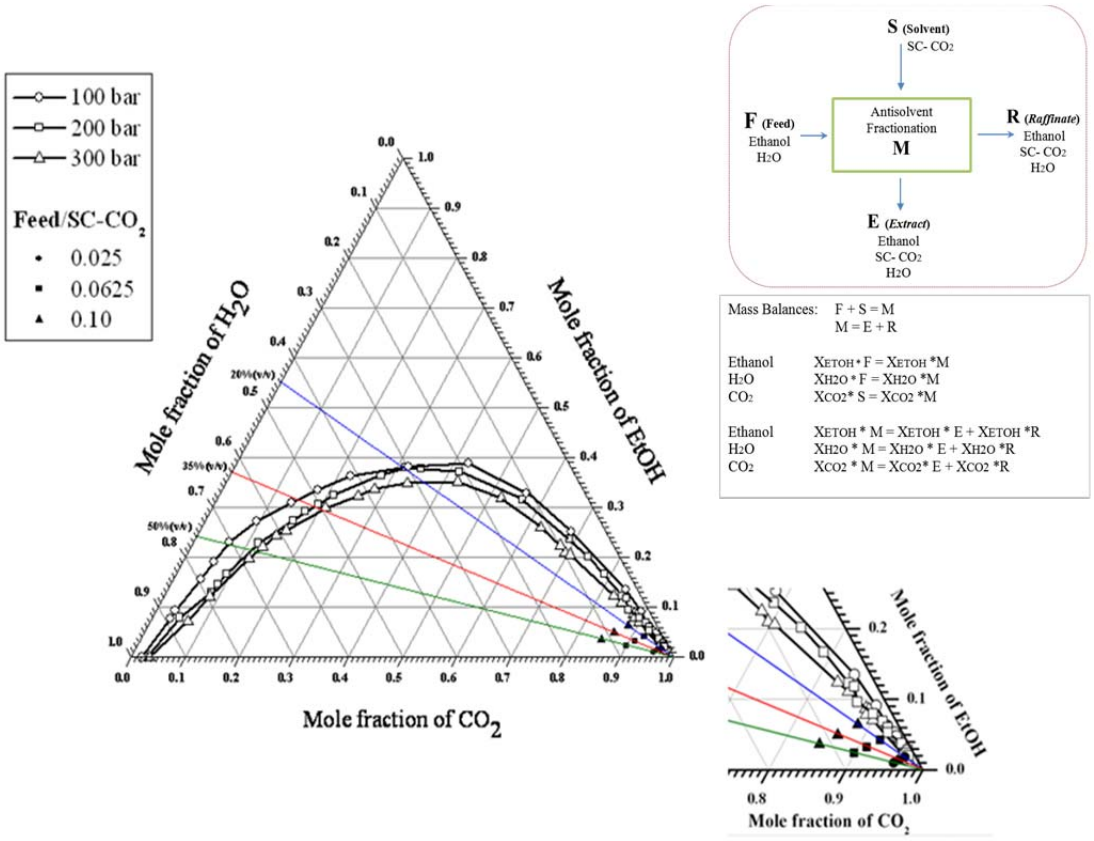

Table S1. Coefficients of regression of third order model for the variables response of factorial design for the analysis of RSM for *extract* fraction

	CA+CS content		Total Phenolic Content		Antioxidant activity (TEAC)		Antioxidant activity EC_{50}		% Cell Viability	
Coefficient	**Estimate**	**p-value**	**Estimate**	**p-value**	**Estimate**	**p-value**	**Estimate**	**p-value**	**Estimate**	**p-value**
β_0	-328.304		21.956		0.799		21.928		13.102	
β_1	2.184	0.994	0.480	0.096	0.003	0.304	-0.043	0.096	0.152	0.482
β_2	1986.480	0.059	728.711	0.647	5.356	0.304	-85.911	0.110	1249.490	0.094
β_3	19.613	0.0001*	3.734	0.015*	0.039	0.013*	-0.384	0.004*	-0.091	0.018*
$\beta_{1,2}$	-8.457	0.002*	-4.123	0.169	-0.023	0.257	0.289	0.228	-3.126	0.143
$\beta_{1,3}$	-0.070	0.0001*	-0.014	0.088	0.000	0.457	0.001	0.010	-0.001	0.361
$\beta_{2,3}$	-80.496	0.022*	-26.856	0.444	-0.204	0.818	2.502	0.065	-22.231	0.091
$\beta_{1,2,3}$	0.360	0.001*	0.152	0.058	0.001	0.244	-0.009	0.028	0.048	0.357
Lack-of-fit	1869.100	0.001*	31.776**	0.452	0.056**	0.161	0.435**	0.126	166.505**	0.185
R^2	94.302		97.353		92.467		98.165		93.188	
R^2 adjust	81.215		91.177		74.889		93.885		77.294	
Pure error**	3.989		36.955		0.012		0.067		83.459	

* Statistically significant values: $p < 0.05$
** Mean Square

4.1.4 Comparative study of green sub- and supercritical processes to obtain carnosic acid and carnosol-enriched rosemary extracts with *in vitro* antiproliferative activity on colon cancer cells

A.P. Sánchez-Camargo, V. García-Cañas, M. Herrero, A. Cifuentes & E. Ibáñez

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Comparative Study of Green Sub- and Supercritical Processes to Obtain Carnosic Acid and Carnosol-Enriched Rosemary Extracts with in Vitro Anti-Proliferative Activity on Colon Cancer Cells

Andrea del Pilar Sánchez-Camargo, Virginia García-Cañas, Miguel Herrero, Alejandro Cifuentes and Elena Ibáñez *

Laboratory of Foodomics, Institute of Food Science Research, Instituto de Investigación en Ciencias de la Alimentación, Consejo Superior de Investigaciones Científicas, Nicolas Cabrera 9, 28049 Madrid, Spain; andreap.sanchez@csic.es (A.d.P.S.-C.); virginia.garcia@csic.es (V.G.-C.); m.herrero@csic.es (M.H.); a.cifuentes@csic.es (A.C.)

* Correspondence: elena.ibanez@csic.es; Tel.: +34-910-017596; Fax: +34-910-017905

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Abstract: In the present work, four green processes have been compared to evaluate their potential to obtain rosemary extracts with in vitro anti-proliferative activity against two colon cancer cell lines (HT-29 and HCT116). The processes, carried out under optimal conditions, were: (1) pressurized liquid extraction (PLE, using an hydroalcoholic mixture as solvent) at lab-scale; (2) Single-step supercritical fluid extraction (SFE) at pilot scale; (3) Intensified two-step sequential SFE at pilot scale; (4) Integrated PLE plus supercritical antisolvent fractionation (SAF) at pilot scale. Although higher extraction yields were achieved by using PLE (38.46% dry weight), this extract provided the lowest anti-proliferative activity with no observed cytotoxic effects at the assayed concentrations. On the other hand, extracts obtained using the PLE + SAF process provided the most active rosemary extracts against both colon cancer cell lines, with LC_{50} ranging from 11.2 to 12.4 μg/mL and from 21.8 to 31.9 μg/mL for HCT116 and HT-29, respectively. In general, active rosemary extracts were characterized by containing carnosic acid (CA) and carnosol (CS) at concentrations above 263.7 and 33.9 mg/g extract, respectively. Some distinct compounds have been identified in the SAF extracts (rosmaridiphenol and safficinolide), suggesting their possible role as additional contributors to the observed strong anti-proliferative activity of CA and CS in SAF extracts.

Keywords: rosemary; supercritical fluid extraction (SFE); process intensification; subcritical fluids; supercritical fluids; anti-proliferative; colon cancer cell; HT-29; HCT116

1. Introduction

Nowadays, supercritical fluid extraction (SFE) employing CO_2 is an established industrial process for the production of high added-value products. By 2014, there were more than 150 SFE industrial plants with a total extraction volume of more than 500 L around the world. Many of these production plants are generally devoted to the SFE of natural products, leading to the recovery of high-value products which provide interesting options for their use in the nutraceutical and functional food industry [1]. In this sense, the search for bioactive compounds or "target molecules" from natural sources (marine-derived and agro-industrial products or their by-products) has become the most important application of the scientific research of SFE [2–8]. According to the review work recently published by Da Silva et al. (2016), the bioactivities from natural compounds obtained by SFE from 2010 to 2015 were mainly antioxidant (41%), antitumor (18%) and antibacterial (10%),

followed by antiviral, antimicrobial, anti-inflammatory and anticholinesterase activity (in a total of 5%) [2]. In this regard, the recovery of bioactive compounds from herbs and spices (especially those belonging to the *Lamiaceae* family) employing supercritical and subcritical fluids has been intensified in the last 10 years [5,9,10], mainly due to several biological properties such as: antioxidant [11–13], antimicrobial [14–17], anti-proliferative [18,19], antitumor [20,21], anti-inflammatory [22–24] and anti-obesity [25,26], among others. Rosemary-leaf extracts (*Rosmarinus officinalis* L.) have received special attention since European Food Safety Authority and U.S. Food and Drug Administration approved their use as food additive, demonstrating to be safe for human health at specific compositions [27,28]. Several rosemary compounds, principally carnosol (CS) [29–32], carnosic acid (CA) [33–35], and ursolic acid [36–38], have demonstrated different anticancer activities, such as anti-proliferative, antiinvasive, and antitumorigenic effects, in a dose-dependent manner. Interestingly, although several of the observed anti-proliferative effects of rosemary extracts have been commonly attributed to some of these components, in many cases the effect of the complete extract is higher than that exerted by the individual compounds [20,21,30,33,39,40]. In some cases, this general observation has been attributed to the synergistic effect of the combination of the main bioactive compounds with other unknown and minor compounds present in the extract [20,21]. These functional aspects of rosemary extracts make necessary to standardize the extraction methodology to achieve an extract composition that exhibits the pursued anti-proliferative activity. Some authors have demonstrated the superior anti-proliferative activity of the SFE rosemary extracts compared to aqueous and methanolic extracts in leukemia, lung, liver, prostate, breast and colon cancer cells [18,33]. In our laboratory, the investigation of green processes extraction methodologies has mainly been focused on the concentration enhancement of bioactive compounds in rosemary extracts by means of multistage fractionation or combination of sub- and supercritical fluid methodologies with upstream process optimization [41–43]. These strategies are in good agreement with the recent trends directed to the development of new intensified and integrated processes, which seems to be more suitable for complex vegetable matrixes such as rosemary [1,42,44,45]. In process intensification, the same multipurpose equipment is used for different unit operations, while in the integrated process, the best process for obtaining each product is sought, where different equipment is commonly employed [44]. The process intensification concept using supercritical fluids has been recently applied in several areas as an option for the future production of substitutes for petrochemical derivatives from biomass, mainly as source of energy and biofuels [46,47]. We recently reported the use of a pilot scale SFE equipment to study a two-sequential step SFE as intensification process. Following this strategy, it was possible to obtain two fractions, one rich in volatile oil (containing 1,8-cineole and camphor) and the other rich in CA and CS, being this latter fraction tested for its inhibitory activity against colon cancer cell proliferation [42]. On the other hand, process integration includes prior unit operation (fermentation, extraction, enzyme pre-treatment, physical fractionation or size reduction) followed by sub-or supercritical extraction or fractionation processes (supercritical chromatography, enzymatic conversion, precipitation and coating of solutes, among others) [45]. Regarding this approach, recent reports suggest the combined use of extraction processes, such as supercritical CO_2 followed by pressurized liquid extraction (PLE, employing ethanol and water) for improving the recovery of compounds with different polarities and bio-functionalities [44,48–50]. Another exceptionally versatile process that has been used in the integrated SF-processes is the Supercritical Antisolvent Fractionation (SAF), which benefits from the antisolvent properties of supercritical carbon dioxide (SC-CO_2) and allows for the precipitation of insoluble compounds in the SC-CO_2 organic solution mixture [45,51]. Following these ideas, an integration of two lab-scale processes, PLE and SAF was carried out to obtain two fractions enriched in different families of compounds: a raffinate fraction (enriched in phenolic acids, mainly rosmarinic acid) and an extract fraction (enriched in phenolic diterpenes, mainly CA and CS) [41]. The integrated process was optimized and the extract fraction showed improved in vitro anti-proliferative activity against human colon adenocarcinoma cells. Once the processes have been optimized at lab-scale, it is

mandatory to test their efficacy at large scale; this step is crucial for the future implementation of standardized processes leading to a product with well-known composition and bioactivity.

Therefore, in the present work, a comparative study of the four types of green processes previously developed at lab-scale was carried out to evaluate their potential to obtain rosemary extracts with in vitro anti-proliferative activity against two colon cancer cell lines (HT-29 and HCT116). The comparison was performed on several rosemary extracts obtained by PLE using an hydroalcoholic mixture as solvent with standard lab-scale equipment (a); and three different processes at pilot scale, namely, single-step SFE (b), two-step sequential SFE (process intensification, c), and PLE + SAF (process integration, d). To achieve that, PLE + SAF process (d) was assessed at higher scale in the present study, and the resulting extracts were compared in terms of yield, chemical composition, and antioxidant and anti-proliferative activities to the extracts obtained by the other three previously optimized processes (a–c). All the rosemary extracts were chemically characterized using different MS-based analytical techniques in an attempt to correlate the presence of specific rosemary constituents with the observed bioactivities.

2. Results and Discussion

2.1. Yield of Phenolic Compounds and Antioxidant Properties of the Extracts

In the present study, four green processes were selected based on previous works focused on the enrichment of rosemary extracts on CA and CS [41,42], to investigate their potential for the production of rosemary leaves' extracts and fractions with potent in vitro anti-proliferative activity against colon cancer cells. Thus, PLE process was chosen since it is suitable for providing a rosemary extract at lab scale that can be also used to obtain the starting material required for other processes (see below). In addition to this, we selected three different processes performed at pilot scale. Namely, a single-step SFE process using ethanol as co-solvent (7%, *w*/*w*) [41], a two-step SFE process for further CA enrichment of the extract [41] and an integrated process that involves PLE and supercritical antisolvent fractionation [41]. A total of six rosemary extracts were obtained in duplicate using the conditions summarized in Table 1. As mentioned, the SAF process, previously optimized at lab scale to obtain extracts fractions enriched in the phenolic diterpenes [41], was performed at pilot scale in the present work to obtain three different extracts (SAF1-3) to make them comparable with the other extracts obtained at pilot scale. As can be observed in Table 2, PLE process provided the highest extraction yield (38.46 g/100 g rosemary-leaf dry) due mainly to the polarity solvent and the lower selectivity of this procedure. On the other hand, despite the co-solvent and other distinct SFE extraction conditions, the low extraction yields of SFE1 and SFE2 processes were not significantly different. The extraction yields (or recoveries, in this case) of the SAF processes were higher when the feed to SC-CO_2 mass flow ratio was the lowest. Thus, around 21% (*w*/*w*) of dry PLE extract was recovered employing SAF1 conditions, in accordance with the values obtained at lab-scale [41]. Total phenol values were statistically different among processes studied and did not correlate linearly (at confidence level of 95%) with the antioxidant activity, expressed as Trolox equivalent antioxidant capacity (TEAC) assay (r = 0.64) and EC_{50} (half maximal effective concentration) (r = −0.23). The highest phenol amount was achieved in PLE sample (233.9 mg Galic acid equivalent (GAE)/g extract), which is in agreement with the nature of the solvent (ethanol/water mixture) employed to extract this kind of compounds. However, this high total phenolic content (TPC) value for PLE extract does not correspond with the highest TEAC and lowest EC_{50} values among the extracts obtained. For instance, SAF1 exhibited a lower TPC value than PLE sample; however, their TEAC and EC_{50} values were 49% higher and 56% lower, respectively. This evidence could suggest that other types of compounds present in this extract had a positive influence on the antioxidant activity. A similar observation can be done for SFE2 and SAF2 extracts. Interestingly, SAF2 and SAF3 showed very similar TEAC values but the concentrations required to inhibit 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by half were very different among them. A similar discrepancy was observed in the analysis of SFE2 and PLE extracts. In support of

these observations, Yesil-Celiktas et al. (2007) reported similar results, indicating that TEAC assay did not show any correlation with phenol content ($r = -0.17$) and DPPH assay ($r = 0.16$), in rosemary extracts obtained by SFE [52]. These phenomena could be explained by the mechanism employed by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical which is not able to allow for discrimination between the genuine antioxidant compounds and just expresses well by reducing agents that react primarily by a single electron transfer mechanism [53]. Comparing the six extracts, the conditions used to obtain SAF1 samples seemed to be the most adequate green alternative in order to maximize total phenolic content and antioxidant activity. The potency of all the extracts to inhibit colon cancer cell proliferation was next tested on HT-29 and HCT116 cell lines.

Table 1. Experimental conditions employed for the extraction of the different rosemary extracts.

Process	Sample Name	Pressure (Bar)	Temperature (°C)	Feed/SC-CO_2 Ratio	%H_2O (*w*/*w*)	%Ethanol (*w*/*w*)	Process Time (min)
PLE	PLE	100	150	-	24.0	76.0 **	20
Single-step SFE	SFE1	150	40	-	-	7.0 *	300
Two-step SFE	SFE2	300	40	-	-	0	60
		150	40	-	-	7.0 *	120
SAF	SAF1	100	40	0.025	55.8	44.2 **	180
SAF	SAF2	100	40	0.100	55.8	44.2 **	60
SAF	SAF3	100	40	0.025	24.0	76.0 **	180

*: Ethanol as co-solvent; **: ethanol in the solvent mixture. SC-CO_2: Supercritical carbon dioxide; PLE: Pressurized liquid extraction; SAF: Supercritical antisolvent fractionation; SFE: Supercritical fluid extraction.

Table 2. Extraction yield (% dry weight), total phenolic content (TPC) and antioxidant activity (Trolox equivalent antioxidant capacity (TEAC) and 1,1-diphenyl-2-picrilhidrazyl (DPPH) assays) obtained for the different extracts.

Sample	Yield (g/100 g Sample)	TPC [1]	TEAC [2]	EC_{50} [3]
PLE	38.46 ^{d} ± 1.99	233.88 ^{f} ± 4.43	2.75 ^{b} ± 0.04	7.70 ^{d} ± 0.33
SFE1	6.74 ^{a} ± 0.33	134.42 ^{a} ± 4.51	1.87 ^{a} ± 0.05	7.03 ^{e} ± 0.15
SFE2	4.68 ^{a} ± 0.01	169.01 ^{b} ± 8.61	2.64 ^{b} ± 0.03	5.61 ^{c} ± 0.11
SAF1	20.65 ^{c} ± 1.74	220.05 ^{e} ± 5.82	4.09 ^{d} ± 0.15	3.39 ^{a} ± 0.08
SAF2	5.74 ^{a} ± 0.45	203.04 ^{d} ± 7.81	3.67 ^{c} ± 0.09	4.12 ^{b} ± 0.12
SAF3	15.36 ^{b} ± 1.41	188.55 ^{c} ± 1.06	3.80 ^{c} ± 0.20	7.83 ^{d} ± 0.22

In each column, superscripts letters mean groups not statistically different ($p > 0.05$), as analyzed by one-way ANOVA. [1] mg gallic acid equivalents (GAE) g^{-1} extract; [2] mmol trolox equivalents g^{-1} extract; [3] Efficient concentrations, μg extract mL^{-1} obtained by DPPH assay.

2.2. Anti-Proliferative Activity of the Extracts

To determine the anti-proliferative effect of the polyphenol-enriched extracts, HT-29 and HCT116 cells were incubated with increasing concentrations of extracts (from 0 to 50 μg/ mL) for 24 and 72 h and cell proliferation was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All the extracts exhibited a concentration-dependent anti-proliferative effect after both exposure times (Figure S1, Supplementary Materials). In order to characterize the anti-proliferative activity of these extracts in more detail and compare their potencies, the growth inhibition (GI_{50}, as an indicator for cytostaticity) and the lethal concentration (LC_{50}, as an indicator for cytotoxicity), were also determined in both cell lines in time course experiments at 24 and 72 h incubation times. As it is shown in Figure 1, HT-29 and HCT116 cell lines showed different sensitivity to the extracts. Specifically, HCT116 cells were less refractory to the inhibitory and cytotoxic effect of the three SAF extracts than HT-29 cells. Thus, these particular extracts exhibited good cytostatic potential at concentrations below 10 μg/mL in HCT116 cells after 24 h-exposures, whereas GI_{50} values obtained in the assays with HT-29 cells were above that concentration (Figure 1A). The superior potency of SAF extracts towards HCT116 cells is also illustrated in Figure 1B, where it is shown that extract concentrations ranging

from 11.2 to 12.4 μg/mL were sufficient to induce 50% HCT116 cell death (LC_{50}), whereas higher concentrations (from 21.8 to 31.9 μg/mL) were needed to exert a comparable cytotoxic effect in the HT-29 cell line. To note, the activity of the three SAF extracts was statistically comparable in HCT116 cells, whereas one of them, SAF3 extract, showed to be more effective than the other two extracts against HT-29 cells. Regarding the latter cell line, it showed an extraordinary tolerance to cytostatic concentrations of SFE1 extract after 72 h-exposure (Figure 1C). In general, the inhibitory effects of the extracts did not significantly vary by the exposure time (Figure 1C,D). An exception to this was observed with SFE1 extract whose effect was significantly stronger in HT-29 cells at 24 h than at 72 h. These results suggest that both cytostatic and cytotoxic concentrations of SFE1 extract allow prompt cell recovery with partial restoration of proliferation. On the other hand, PLE extract exhibited the lowest anti-proliferative activity among the studied extracts, providing GI_{50} values above 30 μg/mL for HCT116 cells whereas GI_{50} values for HT-29 cells were outside the testing range. In addition, PLE extract did not exert cytotoxic effects at the assayed concentrations. In vitro studies have often confirmed strong anti-proliferative effects to be associated with phenolic-rich rosemary extracts [18,33,40,54–56]. Interestingly, in the present study, examination of the total phenolic content of the six extracts (Table 2) indicated that there is a lack of positive correlation between the total phenolics in the extracts and their anti-proliferative activity in both cell lines. This observation is illustrated by PLE extract, which showed the highest phenolics content but exhibited the lowest anti-proliferative activity in both cell lines. This lack of correlation suggests that the effect of the extracts on cell proliferation and viability may be due to certain extract constituents. In this regard, the anti-proliferative activity (and also the antioxidant activity) of rosemary extracts has frequently been attributed to the presence of major diterpenes, CA and CS [19,57]. Nevertheless, individual CA and CS in pure solutions or in binary mixtures at the same concentrations as those found in rosemary extracts appear to exert lower inhibitory effects than the whole extracts [20,56]. In our present study, to investigate the selectivity of each process for the extraction of specific bioactive compounds (or groups of compounds) and their potential correlation with the anti-proliferative activity, the chemical compositions of the extracts were assessed using different MS-based analytical techniques.

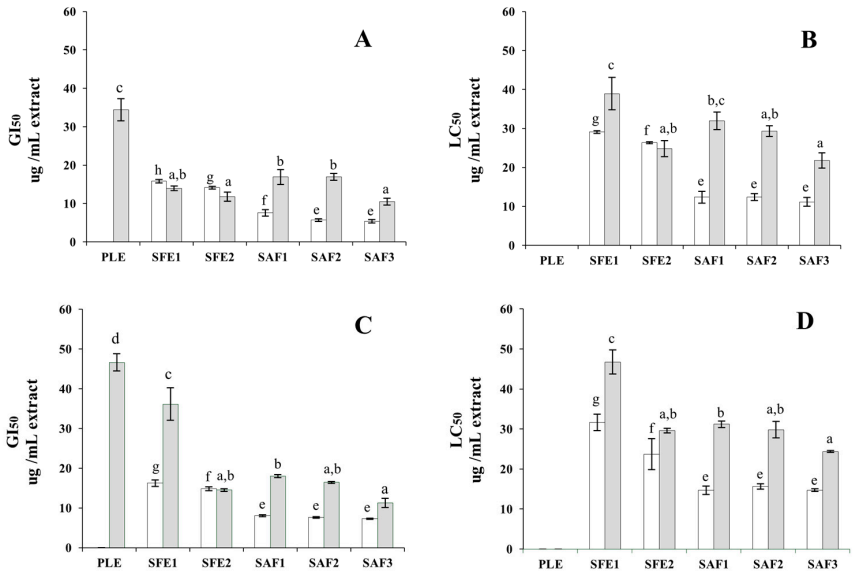

Figure 1. Cytostatic (**A**,**C**) and cytotoxic (**B**,**D**) activities of the rosemary extract on HCT116 (white) and HT-29 cells (grey) at different exposure times. Calculated GI_{50} values at 24 h (**A**) and 72 h (**C**); calculated LC_{50} values at 24 h (**B**) and 72 h (**D**). Error bars represent standard error of the mean (SEM). In each bar graph, mean values that do not share superscripts letters indicate that they differ by $p < 0.05$ as analyzed by one-way analysis of variance (ANOVA). Superscript letters (e–h) and (a–d) have been used to indicate ANOVA results in HCT116 and HT-29 cell lines, respectively.

2.3. Chemical Characterization of the Extracts by LC-DAD-MS, LC-Q/TOF-MS, and GC-MS

The quantification of main phenolic (CA and CS, carried out by liquid chromatography coupled to Diode Array Detector and mass spectrometry (LC-DAD-MS)) and volatile compounds (1,8-cineole and camphor, by gas chromatography coupled to mass spectrometry (GC-MS)) in the rosemary extracts is presented in the Supplementary Materials (Table S1). Chromatographic data revealed that high enrichment in CA and CS was achieved in all the extracts, ranging from 263.7 to 443.3 and from 33.9 to 88.9 mg/g extract, respectively (Figure 2A). An exception to this was PLE extract in which CA and CS were only found at 105.0 and 10.7 mg/g extract, respectively. SFE2 extracts was the most enriched in both diterpenes (443.3 and 88.9 mg/g extract, respectively), achieving an improved extract with more than 50% (*w*/*w*) of these phenolic diterpenes in their total composition. Also, similar to the lab scale approach [42], the CA + CS concentration, TPC and antioxidant activity in the pilot SAF scale were found to be in the following order: SAF1 > SAF2 > SAF3. However, in terms of recovery, the lab-scale process showed lower values. On the other hand, a comparison among SAF and the other processes indicated a new order for CA + CS enrichment in the extracts as follows: SFE2 > SAF1 > SAF2 $\geq$ SFE1 > SAF3 > PLE.

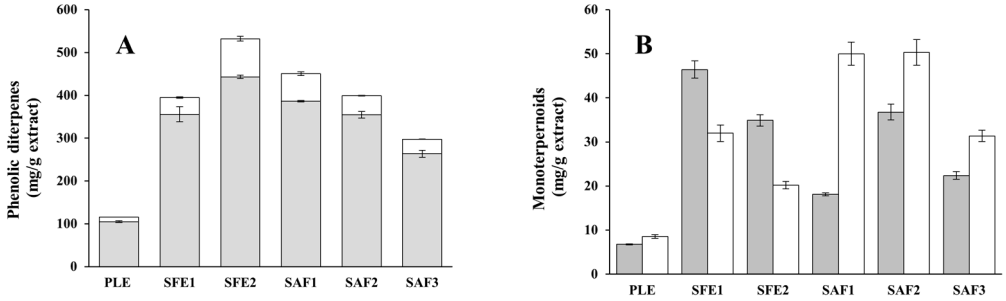

Figure 2. Concentration of main phenolic diterpenes and monoterpenes (mg/g) in the rosemary extracts. (**A**) carnosic acid (CA) (grey) and carnosol (CS) (white); (**B**) 1,8-cineole (grey) and camphor (white). Error bars represent standard deviation (SD).

Next, CA and CS concentrations, as well as the sum of both concentrations in the extracts, were compared to their anti-proliferative activity. As expected, the less active extract (PLE) was the one containing the lowest CA and CS concentration. However, excluding the observation for PLE extract, a lack of positive correlation between the extract potency and the CA and CS concentrations was observed in the rest of the rosemary extracts. Interestingly, SAF3 extract was less enriched in CA and CS than those obtained using antisolvent fractionation technology, but was the most active (among SAF extracts) inhibiting cell proliferation and inducing cytotoxic effects in HT-29 cells. Furthermore, SFE1 and SAF2 extracts showed comparable CA and CS concentrations (p-value > 0.05; Supplementary Materials, Table S1); however, their activities were strikingly different in both cell lines. These results suggest the potential presence of other unidentified rosemary constituents that may also contribute to the observed anti-proliferative effect of the extracts (especially in SAF3). This lack of positive correlation between the concentration of the two major diterpenes in the extracts and the level of inhibition of cell proliferation prompted us to examine other chemical constituents in the extracts. Thus, UHPLC-qTOF-MS analysis of the extracts was performed to identify potential active compounds present in the extracts. Although all extracts provided similar chromatographic profiles (Supplementary Materials, Figure S2), interesting qualitative and quantitative differences were observed among them. Table 3 summarizes the 29 resolved peaks and 17 compounds that have been tentatively identified. The identified compounds could be classified according to their nature as follows: phenolic acids (syringic acid, rosmarinic acid and tryhydroxycinnamic acid derivate), flavonoids (gallocathechin and genkwanin), phenolic terpenes (rosmanol, epirosmanol/isorosmanol, rosmadial, carnosol, carnosic acid, rosmaridiphenol, methyl carnosate/12-methoxycarnosic acid,

betulinic acid, oleanolic acid and ursolic acid), dihydrocoumarins (safficinolide) and diterpene lactones (11,12-dimethylrosmanol). Some works have previously described these families of compounds typically found in rosemary extracts [10,27,43,58,59]. In order to detect if there exists a similarity among extracts with potent anti-proliferative activity, results were examined to identify the compounds that were exclusively present in the most potent extracts compared to those extracts with lower activities. Interestingly, three signals with masses 207.064 (tryhydroxycinnamic acid derivate), 331.156 (NI3) and 315.197 (rosmaridiphenol) Da were only found in the three most potent extracts (SAF1-3), showing maximum peak area values in the chromatographic analysis of SAF1 extract. With regard to the analysis of this extract, it revealed a signal with mass 343.156 Da (safficinolide) that was not detected in any other extract. Other common features observed in the three supercritical antisolvent fractionation extracts were the higher enrichment in 11,12-dimethylrosmanol and the lower rosmanol peak area compared to SFE1 and SFE2, suggesting that this latter diterpene is better extracted using SC-CO_2 and SC-CO_2 with ethanol as solvents. To note, betulinic, oleanolic and ursolic acids show identical molecular formula, and thus, the same m/z; however, they were tentatively identified by their retention times according to a similar separation carried out by Kontogianni et al. (2013) [19]. The chromatographic profile of SAF3 extract analysis showed maximum peak areas for signals at m/z 455.352 that may correspond to ursolic acid (455.352 Da), a compound with demonstrated cytotoxic activity [19], and four other masses that correspond to less polar compounds (467.317, 467.318, 615.406, and 551.374 Da).

Besides the low CA and CS content in PLE sample, this extract showed a distinctive chromatographic profile compared to the rest of the extracts. For instance, peak signals with masses corresponding to syringic acid, gallocathechin, rosmarinic acid and other two non-identified compounds (NI1 and NI2) were exclusively detected in the analysis of PLE extract. The distinctive presence of these phenolic acids and flavonoid evidences a higher selectivity for polar compounds of the PLE process compared to the other extraction procedures. Furthermore, the analysis of PLE extract also revealed that betulinic acid was only detected in this extract. Interestingly, Kontogianni et al. (2013) attributed part of the cytotoxic activity observed in a CA-enriched rosemary extract obtained using solid-liquid extraction to the presence of the triterpenoids betulinic and ursolic acids in addition to CA [19]. More recently, in a study based on the fractionation of a rosemary extract obtained by SFE, CA was the major contributor to the anti-proliferative activity, followed by CS and also betulinic acid [58]. In that case, betulinic acid concentration in the extract was 2.1 μM, and the incubation of HT-29 cells with a purified fraction from the same extract containing 81% betulinic acid and 19% hinokione showed the same cytotoxicity as the whole extract. In another published work, Rzeski et al. (2006) demonstrated that betulinic acid acts as an effective anticancer agent by inducing growth arrest and apoptosis in concentration-dependent manner, with HT-29 cells being particularly sensitive to this pentacyclic triterpenoid [60]. However, reported IC50 values for betulinic acid by different research groups for the same cell model (HT-29) are discordant (2.7 μM, [60]; 13.9 μM, [61]; and 32.7 μM, [62]). The extremely low aqueous solubility (<1 μM), high protein binding (>99%) and poor membrane permeability of this compound [63,64] could explain the lack of robustness in data obtained under slightly different culturing conditions. Interestingly, our findings indicate that betulinic acid was only present in the less active extract, suggesting that this compound is not among the most relevant active constituents in the rosemary extracts obtained in the present study. To gain further insight into the chemical differences among the extracts, the main volatile compounds were identified and quantified by GC-MS analysis.

Table 3. Tentatively identification by Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (LC-Q/TOF-MS) of compounds present in rosemary leaf extracts.

Peak	Rt (min)	[H-M]$^-$	Molecular Formula	Identification	Peak Area (Mean ± SD) (× 10^6)					
					PLE	SFE1	SFE2	SAF1	SAF2	SAF3
1	0.479	387.1171	$C_{13}H_{24}O_{13}$	NI1	3.91 ± 0.24	-	-	-	-	-
2	0.994	198.0528	$C_9H_{10}O_5$	Siringic acid	0.36 ± 0.02	-	-	-	-	-
3	1.737	306.0798	$C_{15}H_{14}O_7$	Gallocathechin	0.67 ± 0.04	-	-	-	-	-
4	1.797	387.1678	$C_{18}H_{28}O_9$	NI2	0.99 ± 0.03	-	-	-	-	-
5	2.274	360.0845	$C_{18}H_{16}O_8$	Rosmarinic acid	4.10 ± 0.12	-	-	-	-	-
6	2.730	207.0636	$C_{11}H_{12}O_4$	Tryhydroxycinnamic acid derivate	-	-	-	1.024 ^{c} ± 0.006	0.71 ^{b} ± 0.01	0.61 ^{a} ± 0.05
7	3.178	345.1737	$C_{20}H_{26}O_5$	Rosmanol	0.930 ^{a} ± 0.009	6.16 ^{f} ± 0.42	4.80 ^{e} ± 0.15	3.90 ^{d} ± 0.02	2.37 ^{b} ± 0.01	2.87 ^{c} ± 0.11
8	3.275	345.1706	$C_{20}H_{26}O_5$	Epirosmanol/Isorosmanol	-	1.72 ^{b} ± 0.06	1.53 ^{a} ± 0.01	2.68 ^{c} ± 0.15	1.80 ^{b} ± 0.007	1.69 ^{a,b} ± 0.07
9	3.392	283.0617	$C_{16}H_{12}O_5$	Genkwanin	0.78 ^{a} ± 0.05	2.29 ^{d} ± 0.02	1.79 ^{b} ± 0.01	2.02 ^{c} ± 0.01	1.88 ^{b} ± 0.02	1.90 ^{b,c} ± 0.06
10	3.843	343.1563	$C_{20}H_{24}O_5$	Safficinolide	-	-	-	0.57 ± 0.01	-	-
11	3.883	331.1562	$C_{19}H_{24}O_5$	NI3	-	-	-	2.06 ^{c} ± 0.09	1.26 ^{b} ± 0.02	0.99 ^{a} ± 0.05
12	3.960	329.1754	$C_{20}H_{26}O_4$	Carnosol	3.76 ^{a} ± 0.12	15.49 ^{b} ± 0.17	30.90 ^{e} ± 0.90	29.76 ^{e} ± 0.88	22.63 ^{d} ± 0.07	18.84 ^{c} ± 0.16
13	4.111	343.1630	$C_{20}H_{24}O_5$	Rosmadial	-	0.92 ^{b} ± 0.06	-	0.99 ^{c} ± 0.01	0.91 ^{b} ± 0.01	0.708 ^{a} ± 0.002
14	4.158	373.2037	$C_{22}H_{30}O_5$	11,12-Dimethylrosmanol	-	-	2.17 ^{a} ± 0.14	4.53 ^{c} ± 0.23	3.22 ^{b} ± 0.03	3.26 ^{b} ± 0.02
15	4.258	325.1865	$C_{21}H_{26}O_3$	NI4	1.39 ^{a} ± 0.06	2.29 ^{c,d} ± 0.25	1.86 ^{b} ± 0.14	1.96 ^{b,c} ± 0.24	2.51 ^{d} ± 0.03	1.37 ^{c} ± 0.08
16	4.338	331.1952	$C_{20}H_{28}O_4$	Carnosic Acid	21.80 ^{a} ± 1.04	42.86 ^{b} ± 0.36	50.78 ^{d} ± 2.44	49.28 ^{d} ± 1.09	50.01 ^{d} ± 0.25	45.95 ^{c} ± 0.53
17	4.575	345.2133	$C_{21}H_{30}O_4$	Methyl carnosate/12-methoxy-carnosic acid	1.50 ^{a} ± 0.04	8.04 ^{b} ± 0.25	12.63 ^{d} ± 0.65	12.42 ^{d} ± 0.89	12.33 ^{d} ± 0.14	9.95 ^{c} ± 0.43
18	4.753	315.1965	$C_{20}H_{28}O_3$	Rosmaridiphenol	-	-	-	0.50 ^{b} ± 0.02	0.509 ^{b} ± 0.008	0.21 ^{a} ±0.02
19	4.799	317.2107	$C_{20}H_{30}O_3$	NI5	-	2.27 ^{a,b} ± 0.07	2.47 ^{b} ± 0.14	2.15 ^{a} ± 0.08	2.138 ^{a} ± 0.006	2.40 ^{b} ± 0.11
20	4.818	455.3422	$C_{26}H_{48}O_6$	Betulinic Acid *	2.304 ± 0.003	-	-	-	-	-
21	4.976	455.3650	$C_{30}H_{48}O_3$	Oleanolic acid *	7.99 ^{c} ± 0.52	5.56 ^{b} ± 0.22	5.15 ^{b} ± 0.08	4.08 ^{a} ± 0.14	5.25 ^{b} ± 0.03	5.14 ^{b} ± 0.32
22	5.110	455.3515	$C_{30}H_{48}O_3$	Ursolic acid *	-	1.84 ^{c} ± 0.19	1.51 ^{b} ± 0.05	1.368 ^{a} ± 0.009	1.542 ^{b} ± 0.007	2.24 ^{d} ± 0.02
23	5.441	479.2785	$C_{30}H_{40}O_5$	NI6	-	1.07 ^{d} ± 0.02	0.60 ^{a} ± 0.04	0.94 ^{c} ± 0.07	0.759 ^{b} ± 0.007	1.15 ^{d} ± 0.08
24	5.621	331.1921	$C_{20}H_{28}O_4$	NI7	-	0.12 ^{a} ± 0.02	1.61 ^{d} ± 0.10	1.89 ^{e} ± 0.01	0.521 ^{c} ± 0.004	0.33 ^{b} ± 0.03
25	6.414	467.3168	$C_{30}H_{44}O_4$	NI8	1.23 ^{a} ± 0.01	4.42 ^{d} ± 0.16	4.99 ^{e} ± 0.31	1.63 ^{b,c} ± 0.04	1.79 ^{c} ± 0.03	5.48 ^{f} ± 0.02
26	6.658	467.3184	$C_{30}H_{44}O_4$	NI9	0.83 ^{a} ± 0.06	3.32 ^{c} ± 0.11	2.38 ^{b} ± 0.08	1.06 ^{d} ± 0.03	1.03 ^{a} ± 0.02	4.48 ^{d} ± 0.17
27	6.840	615.4061	$C_{33}H_{60}O_{10}$	NI10	0.075 ^{a} ± 0.018	0.50 ^{c} ± 0.04	0.33 ^{b} ± 0.02	-	-	0.57 ^{d} ± 0.03
28	6.997	551.3749	$C_{35}H_{52}O_5$	NI11	-	0.305 ^{b} ± 0.005	0.20 ^{a} ± 0.02	-	-	0.46 ^{c} ± 0.03
29	7.290	535.3794	$C_{35}H_{52}O_4$	NI12	-	-	0.34 ± 0.02	-	-	-

a–f, for each peak (row), peak area mean values that do not share subscripts differ by $p < 0.05$ as analyzed by one-way ANOVA; * The order of these compounds is suggested according to the identification performed by Kontogianni et al., 2013 [19].

In all cases, 1,8-cineole and camphor were the two more abundant volatile compounds found in the GC chromatograms profiles. These results are in good agreement with others reported in the literature for supercritical fluids and hydrodistillated rosemary extracts [14,44,65]. As it is shown in Figure 2B, quantitative data indicated that 1,8-cineole and camphor were present at levels ranging from 6.8 to 46.4 and from 8.6 to 50.3 mg/g extract, respectively. As a general trend, both monoterpenes were more abundant in the more active extracts. Single-step and two-step sequential extraction procedures seemed to be more selective for 1,8-cineole than camphor; the concentration of both volatile monoterpenoids was lower in SFE2 (approximately 25% and 37% lower). This can be explained by the removal of most aromatic and highly volatile components (mono- and sesquiterpenes, and their oxygenated derivates) in the two-step sequential SFE process, leading to a more volatile-free extract (SFE2). The opposite behavior occurred with the antisolvent fractionation methodology (SAF) where extracts showed higher camphor content, which is less volatile than 1,8-cineole.

The cytotoxic activity of this latter compound has been demonstrated to be within the low millimolar range against human colorectal HCT116 cells [66], which is well above (e.g., in 30 μg extract/mL, the concentration of 1,8-cineole ranged from 1.32 to 9.03 μM) its concentration in the rosemary extracts of our present study. A similar potency has been recently reported for camphor monoterpene, exerting a 50% reduction in viability at concentrations of 5.5 and 4.5 mM, respectively in HT-29 and HCT116 cells [67]. These published data suggest that both monoterpenes, when individually assayed, have only modest cytotoxic activity compared to other compounds in the rosemary extracts. According to our quantitative data, their content in the extracts seems to be below their reported inhibitory concentrations. In spite of that, camphor and 1,8 cineole were, with respect to PLE extract, enriched more than three-fold in the rest of the extracts with a comparably good activity. This is in accordance with findings by other groups [55], indicating that the contribution of volatile monoterpenes to the anti-proliferative activity of the extracts, particularly those with high CA content, cannot be totally dismissed.

3. Materials and Methods

3.1. Samples and Reagents

Raw material consisted of rosemary (*Rosmarinus officinalis* L.) dried leaves obtained from Herboristeria Murciana (Murcia, Spain) that were ground at low temperature (by its mixing with small rocks of dry ice) employing a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany). Sample particle size was in the range of 500 and 900 μm. Next, grinded samples were vacuum packed and stored at 4 °C until further use. Carbon dioxide (99% purity) purchased from Carburos Metálicos (X50S, Barcelona, Spain) was employed for the anti-solvent fractionation and supercritical fluids extractions. Ethanol (99.5%), provided by VWR Chemicals (Fontenay-sous-Bois, France), and ultrapure water, obtained from a Millipore system (Billerica, MA, USA), were used for PLE. Phenols standards such as rosmarinic acid (RA, ≥98%), CA (≥97%), CS (≥98%), gallic acid, as well as other chemicals as 1,8-cineole (99%), camphor (95%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%), ABTS (≥99%), DPPH (99%) were purchased from Sigma-Aldrich (Madrid, Spain). Folin-Ciocalteu phenol reagent (2N) was provided by Merck (Darmstadt, Germany). For the UHPLC-qTOF-MS analyses, MS grade ACN and water from LabScan (Dublin, Ireland) were employed. Dry extracts were dissolved in DMSO (Sigma-Aldrich) at the appropriate concentrations and stored as aliquots at −80 °C until their use in cell proliferation inhibition assays.

3.2. Rosemary-Leaf Extraction Procedures

The extraction procedures used in the present work to obtain the different rosemary extracts are described in this section (main extraction parameters are also summarized in Table 1).

3.2.1. Pressurized Liquid Extraction (PLE)

Hydroalcoholic rosemary extracts were obtained using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. The extraction protocol has been described in depth previously [41]. Briefly, a mixture of ethanol + water (80:20, *v*/*v* or 76:24 *w*/*w*) was employed as solvent at 100 bar and 150 °C for 20 min. Successive extractions were performed until complete 1000 mL of total extract. An aliquot was submitted to rotary evaporation and freeze-drying (Labconco Corporation, Kansas City, MO, USA) to eliminate the ethanol and water, respectively, thus obtaining the dry extract. The remaining liquid extract was stored with N_2 atmosphere in the dark at −20 °C until their use for the antisolvent fractionation experiments.

3.2.2. Single-Step SFE Process

Single-step SFE process was performed as previously reported in detail by Herrero et al. 2010 [43]. Extraction was carried out in triplicate in a pilot scale supercritical fluid extractor (model SF2000, Thar Technology, Pittsburgh, PA, USA) including a 2 L cylinder extraction cell and two different separators with independent control of temperature and pressure. The extraction conditions employed for the single-step SFE process were 150 bar, 40 °C for 300 min, as described in Table 1. CO_2 flow rate was set to 60 g·min^{-1} and 7% ethanol was employed as co-solvent. After the extractions, ethanol was eliminated by rotary evaporation (R-210, Büchi Labortechnik AG, Flawil, Switzerland). The extracts were stored with N_2 atmosphere in the dark at −20 °C.

3.2.3. Two-Step Sequential SFE

The same equipment employed for the single-step SFE process was used for the two-sequential SFE extraction as described by Sánchez-Camargo et al. (2014) [42]. The extraction protocol involved two consecutive steps with the following conditions: (1) 300 bar for 60 min and (2) 150 bar, 7% ethanol (*w*/*w*) for 120 min. Both stages were performed at 40 °C and using 60 g·min^{-1} of CO_2 flow. Once the extracts were obtained in triplicate, ethanol was also eliminated by rotary evaporation. Dried extracts were kept at −20 °C, in the dark and in N_2 storage atmosphere.

3.2.4. Supercritical Antisolvent Fractionation (SAF) at Pilot Scale

A semi-continuous SAF was carried out in a Spe-ed Helix™ supercritical fluid extractor from Applied Separations (Allentown, PA, USA). Originally, this equipment was designed to carry out SFE (with or without a co-solvent) and subcritical water extraction (SWE); in the present work, some modifications were introduced in the system in order to use it as a SAF at pilot scale (Figure 1). Owing to the unavailability of a pilot-scale PLE equipment and in order to obtain enough volume of PLE extract to feed SAF processes, PLE extract was made of a pool of extracts obtained in various independent extractions using lab-scale PLE equipment. Then, based on previous lab scale results [41] three different extraction conditions (see Table 1) were selected to be compared, and a 60-fold scale up from bench to pilot scale was achieved. After the PLE process, the resulting hydroalcoholic rosemary extract was properly diluted to obtain the extracts with 44.2% and 76.0% (*w*/*w*) of ethanol (or 50% and 80% (*v*/*v*) ethanol, respectively), for SAF1-2 and SAF3, respectively and according to the previously optimized conditions. Then, diluted extracts were filtered through Whatman cellulose filter paper and 100 mL samples were employed for each fractionation, accomplished in triplicate. In the Figure 3 is showed an scheme of the SAF pilot scale equipment employed. Briefly, CO_2 was provided from a pressurized cylinder (1), subcooled in a chiller (2) and then conducted to a high pressure pump (3). Immediately, the CO_2 was compressed at 100 bar and continuously pumped at a flow of 10 L·min^{-1} adjusted by a heated micrometering valve (HMMV) (8) at the exit of the system through of CO_2 mass flowmeter (10). At the same time, PLE extract (5) was fed through another high pressure pump at suitable flow according to feed/SC-CO_2 rate (6). In a T-tube device, the CO_2 (4) and the feed (rosemary extract) was mixed before reaching the raffinate chamber separation. A polypropylene

vessel (250 mL) was placed inside the high-pressure stainless-steel extraction cell to act as precipitation raffinate vessel (7). The temperature of the raffinate separation chamber was kept at 40 °C by a heating jacket and measured by an internal thermocouple. Once the aqueous fraction was separated, the fraction soluble in SC–CO_2 + ethanol was precipitated in a glass bottle (9) acting as extract chamber separation, which was kept at room temperature (25 °C). During the SAF time, a whole process overview was carried out through to a software and screen coupled to the system. After obtaining the extract fraction, the ethanol was removed by rotary evaporation and the water was eliminated by freeze-drying (Labconco Corporation, Kansas City, MO, USA). Dried extracts were kept under freezing at −20 °C N_2 atmosphere until analysis. The extraction yield (or recovery) was determined gravimetrically, as the ratio of the mass of dry extract recovered in the separators and the mass of dry PLE extract fed, and expressed as a percentage.

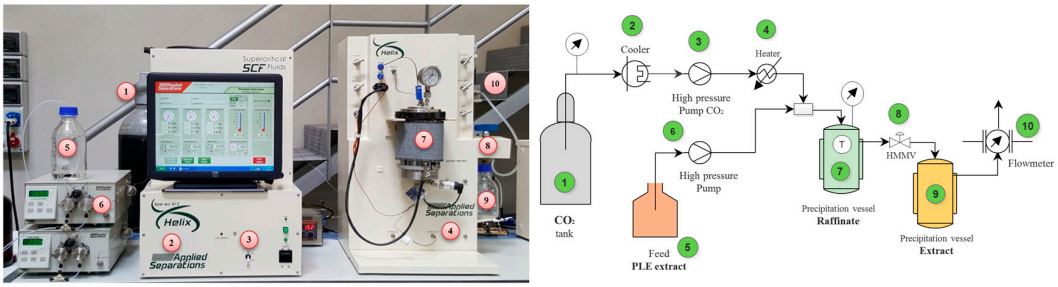

Figure 3. Scheme of the supercritical antisolvent fractionation (SAF) pilot scale system used in this work.

3.3. Chemical Characterization of Rosemary Extracts

3.3.1. Liquid Chromatography-Diode Array Detection-Mass Spectrometry

Major phenolic compounds typically present in rosemary extracts (CA and CS) were quantified employing an ACCELA UHPLC system (Thermo Scientific™, San Jose, CA, USA) coupled to a TSQ Quantum (Thermo Scientific™) triple quadrupole analyzer via an electrospray interface. Briefly, the analytical conditions employed consisted on the use of a Hypersil Gold column (50 mm × 2.1 mm, d.p. 1.9 µm) (Thermo Scientific™) using as mobile phases 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) eluted according to the following gradient: 0 min, 95% B; 0.35 min, 95% B; 3.5 min, 40% B; 6.2 min, 5% B; 7 min, 95% B; 10 min, 95% B. The flow rate was 0.4 mL/min and the injection volume was 5 µL. The diode array detector recorded the spectra from 200 to 450 nm. Calibration curves were constructed using external calibration method to quantify CA and CS (0.0625–2.0 µg/mL and 0.313–5.0 µg/mL, respectively). The mass spectrometer was operated in the negative electrospray ionization (ESI) mode using multiple reaction monitoring (MRM) with a Q1 and Q3 resolution of 0.7 Da FWHM, scan width 0.010 Da and scan time of 0.10 s. The values corresponding to the tube lens voltage and collision energy for each ion transition were optimized for each quantified compound: carnosic acid m/z 331.14 ([M-H]$^-$) and m/z 287.19 ([M-H]$^-$, product ion using 62 V and 26 V as tube lens value (TLV) and CE, respectively), and for carnosol m/z 329.16 ([M-H]$^-$) and 285.15 m/z ([M-H]$^-$, product ion using 45 V and 20 V as TLV and CE, respectively).

3.3.2. Liquid Chromatography-Quadrupole Time-Of-Flight Mass Spectrometry (LC-Q/TOF-MS)

To obtain a more complete chemical characterization of the rosemary extracts, these were analyzed by liquid chromatography coupled to a high-resolution mass spectrometer. It consisted of an ultrahigh performance liquid chromatography (UHPLC) system 1290 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of-flight mass spectrometer (Q/TOF MS) Agilent 6540 that was equipped with an orthogonal ESI source (Agilent Jet Stream, AJS, Santa Clara, CA, USA), and controlled by a PC running the Mass Hunter Workstation software 4.0 (MH) from Agilent. The analyses were performed in negative ion mode. Chromatographic separation of the extracts was

achieved using a Hypersil Gold column (50 mm × 2.1 mm, d.p. 1.9 µm) (Thermo Scientific™) with a mobile phase composition of acetonitrile (+0.1% formic acid, A) and water (+0.1% formic acid, B). The gradient program was as follows: 0 min, 95% B; 0.35 min, 95% B; 3.5 min, 30% B; 6.2 min, 5% B; 9 min, 95% B. The flow rate was 0.4 mL/min with an injection volume of 2 µL. The diode array detector recorded the spectra from 200 to 450 nm. The extracts were injected to a concentration of 50 µg/mL. MS parameters were the following: capillary voltage, 4000 V; nebulizer pressure, 30 psi; drying gas flow rate, 10 L/min; gas temperature, 300 °C; skimmer voltage, 45 V; fragmentor voltage, 125 V. The QTOF-MS was set to acquire m/z ranging between 50 and 1100 amu at a scan rate of 5 spectra per s. External calibration of the TOF MS was carried out using a commercial mixture from Agilent with the following m/z values: 301.9981, 601.9790, 1033.9881, 1333.9689, 1633.9498, 1933.9306, 2233.9115, 2533.8923 and 2833.8731. The identification of CA and CS was based on the standard samples. The other compounds were tentatively identified in accordance with the molecular formula and the exact mass of the compound.

3.3.3. Gas Chromatography-Mass Spectrometry (GC-MS)

The main volatile compounds (camphor and 1,8-cineole) in rosemary extracts were quantified using the GC-MS method developed by Sánchez-Camargo et al. (2014) [42] with some modifications. A GCMS-QP2010 plus system (Shimadzu, Kyoto, Japan) equipped with a DB-5ms column (30 m × 0.25 mm I.D. × 0.25 µm df, Quadrex Corporation, Woodbridge, CT, USA) was used. The oven temperature program for the separation was carried out as follow: 60 °C, held for 4 min, and then raised to 100 °C at 3 °C/min followed by an increase to 110 °C at 1 °C/min, and then to 150 °C at 5 °C/min. Finally, the temperature was raised to 300 °C at 15 °C/min and this value was held for 25 min. Injection volume was 1.6 µL in split mode (split ratio 1:10) maintaining an injector temperature of 250 °C. The carrier gas employed was He at 36.4 $cm \cdot s^{-1}$. MS detection parameters were: interface and source temperatures were 280 °C and 230 °C, respectively; mass range, m/z 40–500; scan speed: 2500 amu/s; event time 0.20 s. Collection and handling of data was performed using the GCMS solution (ver. 2.50 SU3, Shimadzu) software. A commercial mass spectral database (Wiley) and the linear retention indices (LRI) of the resolved peaks were used to identify the different compounds. For the determination of LRIs, a hydrocarbon mixture ranging from C_8 to C_{30} (Hydrocarbons/C5–C30, straight-chain alkanes, Sigma-Aldrich) was employed and analyzed under the same experimental conditions as the sample. After identification, calibration curves of camphor and 1,8-cineole were employed to quantify their content in the sample (2.5–25.0 µg/mL, for both compounds). GC-MS analyses were carried out in triplicate.

3.4. Total Phenols Content (Folin-Ciocateu Method)

The quantification of total phenols content (TPC) in the rosemary extracts was carried out using the Folin–Ciocalteu method with some modifications [68]. Briefly, 600 µL of water were mixed with 10 µL of each extracts (2.5–5 mg/mL of rosemary extract in ethanol or ethanol: water mixtures) to which 50 µL of undiluted Folin–Ciocalteu reagent (2N) was subsequently added. After 1 min, 150 µL of 20% (w/v) Na_2CO_3 were added and the volume was made up to 1.0 mL with water. After 2 h of incubation at 25 °C, 300 µL of the mixture was transferred into a well of a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader (Synergy HT, Bio Tek Instruments, Winooski, VT, USA). A gallic acid calibration curve (0.032–2.00 mg/mL) was elaborated in the same way and the TPC was expressed as mg of gallic acid (GAE) per g of extract. All analyses were done in triplicate.

3.5. Antioxidant Activity In Vitro Assays

3.5.1. DPPH Radical Scavenging Assay

The antioxidant activity was determined following the adjusted procedure described by Brand-Williams, Cuvelier and Berset (1995) [69] employing 1,1-diphenyl-2-picrilhidrazyl (DPPH)

reagent. A stock solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol which was further diluted 1:10 with methanol to give the working solution. Both stock and working solutions were stored at 4 °C until use. A volume of 975 µL of DPPH diluted solution was added to 25 µL of each extract concentration solution and the reaction was kept at darkness for 4 h at room temperature. Different concentrations (from 0.0625 to 0.5 mg/mL) of each extract were tested. Once the reaction was finished, 300 µL of this mixture was transferred into a well of a microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (Synergy HT). DPPH–methanol solution was used as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The extract concentration (expressed in µg/mL) responsible for a 50% decrease in the initial activity of the DPPH (EC_{50}, µg/mL) was calculated by linear regression of the percentage remaining DPPH curve obtained for all the extract concentrations. Therefore, the lower the EC_{50} value, the higher the antioxidant capacity. Measurements were done in triplicate.

3.5.2. TEAC Assay

The antioxidant capacity of the different rosemary extracts was determined by TEAC assay following the ABTS radical method based on the procedure described by Re et al. (1999) with some modifications [70]. $ABTS^{\bullet+}$ radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature during 16 h before its use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) until achieve an absorbance of 0.7 (±0.02) at 734 nm. One mL of $ABTS^{\bullet+}$ solution was mixed with 10 microliters of sample (five different concentrations) in a 1.5-mL vial and 300 µL of the mixture were transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min during 45 min in a microplate spectrophotometer reader (Synergy HT). Trolox was used as a reference standard and results were expressed as TEAC values (mmol of Trolox/g extract). These values were obtained from five different concentrations of each extract tested (between 0.0625–1 mg/mL) in the assay giving a linear response between 20% and 80% blank absorbance. All analyses were done in triplicate.

3.6. Cell Culture

Colon adenocarcinoma HT-29 and HCT116 cell lines were purchased from ATCC (American Type Culture Collection, LGC Promochem, UK). Cells were cultured in McCoy's 5A supplemented with 10% (*v*/*v*) heat inactivated FBS (Fetal Bovine Serum), 50 U/mL streptomycin, and 50 U/mL penicillin G, in humidified atmosphere and 5% CO_2 at 37 °C. Cells were trypsinized when reached ~50% confluence, neutralized with culture medium, seeded at 5000 cells/well, and allowed to adhere overnight at 37 °C. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method was used to assess the antiproliferative activity of the extracts. Briefly, cells were incubated with the vehicle (0.2% (*v*/*v*) DMSO) regarded as untreated controls or with different extract concentrations and incubated for different times (24 and 72 h). After incubations, cells were incubated with MTT solution (0.5 mg/mL) at 37 °C for 3 h. Then, the medium was aspirated, and the purple formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured in a microplate reader (Synergy HT). Based on the NIH definitions [71], the percentage of growth (PG) was calculated with the formula $PG = 100\ [(T - T_0)/(C - T_0)]$ when $T \geq T_0$, or $PG = 100\ [(T - T_0)/C]$ when $T < T_0$, T being the optical density of treated cells, C the optical density of control cells, and T_0 the optical density at time zero. Then, PG values were used to calculate the parameters related with cell proliferation after 24 h of treatment (GI_{50}, 50% growth inhibition; and LC_{50}, 50% lethal concentration) using SigmaPlot v12.5 software (Systat Software Inc., Erkrath, Germany). The results are provided as the mean ± SEM of at least three independent experiments, each performed in triplicate.

3.7. Statistical Analysis

Data analysis were carried out using the software Statgraphics Centurion XVI® (StatPoint Technologies, Inc., Warrenton, VA, USA) using a level of significance set at 95%. One-way

analysis of variance (ANOVA), together with *F*-test, was employed to group extracts, based on statistically significant differences. Mean values were compared using Student-Newman-Keuls multiple comparison procedure and differences were considered statistically significant if $p < 0.05$.

4. Conclusions

Taken together, our data highlight the good potential of novel green extraction strategies for obtaining rosemary extracts with potent inhibitory activity of cancer cell proliferation. The most active rosemary extracts were characterized by containing CA and CS at concentrations above 263.7 and 33.9 mg/g extract, respectively. However, above those concentrations, a lack of positive correlation between the extract potency and the CA and CS content was observed, which suggest the potential presence of other unidentified constituents that may also contribute to the observed anti-proliferative effect of the rosemary extracts. The extracts obtained using an integrated process that involved PLE and SAF provided the most active rosemary extracts against both colon cancer cell lines. The compounds tentatively identified as rosmaridiphenol and safficinolide were exclusively identified in SAF1-3 and SAF1 extracts, respectively, suggesting that they are possible additional contributors to the observed strong anti-proliferative activity of CA and CS in SAF extracts. Contrasting with published data that suggest betulinic acid as a relevant contributor to the anti-proliferative activity of rosemary extracts, our data indicate this compound is not among the most active constituents in the rosemary extracts obtained in the present work. In addition, although the concentrations of the two major monoterpenes in the extracts are well below their reported active concentration in the same cell models, their potential synergistic contributions to the anti-proliferative activity of the extracts should not be dismissed. This study also illustrates the complexity of assigning the anti-proliferative activity to individual or group of compounds in complex extracts when more than one compound is active. This challenging task is crucial for the development of extraction processes that provide optimal content of active compounds.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/17/12/2046/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials: Comparative Study of Green Sub- and Supercritical Processes to Obtain Carnosic Acid and Carnosol-Enriched Rosemary Extracts with In Vitro Anti-Proliferative Activity on Colon Cancer Cells

Andrea del Pilar Sánchez-Camargo, Virginia García-Cañas, Miguel Herrero, Alejandro Cifuentes and Elena Ibáñez

Table S1. Quantification of main phenolic and volatiles compounds in the rosemary extracts. Carnosic acid (CA), Carnosol (CS). Concentrations indicated as mg/g extract ± SD. Values are the mean of three replicates.

Sample	mg/g Extract				
	CA	CS	CA + CS	1.8-Cineole	Camphor
PLE	104.96 ± 1.87 ^{c}	10.66 ± 0.06 ^{c}	115.63 ± 1.93 ^{c}	6.78 ± 0.14 ^{c}	8.57 ± 0.43 ^{c}
SFE1	355.73 ± 17.44 ^{a}	39.23 ± 1.91 ^{a}	394.97 ± 17.91 ^{a}	46.42 ± 1.95 ^{a}	31.97 ± 1.90 ^{a}
SFE2	443.28 ± 4.09 ^{b}	88.88 ± 5.41 ^{b}	532.16 ± 4.73 ^{b}	34.88 ± 1.30 ^{b}	20.23 ± 0.82 ^{b}
SAF1	386.49 ± 1.98 ^{d}	64.24 ± 4.04 ^{d}	450.73 ± 4.70 ^{d}	18.11 ± 0.33 ^{d}	50.02 ± 2.62 ^{d}
SAF2	354.92 ± 7.63 ^{a}	44.04 ± 0.73 ^{a}	398.96 ± 8.00 ^{a}	36.79 ± 1.76 ^{b}	50.34 ± 2.93 ^{d}
SAF3	263.70 ± 8.07 ^{e}	33.89 ± 0.51 ^{e}	297.59 ± 8.57 ^{e}	22.38 ± 0.89 ^{e}	31.38 ± 1.29 ^{a}

PLE, Pressurized liquid extraction; SFE, Supercritical fluid extraction; SAF, Supercritical antisolvent fractionation. In each column, superscripts mean groups not statistically different ($p > 0.05$), as analyzed by one-way ANOVA.

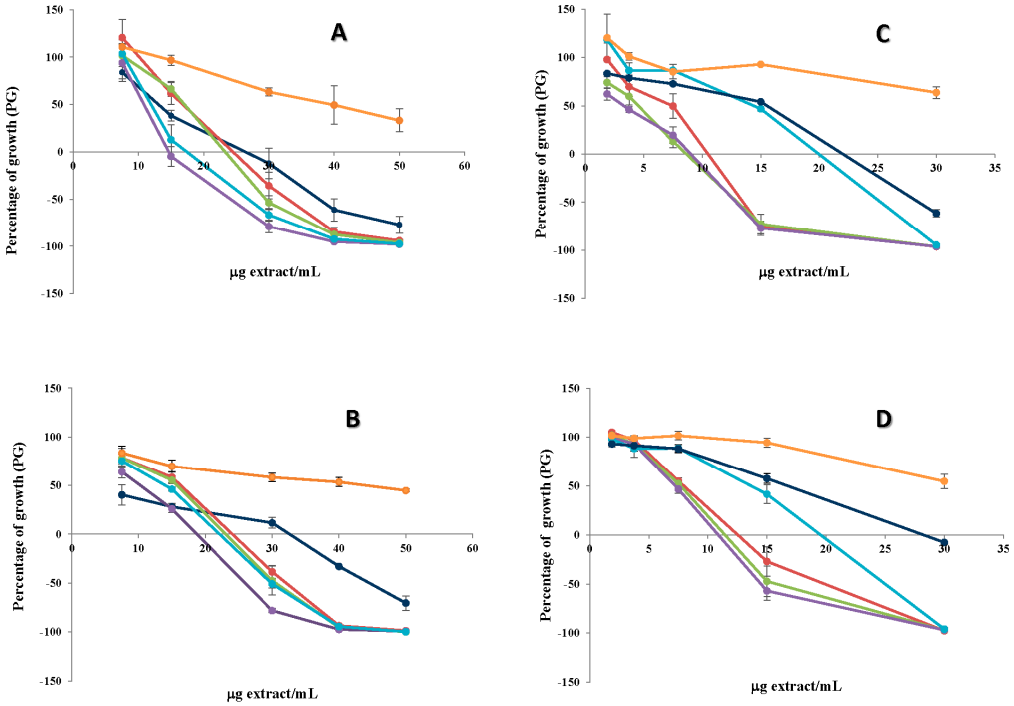

Figure S1. Percentage of growth (PG) of HT-29 and HCT116 colon cancer cells exposed to the different extracts concentrations at different exposure times. Calculated values for HT-29 at 24 h (**A**) and 72 h (**B**); and for HCT116 at 24 h (**C**) and 72 h (**D**). SFE1 (blue line), SFE2 (cyan line), PLE (orange line), SAF1 (red line), SAF2 (green line), SAF3 (violet line). Error bars represent standard error of the mean (SEM).

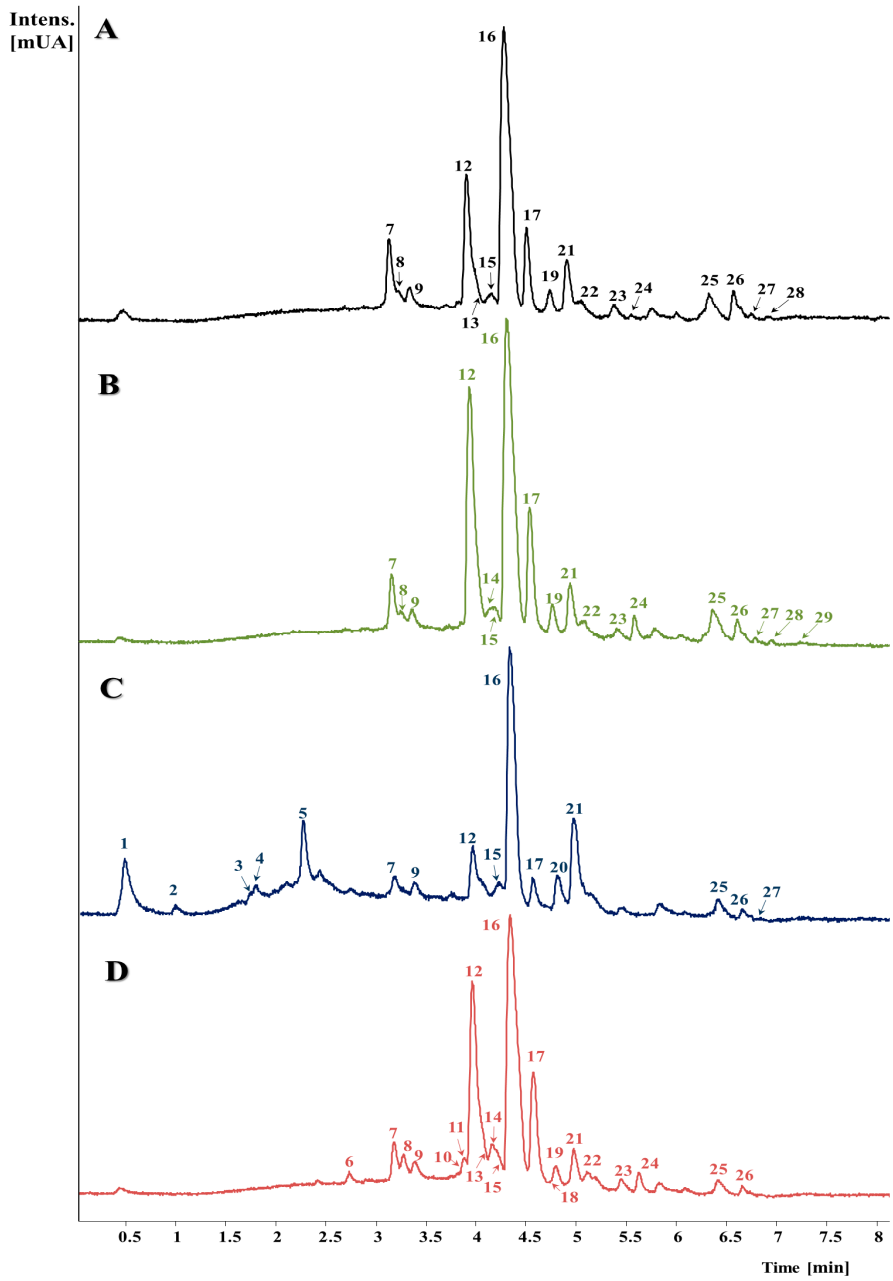

Figure S2. Chromatograms (at 280 nm) corresponding to SFE1 (**A**); SFE2 (**B**); PLE (**C**) and SAF1 (**D**) extracts. SAF1 was selected as example for PLE + SAF integrated process. For peak identification, see Table 3.

4.2 Obtención de extractos ricos en polifenoles y carotenoides a partir de algas marinas empleando fluidos presurizados

4.2.1 Prefacio

Las algas marinas y las microalgas son una fuente poco explorada de compuestos con actividad biológica interesante. Aunque algunos componentes de estos organismos se utilizan actualmente en las industrias alimentaria y farmacéutica, todavía hay miles de especies diferentes que no se han estudiado hasta el momento. Las microalgas son el foco de muchos esfuerzos de investigación en el campo de los biocombustibles y la captura de CO_2; además, la producción de microalgas para la alimentación es una realidad comercial. El hecho de que estos microorganismos puedan ver modificada su composición química de acuerdo a las condiciones de cultivo en las que se producen, aumenta aún más su interés, ya que pueden comportarse como biofactorías para la producción de compuestos bioactivos "target". Por otra parte, las macroalgas también han despertado gran interés por su composición y potencial bioactividad. Si a este hecho se suma que algunas especies de macroalgas son consideradas organismos invasivos, es fácil de entender que su valorización pueda tener un gran impacto ambiental y comercial. Por todos estos motivos, en los últimos años hay un creciente interés en el desarrollo de diferentes estrategias de extracción basadas en el uso de EAE, PLE y SFE empleando bio-disolventes, como alternativa para la obtención de compuestos bioactivos de microalgas y algas.

En la presente Tesis Doctoral se estudió el potencial de la combinación de la extracción asistida por enzimas o la hidrólisis alcalina con PLE con el objetivo de mejorar la eficiencia de extracción para el aislamiento de los florotaninos del alga marrón *Sargassum muticum*. Este estudio se presenta en la sección 4.2.2, y corresponde al trabajo titulado *"Considerations on the use of enzyme-assisted extraction in combination with pressurized liquids to recover bioactive compounds from algae"* de Sánchez-Camargo et al., publicado en la revista Food Chemistry (2016), 192, 67–74. Para la extracción asistida con enzimas se ensayaron dos tipos de enzimas, una carbohidrasa y una proteasa, a diferentes condiciones de hidrólisis. Posteriormente, el

residuo húmedo de alga remanente de la hidrólisis se sometió a PLE empleando una mezcla hidroalcohólica y siguiendo el diagrama de flujo mostrado en el esquema de la Figura 4.3.

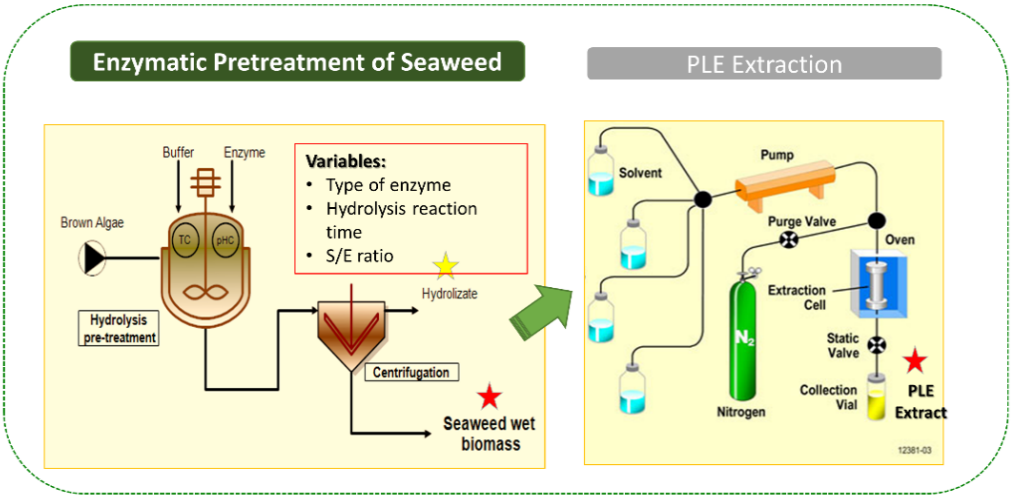

Figura 4.3. Esquema del proceso integrado para obtención de extractos a partir de algas marrones empleando EAE+PLE.

Los resultados mostraron que no existió un efecto positivo de las enzimas sobre la extracción de compuestos fenólicos y que el empleo únicamente de PLE proporcionaba los valores de rendimiento y actividad antioxidante más altos. Posteriormente se utilizó un diseño experimental factorial para optimizar las condiciones de extracción de PLE, teniendo como factores principales el porcentaje de etanol en la mezcla disolvente y la temperatura de extracción. Las variables respuesta del diseño fueron: el rendimiento de extracción, el contenido en fenoles totales (Folin–Ciocalteu), el contenido de florotaninos totales (DMBA) y la actividad antioxidante (ABTS).

Las condiciones óptimas de extracción se emplearon para llevar a cabo el estudio del efecto de la localización geográfica de crecimiento de 13 muestras de alga *Sargassum muticum* recolectadas a lo largo de las costas del Atlántico norte Europeo (Figura 4.4) sobre la composición y bioactividad de las mismas. Los extractos resultantes se caracterizaron en cuanto a rendimiento de extracción, contenido de fenoles y florotaninos totales, y actividad antioxidante.

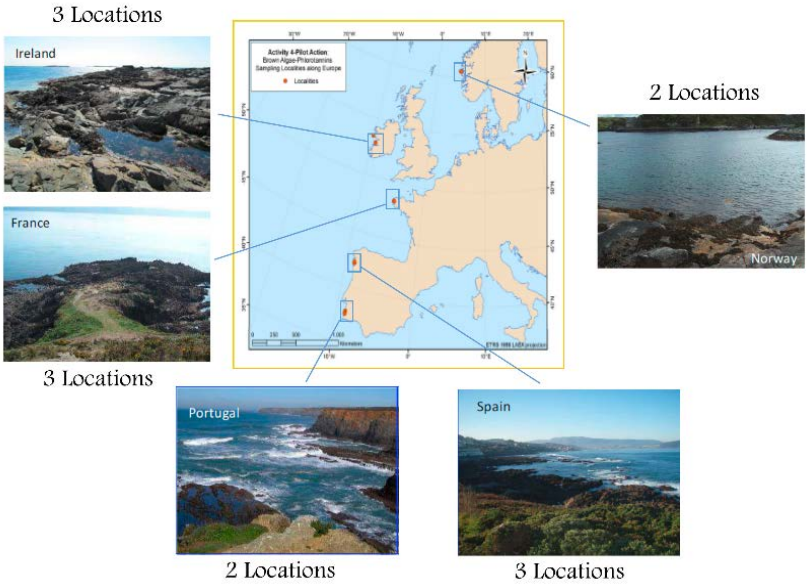

Figura 4.4. Localizaciones de las 13 muestras de *Sargassum muticum* recolectadas a lo largo de las costas del Atlántico Europeo.

Este trabajo se realizó en colaboración con el Laboratoire des Sciences de l'environnement marin (Université de Bretagne Occidentale, Francia) y los resultados están plasmados en el Anexo C, y corresponde al trabajo titulado *"Antiproliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga Sargassum muticum collected on North-Atlantic coasts"* de Montero et al., publicado en la revista Journal of Chromatography A (2016), 1428, 115–125[2]. Una vez seleccionados los extractos con mayor contenido en compuestos fenólicos y actividad antioxidante, se llevó a cabo una purificación de los mismos y las fracciones ricas en florotaninos se caracterizaron por medio de cromatografía de líquidos bidimensional completa acoplada a espectrometría de masas (LC × LC-MS/MS). Además, se estudió por primera vez la actividad antiproliferativa de estos compuestos en células de cáncer de colon HT-29 y se observó la respuesta al tratamiento a cinco concentraciones diferentes, durante 24, 48 y 72 h.

[2] Aunque este trabajo no se incluye dentro de las publicaciones que aparecen en el apartado de Resultados, el trabajo se realizó conjuntamente por la doctoranda y la primera firmante del mismo y, por tanto, se discutirán los resultados más relevantes que conciernen al trabajo realizado por la doctoranda dentro del mismo.

Al igual que *Sargassum muticum*, *Cystoseira abies-marina* es una de las especies de algas marrones más importantes encontradas en los ecosistemas del Mar Mediterráneo y el Océano Atlántico y, tal y como se ha mencionado en la introducción de esta Memoria, se ha descrito como una fuente prometedora de compuestos fenólicos. En estudios previos del grupo de investigación se han desarrollado técnicas de identificación de florotaninos (tipo y grado de polimerización) en extractos de *C. abies-marina* de extractos purificados por SPE (solid phase extraction) y extracción líquido-líquido empleando disolventes orgánicos (Montero et al., 2014). Sin embargo, surge la necesidad de desarrollar nuevos procesos ecológicos para eliminar/reemplazar el uso de estos disolventes contaminantes y a menudo tóxicos y a su vez obtener extractos enriquecidos con florotaninos buscando una mayor eficiencia y cumplimiento de los principios de la química verde. En este sentido, el modelado teórico de los parámetros de solubilidad de Hansen proporciona una estimación útil y precisa para la selección de un disolvente selectivo para la extracción de estos compuestos bioactivos naturales. En la sección 4.2.3 se presenta el estudio titulado *"Application of Hansen solubility approach for the subcritical and supercritical selective extraction of phlorotannins from Cystoseira abies-marina"* de Sánchez-Camargo et al., publicado en la revista RSC Advances (2016), 6, 94884-94895. Con el fin de conducir el proceso hacia la extracción de los compuestos objetivo, se determinó la composición química de los florotaninos de *Cystoseira abies-marina* usando un método previamente desarrollado empleando LC × LC -MS/MS (Montero et al., 2014). Una vez identificados los compuestos mayoritarios, se realizó por primera vez la estimación de sus parámetros de solubilidad en algunos bio-disolventes en condiciones sub- y supercríticas para mejorar su extracción selectiva. Debido a la poca información y a la ausencia de patrones comerciales de estos compuestos, no fue posible obtener experimentalmente los parámetros de solubilidad mediante mediciones indirectas (es decir, pruebas de solubilidad, presión osmótica, turbidez, volumen específico o viscosidad intrínseca). Por tanto, se emplearon métodos de contribución de grupo (GCM) como una buena aproximación para predecir sus propiedades fisicoquímicas y los parámetros de solubilidad de las estructuras moleculares de los florotaninos más abundantes utilizando reglas aditivas. Posteriormente, basados en las predicciones teóricas, se llevaron a cabo ensayos experimentales para corroborar los resultados obtenidos.

Continuando con este mismo enfoque, la estimación de los parámetros de solubilidad de Hansen de fucoxantina en diferentes bio-disolventes puede ser un factor clave en el desarrollo de procesos selectivos de extracción de este compuesto a partir de *Phaeodactylum tricornutum*. Los resultados de este trabajo de investigación se presentan en el artículo titulado *"New approaches for the selective extraction of bioactive compounds employing bio-based solvents and pressurized green processes"* de Sánchez-Camargo et al., sometido a revisión en febrero de 2017 a la revista *Journal of Supercritical Fluids*. El uso de cálculos teóricos reduce la elección de disolventes adecuados y selectivos para la extracción de fucoxantina, incluyendo bio-disolventes como CO_2, acetato de etilo, lactato de etilo, d-limoneno y etanol en condiciones sub- y supercríticas. Las nuevas herramientas de predicción, como el software HSPiP 5.0, fueron de gran utilidad para predecir parámetros como el radio de interacción de la esfera de Hansen. Con posterioridad a la predicción teórica, se desarrollaron procesos de extracción con líquidos presurizados y SC-CO_2 (empleando modificador) con el fin de corroborar los resultados obtenidos y relacionar los datos teóricos predichos con los ensayos experimentales. Los parámetros evaluados en los extractos obtenidos fueron el rendimiento de extracción, el contenido de fucoxantina en el extracto (determinado mediante HPLC-DAD-APCI-MS) y la relación de carotenoides y clorofilas totales, como medida de la selectividad del proceso. Una vez caracterizados los extractos fue posible seleccionar el bio-disolvente más selectivo y optimizar experimentalmente las variables de extracción implicadas en el proceso.

4.2.2 Considerations on the use of enzyme-assisted extraction in combination with pressurized liquids to recover bioactive compounds from algae.

A. P. Sánchez-Camargo, L. Montero, V. Stiger-Pouvreau, A. Tanniou, A. Cifuentes, M. Herrero, & E. Ibáñez

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Considerations on the use of enzyme-assisted extraction in combination with pressurized liquids to recover bioactive compounds from algae

Andrea del Pilar Sánchez-Camargo [a], Lidia Montero [a], Valérie Stiger-Pouvreau [b], Anaëlle Tanniou [b], Alejandro Cifuentes [a], Miguel Herrero [a,*], Elena Ibáñez [a]

[a] Laboratory of Foodomics, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain
[b] LEMAR UMR CNRS UBO IRD IFREMER 6539, Université de Bretagne Occidentale (UBO), Institut Universitaire Européen de la Mer (IUEM), Technopôle Brest-Iroise, Rue Dumont d'Urville, Plouzané 29280, France

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ABSTRACT

Pressurized liquids, PLE, and enzyme-assisted extraction, EAE, have been tested to improve the extraction of phlorotannins from the seaweed *Sargassum muticum*. Enzymatic treatment with proteases and carbohydrases, alkaline hydrolysis and PLE with ethanol:water as extracting solvent have been studied in terms of extraction yield, total phenolic content and antioxidant activity (TEAC assay). Results demonstrated that the application of PLE alone provided the highest yields and relevant antioxidant activity. An experimental design was employed to further optimize the PLE extraction conditions; optimum parameters included the use of 160 °C and 95% ethanol. Under these conditions, values of 21.9%, 94.0 mg gallic acid equivalents g^{-1}, 5.018 mg phloroglucinol equivalents g^{-1} and 1.275 mmol trolox equivalents g^{-1} were obtained for extraction yield, total phenols, total phlorotannins and TEAC, respectively. A preliminary chemical characterization by liquid chromatography coupled to mass spectrometry provided insight in terms of the mechanisms involved in the different processes.

1. Introduction

Nowadays, the modern conception of nutrition implies not only covering all the physical needs in order to prevent nutritional and energetic deficiencies, but also to go further towards the prevention of chronic diseases (Norheim et al., 2012). Within this frame, in the last years a huge amount of new functional foods have been developed and marketed (Khan, Grigor, Winger, & Win, 2013). These novel foods are basically traditional foods to which a functional ingredient, responsible of providing an additional health benefit, has been added. Among the positive health benefits of functional foods, probiotics (Neef & Sanz, 2013), cholesterol-lowering effect (Wong, 2014), improvement of bone health (Arjmandi et al., 2010), anti-hypertensive (Mohamed, 2014) or prevention of gut-related diseases (Oozeer et al., 2010) can be pointed out. On the other hand, from the functional ingredients perspective, antioxidants are one of the most relevant groups of components. Among them, phenolic compounds are an important class of natural antioxidants that can also provide other beneficial effects, including prevention of cancer (Zhou & Raffoul, 2012), cardiovascular diseases (Zuchi, Ambrosio, Lüscher, & Landmesser, 2010) or neurodegenerative diseases (Iriti & Faoro, 2009), which are directly related to oxidative stress. Due to the increasing interest towards these compounds, the search of new natural sources of these potential functional ingredients is, at present, of paramount importance.

Algae are a highly heterogeneous group of organisms that have already been proposed as potential functional food ingredients (Li & Kim, 2011). Brown algae (Phaeophyceae) are well-known for possessing an important class of phenolic compounds, phlorotannins, that are exclusively found in these organisms (Montero, Herrero, Ibáñez, & Cifuentes, 2014). These compounds have been suggested to possess several bioactivities including antioxidant (Tanniou et al., 2013; Tierney et al., 2013; Wang et al., 2012), anti-inflammatory (Lopes et al., 2012), antidiabetic (Lee & Jeon, 2013), anti-proliferative (Nwosu et al., 2011) or antibacterial effects (Lopes, Pinto, Andrade, & Valentao, 2013; Tanniou et al., 2014). Phlorotannins can be found in brown algae in relatively high percentage (up to 15% of dry weight depending on species) and are formed as polymers of phloroglucinol (1,3,5-trihydroxybenzene) (Koivikko, Loponen, Pihlaja, & Jormalainen, 2007); however, their

* Corresponding author.
E-mail address: m.herrero@csic.es (M. Herrero).

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chemical composition is greatly variable, considering that there are different types of phlorotannins with very different degrees of polymerization. Four main types of phlorotannins, including fuhalols and phlorethols (both containing ether linkages), fucols (with phenyl linkages), fucophlorethols (containing ether and phenyl linkages) and eckols (with a benzodioxin linkage) have been described. It is widely accepted that phlorotannins are found within the algal cells forming complexes with different components of the cell walls, such as alginic acid (Kim et al., 2013) therefore, the protocols to obtain extracts enriched in these components should be optimized to improve their extractability.

The use of advanced extraction methods allows the development of faster, more efficient and greener processes than those traditionally employed based on the use of organic solvents. One of these advanced extraction processes is pressurized liquid extraction (PLE). This technique is based on the use of solvents at high temperatures and pressures to maintain their liquid state during the whole extraction procedure (Mendiola, Herrero, Cifuentes, & Ibañez, 2007). PLE has already been shown as a powerful extraction tool to extract a variety of bioactive compounds from different natural samples (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013; Tanniou et al., 2013). On the other hand, enzyme-assisted extraction (EAE) is also considered a green technique that takes advantage of enzymatic hydrolysis processes to assist the release of interesting compounds to the extracting solvent (Puri, Sharma, & Barrow, 2012; Wijesinghe & Jeon, 2012). In the case of plant and algal material, this latter technique could pose the additional advantage of producing the hydrolysis of bioactive components that are linked to cell wall components, thus, increasing their extraction efficiency (Rodrigues et al., 2015).

For this reason, in this work, EAE and alkaline hydrolysis, both combined to PLE are investigated with the aim to study a potential increase the extraction efficiency for the isolation of phlorotannins from the brown alga *Sargassum muticum*. This brown alga has previously been described as a source of interesting phenolic compounds (Tanniou et al., 2013), with small size compared to polymers synthesized by other brown algal species (Stiger-Pouvreau, Jégou, Cérantola, Guérard, & Le Lann, 2014). Moreover, the generated extracts have been characterized in terms of antioxidant activity together with total phenolic and total phlorotannins contents. Furthermore, the preliminary chemical characterization of the obtained extracts by LC-DAD–MS has been attempted.

2. Materials and methods

2.1. Samples and chemicals

Samples of *S. muticum* were composed of thalli of *S. muticum* that were collected in July 2011 at the Dellec (Plouzané), a semi-exposed field site on the western coast of Brittany (France). After collection, seaweeds were washed, first with filtered seawater then with distilled water in order to get rid of residual sediments and salts. The cleaned algal materials were then surface-dried with blotting paper towel, chopped into fragments, freeze-dried, reduced to powder with a Waring Blender and finally sieved at 250 μm.

Carbohydrases (Viscozyme L) and proteases (Alcalase 2.4L FG) used in this study were kindly donated by Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). Gallic acid, phloroglucinol dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ⩾97%) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, ⩾99%) were purchased from Sigma-Aldrich (Madrid, Spain). The Folin–Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany).

2.2. Enzyme treatment and extraction of phenolic compounds

Enzymatic hydrolysis was carried out in 50 mL tubes and using a Thermomixer R (Eppendorf AG, Hamburg, Germany). Twenty-five mL of appropriate buffer solution were added to one gram of dried algae, and then 100 μL of enzyme were mixed (450 rpm). Alcalase and viscozyme enzyme cocktails were studied. The optimum hydrolysis conditions in terms of reaction temperature and pH for alcalase according to the product specifications included 50 °C, pH 7.0 and 0.1 M phosphate buffer, whereas for viscozyme 50 °C, pH 4.5 and 0.1 M sodium acetate-acetic acid buffer were employed. The enzymatic hydrolysis reactions were performed for 2 or 4 h. Afterwards, hydrolyzed (water soluble compounds) materials were separated from the seaweed residual biomass by centrifugation at 5000 rpm, 4 °C for 20 min, freeze-dried and stored at −20 °C until use. The extraction yield after enzymatic hydrolysis was determined by a gravimetric method, subtracting the dried weight of the residue from one gram of brown algal samples dried, and expressed as percentage. The residual biomass was carefully weighted and stored at −20 °C protected from light until extraction by PLE.

2.3. Alkaline hydrolysis

For comparison, alkaline hydrolysis treatments were performed following the method described by Koivikko, Loponen, Honkanen, and Jormalainen (2005) with some modifications. Briefly, one gram of dry algae was suspended in 25 mL of 1 M aqueous NaOH solution (80 °C) and stirred for 2.5 h at room temperature. After hydrolysis, samples were neutralized with 1 mL of H_3PO_4 (85% w/w) until pH 6.5–7.0 and centrifuged at 5000 rpm at 4 °C for 20 min. Remaining biomass was stored at −20 °C protected from light for subsequent PLE extraction, whereas the hydrolyzate was freeze-dried and kept at −20 °C until analysis.

2.4. Pressurized liquid extraction (PLE)

Extractions were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. Ultrapure water and ethanol were used as solvents. At the beginning of the day, the solvents were sonicated for 10 min. For each extraction, 2 g of seaweed residual biomass from hydrolysis pre-treatment or intact algae were loaded into an 11 mL stainless steel extraction cell after being mixed with sea sand, employed as dispersive agent. Preliminary PLE experiments were performed according to the extraction conditions previously employed for the extraction of phenolic compounds from *S. muticum* (Tanniou et al., 2013): static extraction time, 20 min; extraction pressure, 1500 psi; extraction temperature, 120 °C; extraction solvent, 75:25 ethanol:water (v/v). An instrumentally preset warming-up time of 6 min was also accomplished before the static extraction period. After extraction, ethanol was eliminated using a rotary evaporator (Büchi R-3000, Flawil, Switzerland) and then brown algal extracts were freeze-dried (Labconco Corporation, Missouri, USA) prior to further analyses.

2.5. Experimental design

PLE was further optimized using a 3-level factorial design 3^2 (including two center points). The effects of temperature (50–200 °C) and percentage of ethanol in the mixture solvent (0–100%) (v/v) on extraction yield (X_0%), total phenolic content (mg GAE/g extract), antioxidant capacity (mM TE/g extract) and phlorotannins content (mg PGE/g extract) were investigated. A total of 11 experiments were conducted in a randomized order. The experimental design and data analysis were carried out using

response surface methodology with the software Statgraphics Centurion XVI (StatPoint Technologies, Inc., Warrenton, VA 20186, USA). The effects of the independent variables on the response variables in the extraction process were assessed using the pure error, considering a level of confidence of 95% for all the variables. The quadratic model proposed for each response variable (Y_i) was:

$$Y_i = \beta_0 + \beta_1 T + \beta_2 S + \beta_{1,1} T^2 + \beta_{1,2} T * S + \beta_{2,2} S^2 + \text{error} \quad (1)$$

where T is the temperature, S is the solvent composition (percentage of ethanol in the mixture), β_0 is the intercept, β_1, β_2 are the linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ are the quadratic coefficients, $\beta_{1,2}$ is the linear-by-linear interaction coefficient, and error is the error variable. Polynomial models (Eq. (1)) were evaluated considering the percent variation explained by the correlation coefficient (R^2), the residual standard deviation (RSD), and the lack-of-fit test for the model from the analysis of variance table, as the significance criteria. The effect of each factor and its statistical significance, for each of the response variables, was analyzed from the standardized Pareto chart. The response surfaces of the respective mathematical models were also obtained, and the significances were accepted at $p \leqslant 0.05$ (considering a level of confidence of 95%). A multiple response optimization was carried out by the combination of experimental factors, looking for maximizing the desirability function.

2.6. In vitro determinations

2.6.1. Total phenols content (Folin–Ciocalteu method)

Total phenols content (TPC) on the freeze-dried hydrolyzates and on the PLE extracts was determined spectrophotometrically by using the Folin–Ciocalteu method according to Kosar, Dorman, and Hiltunen (2005), with some modifications. Briefly, 10 μL aliquot of extract solution (concentration range from 5 to 10 mg mL^{-1}) and 600 μL ultrapure water were mixed, to which 50 μL undiluted Folin–Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 20% (w/v) Na_2CO_3 were added and the volume was made up to 1.0 mL with water. The samples were incubated for 2 h at 25 °C in the darkness. Later on, 300 μL of each reaction mixture were transferred to a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader Powerwave XS (Bio Tek Instruments, Winooski, VT). Standard curves with serial gallic acid solutions were used for calibration. The phenolic content was expressed as mg of gallic acid (GAE) per g of extract. All analyses were done by triplicate.

2.6.2. Total phlorotannins content (DMBA assay)

DMBA colorimetric assay was employed as a response variable of the experimental design to estimate the amount of total phlorotannins content in the brown alga extracts (Lopes et al., 2012). DMBA solution was prepared just prior use by mixing equal volumes of 2% DMBA reagent in acetic acid m/v and 6% hydrochloric acid in acetic acid v/v. A total of 50 μL of sample (5 mg mL^{-1}) was mixed with 250 μL of DMBA solution in a 96-well microplate and the reaction was conducted at room temperature for 60 min in the dark. Then, the absorbance was read at 515 nm using a microplate spectrophotometer reader Powerwave XS (Bio Tek Instruments, Winooski, VT). Water was used as blank and control samples without DMBA solution were also included. A calibration curve using phloroglucinol dihydrate (PG) (0–62.5 μg mL^{-1}) was employed to estimate of total phlorotannins content. All samples, blanks, and controls were prepared in triplicate. Data were presented as the average of triplicate analyses expressed as milligram phloroglucinol equivalents (PGE) per gram of dry extract.

2.6.3. Trolox equivalents antioxidant capacity assay (TEAC)

TEAC assay was employed to measure antioxidant capacity following the method based on the procedure described by Re et al. (1999). $ABTS^{\cdot+}$ radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature during 16 h before use. The aqueous $ABTS^{\cdot+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7 (±0.02) at 734 nm. Ten microliters of sample (5 different concentrations ranging from 0.25 to 2 mg mL^{-1}) and 1 mL of $ABTS^{\cdot+}$ solution were mixed in an eppendorf vial and 300 μL of the mixture were transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min during 45 min in a Powerwave XS microplate spectrophotometer reader (Bio Tek Instruments, Winooski, VT). Trolox was used as reference standard and results were expressed as TEAC values (mmol of trolox/g extract). These values were obtained from five different concentrations of each extract tested in the assay giving a linear response between 20% and 80% of the blank absorbance. All analyses were done in triplicate.

2.7. HPLC-DAD–MS-based chemical characterization of S. muticum extracts

The chromatographic separation of the extracts was performed on an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) consisting in a binary pump, an autosampler and a diode-array detector (DAD), directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) with an electrospray interface. A Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 μm particle diameter, Agilent Technologies, Santa Clara, CA) column was employed using (A) water (0.1% formic acid) and (B) acetonitrile as mobile phases following a gradient elution of: 0 min, 2% B; 20 min, 5% B; 30 min, 20% B; 35 min, 20% B. A flow rate of 0.2 mL min^{-1} and an injection volume of 20 μL were employed. The detection wavelength was set at 280 nm and the UV–Vis spectra were acquired from 190 to 550 nm. The MS was operated under ESI negative and positive ionization modes using the following settings: dry temperature, 350 °C; mass range, m/z 90–2200 Da; dry gas flow rate, 12 L min^{-1}; nebulization pressure, 40 psi.

2.8. Statistical analysis

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95%. One-way analysis of variance (ANOVA), together with Stude nt–Newman–Keuls test, was employed to group extracts, based on statistically significant differences. Mean values were compared using the Tukey's test and differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. EAE of S. muticum brown macroalga

In order to study the possibility of coupling a EAE process to PLE to obtain a higher amount of bioactive phenolic compounds from *S. muticum*, different enzymatic treatments were explored based on the use of several enzyme cocktails. Table 1 presents the results obtained in terms of extraction yield after hydrolysis, TPC and TEAC for each treatment. An alkaline hydrolysis procedure was also included for comparison. Phenolic compounds were the main target compounds as brown algae have been described as particularly rich sources of these interesting components (Li, Wijesekara, Li, & Kim, 2011; Stiger-Pouvreau et al., 2014). Two commercial enzymes were employed, namely alcalase and viscozyme. These two

Table 1
Extraction yield (%), total phenols content (mg GAE g^{-1} extract) and antioxidant activity (mmol TE g^{-1} extract, TEAC assay) of the brown macroalga *Sargassum muticum* extracts recovered after the different hydrolysis treatments studied. Values presented are mean ± sd.

Treatment	Extraction yield (%)	Total phenols content (mg GAE g^{-1})[1]	TEAC (mmol TE g^{-1})[2]
Alcalase 2 h	13.6 ± 1.4^{a}	7.91 ± 0.65^{a}	0.262 ± 0.006^{a}
Alcalase 4 h	17.81 ± 2.8^{b}	6.08 ± 0.54^{b}	0.205 ± 0.015^{b}
Viscozyme 2 h	20.6 ± 1.7^{c}	10.33 ± 1.16^{c}	0.178 ± 0.005^{c}
Viscozyme 4 h	23.5 ± 0.1^{d}	10.01 ± 0.66^{c}	0.172 ± 0.025^{c}
Alkaline hydrolysis	12.8 ± 0.1^{a}	9.88 ± 0.96^{c}	0.187 ± 0.011^{b,c}

Superscripts mean groups not statistically different ($p > 0.05$) for each response.
[1] mg gallic acid equivalents g^{-1} extract.
[2] mmol trolox equivalents g^{-1} extract.

enzymes were selected by their different selectivity in order to study to which components algal polyphenols may be bound. Cell walls of brown algae are mainly composed of polysaccharides including alginic acid, alginates (carboxylated polysaccharides, salts of alginic acid), and fucans (sulfated polysaccharides) (Balboa, Conde, Moure, Falque, & Dominguez, 2013a). The presence of these compounds in the cell wall limits the accessibility to bioactive compounds, reducing the extraction efficiency during application of traditional extraction methods. Thereby, the degradation of cell wall polysaccharides may be an important step in the release of active components as phlorotannins (Jeon, Wijesinghe, & Kim, 2012). On the other hand, it has been already described that phlorotannins may be naturally found forming strong complexes with proteins, either reversible by hydrogen bonding or irreversible by covalent condensation (Stern, Hagerman, Steinberg, & Mason, 1996). Alcalase is a protease from *Bacillus licheniformis*, whereas viscozyme is an enzymatic preparation composed by carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. Thus, the use of these two enzymes could provide with information regarding the possible phlorotannin complexes found in *S. muticum*. For each enzyme two different treatment times were studied at appropriate pH conditions (as suggested by the manufacturer), in order to determine the influence of the treatment time on the release of phenolic compounds. Moreover, as already mentioned, an alkaline hydrolysis treatment was also performed for comparison. Thus, the five mentioned treatments were carried out (two treatments per enzyme and the alkaline hydrolysis) as described in Section 2.

As can be observed in Table 1, highest extraction yields, expressed as dry weight of the supernatant with respect to the initial alga dry weight were obtained for viscozyme treatments. Slightly but significant ($p < 0.05$) higher yields were attained using the 4 h treatments compared to 2 h. Alkaline hydrolysis produced similar results than a 2 h alcalase treatment. The first conclusion is that carbohydrases are more effective than proteases on weakening cell wall structure, although the effect of hydrolysis solutions is also important when considering the extraction yield. In fact, buffer solutions without enzymes were employed as controls, and it could be confirmed that similar extraction yields were obtained in each case (Table S1 – Supplementary material). The difference among enzymes and hydrolysis solutions is in line with the fact that *S. muticum* cell wall is thought to be composed by a variety of polysaccharides, such as fucoidans, alginate and laminarin and their derivatives (Balboa, Rivas, Moure, Domínguez, & Parajó, 2013b). In this regard, the lower pH employed for viscozyme could promote a better release of compounds contained in algal cells. The functional characterization of the extracts was completed with the determination of total phenols amount and total antioxidant activity. As can be seen in Table 1, viscozyme extracts contained a similar amount of total phenols than those obtained using the alkaline hydrolysis procedure. On the other hand, alcalase extracts contained the lowest amount of phenols. These values might suggest that hydrolysis conditions during the treatment with carbohydrases were more effective on releasing bound polyphenols. Moreover, a direct effect on the cell wall, as mentioned, cannot be discarded. Likewise, strong alkaline conditions were also favorable to the release of those components. Interestingly, results obtained from the antioxidant capacity assay did not follow the same trend. In that case, alcalase extracts possessed higher antioxidant activity than the rest of extracts, being the antioxidant activity of the viscozyme extracts the lowest among the different treatments. In any case, it is worth to mention that although significant ($p < 0.05$), the quantitative differences between alcalase and viscozyme extracts were not high in terms of antioxidant activity, being in all cases rather modest. Those results are in agreement with those reported from enzymatic hydrolysates obtained from *S. muticum* collected on North Brittany (France) (Hardouin et al., 2014). Compared to controls, total phenols amount after the use of both enzymes was higher. The antioxidant activity was also enhanced even in a more marked way (Table S1 – Supplementary material). This demonstrates that even if the extraction yield was very similar, as already commented, the activity of enzymes favored the release of compounds that could have a positive influence on the total antioxidant activity of the extracts.

3.2. EAE–PLE combined extraction of S. muticum brown alga

Following the hydrolysis treatment, the coupling to PLE was performed, in order to more precisely study the potential influence of each treatment on the final PLE extract attainable. In fact, an effective cell disruption would potentially increase the recovery during the subsequent PLE process, thus, making the whole combined process more effective. The solid residue obtained after hydrolysis was extracted, and the PLE conditions employed were selected according to our previous experience with this material (Tanniou et al., 2013) including the use of a mixture of ethanol:water (25:75, v/v) as extracting solvent at 120 °C for 20 min. Table 2 summarizes the results obtained from the different hydrolysis-PLE combined processes as well as those obtained from the PLE alone in terms of extraction yield, total phenols and antioxidant activity. As it can be observed, the highest yield was obtained from the 2 h alcalase-PLE combined treatment, although it was not statistically different from that obtained using PLE alone ($p > 0.05$). On the other hand, viscozyme treatments as well as alkaline hydrolysis coupled to PLE extraction produced similar lower yields. Clearly, cell wall weakening produced during the hydrolysis treatments could potentially increase the effect of high pressure in the subsequent extraction. This effect could facilitate the recovery of the different compounds increasing the total extraction yield attained.

Table 2
Extraction yield (%), total phenols content (mg GAE g^{-1} extract) and antioxidant activity (mmol TE g^{-1} extract, TEAC assay) of the brown macroalga *Sargassum muticum* PLE extracts (120 °C, 20 min, ethanol:water 75/25 (v/v)) performed after the indicated pre-treatment. Values presented are mean ± sd.

Pre-treatment	Extraction yield (%)	Total phenols content (mg GAE g^{-1})[1]	TEAC (mmol TE g^{-1})[2]
No treatment	40.1 ± 0.7^{a,b}	47.55 ± 2.28^{a}	0.585 ± 0.019^{a}
Alcalase 2 h	46.1 ± 5.6^{b}	27.17 ± 1.54^{b}	0.581 ± 0.045^{a}
Alcalase 4 h	34.6 ± 1.0^{a,c}	21.86 ± 1.05^{c}	0.469 ± 0.051^{b}
Viscozyme 2 h	29.6 ± 2.4^{c}	39.20 ± 3.55^{d}	0.687 ± 0.050^{c}
Viscozyme 4 h	29.2 ± 0.8^{c}	38.74 ± 3.58^{d}	0.629 ± 0.089^{a,c}
Alkaline hydrolysis	28.0 ± 0.4^{c}	28.88 ± 2.26^{b}	0.472 ± 0.027^{b}

Superscripts mean groups not statistically different ($p > 0.05$) for each response.
[1] mg gallic acid equivalents g^{-1} extract.
[2] mmol trolox equivalents g^{-1} extract.

However, in our experiments, the use of a pre-treatment before extraction did not significantly improve the results. This observation was also evident considering the total phenols found in the extract. In this case, the PLE process without any other previous treatment provided the best results. Viscozyme-treated PLE extracts contained similar amounts of phenols, whereas alcalase-treated and alkaline hydrolyzed PLE extracts contained the lowest amount of phenols. This behavior can be partly explained due to the formation of protein polyphenol complexes during the extraction, as suggested by Siriwardhana et al. (2008). These authors hypothesized that when the algal cell wall is disrupted by proteases or carbohydrases, the intracellular constituents including proteins are released from the cells, being prone to form complexes with polyphenols, leading to aggregation and ultimate precipitation; in this sense, strong interactions have been previously reported (Stern et al., 1996) between phlorotannins and proteins that would somehow explain the low amount of TPC detected. Another explanation for the decreased TPC in carbohydrase-treated extracts could be due to the release of oligosaccharides and simple sugars during the degradation of cell wall polysaccharides, resulting in lower overall levels of polyphenols in the extracts (Hardouin et al., 2014; Siriwardhana et al., 2008).

In terms of antioxidant activity values did not exactly correlate with total phenols content. In this sense, alcalase treatment for 4 h as well as alkaline hydrolysis-PLE extracts provided the lowest antioxidant activity of all extracts. The rest of extracts showed close antioxidant activities ($p < 0.05$) that were clearly higher, except for the PLE extracts obtained after a 2 h viscozyme treatment. These latter extracts possessed slightly higher antioxidant activity. In any case, all the antioxidant activities obtained as well as the total phenols content of the PLE extracts were by far higher compared to their respective hydrolysis processes alone. From these results, it is interesting to note that PLE alone, without the need of any pre-treatment, provided with very high extraction yields, total phenols amount and antioxidant activities. In fact, although viscozyme-treated PLE extracts possessed a slightly higher antioxidant activity, the total yields as well as the total phenol contents obtained suggested that the use of a PLE procedure was more favorable in this case. An enzymatic treatment could somewhat increase the antioxidant activity of the extracts (but not the total phenols content), although this increment was not sufficiently high to justify the time needed to obtain the extracts comparing PLE and the combined EAE–PLE process (20 min vs ca. 200 min, respectively). Consequently, the use of PLE alone was selected for further optimization to maximize the extraction of phlorotannins from *S. muticum* samples. This decision was further supported by the fact that PLE extracts from controls (samples extracted with hydrolysis solutions without enzymes) demonstrated very similar TEAC and TPC (Table S1 – Supplementary material) values compared to their corresponding enzyme treatments.

Considering that the influence of the enzymatic and alkaline hydrolysis was not sufficiently clear in terms of the real effect on cell wall's structure and on the compounds released in the media, a HPLC-DAD–MS-based preliminary chemical characterization was attempted to shed more light on the possible chemical changes produced in the sample during those treatments.

3.3. HPLC-DAD–MS comparison of the generated extracts

A method based on RPLC-DAD–MS was employed to carry out a preliminary characterization of the obtained extracts with the aim to observe possible differences among them. Fig. 1 shows the chromatograms (280 nm) corresponding to the extracts obtained using PLE alone (Fig. 1A) and the different combined processes (EAE–PLE, alkaline hydrolysis-PLE) (Fig. 1B–D corresponding to PLE in combination with viscozyme, alcalase and alkaline hydrolysis, respectively). As it can be observed in the figure, some differences in the profiles of the compounds extracted after the application of the studied processes can be observed. Those differences were not as big as expected, considering that the enzymes were selected to target the degradation of specific families of compounds in the algal cell wall structure. The detected compounds were tentatively assigned according to their UV–Vis and MS features. Nevertheless, unfortunately, a positive complete identification was not possible due to the lack of standards and the absence of information related to the extent of such treatments in other type of brown algae belonging to Fucales. However, some observations can be made: (1) the main peak in the non-treated PLE extract (peak 9) was also present in the PLE extracts previously treated with viscozyme but almost disappeared in the alcalase-treated PLE extract and was not found at all in the alkaline hydrolysis treated extract. The UV–Vis spectra of this compound had a maximum wavelength at 270 nm and produced an ion at m/z 113.8 ($[M+H]^+$). Although no conclusive identification could be reached with the available information, the stability of this component could be related to pH, being more easily extracted at acidic pHs (viscozyme conditions) and totally degraded under strong alkaline conditions. This compound could also be strongly related to the antioxidant activity of the extracts (data shown in Table 2); as it can be observed, the antioxidant activity of the different extracts can be correlated to the concentration of this component being higher in the viscozyme-treated PLE extracts, followed by PLE extracts, alcalase and alkaline hydrolysis-treated extracts; (2) other compounds increased their intensities in the treated extracts with respect to the non-treated one, such as peak 1 that presented a maximum at 260 nm. Unfortunately, this component was not properly ionized in the MS and no further information could be obtained; (3) new peaks

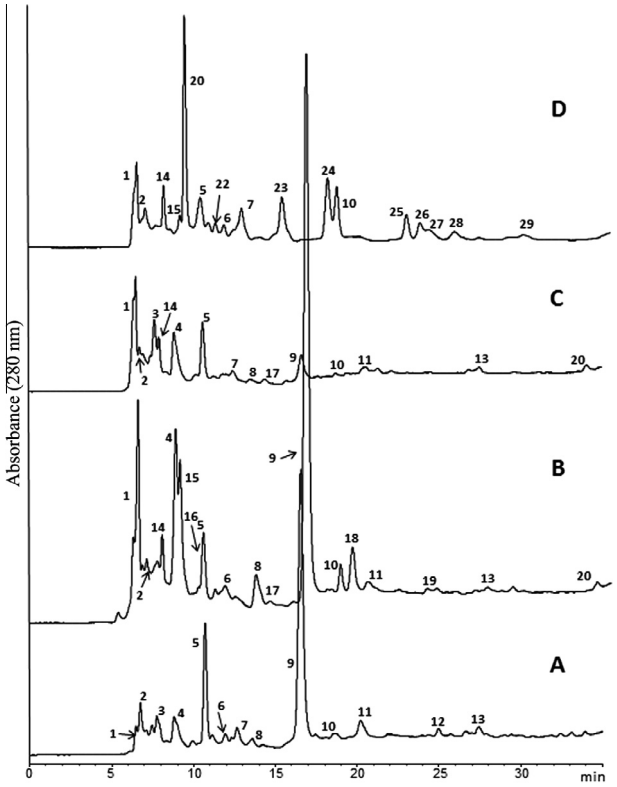

Fig. 1. Chromatograms (280 nm) corresponding to the different extracts produced using PLE alone (A) or PLE in combination with viscozyme (B), alcalase (C) and alkaline hydrolysis (D) treatments.

(peaks 14–18, Fig. 1B and C) appeared when the extracts were treated with enzymes. The presence of these components could be related to the catalytic activity of the employed enzymes that would release them or make them more available for the subsequent PLE extraction. For instance, a new peak was detected in the MS detector (peak 16 in the chromatogram) after viscozyme treatment, with a molecular ion at m/z 389.5 ($[M-H]^-$) that gave fragments at m/z 371, 315, 285, 243 and 138 that have been previously associated to a fucoidan (monosulfated difucose) (Daniel et al., 2007). Other peaks were also detected in the MS chromatogram (data not-shown) that did not possess UV absorption at 280 nm but yielded fragment ions similar to those of peak 16 allowing their tentative assignment as members of the fucoidans family; (4) the most different chromatogram was obtained for the alkaline hydrolysis-treated PLE extract (Fig. 1D). The harsh hydrolysis conditions performed produced the disappearance of some components as well as the appearance of new peaks exclusively detected in this extract, according to their UV–Vis and MS spectra characteristics (peaks 20–29). In fact, it is widely accepted that alkali may generate new compounds that are not found in the native algal composition through different reactions including degradations, rearrangement, condensation and base-catalyzed reactions (Craigie, 2011). In any case, considering the results shown in Table 2, these new components seemed to be less active in terms of antioxidant activity than those present in the non-treated and viscozyme-treated PLE extracts, as the antioxidant activity found in the alkaline hydrolysis extracts was the lowest. Indeed, the strong alkaline conditions employed could have caused the observed decrease on the antioxidant activity of the components extracted, as also previously described (Craigie, 2011); (5) Other compounds detected possessed UV–Vis and MS features compatible with some phlorotannins, although no further information from their MS/MS spectra could be used to increase the certainty on their identification. Thus, peak 3 was tentatively assigned to hydroxytetrafuhalol (m/z 529.9, $[M-H]^-$), peak 10 to triphlorethol (m/z 373.9, $[M-H]^-$) whereas peak 11 was tentatively identified as dihydroxypentafuhalol (m/z 671.0, $[M-H]^-$).

Even considering the discussed singularities among the different profiles, it seems clear the small influence of the studied treatments prior PLE compared to the use of this extraction technique alone.

3.4. Optimization of the extraction of phlorotannins from S. muticum

Once the use of the PLE process alone was confirmed as the most-suitable for the extraction of phlorotannins from the brown macroalga *S. muticum* samples, the search for the best extraction conditions was continued. To do that, an experimental design was devised in order to further optimize the PLE conditions to maximize the amount of recovered phlorotannins. To do that, a three-level factorial design was set-up using two factors, extraction temperature (50, 125 and 200 °C) and ethanol:water solvent mixture composition (0%, 50% and 100% ethanol). These parameters are well-known to be the most influencing parameters in PLE extractions (Herrero et al., 2013). The rest of extraction conditions (extraction time, 20 min, and extraction pressure, 1500 psi) were selected according to our previous experience. Four different response variables were selected: extraction yield, total phenols amount, antioxidant activity as well as total phlorotannins content. Results are summarized in Table 3. As can be seen, different behaviors can be observed for the diverse selected response variables. Extraction yield increased with temperature, as expected, due to a mass transfer rate enhancement. Higher extraction yields were obtained using 100% water as solvent for all tested temperatures, except for 200 °C in which 50% ethanol provided slightly higher yields. In any case, the use of 100% ethanol always provided the lowest extraction yields. However, this extracting solvent was the most favorable to maximize the total phenols and total phlorotannins recovery, as well as to provide extracts with higher antioxidant activity. Considering these results, it can be inferred that ethanol was more selective towards the extraction of active polyphenols (antioxidants), whereas water was efficient to extract more gross material.

In Fig. 2, the standardized Pareto charts for the four response variables studied are shown as well as their corresponding response surfaces. Different bar shadings indicate positive and negative effects of the factors in the response variables and the vertical line tests the significance of the effects at the 95% confidence level. As it can be observed, ethanol proportion in the solvent mixture was always the most important factor. Extraction temperature also had an important influence on the response variables except for antioxidant activity. Besides this information, analyzing the surface plots for each variable it is possible to deduce that the extraction conditions to maximize the different response variables were not the same. Response surface of those variables related to antioxidant activity, total phenols and total phlorotannins content were similar; values increased according to an increment on ethanol content and also, to a lesser extent, with temperature. On the other hand, extraction yield was maximized with the highest amount of water in the extraction solvent and with an increase of temperature. Thus, in order to consider the four response variables at the same time, a multiple response optimization was carried out giving higher weight in the optimization to total phenols, total phlorotannins and antioxidant activity over the extraction yield. This way, the selective extraction of the target compounds

Table 3
Experimental matrix design conditions (factor levels between parentheses) and results for each response variable studied for the optimization of the PLE of the brown macroalga *Sargassum muticum*. Values presented are mean ± sd.

Exp.	PLE extraction conditions		Extraction yield (%)	Total phenols content (mg GAE g^{-1})[1]	Total phlorotannins (mg PGE g^{-1})[2]	Antioxidant activity TEAC (mmol TE g^{-1})[3]
	Temp. (°C)	Ethanol%				
1	50 (−1)	0 (−1)	29.5	29.61 ± 1.32	1.153 ± 0.110	0.489 ± 0.096
2	50 (−1)	100 (1)	5.3	94.20 ± 4.01	4.946 ± 0.257	1.468 ± 0.282
3	50 (−1)	50 (0)	30.7	58.10 ± 0.17	2.243 ± 0.105	0.866 ± 0.012
4	125 (0)	0 (−1)	42.3	52.26 ± 0.80	2.524 ± 0.078	0.766 ± 0.024
5	125 (0)	100 (1)	9.8	93.84 ± 5.14	4.202 ± 0.315	1.070 ± 0.001
6	125 (0)	50 (0)	40.3	76.62 ± 6.90	3.841 ± 0.046	1.081 ± 0.076
7	125 (0)	50 (0)	41.9	79.61 ± 6.44	3.778 ± 0.646	1.219 ± 0.649
8	125 (0)	50 (0)	41.5	77.32 ± 5.60	3.672 ± 0.024	1.307 ± 0.048
9	200 (1)	0 (−1)	39.6	69.31 ± 6.16	2.766 ± 0.289	0.936 ± 0.011
10	200 (1)	100 (1)	20.2	93.16 ± 6.47	5.693 ± 0.161	1.329 ± 0.051
11	200 (1)	50 (0)	44.8	82.22 ± 6.37	3.988 ± 0.422	1.081 ± 0.028

[1] mg gallic acid equivalents g^{-1} extract.
[2] mg phloroglucinol dihydrate equivalents g^{-1} extract.
[3] mmol trolox equivalents g^{-1} extract.

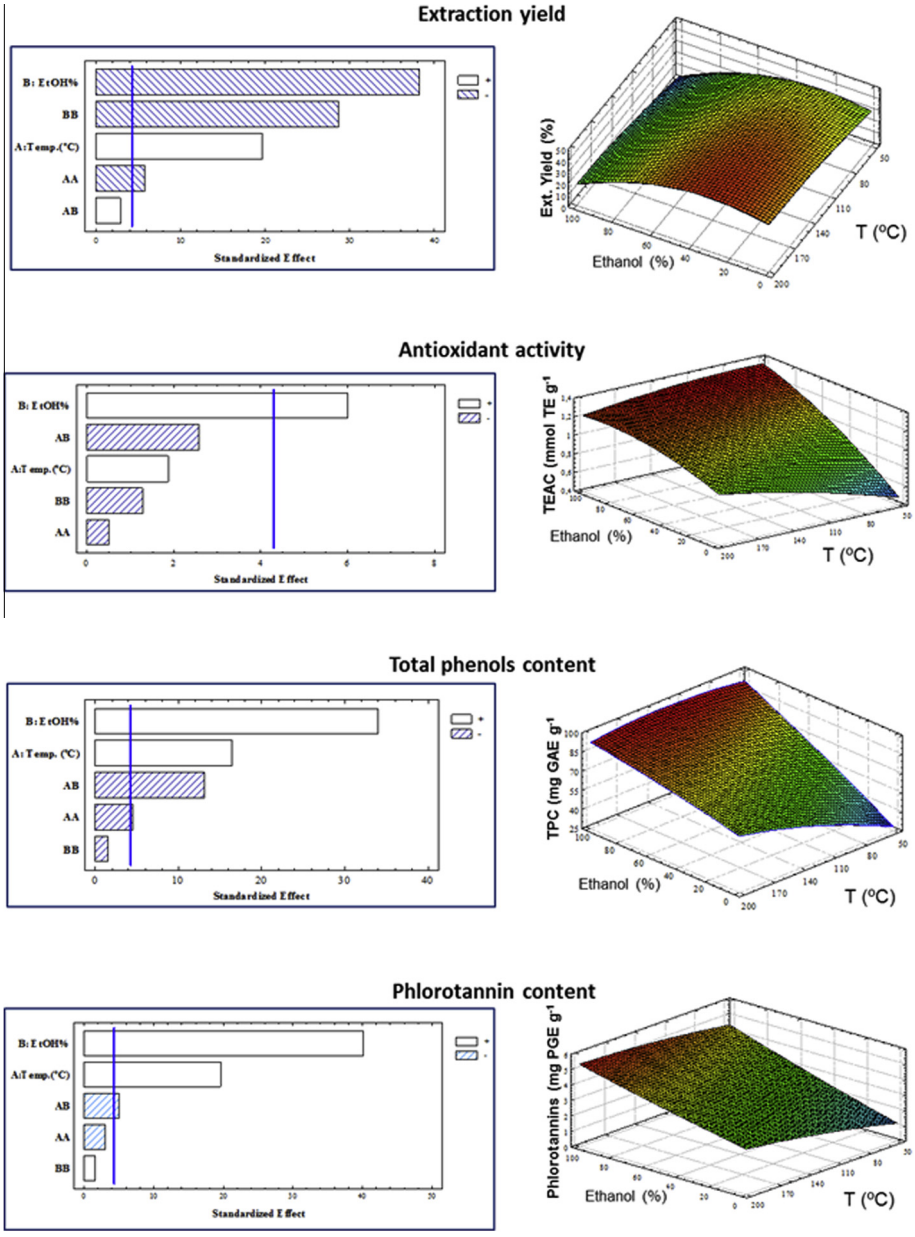

Fig. 2. Standardized Pareto charts for the four response variables studied in the experimental design (stripped and white bars show negative and positive effects, respectively), and their corresponding response surfaces.

(polyphenols and phlorotannins) is favored. The optimum global extraction conditions provided by the statistical model included the use of 160 °C as extraction temperature and 95% ethanol as pressurized solvent. Under these conditions, predicted values of 21.9%, 94.0 mg GAE g^{-1}, 5.018 mg PGE g^{-1} were obtained for extraction yield, total phenols and total phlorotannins contents, respectively, whereas the predicted TEAC value was 1.275 mmol TE g^{-1}. Moreover, an overall desirability value of 0.949 was obtained. Experimental values obtained in the optimum presented an average RDS% of 5.85%, 7.55%, 17.69% and, 4.24%, for the extraction yield%, TPC, phlorotannin content and TEAC, respectively, thus demonstrating the usefulness of the proposed model to assess the PLE process.

4. Conclusions

In the present work we have demonstrated that the application of PLE is a more straightforward way to obtain active phlorotannins from the brown macroalga *S. muticum*. Enzyme-assisted extraction process using either proteases or carbohydrases before PLE did not improve the attainable results in terms of total polyphenols and phlorotannins recoveries. Nevertheless, the use of carbohydrases (viscozyme) during 2 h combined with pressurized liquids allowed the attainment of slightly more antioxidant extracts compared to the use of PLE alone. However, the amount of total phenols obtained was lower. Thus, the use of longer processes combining both extraction mechanisms was not worth in this particular application. As a consequence, the further optimization of the phlorotannins extraction from *S. muticum* was performed using a single PLE process as extraction technique. After the application of an experimental design, it could be determined that the best extraction conditions to maximize the response variables selected (extraction yield, total phenols, total phlorotannins and antioxidant activity) included the use of 160 °C as temperature and 95% ethanol as pressurized solvent. Independently of these results, the use of combined EAE–PLE processes may be successful in other applications, although the study of more selective

enzymes directed to algal polysaccharides from the cell wall would be required to selectively release the target compounds.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.06.098.

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Table S1. Extraction yield (%), total phenols content (mg GAE g^{-1} extract) and antioxidant activity (mmol TE g^{-1} extract, TEAC assay) of the brown macroalga *Sargassum muticum* extracts recovered after EAE treatment and combined EAE-PLE process compared to controls (buffered hydrolysis solutions without enzymes). Values presented are mean ± sd.

Process	Extraction yield (%)	Total phenols content (mg GAE g^{-1})[1]	TEAC (mmol TE g^{-1})[2]
EAE			
Alcalase 2 h	13.6 ± 1.4	7.91 ± 0.65	0.262 ± 0.006
Alcalase hydrolysis solution	13.1 ± 0.4	4.13 ± 0.20	0.092 ± 0.001
Viscozyme 2 h	20.6 ± 1.7	10.33 ± 1.16	0.178 ± 0.005
Viscozyme hydrolysis solution	23.1 ± 0.2	9.15 ± 0.43	0.096 ± 0.004
EAE+PLE			
Alcalase 2 h	46.1 ± 5.6	27.17 ± 1.54	0.581 ± 0.045
Alcalase hydrolysis solution	34.2 ± 0.7	24.60 ± 0.98	0.387 ± 0.021
Viscozyme 2 h	29.6 ± 2.4	39.20 ± 3.55	0.687 ± 0.050
Viscozyme hydrolysis solution	19.5 ± 0.7	44.60 ± 2.80	0.703 ± 0.045

[1]mg Gallic Acid Equivalents g^{-1} extract; [2]mmol Trolox Equivalents g^{-1} extract.

4.2.3 Application of Hansen solubility approach for the subcritical and supercritical selective extraction of phlorotannins from *Cystoseira abies-marina*.

A. P. Sánchez-Camargo, L. Montero, A. Cifuentes, M. Herrero, & E. Ibáñez

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Application of Hansen solubility approach for the subcritical and supercritical selective extraction of phlorotannins from *Cystoseira abies-marina*†

A. P. Sánchez-Camargo, L. Montero, A. Cifuentes, M. Herrero and E. Ibáñez*

Cystoseira abies-marina is one of the most important brown algae species found in the Mediterranean Sea and Atlantic Ocean ecosystems and has been reported as a promising source of phenolic compounds, including phlorotannins, with important biological activities. In the present work, the possibility of developing new green processes to eliminate/replace the use of traditional polluting and often toxic solvents to obtain phlorotannin-enriched extracts is explored, looking for higher efficiency and compliance with the rules of green chemistry. The theoretical modelling of the Hansen solubility parameters could provide a useful and accurate estimation for the solvent selection and prediction of the solubility of these natural bioactive compounds. In order to drive the process towards the extraction of the target compounds, the chemical composition of phlorotannins from *Cystoseira abies-marina* was determined using a comprehensive two-dimensional liquid chromatography (LC × LC-MS/MS) method. Phlorethols, fucols or fucophlorethols containing from 3 to 14 phloroglucinol units (PGU) were tentatively identified. The estimation of Hansen solubility parameters of the most abundant phlorotannins (7 PGU) in some green solvents (ethanol, water, ethyl lactate and CO_2 + ethanol) under sub- and supercritical conditions was carried out to improve their selective extraction and is presented for the first time. According to the theoretical approach, low temperatures of the extraction solvents provide the minimum R_a (difference in solubility parameter). However, this work experimentally demonstrates that pure ethanol at 100 °C showed the highest selectivity to extract phlorotannins. The Hansen approach proves to be a profitable tool to select a suitable solvent for extraction purposes.

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1. Introduction

The interest in natural bioactive compounds from marine algae able to provide health benefits has intensified in the past few years in the area of functional foods/nutraceuticals.[1–4] This increase is due to the fact that seaweeds are a good supply of key nutrients including carbohydrates and protein as well as soluble dietary fibers, peptides, polyphenols, carotenoids and minerals, which are amongst the most promising compounds found in macroalgae.[5,6] *Cystoseira* is one of the most important species of brown algae found in the Atlantic Ocean and Mediterranean Sea ecosystems.[7] It has been described to have pharmacological potential since its extracts exhibit high and selective antioxidant,[8,9] anti-proliferative[10–12] and anti-inflammatory activities.[11,13] These interesting bioactive properties have mainly been related to the presence of phlorotannins, which are complex polymeric phenolic compounds based on phloroglucinol monomers, linked to each other by different bonds. Depending on the type of the bond present on their structure, phlorotannins can be classified in phlorethols and fuhalols (ether linkages), fucols (phenyl linkages), fucophlorethols (ether and phenyl linkages) and eckols (benzodioxin linkages).[14] In a recent work carried out in our laboratory, we have identified more than 50 different phlorotannins in a *C. abies-marina* brown algae extract employing comprehensive two-dimensional liquid chromatography (HILIC × RP-DAD-MS/MS).[15] Besides, employing this powerful analytical technique it was possible to identify fuhalols, hydroxyfuhalols and mainly phlorethols with different degree of polymerization from purified extracts of *Sargassum muticum* collected on the Norwegian coast. Furthermore, these extracts showed a prominent anti-proliferative activity against HT-29 adenocarcinoma colon cancer cells ($IC_{50} = 32.2 \pm 1.7$ μg mL^{-1}).[16]

Considering the interest of bioactive compounds from microalgae and seaweeds from a bioeconomical perspective, our research has been focused on the development of green integrated processes (*e.g.* enzyme assisted extraction (EAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE) and their combinations) to improve the efficiency of the extraction while

Laboratory of Foodomics, Bioactivity and Food Analysis Department, Institute of Food Science Research, CIAL (UAM-CSIC), C/Nicolás Cabrera 9, Campus UAM Cantoblanco, 28049 Madrid, Spain. E-mail: elena.ibanez@csic.es

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replacing toxic organic solvents with environmental-friendly solvents such as water, ethanol and/or CO_2.[16–21] Even though some improvements have been achieved, usually it becomes necessary to carry out some purification procedures after extraction (L–L and S–L extractions) to remove lipids, carbohydrates and proteins fractions and to obtain phlorotannins-enriched extracts.[11,15,16,22] In this sense, the complex interplay between thermodynamics (solubility) and kinetics (mass transfer) has to be understood to perform a selective extraction process.[23] Although several models to predict the mass transfer rates for sub- and supercritical extraction from different natural matrices have been developed and discussed in detail in several recent publications,[24–27] in the present work our approach was to use the solubility parameters as a design tool to select the most convenient solvents for extraction of bioactive ingredients, thus becoming an interesting alternative to achieve selective extractions.[28–34] The Hildebrand solubility parameter (δ) provides a numerical estimate of the degree of interaction between materials, and as a consequence, materials with similar δ values are likely to be miscible.[35] The division of the Hildebrand parameter into three components (dispersive, polar and hydrogen bonding forces) by Hansen in 1967, lastly named as Hansen solubility parameters (HSP), has been widely applied from academic labs to industrial applications to predict the solubility of polymers, bio-polymers, drugs, pigments, dyes, and some biological materials in different types of solvents.[35] The estimation of the individual Hansen parameters depends greatly upon the availability of data. In cases where there is no possibility to experimentally obtain the solubility parameters by indirect measurements (*i.e.* solvency testing, osmotic pressure, turbidity, specific volume or intrinsic viscosity), the group contribution methods (GCM) is a good approximation to predict physicochemical properties and solubility parameters from molecular structures using additive rules. For the estimation of properties of pure compounds,[36] GMC such as those given by Lydersen,[37] Klincewicz and Reid[38] and Joback and Reid,[39] among others, have been widely used with limited applicability. However, some newer methods have introduced even second-order and third-order groups to improve the predictive capability for representing complex molecules.[40,41] On the other hand, following the same Hansen approach, Hoftyzer and Van Krevelen, Small, Fedors and Hoy have developed some group contribution techniques to estimate the solubility parameters for a wide variety of compounds.[35,42] Although some of these predictions are only valid at normal conditions, some methods have been developed to correct the pressure and temperature effect for supercritical and subcritical conditions.[43,44] Several theoretical studies of the Hansen solubility parameters stated that under subcritical conditions, solubility parameters for water and ethanol were mainly dependent on the temperature since the pressure exerted only a minor influence on the total solubility parameter below its critical point.[29,45] Nevertheless, at very high pressures under near-critical and supercritical conditions, the solubility parameter of water and other solvents, including CO_2, showed a rise with increasing pressure and a drop with decreasing temperature.[43,46] Some recent research works employing these solvent conditions for green processes have estimated the solubility parameter of some natural bioactive compounds. For instance, Srinivas *et al.*[29] applied the Hansen solubility parameters approximation for the betulin–ethanol and betulin–water systems under subcritical conditions. In that work it could be verified that ethanol is a better solvent for betulin, being in agreement with the experimental data reported. A similar approach was used in predicting the extraction conditions of target solutes form natural matrices: silymarins from milk thistle, vitamins B from Brewer's yeast and anthocyanins from grape pomace.[29] Also, the estimation of solubility parameters of some carotenoids (present in some selected spices) such as lutein, β-carotene, violaxanthin, zeaxanthin and curcumin in supercritical carbon dioxide (SC-CO_2) at different pressure (10–100 MPa) and temperature (25–75 °C) were advantageous to optimize their extraction.[47] Furthermore, the increment of the solvent power of SC-CO_2 with an addition of co-solvents and the prediction of their solubility parameters have resulted in successful selective extractions of caffeine from green tea.[31] Regarding the marine algal polyphenols, theoretical information about the solubility parameters in green solvents has not been described yet. Therefore, the objective of the present work was the estimation of Hansen solubility parameters of the most abundant phlorotannins found in the brown algae *Cystoseira abies-marina* in green subcritical and supercritical solvents in order to devise and optimize a new extraction and purification protocol for their selective isolation.

2. Materials and methods

2.1 Sample and chemicals

Thalli of *Cystoseira abies-marina* (S. Gmelin) C. Agardh were provided in April, 2012 by The Spanish Bank of Algae from Las Palmas, Gran Canarias. After collection, dry seaweeds were ground using a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at low temperature (10 °C) employing small rocks of dry ice for this purpose. The particle size was determined by sieving the ground material to appropriate size (between 999 and 500 μm). Then, the whole sample was vacuum-packed and stored at 4 °C until its use.

Phloroglucinol, acetic acid, formic acid, 2,4-dimethoxybenzaldehyde (DMBA) were purchased from Sigma-Aldrich (Madrid, Spain). Hydrochloric acid was obtained from Probus (Barcelona, Spain), whereas dichloromethane was acquired from Fluka AG (Buchs, Switzerland) and ethyl acetate from Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA). Acetonitrile, ethanol, methanol and acetone employed were HPLC-grade and were acquired from VWR Chemicals (Barcelona, Spain).

2.2 Extraction equipment and procedures

Initially, in order to obtain the maximum quantity of phlorotannins to be purified and subsequently characterized by comprehensive two-dimensional liquid chromatography (LC × LC-MS/MS), dry algae were submitted to pressurized liquid extraction (PLE). PLE conditions were selected according to previous research works developed in our laboratory for other

brown algae species.[15–17] A mixture of acetone/water (70 : 30, v/v) at 100 and 160 °C was tested at 10.3 MPa for 20 min. The extraction procedure was carried out employing an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. For each extraction, an 11 mL stainless steel extraction cell was employed to load 1 g of sea sand, followed by 1 g of dried brown alga mixed with the same quantity of sea sand.

For comparison purposes, a conventional solid–liquid extraction was carried out using acetone/water (70 : 30, v/v) at 25 °C. Briefly, 3 g of dry algae were mixed with 30 mL of this solvent mixture employing a magnetic stirring during 45 min in the darkness. The supernatant was centrifuged, the remaining residue was extracted 3 more times with 10 mL of solvent mixture and finally after the extractions the supernatants were pooled. Acetone was removed using rotary evaporation and aqueous crude extracts were freeze-dried to determine the extraction yield (gravimetric method, defined as g extract per 100 g dry algae). All of the assays were carried out by triplicate.

2.3 Phlorotannins purification procedure

In order to obtain concentrated phlorotannin extracts, a liquid–liquid solvent purification protocol reported by Stiger-Pouvreau *et al.*[22] was employed. The dry crude extract was re-diluted in a given volume of water and then, dichloromethane (1 : 1, v/v), acetone (3 : 1, v/v) and ethanol (3 : 1, v/v) were used to remove consecutively lipid, protein and carbohydrates fractions present in the crude extracts. Finally, phlorotannins were extracted from the water fraction with ethyl acetate (1 : 1, v/v). After, the extract was dried using N_2.

2.4 Total phlorotannin content determination

The total phlorotannin content in the crude algae extracts and purified extracts (phlorotannins fraction) were quantified using the DMBA (dimethoxybenzaldehyde) colorimetric assay following the method described by Lopes *et al.*[13] The working reagent was prepared mixing equal volumes of hydrochloric acid (6%, v/v) and DMBA (2%, m/v) dissolved in glacial acetic acid. Then, 50 μL of each extract (2.0–5.0 mg mL^{-1}) or phloroglucinol standard (0.98–62.5 μg mL^{-1}) were mixed with 250 μL of the working reagent in a 96-wells plate for 60 min, at room temperature in darkness. After the reaction time, the absorbance was measured at 515 nm in a microplate spectrophotometer reader Powerwave XS (Bio Tek Instruments, Winooski, VT). Water was used as blank and control samples without DMBA solution were also included. A calibration curve using phloroglucinol was employed to estimate of total phlorotannins content. Data were presented as the average of triplicate analyses expressed as milligram phloroglucinol equivalents (PGE) per gram of dry extract. All blanks, samples, and controls were prepared in triplicate.

2.5 Characterization of phlorotannins by comprehensive two-dimensional liquid chromatography (LC × LC-DAD-MS/MS)

In order to carry out the chemical characterization of the purified phlorotannin fraction, a comprehensive two-dimensional liquid chromatography method (LC × LC) previously developed in our laboratory[15] (with some modifications) was employed. The sample was diluted at a concentration of 45 mg mL^{-1} in MeOH/ACN (3 : 7, v/v) and filtered through 0.45 μm nylon syringe filters (Análisis vínicos, Tomelloso, Spain) before injection. The separation was developed employing a HILIC column (Lichrospher diol-5 150 × 1.0 mm, 5 μm particle diameter, HiChrom, Reading, UK) with a precolumn with the same stationary phase, for the first dimension (^{1}D). A short partially porous C_{18} reversed phase column (50 × 4.6 mm, 2.7 μm particle diameter, Supelco, Bellefonte, CA) with a C_{18} precolumn was used for the second dimension (^{2}D). In the ^{1}D, the flow rate and the injection volume employed were 15 μL min^{-1} and 20 μL, respectively. The mobile phases were (A) ACN/acetic acid (98 : 2, v/v) and (B) methanol/water/acetic acid (95 : 3 : 2, v/v/v) eluted according to the following gradient: 0 min, 3% B; 3 min, 10% B; 5 min, 15% B; 30 min, 35% B; 70 min, 35% B. A 2-position 10-port switching valve acted as modulator or interface between the two dimensions, with a modulation time of 1.3 min. The interface was equipped with two identical 30 μL injection loops. For the ^{2}D, repetitive 1.3 min analysis were used, employing as mobile phases water (0.1% formic acid, (A) and ACN (B) eluted according to the following gradient: 0 min, 0% B; 0.1 min, 5% B; 0.3 min, 20% B; 0.8 min, 40% B; 0.9 min, 70% B; 1 min, 90%; 1.01 min, 0% B). The ^{2}D flow rate employed during the whole LC × LC analysis was 3 mL min^{-1}. The flow eluting from the ^{2}D column was splitted before entering the MS instrument, so that the flow rate introduced in the MS detector was 600 μL min^{-1}. The wavelength used to monitor the separations was 280 nm, although UV-Vis spectra were collected from 190 to 550 nm during the whole analysis using a sampling rate of 20 Hz in the DAD. The MS was operated under negative ESI mode and employing the following conditions: dry temperature, 350 °C; mass range, m/z 90–2200 Da; dry gas flow rate, 12 L min^{-1}; nebulization pressure, 40 psi. LC data were elaborated and visualized in two and three dimensions using LC Image software (version 1.0, Zoex, Houston, TX, USA).

3. Solubility parameter estimation: modelling

Once the phlorotannins profile present in the purified extract was identified, the Hansen solubility parameters of the most abundant phlorotannins in different green solvents were estimated. The basic equation governing the assignment of Hansen parameters is the total cohesion energy, E, which is defined by the contribution of three energies: E_D, dispersion energy (related to the van der Waals forces), E_P, polarity energy (related to dipole moment), and E_H, hydrogen bonding energy. Dividing this by the molar volume (V) gives the square of the total (δ_T^2) solubility parameter as the sum of the squares of the Hansen (D, P, and H components) as defined by eqn (1) and (2):[35]

$$\frac{E_T}{V} = \frac{E_D}{V} + \frac{E_P}{V} + \frac{E_H}{V} \quad (1)$$

$$\delta_T \ \left(\mathrm{MPa}^{1/2}\right) = \sqrt{{\delta_D}^2 + {\delta_P}^2 + {\delta_H}^2} \quad (2)$$

The Hansen three-dimensional solubility parameter model considers that the mutual solubility parameter between a solute i and a solvent j, called R_a "distance", is based on their respective partial solubility parameter components, as follow:[35]

$$R_a = \sqrt{4\left(\delta_{Di} - \delta_{Dj}\right)^2 + \left(\delta_{Pi} - \delta_{Pj}\right)^2 + \left(\delta_{Hi} - \delta_{Hj}\right)^2} \quad (3)$$

The particular region where solvent–solute combinations occur as a solution is named the "solubility sphere". Through trial and error, solvents tested are plotted in Hansen space to create the solubility sphere with the radius of the sphere indicated, which is known as the "interaction radius" and denoted as R_0. Thus, the ratio R_a/R_0 has been called the Relative Energy Difference (RED): if RED = 0, no energy difference is found meaning a "perfect solvent"; if RED < 1.0 indicates high affinity, if RED = 1.0 indicates boundary condition; and if RED > 1.0 indicate low affinity. However, R_0 is based only on experimental data of the observation of the interaction between studied solutes and well-known solvents and therefore it can only be used when solubility experiments can be carried out.[35]

On the other hand, in order to help visualizing the three parameters on a plane, Teas[48] developed a triangular plotting technique in which the individual Hansen parameters were normalized by the sum of the three parameters, as defined by eqn (4) to (6). In each side of the triangle, the contribution of each parameter is located; the sum of these three fractional parameters should be 1.0. Teas plot can be a suitable tool to study the solubility behavior of a specific compound in untested solvents by determining the solvent's position with respect to the solute's position in Teas plot.

$$F_D = \frac{\delta_D}{\delta_D + \delta_P + \delta_H} \quad (4)$$

$$F_P = \frac{\delta_P}{\delta_D + \delta_P + \delta_H} \quad (5)$$

$$F_H = \frac{\delta_H}{\delta_D + \delta_P + \delta_H} \quad (6)$$

3.1 Solubility parameter of the solute

For the solutes studied in this work (phlorotannins), there are no previous theoretical or experimental data in the literature about their physical properties or solubility parameters. Moreover, standard compounds are not available. In this sense, Marrero & Gani[41] group contribution method was used for the estimation of critical data (third-order group) and the Yamamoto-molecular break method using its Simplified Molecular Input Line Entry Syntax (SMILES) (HSPiP Version 5.0, Denmark) was employed for the evaluation of the molar volume. For the partial solubility parameters of the solutes (dispersion, polarity and hydrogen bonding), the Hansen[35] approach was chosen to estimate them at room temperature. The total solubility parameters were calculated according to eqn (2). On the other hand, to evaluate the temperature dependence of the solute solubility parameter (since pressure does no exert a large influence on the properties of solid), the Jayasri and Yaseen[44] method was employed (eqn (7)), where T_r means the reduced temperature at room temperature (1) at a given sub- or supercritical temperature (2).

$$\delta_2 = \delta_1 \left(\frac{1 - T_{r2}}{1 - T_{r1}}\right)^{0.34} \quad (7)$$

3.2 Solubility parameter of the solvents

The green solvents selected to carry out the modelling and the solubility parameter estimation were: (a) ethanol, (b) water, (c) ethyl lactate, (d) CO_2 + ethanol. Water, ethanol and ethyl lactate, were evaluated at subcritical conditions considering temperatures equal to 25, 100 and 150 °C and keeping the pressure constant at 1.0 MPa. For supercritical conditions, mixtures of CO_2 + ethanol (70 : 30, 60 : 40, and 50 : 50%, w/w), at 10, 20, 30 MPa were studied, maintaining the temperature constant at 40 °C.

The physical properties of the subcritical solvents were taken and calculated following the Gunn–Yamada method as described by Pereira *et al.*[45] Their three-dimensional solubility parameters at room temperature were obtained from Hansen.[35] For supercritical conditions, the solvent densities at the specified pressure and temperature were obtained from NIST REFPROP database 2015.[49] The effect of pressure and temperature of the sub- and supercritical solvents on their solubility parameters were estimated employing the approach provided by Williams *et al.*,[43] as described in eqn (8)–(10):

$$\delta_D = \delta_{D,ref} \left(\frac{V_{ref}}{V}\right)^{1.25} \quad (8)$$

$$\delta_P = \delta_{P,ref} \left(\frac{V_{ref}}{V}\right)^{0.5} \quad (9)$$

$$\delta_H = \delta_{H,ref} \left[e^{\left(-1.32\times10^{-3}\left(T_{ref} - T\right) - \left(\frac{V_{ref}}{V}\right)^{0.5} \right)} \right]^{-1} \quad (10)$$

where the subscript ref means the relative property at room temperature (25 °C), V is the molar volume and T the sub or supercritical temperature. For a mixture consisting of supercritical CO_2 (SC-CO_2) and ethanol (co-solvent), the solubility parameters for the mixed fluid, was determined as follow:

$$\delta_{Mix\text{-}D,P,H} = \Phi_{SC\text{-}CO_2} \times \delta_{SC\text{-}CO_2\text{-}D,P,H} + \Phi_{Ethanol} \times \delta_{Ethanol\text{-}D,P,H} \quad (11)$$

where Φ is the volume fraction of SC-CO_2 and ethanol.

4. Results and discussion

4.1 Extraction of polyphenols from brown algae

As mentioned, marine algae have been considered as the "plant-based food of the future"[5] because of their composition in

nutrients and micronutrients.[19,50] A typical class of polyphenols exclusive from brown seaweeds are phlorotannins. These compounds are derived from polymerized phloroglucinol units (1,3,5-trihydroxybenzene).[51,52] Due to the relevant biological activities attributed to phlorotannins, in the last few years we have been developing different green extractions techniques for their extraction/isolation. For instance, PLE[16,17,53,54] is among the most promising processes to carry out this purpose. Taking into account that the solvent most-commonly used to extract phlorotannins has been aqueous mixtures of acetone,[55–57] in the present study, 3 different extraction conditions have been selected in order to obtain phlorotannins-enriched extracts to determine its chemical composition; the knowledge of their exact composition is the first step towards their selective isolation by using the Hansen solubility parameters approach.

Fig. 1 shows the results of the extraction yield (g extract per 100 g dry algae) and the total phlorotannin content (mg phloroglucinol equivalent (PGE) per g extract) (on the left and right axes, respectively) obtained under the different extraction conditions tested. Regarding solid–liquid extraction, the extraction yield, as well as the phlorotannin content, was similar to those obtained by PLE at 100 °C. A further increase of temperature up to 160 °C leads to an increase on the extraction yield from 27.0 to 41.9% (w/w); however, under those conditions the phlorotannins extraction decreases 48%, probably due to their degradation or to a change in their solubility. Taking into account these results, only PLE extract obtained at 100 °C was selected to carry out the purification procedure due to the reduced use of solvents and less time required for their extraction.

4.2 Isolation of phlorotannins from algae crude extracts

As already commented, brown algae contain a wide variety of compounds including lipids, proteins and carbohydrates,[6] which are present in significant amounts in the extracts. Therefore, after obtaining the extract, liquid/liquid purification is required to remove these fractions and to obtain phlorotannins-enriched extracts. These fractions were gravimetrically quantified and the results showed that dichloromethane, acetone, ethanolic and aqueous fractions represented 10.8 ± 0.5, 6.7 ± 0.3, 1.5 ± 0.1 and 74.6 ± 3.7% (w/w) of the crude extract, respectively. Regarding to the phlorotannin purified fraction (corresponding to the ethyl acetate (EA) fraction), a purification yield of 6.4 ± 0.3% was obtained. The phlorotannins content in this purified fraction was 20.5 ± 1.6 mg PGE per g, increasing the concentration of the phlorotannins 3-fold, related to the crude extract.

4.3 Chemical characterization of phlorotannins by LC × LC-ESI-MS/MS

Despite the DMBA colorimetric method is used to provide a general estimation of the phlorotannin content found in the extract, this assay is not able to provide any information about the chemical structure of these compounds. As already mentioned, from the chemical and structural point of view, phlorotannins are complex polymeric phenolic compounds of phloroglucinol units, linked to each other by different bonds. As the number of phloroglucinol units becomes bigger, the complexity of their structure and diversity greatly increases.[58] This level of complexity makes the identification and chemical characterization of phlorotannins a difficult challenge. In this sense, different analytical techniques have been employed to separate and identify phlorotannins from different brown algae, such as HPLC-DAD-MS,[55,57,59] UHPLC-DAD-MS,[54,60] or even comprehensive two dimensional liquid chromatography coupled to MS (LC × LC-MS/MS). This technique has been successfully employed to separate phlorotannins with different chemical structure and degree of polymerization from *Cystoseira abies-marina* and *Sargassum muticum* brown algae.[15,16] In this work, a HILIC separation in the first dimension (^{1}D) was on-

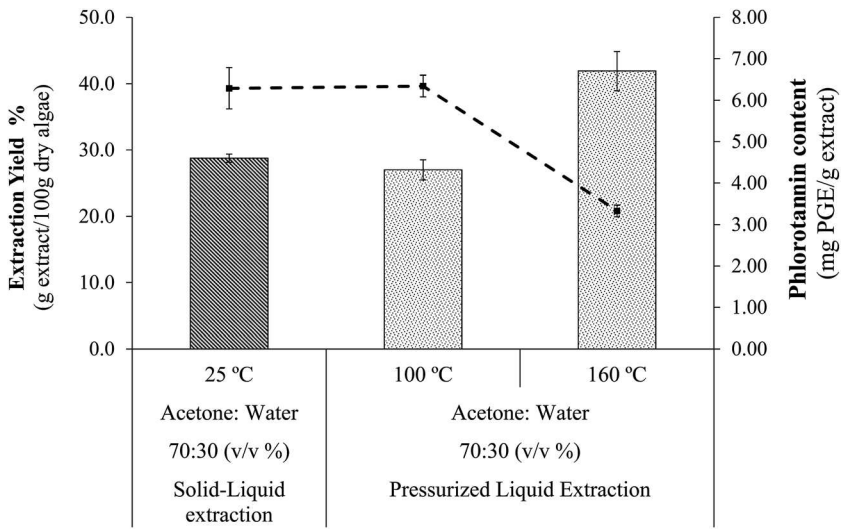

Fig. 1 Extraction yield% and phlorotannin content of *C. abies-marina* extracts obtained by solid–liquid extraction and PLE before purification procedure.

line coupled to a RP-based separation in the second dimension (^{2}D). In HILIC mode, phlorotannins are eluted according to the increased degree of polymerization (DP); therefore, phlorotannins with lower DP elute at the beginning of the analysis, whereas phlorotannins with higher DP are more retained and are eluted progressively with the increase of the aqueous mobile phase in the gradient. On the other hand, the RP-based ^{2}D allows the separation of different phlorotannins with the same DP in agreement to their relative hydrophobicity.

As it can be observed in Fig. 2, with the HILIC × RP-MS/MS method developed in this work, 35 compounds present in the purified extract of *Cystoseira abies-marina* were separated. Twenty-nine compounds were tentatively identified as phlorotannins with a DP from 3 to 14 phloroglucinol units (PGU). In Table SI-1(ESI†), a list with the separated peaks including retention times, $[M - H]^-$, main MS/MS fragments and their tentative identification is presented. Thereby, the smaller phlorotannins detected were trimers, detected at *m*/*z* 375.6 (peak 1) with typical fragments at *m*/*z* 247 (loss of 126 Da, corresponding to a phloroglucinol unit) and *m*/*z* 125 (loss of two phloroglucinol units). Then, phlorotannins with a growing DP were detected up to 14 DP (peak 35) which presented a double charged ion $[M - 2H]^{2-}$ at *m*/*z* 869.1.

Besides, in Table SI-1,† the relative peak area of each compound is shown. Clearly, the most abundant compounds in the *Cystoseira* extract are phlorotannins with 7 PGU presenting a relative area of 17.53 and 39.52% (peaks 15 and 16, respectively), followed by phlorotannins of 5 DP (peak 7 showing a relative peak area of 8.03%). These data can be also visualized in the 2D plot, where the most abundant compounds correspond with the most intense plots (peaks 7, 15 and 16).

As mentioned in the introduction, phlorotannins can be classified according to the link between the phloroglucinol units. In particular there are three phlorotannins types that are structurally very similar, namely phlorethols (phlorotannins with ether bound), fucols (phenyl linkages) or fucophlorethols (ether and phenyl linkages). Unfortunately, the differentiation between these three groups could not be established with the available analytical tools, mainly due to the fact that all of them have the same molecular weight and similar fragmentation pathway. For this reason we considered that phlorotannins detected in *Cystoseira abies-marina* could belong to the three groups, for instance the main compound present in this extract (phlorotannin with 7 PGU) could be heptaphlorethol, heptafucol or heptafucophlorethol. Due to the impossibility of knowing the exact molecular structure present in the purified extract, we selected heptaphlorethol and heptafucol as models for the estimation of Hansen solubility parameters because they include both type of bounds (ether and phenyl) that could be found in *Cystoseira*'s phlorotannins.

4.4 Solubility parameters estimation

As mentioned, the Hansen solubility parameters provide a numerical estimate of the degree of interaction between materials, and can be a good indication of their solubility. The estimation of solubility should be taken into account for the selection of the critical solvent extraction since this theoretical approach is useful as a first approximation, avoiding time-consuming experimental procedures. Recent works have successfully used the solubility parameters approach for the estimation of biocompounds such as amino acids, acetamides, fatty acids, pigments, pesticides, polyphenols and volatile aroma compounds, among others, in alternative solvents like vegetable oils and CO_2.[28,29,61–63] In the present work, once identified the most abundant compound present in the enriched-phlorotannin extract, heptaphlorethol or heptafucol (shown in Fig. 3(a) and (b), respectively), these structures were selected as solute molecular models to estimate their solubility parameters in different green solvents. In this regard, SMILES notation is a very useful tool for entering and representing molecules and reactions, which allows representing a string from a 2D or 3D chemical drawing, precise enough to be a unique identifier. It is essentially a language which employs characters-encoding based on ASCII (American Standard Code for Information Interchange). By entering the SMILES values of the target

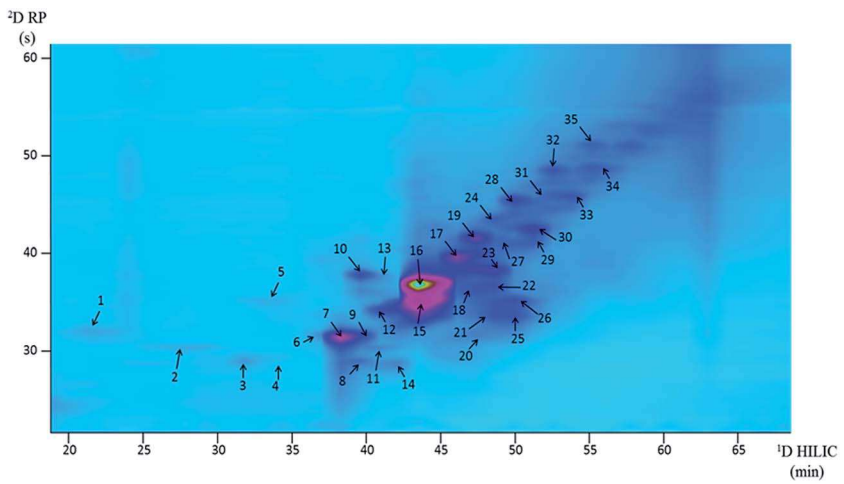

Fig. 2 2D plot (280 nm) of the *C. abies-marina* purified extracts using the HILIC × RP-MS/MS method. DP: degree of polymerization.

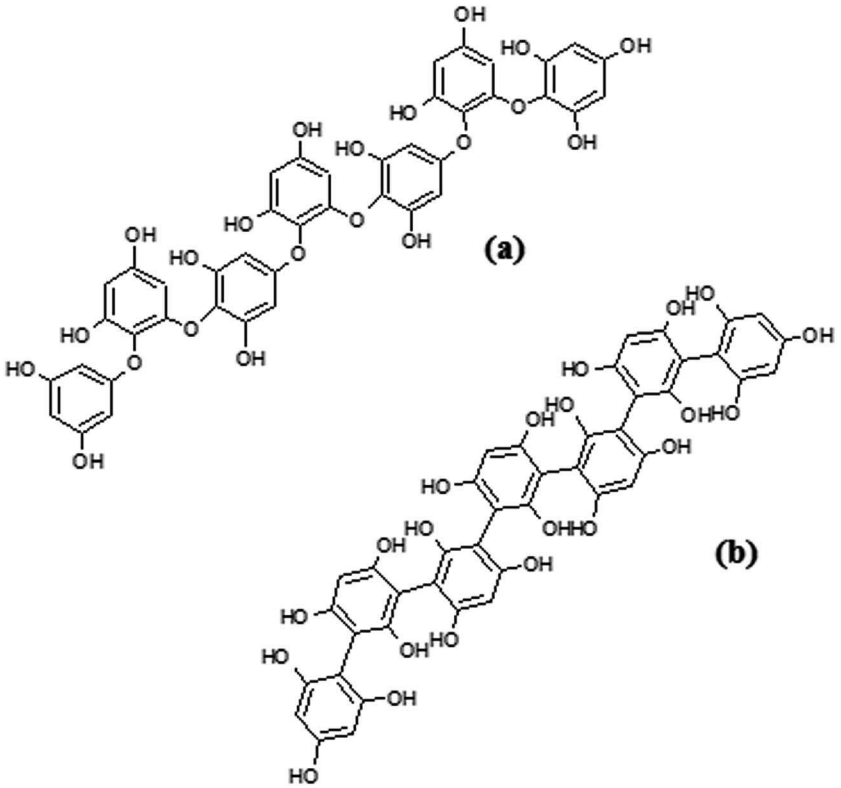

Fig. 3 The structures of phlorotannins, (a) heptaphlorethol and (b) heptafucol.

molecular in HSPiP software, the Y-MB method provides a whole range of predicted values. Following this approach, selected phlorotannins chemical structures were transformed to their SMILE notation (heptaphlorethol, Oc\7c\c\(O)c\(Oc\6c\c\(O)c\c\(O)c\6(Oc\5c\c\(O)c\(Oc\4c\c\(O)c\c\(O)c\4(Oc\3c\c\(O)c\(Oc\2c\c\(O)c\c\(O)c\2(Oc\1c\c\(O)c\c\(O)c\1)c\(O)c\3))c\(O)c\5))c\(O)c\7 and heptafucol, Oc\1c\c\(O)c\(c\(O)c\1)c\2c\(O)c\c\(O)c\(c\2(O))c\3c\(O)c\c\(O)c\(c\3(O))c\4c\(O)c\c\(O)c\(c\4(O))c\5c\(O)c\c\(O)c\(c\5(O))c\6c\(O)c\c\(O)c\(c\6(O))c\7c\(O)c\c\(O)c\c\7(O)) in order to calculate their cohesion energy (E) using the group contribution method and their molar volume using Yamamoto-Molecular Break (Y-MB). Results on the calculation of E in terms of dispersion, polar and hydrogen bonding energies for both solutes are shown in Table 1. Furthermore, using the Hansen group contribution method, it was possible to calculate the solubility parameters of heptaphlorethol and heptafucol. Results of HSP for both solutes and the different green solvents tested are given in Table 2.

As can be seen, similar results for the estimation of the solubility parameters for heptaphlorethol and heptafucol structures were obtained. In addition, it is important to remark that their HSP values varied only marginally with the temperature. The total solubility parameter is highly influenced by dispersion (δ_D) and hydrogen-bonding forces (δ_H), as was expected due to presence mainly of benzene ring and, at least, two or three hydroxyl group in each one. In the estimation of solubility parameters of solvents, considering that pressure exerts only a minor influence on the total solubility parameter below its critical point; only values at 1.0 MPa are given. On the other hand, under subcritical conditions, an increase in the temperature leads to a decrease in the partial solubility parameters and therefore, in the total solubility parameter. For ethanol and ethyl lactate, the polar interaction (δ_P) remains almost constant with the increase in temperature, whilst the hydrogen-bonding and dispersive interaction showed more influence. On the other hand, the decrease in the solubility parameter components of water with increasing temperature is mainly due to a reduction in hydrogen-bonding parameter. Under supercritical conditions, the solubility parameter of CO_2 raised with increasing of pressure at constant temperature, due to a density effect.

One interesting and clearer way to get a better idea about the optimal solvents to be selected for the selective extraction is by calculating the difference in the solubility between the subcritical and supercritical solvent and the solutes, as defined by the eqn (3) (R_a distance). Fig. 4(a) and (b) shows the plotted R_a values for the conditions described in Table 2. The smaller the R_a, the better is the solvent for the solute. For ethyl lactate and ethanol, an increase in the extraction temperature will induce drastically a decrease in the solubility of heptaphlorethol and heptafucol. Interestingly, low temperatures improve their solubility in these solvents, implying that are "good solvents". An opposing behavior is shown by water, where an increase in the temperature slightly decreases the R_a value, for both solutes. Water at 150 °C seems to be more efficient for extracting such polar compounds from natural matrices when compared to ethanol and ethyl lactate at the same temperature. However,

Table 1 Calculation of the cohesion energy (E) of heptaphlorethol and heptafucol using the group contribution method

Phlorotannin	Functional groups	Number of functional groups	Dispersion energy E_D (cal mol^{-1})	Polar energy E_P (cal mol^{-1})	Hydrogen bonding energy E_H (cal mol^{-1})
Heptaphlorethol	Phenyl (trisubstituted)	1	7530	50	50
	Phenyl (tetrasubstituted)	6	7530	50	50
	–O– ether	6	0	500	450
	OH– (aromatic)	15	1870	800	4650
Heptafucol	Phenyl (tetrasubstituted)	2	7530	50	50
	Phenyl (pentasubstituted)	5	7530	50	50
	OH– (aromatic)	21	1870	800	4650

Table 2 Hansen solubility parameters consisting of dispersive interactions (δ_D), polar interactions (δ_P), and hydrogen bonds (δ_T) for solutes and green solvents selected

Compound/solvent	Molar volume (cm^3 mol^{-1})	Temperature (°C)	Pressure (MPa)	δ_D (MPa$^{1/2}$)	δ_P (MPa$^{1/2}$)	δ_H (MPa$^{1/2}$)	δ_T (MPa$^{1/2}$)
Solutes selected							
Heptaphlorethol	521.6	25	1.0	25.45	11.10	24.17	36.81
		40	1.0	25.32	11.04	24.04	36.61
		100	1.0	24.77	10.80	23.51	35.82
		150	1.0	24.29	10.59	23.06	35.12
Heptafucol	488.2	25	1.0	28.08	12.12	28.98	42.13
		40	1.0	27.94	12.06	28.84	41.92
		100	1.0	27.36	11.81	28.24	41.06
		150	1.0	26.86	11.60	27.73	40.31
Subcritical solvents							
Water	18.05	25	1.0	15.50	16.00	42.30	47.81
	20.08	100	1.0	13.56	15.17	36.32	41.63
	21.68	150	1.0	12.32	14.60	32.72	37.89
Ethanol	58.17	25	1.0	15.50	8.80	19.40	26.52
	69.81	100	1.0	12.34	8.03	16.04	21.91
	80.53	150	1.0	10.32	7.48	13.98	19.03
Ethyl lactate	113.99	25	1.0	16.00	7.60	12.50	21.68
	134.39	100	1.0	13.02	7.00	10.43	18.09
	151.18	150	1.0	11.24	6.60	9.20	15.96
Supercritical conditions							
SC-CO_2	70.00	40	10.0	7.50	3.89	4.34	9.50
	52.39	40	20.0	10.77	4.50	4.92	12.67
	50.55	40	30.0	11.27	4.58	4.14	12.85
Ethanol[a]	60.22	40	1.0	14.84	8.65	18.69	25.56
SC-CO_2 + ethanol[a] (70 : 30 v/v)	NC	40	10.0	9.70	5.32	8.65	14.04
	NC	40	20.0	11.99	5.75	9.05	16.08
	NC	40	30.0	12.34	5.80	8.51	16.07
SC-CO_2 + ethanol[a] (60 : 40 v/v)	NC	40	10	10.55	5.79	10.08	15.62
	NC	40	20	12.51	6.16	10.43	17.33
	NC	40	30	12.81	6.21	9.96	17.29
SC-CO_2 + ethanol[a] (50 : 50 v/v)	NC	40	10.0	11.17	6.27	11.52	17.22
	NC	40	20.0	12.81	6.58	11.81	18.62
	NC	40	30.0	13.06	6.62	11.42	18.56

[a] Data taken using ethanol under subcritical conditions. NC: not calculated.

when ethanol, water and ethyl lactate are used at 100 °C to extract heptaphlorethol very close values were found. A fact which is often noted in practice is that usually higher temperatures increase the rate of solubility/diffusion/permeation. However, in terms of solubility parameters, δ_D, δ_P, and δ_H decrease with an increase in temperature, meaning that solvents such as alcohols, phenols, glycols, and glycol ethers become better solvents for compounds of lower solubility parameters (contrary to what is observed for phlorotannins). Thus, increasing the temperature can cause a non-solvent to become a "good solvent". Also, it is possible that a boundary solvent can be a good solvent at a given temperature, but become bad solvent with either an increase or a decrease in temperature.[35] In any case, pure ethanol at low temperature

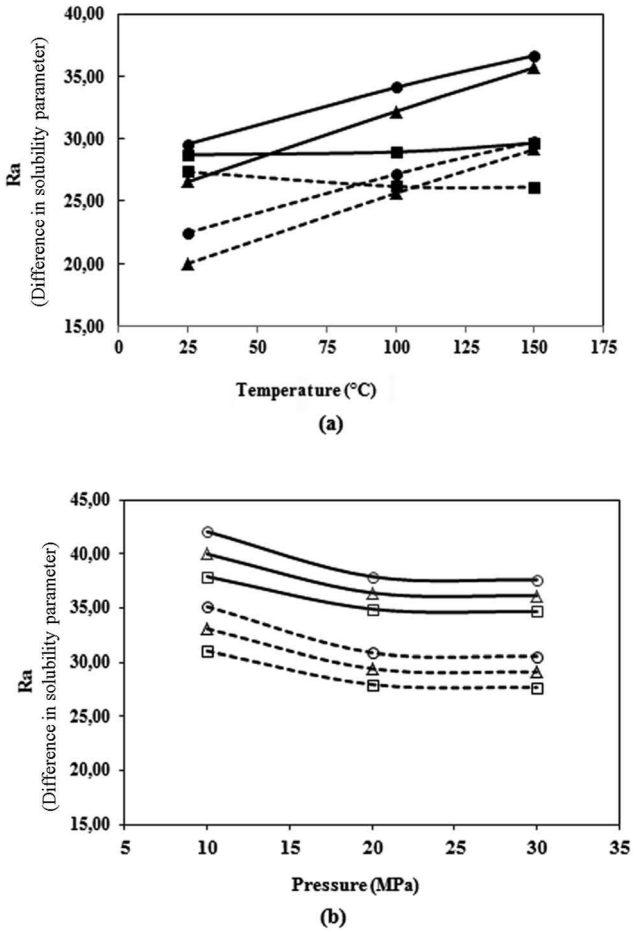

Fig. 4 Influence of the temperature and pressure in R_a value for heptaphlorethol and heptafucol and different (a) subcritical solvents at 1.0 MPa and (b) supercritical solvents at 40 °C. (●) Ethyl lactate, (▲) ethanol, (■) water, (○) CO_2 + EtOH (70 : 30 v/v), (△) CO_2 + EtOH (60 : 40 v/v), (□) CO_2 + EtOH (50 : 50 v/v). Dashed line (-·-), heptaphlorethol; continuous line (---), heptafucol.

(25 °C) is the most suitable solvent for the model solutes because it provides the smallest difference between the solubility parameters of both solutes and the solvent, compared to the other green solvents. These results are in accordance to the experimental results described in a previous work with the brown algae *Sargassum muticum*, where the highest phlorotannin extraction selectivity was observed by employing subcritical pure ethanol (50 °C, 10.3 MPa).[17]

On the other hand, Fig. 4(b) shows the R_a values for the mixture CO_2 + ethanol under supercritical conditions; as can be seen, increasing the percentage of co-solvent and pressure until 20 MPa provides smaller R_a, that is, better solubility. This increase in the solubility parameter is due to a raise in the hydrogen-bonding solubility parameter with an increasing concentration of ethanol in SC-CO_2. Besides, increasing the pressure above 20 MPa do not significantly decreases the value of R_a. On the other hand, similar results using pure ethanol at 150 °C or CO_2 + ethanol (50 : 50 v/v, 40 °C) at 20 MPa and ethyl lactate at 150 °C or CO_2 + ethanol (50 : 50 v/v, 40 °C) at 20 MPa were obtained. In this sense, by tuning the temperature and pressure of subcritical and supercritical fluids and the percentage of co-solvent it is possible to have a change in the contribution of the various intermolecular forces which govern solute solubility.

Another approach tested to improve the solubility of phlorotannins was assessing mixtures of the selected solvents. Table SI-2† shows the theoretical optimal volume fractions Φ for the mixture of ethanol, water and ethyl lactate and the R_a value for heptaphlorethol and heptafucol. As can be seen, low temperatures induce a decrease in the R_a value, condition in which their solubility improves. Mixtures of ethanol/water (80 : 20 v/v) and ethyl lactate/water (63 : 37 v/v) present a similar value than the one obtained with pure ethanol at 25 °C. As temperature increases, the volume fraction of water also increases considerably in the solvent mixtures, due to the decrease of their partial and total solubility parameters under these conditions, thus approaching the solute solubility parameter at the respective temperature. Also, it is worth noting that the use of these mixtures can modify the extraction yield and affect the selectivity of the process.

On the other hand, by using the tool Solvent Optimizer from HSPiP (Version 5.0, Denmark) it was possible to test up to 101 organic solvents (alcohols, esters, ketones, aromatic hydrocarbons, among others) to estimate the solubility of the model phlorotannins considered in the study; results showed that other organic solvents can dissolve heptaphlorethol and heptafucol more effectively than ethanol, such as ethylene glycol (R_a = 15.01; R_a = 22.39, respectively for heptaphlorethol and heptafucol), benzyl alcohol (R_a = 18.21, R_a = 25.34) and tetrahydrofurfuryl alcohol (R_a = 19.22, R_a = 26.39). However, due their toxicity and hazardous solvent conditions, they have not been considered suitable for employing in our green processes. Also, it is worth mentioning that these values are very close to the optimal solvent (ethanol, R_a = 20.01, R_a = 26.57) found to extract those compounds.

4.4.1 Teas ternary plot. Teas ternary plot has been used in the present work to help visualizing the most-suitable solvents to extract heptaphlorethol and heptafucol; as mentioned in the introduction, the method is based on the use of the Teas fractional solubility parameters. Although this is a neat way to condense 3D data into 2D, the significance of the plotted values just provide an idea about the influence of the solvent for each individual structure.[35] Fig. SI-1† shows the Teas ternary plot together with a table (at the top right) with the corresponding number of the target compounds and solvents. The analysis of the graph is based on the fact that the higher solubility of the solute in the solvent should occur when the solubility of the solute and solvent is closer to each other. Numbers 1 and 2 (red star icons) correspond to heptaphlorethol and heptafucol, respectively. The graph shows that the nearest pure solvent for the structures proposed is ethanol (at 25 °C) confirming the results obtained in the estimation of the HSP approach. Due to the high value of the hydrogen-bonding fractional parameter (F_h) for pure water (points 3, 4, 5 in the plot), this solvent seems to be the less appropriate (the farthest) to extract phlorotannins. In any case, from the theoretical approach, it can be inferred that low temperatures are necessary to get a better solubility of

phlorotannins. Other solvents like CO_2 + ethanol (50% v/v) (points 18, 19 and 20) seems to be also useful to extract the phlorotannins in one step.

4.4.2 Experimental assay of theoretical optimized conditions. By using the information provided by the theoretical approach the optimum solvent was selected and extractions were carried out under PLE conditions using pure ethanol at 25 °C (10.3 MPa, 20 min). Ethanol was also tested under subcritical conditions considering 100 °C and 150 °C in order to study the global effect of the increase in the temperature on the final solubility of the phlorotannins. As it was expected, the extraction yield was positively affected by an increase of temperature, obtaining values of 1.00 ± 0.02, 9.4 ± 0.1 and $14.1 \pm 1.4\%$ (w/w), respectively. As for total phlorotannins content (measured using the DMBA colorimetric assay) it was interesting to observe that the experimental behavior at high temperatures was the opposite to the theoretically expected; that is, in terms of R_a distance, an increase in temperature should increase the R_a, thus meaning a decrease in solubility of the phlorotannins. Nevertheless, as shown in Fig. 5, the increment of the temperature from 25 to 100 °C provokes an increment of 25.4% in the phlorotannins extraction (from 4.8 to 6.7 mg PGE per g extract). This increment could be due to the described effect of the temperature stimulates on the mass transfer rate that affects different parameters of the solvent such as density, viscosity or diffusivity. The differences observed between experimental and theoretical approach can be explained since the HSP are based on thermodynamic data and kinetics phenomena, which are highly influenced by temperature, are not taken into account. This can be considered as the biggest limitation of the HSP approach to study the extraction processes. On the other hand, the increment in the temperature up to 150 °C, showed a decrease in the phlorotannin extraction (4.0 mg PGE per g crude extract) following the prediction of Hansen solubility parameters. This effect can be attributed either to a drop of the solubility of the phlorotannins and/or to a degradation of these compounds with the temperature. Despite of above discussed, Hansen solubility parameters can be used *a priori* to evaluate the possibility to dissolve a solute in the most suitable solvent for a given application.

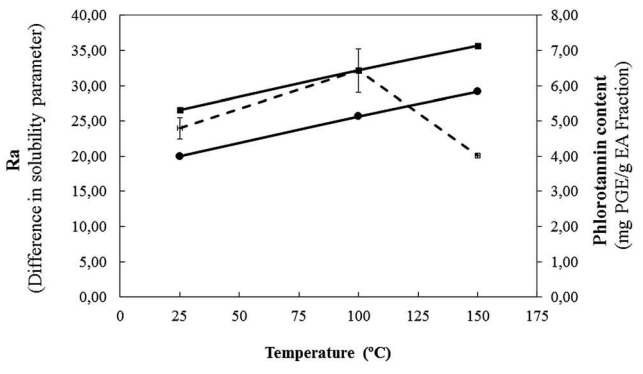

Fig. 5 R_a value and phlorotannin content (mg PGE per g extract) for experimental assays using ethanol pure as solvent at different temperatures. Dashed line (-·-), phlorotannin content of crude extract (right axis); continuous line (—), theoretical R_a values for (●) heptaphlorethol, (■) heptafucol (left axis).

5. Conclusions

The chemical characterization of the purified extracts from *Cystoseira abies-marina* showed that the main types of phlorotannins found were phloroethols, fucols or fucophlorethols with a degree of polymerization from 3 to 14 PGU, being the 7 PGU the most abundant biopolymer. Once the profile of compounds in the extract is known, the group contribution methods and Hansen solubility parameters were interesting predicting approaches to estimate the solubility of structurally complex and biological solutes in sub- and/or supercritical fluid, optimizing theoretically the conditions for their extraction from natural matrices. By estimating the R_a distances or by calculating and constructing the Teas ternary plot, the best existing solvent (or mixture of solvents) can be found. According to this theoretical approach, temperature does not have a strong effect on the solubility parameters of the phlorotannins studied. Besides, by adjusting the subcritical fluids' temperature and supercritical fluids' pressure and percentage of co-solvent, it is possible to have a change in the contribution of various intermolecular forces which govern solute solubility. Theoretically, pure ethanol at low temperature (25 °C) was shown to be the most suitable solvent because it provided the smallest R_a, compared to the other green solvents. However, this work experimentally demonstrates that pure ethanol at 100 °C in subcritical state (10.3 MPa) showed the highest selectivity to extract phlorotannins among different solvents studied. This difference between theoretical and experimental results could be due to the transport phenomena that take place during the extraction process and that are not considered into the HSP approach. Theoretically, green solvents such as ethyl lactate and CO_2 + ethanol (50 : 50 v/v) could also be suitable to design a new green purification method for phlorotannins extraction.

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SUPPLEMENTARY INFORMATION

Table SI-1.Tentative identification of the separated compounds of *Cystoseira abies-marina* by HILIC x RP-DAD-MS/MS analysis.

Peak	tr 2D (s)	Total tr (min)	[M-H]-	Main MS/MS fragments	Relative peak area (%)	Identification
1	32.00	21.33	373.7	247, 125	2.20	Phlorotannin-3 PGU
2	30.05	25.20	329.8	249, 205, 163	0.19	NI
3	30.35	29.11	497.8	479, 372, 339, 249, 229	0.57	Phlorotannin-4 PGU
4	28.85	31.68	497.7	479, 453, 373, 249	0.53	Phlorotannin-4 PGU
5	35.10	31.79	497.9	479, 413, 373, 339, 245, 229	0.72	Phlorotannin-4 PGU
6	31.60	36.93	621.8	603, 559, 479, 247, 229	0.25	Phlorotannin-5 PGU
7	31.35	38.22	621.4	603, 479, 373, 247, 229	8.03	Phlorotannin-5 PGU
8	28.95	39.48	621.5	603, 479, 371, 247, 229	0.50	Phlorotannin-5 PGU
9	31.60	39.53	621.4	603, 495, 371, 263, 229	0.21	Phlorotannin-5 PGU
10	37.80	39.63	621.4	603, 495, 479, 371, 229	1.23	Phlorotannin-5 PGU
11	30.20	40.80	745.9	727, 621, 495, 479, 245, 229	0.23	Phlorotannin-6 PGU
12	34.10	40.87	745.5	727, 601, 479, 339, 229	2.62	Phlorotannin-6 PGU
13	35.70	40.90	745.5	727, 709, 229	0.42	Phlorotannin-6 PGU
14	28.60	42.08	745.7	728, 711, 586, 479, 355, 229	0.70	Phlorotannin-6 PGU
15	35.15	43.49	869.7	772, 712, 634, 621, 497, 245	17.53	Phlorotannin-7 PGU
16	36.70	43.51	869.6	854, 713, 620, 550, 447, 245	39.52	Phlorotannin-7 PGU
17	39.60	46.16	993.9	975,869, 849, 745, 621, 495	3.44	Phlorotannin-8 PGU
18	36.65	47.41	993.3	975, 957, 932, 620	0.95	Phlorotannin-8 PGU
19	41.75	47.50	1117.6	1099, 993, 869, 849, 745, 619	2.12	Phlorotannin-9 PGU
20	31.55	47.33	893.3	875, 785, 727, 661, 497	0.53	NI
21	33.55	48.66	999.6	981, 963, 909, 891, 749, 601	0.58	NI
22	36.45	48.71	1241.6	1223, 1205, 993, 975, 851, 620	0.45	Phlorotannin-10 PGU
23	38.20	48.74	1242.3	1223, 1205, 993, 973, 867, 725	1.88	Phlorotannin-10 PGU
24	43.45	48.83	1241.6	1223, 1205, 993, 975, 851, 743	0.57	Phlorotannin-10 PGU
25	33.80	49.96	1017.3	999, 979, 927, 909, 851, 619	1.46	NI
26	35.20	49.99	1017.7	999, 981, 909, 869, 851, 619	1.78	NI
27	40.90	50.08	1017.9	999, 909, 851, 769, 663	0.52	NI
28	45.45	50.16	1365.4	1117, 993, 975, 867, 849, 727, 479	1.78	Phlorotannin-11 PGU
29	40.95	51.38	745.3*	1241, 1223, 1100, 993,	0.51	Phlorotannin-12 PGU

				726, 229		
30	42.40	51.41	745.5*	1241, 1223, 991, 867, 726, 619, 229	0.87	Phlorotannin-12 PGU
31	44.45	52.74	744.6*	1224, 1118, 995, 939, 614, 339, 228	0.35	Phlorotannin-12 PGU
32	48.55	52.81	806.7*	1365, 1224, 993, 867, 745, 619, 351, 229	1.12	Phlorotannin-13 PGU
33	45.75	54.06	869.3*		1.03	Phlorotannin-14 PGU
34	48.80	55.41	869.1*		0.86	Phlorotannin-14 PGU
35	51.15	55.45	930.7*		0.46	Phlorotannin-15 PGU

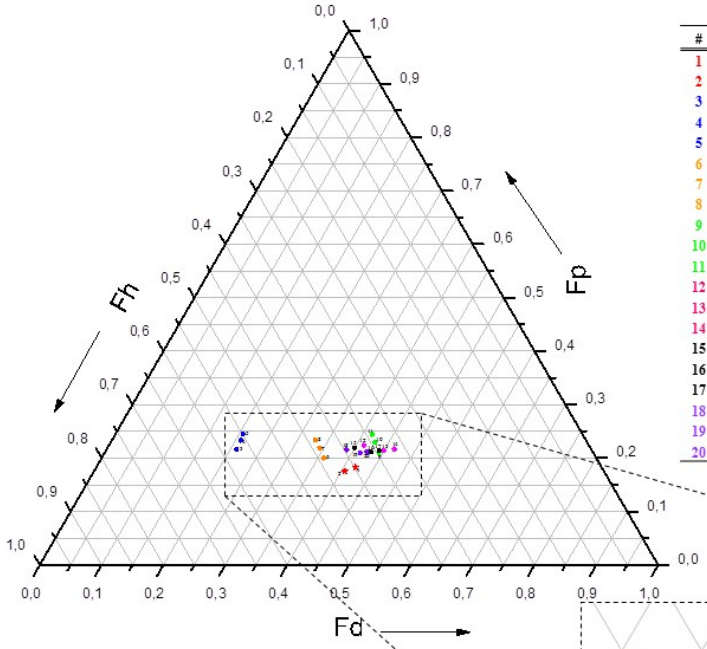
NI, Not identified; *, ions detected as $[M-2H]^{2-}$.

Each identified phlorotannin can belong to the phloretol, fucol or fucophlorethol type.

Table SI-2. Optimal theoretical volume fractions (Φ) for the mixture of ethanol, water and ethyl lactate and their Ra value for heptaphlorethol and heptafucol.

	Heptaphlorethol								
	Mix1			**Mix2**			**Mix3**		
Temperature (ºC)	$\Phi_{Ethanol}$	Φ_{water}	**Ra**	$\Phi_{Ethyl\ lactate}$	Φ_{water}	**Ra**	$\Phi_{Ethyl\ lactate}$	$\Phi_{Ethanol}$	**Ra**
25	0.80	0.20	19.44	0.63	0.37	19.28	0	1.0	20.01
100	0.53	0.47	23.56	0.47	0.53	22.94	0	1.0	25.65
150	0.30	0.70	25.41	0.34	0.66	24.76	0	1.0	29.16
	Heptafucol								
	Mix1			**Mix2**			**Mix3**		
Temperature (ºC)	$\Phi_{Ethanol}$	Φ_{water}	**Ra**	$\Phi_{Ethyl\ lactate}$	Φ_{water}	**Ra**	$\Phi_{Ethyl\ lactate}$	$\Phi_{Ethanol}$	**Ra**
25	0.60	0.40	24.81	0.47	0.53	24.70	0	1.0	26.571
100	0.29	0.71	28.28	0.28	0.72	27.94	0	1.0	32.201
150	0.02	0.98	29.65	0.12	0.88	29.49	0	1.0	35.693

Figure SI-1. Teas ternary plot. The position of the phlorotannins analyzed is shown with a star icon (★) and the solvent studied with a filled circle (●). The corresponding numbers are listed in the table on the right top of the figure.



#	Compound/solvent	(% v/v)	T (°C)	Fd	Fp	Fh
1	Heptaphlorethol	100	25	0.419	0.183	0.398
2	Heptafucol	100	25	0.406	0.175	0.419
3	Water	100	25	0.210	0.217	0.573
4	Water	100	100	0.208	0.233	0.558
5	Water	100	150	0.207	0.245	0.549
6	Ethanol	100	25	0.359	0.200	0.441
7	Ethanol	100	100	0.343	0.219	0.438
8	Ethanol	100	150	0.329	0.234	0.437
9	Ethyl Lactate	100	25	0.443	0.211	0.346
10	Ethyl Lactate	100	100	0.428	0.230	0.343
11	Ethyl Lactate	100	150	0.416	0.244	0.340
12	CO_2+Ethanol (10 Mpa)	70:30	40	0.412	0.224	0.364
13	CO_2+Ethanol (20 Mpa)	70:30	40	0.449	0.214	0.337
14	CO_2+Ethanol (30 Mpa)	70:30	40	0.465	0.217	0.318
15	CO_2+Ethanol (10 Mpa)	60:40	40	0.399	0.219	0.382
16	CO_2+Ethanol (20 Mpa)	60:40	40	0.430	0.212	0.358
17	CO_2+Ethanol (30 Mpa)	60:40	40	0.442	0.214	0.344
18	CO_2+Ethanol (10 Mpa)	50:50	40	0.389	0.215	0.396
19	CO_2+Ethanol (20 Mpa)	50:50	40	0.413	0.210	0.377
20	CO_2+Ethanol (30 Mpa)	50:50	40	0.423	0.212	0.366

4.2.4 New approaches for the selective extraction of bioactive compounds employing bio-based solvents and pressurized green processes.

A.P. Sánchez-Camargo, N. Pleite, M. Herrero, A. Cifuentes, E. Ibáñez, & B. Gilbert-López

Enviado para su publicación a la revista *Journal of Supercritical Fluids*

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Abstract

Solvent selection is a key factor in the development of processes for the extraction of bioactive compounds from natural sources such as algae. The aim of this work was the use of bio-based solvents for the selective extraction of fucoxanthin from P. tricornutum, using pressurized technologies. In this regard, the application of Hansen solubility approach reduced the list of candidate solvents for pressurized liquid extraction (PLE) to four: ethyl acetate, ethyl lactate, d-limonene and ethanol. The use of theoretical calculations narrowed the search of suitable solvents, thus making the process greener. Among the bio-based solvents proposed, d-limonene was the most selective, although it was not able to recover all the fucoxanthin present in the biomass unless a continuous extraction aided by supercritical CO2 was used. The other three solvents tested showed good recoveries of fucoxanthin, but were less selective, following the decreasing order: ethyl acetate > ethyl lactate > ethanol.

Keywords	Pressurized fluid extraction; Hansen solubility parameters; bio-based solvents; fucoxanthin; d-limonene; green process
Taxonomy	Process Technology, Marine Natural Products
Manuscript category	Extractions

Corresponding Author	Elena Ibanez
Corresponding Author's Institution	Institute of Food Science Research
Order of Authors	Andrea del Pilar Sanchez-Camargo, Natalia Pleite, Miguel Herrero, Alejandro Cifuentes, Elena Ibanez, Bienvenida Gilbert-Lopez

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Instituto de Investigación en Ciencias de la Alimentación

Madrid, 19th February 2017

Professor Gerd Brunner
Editor
Journal of Supercritical Fluids

Dear Professor Brunner,

Please, find enclosed copy of the article **"New approaches for the selective extraction of bioactive compounds employing bio-based solvents and pressurized green processes"** by A.P. Sánchez-Camargo et al. to be considered for publication in the Journal of Supercritical Fluids. The work describes the use of Hansen Solubility Parameters (HSP) to theoretically predict the solubility of fucoxanthin in different bio-based solvents (d-limonene, ethyl acetate, ethyl lactate and ethanol) under sub- and supercritical conditions. HSP can be considered a useful tool to guide the solvent selection for the selective extraction of a valuable compound such as fucoxanthin from Phaeodactylum tricornutum microalgae. Theoretical calculations were confirmed experimentally. Results obtained set up the dilemma: selectivity vs recovery and demonstrated that ad-hoc processes can be optimized to target them. I hope you consider the article suitable for publication into the Journal of Supercritical Fluids.

Best regards,

Elena Ibáñez
Laboratory of Foodomics
Institute of Food Science Research (CIAL)
National Research Council (CSIC)
Nicolas Cabrera 9, Campus de Cantoblanco
28049 Madrid, Spain
elena@ifi.csic.es

c/ Nicolás Cabrera, 9.
Campus de la Universidad
Autónoma de Madrid
28049 Madrid

New approaches for the selective extraction of bioactive compounds employing bio-based solvents and pressurized green processes.

Andrea del Pilar Sánchez-Camargo, Natalia Pleite, Miguel Herrero, Alejandro Cifuentes, Elena Ibáñez*, Bienvenida Gilbert-López

Laboratory of Foodomics, Bioactivity and Food Analysis Department, Institute of Food Science Research CIAL (UAM-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain.

(Submission to: The Journal of Supercritical Fluids)

*Corresponding author

Laboratory of Foodomics, Bioactivity and Food Analysis Department

Institute of Food Science Research, CIAL (UAM-CSIC)

C/ Nicolás Cabrera 9, Campus UAM Cantoblanco

28049 Madrid, Spain

e-mail: elena.ibanez@csic.es

Abstract

Solvent selection is a key factor in the development of processes for the extraction of bioactive compounds from natural sources such as algae. The aim of this work was the use of bio-based solvents for the selective extraction of fucoxanthin from *P. tricornutum*, using pressurized technologies. In this regard, the application of Hansen solubility approach reduced the list of candidate solvents for pressurized liquid extraction (PLE) to four: ethyl acetate, ethyl lactate, d-limonene and ethanol. The use of theoretical calculations narrowed the search of suitable solvents, thus making the process greener. Among the bio-based solvents proposed, d-limonene was the most selective, although it was not able to recover all the fucoxanthin present in the biomass unless a continuous extraction aided by supercritical CO_2 was used. The other three solvents tested showed good recoveries of fucoxanthin, but were less selective, following the decreasing order: ethyl acetate > ethyl lactate > ethanol.

Keywords: Pressurized fluid extraction, Hansen solubility parameters, bio-based solvents, fucoxanthin, d-limonene, green process.

1. Introduction

Despite solubility parameter theory has been employed since the first middle of the last century, recently it has received special attention as prior evaluation of the possibility to dissolve a solute in an solvent [1–5]. The solubility theory approach is based on the principle “like dissolves like” and it can be very advantageous to give a first approximation in extraction processes, indicating the most suitable solvent for a given application thus avoiding the selection of impractical experimental conditions. In an effort to improve the applicability of the total solubility parameter, Hansen [6] divided the Hildebrand parameter into three dimensional components, which quantify, individually, the contributions of the dispersion, polar and hydrogen bonding interactions forces[6,7]. The Hansen solubility parameters (HSP) have been widely applied from academic labs to industrial applications and have been employed as a numerical estimation to predict the solubility of many industrial products such as polymers, bio-polymers, drugs, pigments, dyes, and some biological materials in different types of solvents [6]. The estimation of the individual HSP depends greatly upon the availability of data of the target molecule; therefore, when there is no enough information in the databases on the key molecule(s), the group contribution method (GCM) is the most common way employed to predict properties and solubility parameters of molecular structures, using additive rules [8]. On the other hand, due to the growing importance of the sub- and supercritical fluid extraction as green process technologies, several accurately predictions of the HSP in terms of reduced pressure and temperature have been discussed [9–12]. There are few examples in the recent literature in which the estimation of the HSP appears as a useful tool to optimize the pressurized

extraction of bioactive metabolites from natural sources [1,4,13–17]. For instance, Srinivas and King [1] estimated HSP of some carotenoids (e.g. β-carotene, curcumin, lutein, violaxanthin and zeaxanthin) present in spices (e.g. black pepper, cayenne, cinnamon, garlic, ginger) in supercritical carbon dioxide (SC-CO_2) at different temperatures and pressures. On the other hand, Chemat's research group have also employed the HSP to evaluate the performance of several alternative solvents, including SC-CO_2, to extract aroma compounds, fat and oils, and carotenoids, among others, from different food matrices, in order to substitute petroleum based solvents (such as hexane and dichloromethane) [5,13,18–20]. In a recent work carried out in our laboratory, we have estimated the HSP of phlorotannins (polyphenols constituted of phloroglucinol monomers with important bioactivities) obtained from a Mediterranean brown algae in different sub-critical green solvents; this theoretical approach was found as a profitable tool to choose a suitable solvent for selective extraction purposes [17].

The Hansen approach, hence, can be exploited in the development of greener selective processes for the extraction of valuable compounds from natural sources, since the use of a theoretical approach reduces the number of experiments with different solvents. In addition, since the extractions are more selective, purification steps are reduced. Therefore, there is a reduction on waste generation, thus making the processes more environmentally friendly. But to develop a green process, additional criteria for solvent selection should be taken into account. Different organizations have established classifications of solvent "greenness" attending to different standards, e.g.: environmental impact, health, safety, life cycle assessment (LCA), waste, etc [21,22]. However, scarce attention has been paid in the

84 solvent selection guides to bio-based solvents until the last years [21,23,24], when their
85 potential as extraction solvents for the development of sustainable processes has started to
86 be explored [2]. The development of green sustainable processes to place in the market
87 products enriched in bioactive compounds obtained from natural sources is one of the
88 challenges that chemical and pharmaceutical industries have to face nowadays. The trend to
89 a "bio-based world" has put the focus on renewable sources such as algae, recognized as
90 rich source of bioactive and high-added value compounds [25,26]. For instance,
91 fucoxanthin is a xanthophyll (an oxygenated carotenoid) with a unique structure, which
92 includes an allenic bond and a 5,6-monoepoxide (see Figure 1). Fucoxanthin is one of the
93 most abundant carotenoid present in algae, very appreciated for its several biological
94 activities, including antioxidant, antiobesity, antidiabetic, anti-inflammatory,
95 antiphotoaging and anticancer [27]. The commercial production of fucoxanthin has been
96 explored, using *Phaeodactylum tricornutum* (a marine diatom microalga) as biomass [28].
97 In this regard, pressurized liquid extraction (PLE) using a bio-based solvent such as ethanol
98 has been proposed for the recovery of fucoxanthin and other pigments from *P. tricornutum*
99 [29]. Also, ethanol was reported as optimum solvent for the extraction of total carotenoids
100 from *Neochloris oleoabundans*, after an experimental design in which the bio-based solvent
101 d-limonene was also evaluated[30]. The mixture ethanol:d-limonene has been proposed as
102 an alternative to replace n-hexane for the extraction of lipids from microalgae[31]. D-
103 limonene has also been proposed recently as a promising green solvent for the extraction of
104 carotenoids and other bioactive compounds from food [32]. However, in none of these
105 examples the extraction was selective towards a target compound but a family of
106 compounds. The aim of this work was to develop an efficient and selective extraction

method of fucoxanthin from *P. tricornutum* by using pressurized bio-based solvents. According to the theoretical predictive assessment of HSP, ethanol, ethyl acetate, ethyl lactate and d-limonene were the selected solvents and their behavior was experimentally evaluated. In addition, SC-CO_2 employing d-limonene as co-solvent was tested. The quantification of fucoxanthin in the obtained extracts was carried by HPLC-DAD-APCI-MS/MS. The selectivity of the processes was measured by the ratio between total carotenoid and total chlorophylls concentration. The optimization of the SFE and PLE processes was also carried out.

2. Theoretical modelling of solubility parameters

2.1 Estimation of HSP at normal conditions by HSPiP software

Hansen solubility parameters are based on the concept that the total cohesive energy density is calculated by the sum of E_D, dispersion energy (related to the Van der Waals forces), E_P, polarity energy (related to dipole moment), and E_H, hydrogen bonding energy. Dividing this by the molar volume gives the square of the total (δ_T^2) solubility parameter as the sum of the squares of the Hansen (D, P, and H components), as defined by Equation (1)[6]:

$$\delta_T(MPa^{1/2}) = \sqrt{\delta_D^2 + \delta_P^2 + \delta_H^2} \qquad \text{Eq. (1)}$$

HSPiP® software (Version 5.0, UK) offers to calculate HSPs by Yamamoto – Molecular Break (Y-MB) method, which estimates the parameters directly from the molecular structure in computational form employing a neural network (NN) technique and multiple regressions fits. By means of DIY (Do It Yourself) tool available in the software menu, the HSP of fucoxanthin (solute) was calculated. Once its chemical structure was transformed in

its simplified molecular input line syntax (SMILES) notations (CC(=CC=CC=C(C)C=CC=C(C)C(=O)CC12C(CC(CC1(O2)C)O)(C)C)C=CC= C(C) C= C=C3C(CC(CC3(C)O)OC(=O)C)(C)C), it was subsequently used for HSP calculation employing Y-MB method. After, from the solvent optimizer menu 4 bio-based solvents (d-limonene, ethyl lactate, ethyl acetate and ethanol) were selected employing the *Ra* term as criteria, which refers to the distance of a solvent from the center of the Hansen solubility sphere, given by Eq (2). The solvents were selected in ascending order of *Ra*, where the smaller *Ra* corresponds to the greater affinity between solute and solvent.

$$R_a = \sqrt{4(\delta_{Di} - \delta_{Dj})^2 + (\delta_{Pi} - \delta_{Pj})^2 + (\delta_{Hi} - \delta_{Hj})^2} \quad \text{Eq. (2)}$$

In the equation 2, subscript *i* refers to the solute and *j* refers to the solvent. To determine whether the bio-based solvent and the solute are miscible, the relative energy difference (RED) number can be calculated using Eq. (3).

$$RED = R_a/R_0 \quad \text{Eq. (3)}$$

Ro value refers to the radius of interaction of a Hansen solubility sphere; this value must be found experimentally and will be discussed in Section 3.3. As a general guideline: if RED <1, the molecules are alike and will dissolve each other; if RED =1, the system is right on the soluble/insoluble border and if RED >1 the system will not dissolve.

2.2 Estimation of HSP at sub- and supercritical fluid conditions.

150 For the pressurized conditions, Jayasri and Yaseen [33] method can evaluate the
151 temperature dependence of the solute solubility parameter (since pressure does no exert a
152 large influence on the properties of solid) employing the Eq.(4), where *Tr* refers to the
153 reduced temperature at room temperature (1) at a given sub- or supercritical temperature
154 (2).

155 $$\delta_2 = \delta_1 \left(\frac{1 - T_{r2}}{1 - T_{r1}}\right)^{0.34} \quad \text{Eq. (4)}$$

156 For this purpose , the estimation of critical data of fucoxanthin was carried out employing
157 Marrero & Gani [34] group contribution method (third-order group), and the Yamamoto-
158 Molecular Break method using its SMILES notation (HSPiP Version 5.0, UK) was
159 employed for the evaluation of the molar volume. For the bio-based solvents, d-limonene,
160 ethyl acetate, ethyl lactate and ethanol were assessed at subcritical conditions considering
161 two temperatures levels, 40 and 100 ºC, and keeping the pressure constant at 10.0 MPa. For
162 supercritical (or CO_2-expanded liquid) conditions, CO_2 and mixtures of CO_2 + d-limonene,
163 CO_2 + ethyl acetate, CO_2 + ethyl lactate and CO_2 + ethyl ethanol (50:50 %, w/w), at 40ºC
164 and 30 MPa were studied.

165 The physical properties of the subcritical solvents were taken from Capparucci *et al*. [35]
166 and Pereira et al.[36] and were calculated following the Gunn–Yamada method. On the
167 other hand, for supercritical conditions, the CO_2 densities at the specified pressure and
168 temperature were obtained from NIST REFPROP database 2015[37]. Williams *et al*.[9]
169 proposed an integrated form of the effect of pressure and temperature of the sub-and

supercritical solvents on the HSP. The approach provided by these authors was employed, in which Eq. (5), (6) and (7) described the different HSP components:

$$\delta_D = \delta_{D,ref}\left(\frac{V_{ref}}{V}\right)^{1.25} \quad \text{Eq. (5)}$$

$$\delta_P = \delta_{P,ref}\left(\frac{V_{ref}}{V}\right)^{0.5} \quad \text{Eq. (6)}$$

$$\delta_H = \delta_{h,\,ref}\left[e^{\left(-1.32\times10^{-3}\left(T_{ref}-T\right)-\left(\frac{V_{ref}}{V}\right)^{0.5}\right)}\right]-1 \quad \text{Eq. (7)}$$

where the subscript *ref* refers to the relative property at room temperature (25ºC), *V* is the molar volume and T the sub or supercritical temperature. For a mixture consisting of supercritical CO_2 (SC-CO_2) and co-solvent, the solubility parameters of the mixed fluid were determined by the Eq. (8), where Φ is the volume fraction of SC-CO_2 and co-solvent.

$$\delta_{Mix-D,P,H} = \Phi_{SC-CO_2} \times \delta_{SC-CO_{2-D,P,H}} + \Phi_{co-solvent} \times \delta_{co-solvent_{-D,P,H}} \quad \text{Eq. (8)}$$

3. Experimental

3.1 Standards and reagents

All the HPLC-grade solvents employed (acetone, dichloromethane, ethanol, ethyl acetate, methyl *tert*-butyl ether (MTBE), methanol) were acquired from VWR (Leuven, Belgium). (R)-(+)-limonene was from Sigma-Aldrich (St Louis, MO, USA) and ethyl L(-)-lactate from Acros Organic (Thermo Fisher Scientific, Geel, Belgium). Sea sand (0.25–0.30 mm diameter) was from Panreac (Castellar del Vallés, Spain). Butylated hydroxytoluene (BHT)

188 and standards of fucoxanthin and chlorophyll a (from *Anacystis nidulans* algae) were
189 obtained from Sigma-Aldrich (St Louis, MO, USA). The water used was Milli-Q water
190 (Millipore, Billerica, MA, USA).

191 **3.2 Sample**

192 Freeze-dried samples of *P. tricornutum* were obtained from Fitoplancton Marino S.L.
193 (Cadiz, Spain), and stored under dry and dark conditions until use. *P. tricornutum* was
194 grown outdoors in horizontal tubular 2000L reactors. The reactors use pure CO_2 injection to
195 control pH in the culture by pH controller and flowmeters. pH was set at 7.5, while natural
196 light-dark cycles and ambient temperature were used (10-11 h of light, temperatures
197 ranging from 10-22 ºC). The reactors were inoculated with cultures grown in growth
198 chamber with the standard conditions of Fitoplancton Marino S.L. After harvesting, cells
199 were disrupted at high pressure (120 MPa) and then freeze-dried.

200 **3.3 *Ro* value determination**

201 As mentioned before, the radium of the interaction of Hansen sphere (Ro) is based on
202 experimental data obtained of the observation of the interaction between studied solutes and
203 well-known solvents. Thus, 9 organic solvents with a wide range of polarities were used to
204 test the solubility of fucoxanthin: water, methanol, acetone, ethanol, ethyl lactate, ethyl
205 acetate, limonene, dichloromethane and hexane. Two milligrams of fucoxanthin analytical
206 standard were dissolved in 2 mL of each solvent in a vial. Vials with the mixture solute-
207 solvent were hand shaked for 5 min, stored for 1 h at room temperature and visually
208 observed. With these observations, an arbitrary score (as recommended by the HSPiP

209 manual software) between 1 and 5 was awarded, based on the degree of solubility of
210 fucoxanthin in the solvent. The scoring scale for determining the HSP was estimated as
211 follow: 1) easy to be dissolved, 2) take a bit of efforts to be dissolved completely, 3) take a
212 bit of efforts to be dissolved most, 4) dissolve just a bit and 5) no change, just settle down.
213 Those score were introduced in the software and a score lower than 2 was selected as good
214 solvent criteria (defined in HSPiP as "inside" the solubility sphere).
215

216 **3.4 Extraction procedures**

217 *3.4.1 Pressurized liquid extraction (PLE)*

218 Extractions of *P. tricornutum* using pressurized liquid solvents at laboratory scale were
219 performed in an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA,
220 USA). Each solvent was degassed in an ultrasound bath to prevent oxidation during the
221 extraction process before they were placed in the solvent controller. Extractions were
222 carried out at 10 MPa in 11-mL stainless-steel extraction cells containing 1.0 g of
223 microalga mixed with 2.0 g of sea sand; this mixture was sandwiched between portions of 1
224 g of sea sand. Prior to each extraction, a cell heat-up step was performed for 5 min (time
225 fixed by the system, depending on the working temperature set). All extracts were covered
226 in aluminum foil to protect them from light. The ethanol and ethyl acetate extracts were
227 evaporated at a temperature of 30 ºC under a stream of N_2 in a thermostated Turbovap LV
228 evaporation station (Biotage, Uppsala, Sweden). Extraction yields of extracts obtained with
229 limonene and ethyl lactate were calculated by evaporating aliquots of the extracts at 140 ºC

under a stream of N_2 in a thermostated thermoblock Stuart (Bibby Scientific Limited, Stone, UK).

3.4.2 High-pressure extractions in the Carbon dioxide-expanded liquid (CXL) region

The high pressure extractions were carried out in a home-made supercritical fluid extractor. The high-purity CO_2 was provided from a pressurized cylinder and subcooled in a heat exchanger placed within the high pressure pump (model PU-2080 CO_2 Plus from Jasco, Tokyo, Japan). Then it was continuously pumped at a constant flow rate (1.6 mL min^{-1}) and mixed with a continuous flow of limonene (0.8 mL min^{-1}) supplied from a co-solvent pump (model PU-2080 Plus from Jasco, Tokyo, Japan).

For the extraction, 1 g of sample was mixed with 2 g of sea sand, and glass wool was packed at both ends of the SST extraction cell to stop entrainment of the sample. The cell was placed in the oven and the extraction temperature was set at 50 ºC. The pressure was kept at 30 MPa by means of a manual metering valve (Swagelok, Ohio, USA). In order to select the optimal extraction time, a kinetic study was carried out (by duplicate) collecting the extract every 20 min, for a total extraction time of 180 min. An extraction time of 105 min was selected and three replicate extractions were performed at the optimum conditions.

3.4.3 Benchmark extraction method

A conventional acetone extraction was performed (in triplicate) to determine the total extractable compounds in *P. tricornutum*, as described elsewhere[29,38], as a benchmark method. Briefly, 200 mg of freeze-dried microalga were mixed with 20 mL acetone containing 0.1% (w/v) BHT for 24 h under orbital agitation. After centrifugation, the

supernatant was collected and evaporated until dryness using a N_2 stream in a thermostated Turbovap LV evaporation station (Biotage, Uppsala, Sweden). Dry acetone extracts were weighted and stored at −20 ºC.

3.5 Spectrophotometric analysis of total carotenoids and chlorophylls

A spectrophotometric method was used to determine the total carotenoid and total chlorophylls concentration, based on their characteristic absorbance, as described elsewhere [38]. SFE and PLE extracts were dissolved in methanol (concentrations ranging from 5 to 0.05 mg mL^{-1}). Absorbance of these solutions was recorded at two specific wavelengths, 470 nm for carotenoids and 665 nm for chlorophylls. External standard calibration curves of fucoxanthin (0.5 – 10 μg mL^{-1}) and chlorophyll a (0.5 – 7.5 μg mL^{-1}) were used to calculate the total carotenoid and chlorophyll content, respectively. Total carotenoids were expressed as mg fucoxanthin equivalents (FE) g^{-1} extract and, total chlorophylls were expressed as mg chlorophyll g^{-1} extract.

3.6 Quantification of E-fucoxanthin by HPLC-DAD-APCI-MS/MS.

The profile of carotenoids and chlorophylls of *P. tricornutum* extracts was determined by HPLC using a diode-array detector (DAD) connected in series to an ion trap mass spectrometer, according to the method previously described[29,38]. HPLC-DAD analyses of the extracts were performed in an Agilent 1100 series liquid chromatograph (Santa Clara, CA, USA), using a YMC-C_{30} reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size; YMC Europe, Schermbeck, Germany) and a pre-column YMC-C_{30} (10 mm × 4 mm i.d., 5 μm). The mobile phases were mixtures of methanol–MTBE–water (90:7:3

v/v/v) (solvent A) and methanol–MTBE (10:90 v/v) (solvent B), respectively. Pigments were eluted from de column according to the following gradient: 0 min, 0 % B; 20 min, 30 % B; 35 min, 50 % B; 45 min, 80 % B; 50 min, 100% B; 60 min, 100% B; 62 min, 0% B. The injection volume was 10 μL, while the flow rate was 0.8 mL min^{-1}. Absorbance was recorded at 280, 450 and 660 nm, although spectra from 240 to 770 nm were collected using the DAD (peak width > 0.1 min (2 s), slit 4 nm). LC ChemStation 3D Software Rev. B.04.03 (Agilent Technologies, Santa Clara, CA, USA) was used to control the instrument. Extracts were diluted appropriately prior to HPLC analysis. For the calibration curve, twelve different concentrations of E-fucoxanthin in ethanol, ranging from $0.97x10^{-4}$ to 0.2 mg mL^{-1}, were analyzed using the LC-DAD-MS/MS instrument.

The liquid chromatograph equipped with DAD detector was directly coupled to an Esquire 2000 ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) via an atmospheric pressure chemical ionization (APCI) interface. The APCI ion source was operated under positive ionization mode using the following parameters: −3.5 kV capillary voltage, 400°C vaporizer temperature, 350°C drying temperature, 5 L min^{-1} drying gas flow rate, 60 psi nebulizer gas pressure, 4,000 nA APCI corona current. Full scan spectrum was acquired in the range from *m*/*z* 150-1,300, performing automatic MS/MS fragmentation of the highest precursor ion at 1V (Fragmentor amplitude).

4. Results and discussion

4.1 Theoretical selection of the bio-based solvents.

293 Theoretical and experimental approaches can be employed to estimate the HSP of a
294 material. As previously mentioned, when experimental data for the target compound is not
295 available, GCM can be used as a first estimation of its solubility behavior. Despite of this, it
296 is strongly advised to confirm the HSP results for any compound determined by GCM with
297 experimental determination of its HSP[6] (as it will be discussed in section 4.2). The
298 prediction method proposed by Yamamoto-molecular break method (HSPiP) was applied to
299 calculate de Ra values for the solute (fucoxanthin) and the bio-based solvents at different
300 pressurized conditions; results are given in Table 1. As can be seen, the HSP of fucoxanthin
301 were δ_D =18.16, δ_P =4.07 and δ_H =5.06 at 40°C and 10.0 MPa and varied only marginally
302 with the temperature. In addition, it was assumed that the pressure does not exert a big
303 influence on the individual solubility parameter below its critical point, as pointed by
304 several authors[9,36,39]. As expected, according to its molecular structure (Figure 1), the
305 total solubility parameter is highly influenced by the dispersion force (δ_D = 18.16) due to
306 the presence of a long chain backbone made up of four isoprene units and two beta-ionone
307 rings in each end of the molecule. Moreover, fucoxanthin contains oxygen as well as
308 hydroxyl groups and pairs of hydrogen atoms that are substituted by oxygen atoms acting
309 as a bridge (epoxide), which contribute to the polar (δ_P = 4.07) and hydrogen-bonding
310 forces (δ_H =5.06).
311 On the other hand, by using the solvent optimizer tool included in the HSPiP software, a
312 preliminary screening of the more suitable solvents can be obtained; being the ultimate
313 selection done according to the final objective of the work. In this sense, our research
314 approach was focused on the use of alternative solvents to implement more efficient,
315 selective and greener extraction procedures. Therefore, bio-based solvents such as d-

316 limonene, ethanol, ethyl lactate, ethyl acetate and CO_2 were selected; these solvents have
317 the advantage of being obtained from renewable resources, thus providing an alternative to
318 replace the petroleum-based products. However, it is necessary to demonstrate their
319 performance to gain support and to develop a market share[40]. Under subcritical
320 conditions, an increase in the temperature leads to a decrease in the partial solubility
321 parameters and therefore, in the total solubility parameter. This phenomenon causes an
322 increment in the *Ra* value, leading to worst solubility conditions for the target solute (the
323 smaller the *Ra*, the higher the thermodynamic chances of two components being mutually
324 soluble). From the theoretical point of view, d-limonene at low temperature (40 ºC) seems
325 to be the most suitable solvent to dissolve fucoxanthin because it provides the smallest
326 difference (Ra= 3.76) when compared to ethyl acetate, ethyl lactate and ethanol. On the
327 other hand, at supercritical conditions (30 MPa, 40 ºC), *Ra* value for the neat CO_2 is higher
328 than that obtained for limonene, ethyl acetate and ethyl lactate but lower than ethanol.
329 Although similar values of polar (4.68 vs 4.07 $MPa^{1/2}$) and hydrogen-bonding (5.12 vs 5.06
330 $MPa^{1/2}$) partial parameters were obtained for CO_2 and fucoxanthin, the differences observed
331 in the term corresponding to dispersion (δ_D) support the need of adding a co-solvent able to
332 affect the van der Waals forces. Mixtures 50:50 v/v of CO_2 and limonene, ethyl lactate,
333 ethyl acetate and ethanol were theoretically calculated as described by the Eq. (8). Since
334 limonene possessed the highest δ_D term among all the solvent selected, the *Ra* value of the
335 mixture CO_2+d-limonene (50:50 v/v) to fucoxanthin was the lowest (7.73). It is interesting
336 to note that the theoretical *Ra* obtained for this mixture in the CXL region is similar to the
337 Ra for ethyl acetate at 40 ºC (6.22); therefore, a similar behavior in terms of solubility
338 would be expected.

339

340 **4.2 Experimental determination of Radius of interaction (*Ro*) of Hansen sphere.**

341 An interesting way to get a better idea about the optimal solvent to be selected for a
342 selective extraction is by calculating the relative energy difference (RED) number as
343 described by the Eq. (3). The radius of interaction (*Ro*) incorporates all good solvents and
344 excludes the bad solvents in the solubility sphere, and is considered as a fourth parameter in
345 HSP value determinations. The advantage of the solubility sphere approach is that once an
346 interaction radius has been determined, solvents or mixtures that have not been
347 experimentally tested can be quickly screened and, therefore, can be considered for further
348 studies[6]. Table 2 shows the scores for the nine solvents employed to dissolve fucoxanthin
349 and their HSP. The scores were graded based on the interaction between fucoxanthin and
350 the solvents. According to the observations, ethyl acetate, dichloromethane and ethyl
351 acetate dissolved the fucoxanthin almost immediately. For ethyl lactate and limonene, more
352 time was necessary to dissolve it completely. In the case of hexane, only a small fraction
353 was dissolved, while water showed no solubility for fucoxanthin. The HSPiP® software
354 was used to estimate the experimental HSPs values of fucoxanthin based on the data from
355 Table 2. These data were input into the software and the dispersion, polar, hydrogen forces
356 and interaction radius of fucoxanthin were obtained. The estimation indicated that the HSP
357 of fucoxanthin were 16.39, 5.50 and 7.71 and the radius of interaction was 5.3, which
358 means that just a range of solvents located inside the sphere will be able to dissolve it.
359 Similar values were obtained for δ_D (18.16 vs. 16.39 $MPa^{1/2}$) and δ_P (4.07 vs. 5.50 $MPa^{1/2}$)
360 terms for theoretical and experimental approach, respectively. The higher difference was

found on δ_H term (5.06 vs 7.71 $MPa^{1/2}$) and may be due to the unpredictable nature of the chance of forming hydrogen bonds[6]. Figure 2 shows the three-dimensional Hansen solubility sphere for all solvents tested. The graph shows the polar parameter (P-axis) going vertically from 0-40 $MPa^{1/2}$, the hydrogen bonding parameter (H-axis) also goes from 0-40 from left-to-middle right and the dispersion parameter (D-axis) goes from 7.5-27.5 $MPa^{1/2}$ from right-to-middle left. The wire frame green sphere represents the solubility sphere with a radius of interaction (*Ro*). The blue dots inside the sphere show the solvents that dissolve the fucoxanthin and the red cubes show the solvents that do not dissolve easily the fucoxanthin. In this case, from the 9 solvents tested, 5 of them were good and 4 were classified as bad solvents. Once *Ro* value is known, the RED number can be estimated for the HSP of fucoxanthin obtained experimentally. The last column of Table 2 shows the RED number for the organic solvent tested, including the bio-based solvents, at normal conditions, proposed in the present study. RED numbers smaller than 1 indicate that the solute and the solvent are miscible (such as ethyl acetate). RED numbers equal to (or close to) 1 describe a boundary condition (such as ethyl lactate and limonene) and RED numbers higher than 1 mean low affinity between solvent-molecule (such as ethanol). Therefore, based on these results, limonene, ethyl acetate and ethyl lactate would be good solvents to extract fucoxanthin; however, ethanol seems to be a “bad solvent” or a less appropriate solvent to dissolve it. Nevertheless, it is important to mention that HSP are based on thermodynamic data and therefore, kinetics phenomena, which are highly influenced by temperature, are not considered[17]. For this reason, HSP can be used as a prior evaluation

382 to choose the most suitable solvent for a given application but in any case, it is mandatory
383 to experimentally assess the validity of the approach.

384 **4.3 Experimental evaluation of different green solvents by PLE**

385 As aforementioned, based on Hansen solubility parameters, the bio-based green solvents
386 selected for the selective extraction of fucoxanthin were limonene, ethyl lactate, ethyl
387 acetate and ethanol. Two temperatures were assayed: 40 and 100 ºC for a single cycle 20-
388 min PLE at 10.3 MPa. The concentration of fucoxanthin in each extract was quantified by
389 HPLC. Figure 3 shows the chromatographic profiles of the extracts obtained employing the
390 different solvents; their profile was similar to the previously observed for the PLE of *P.*
391 *tricornutum* using EtOH at 50 ºC [29] and it is composed by a main peak of E-fucoxanthin
392 and some minor peaks corresponding to other carotenoids and chlorophyll-related
393 compounds. As can be observed, chlorophylls (marked with *) did not appear in the
394 chromatogram of the extract obtained using limonene at 40 ºC, compared to the extracts
395 attained with the rest of the solvents.

396 <Figure 3>

397 Experimental results obtained for the different tested solvents in terms of extraction yield
398 (%), mg of fucoxanthin g^{-1} extract, mg fucoxanthin g^{-1} algae, recovery (%) of fucoxanthin
399 and selectivity (ratio carotenoids/ chlorophylls) are presented in Table 3. The extraction
400 yield was determined for each extract gravimetrically, and the highest value was achieved
401 by ethanol at 100 ºC (24.68 ± 1.15 g extract/ 100 g dry alga). As can be observed, the
402 extracts more enriched in fucoxanthin were those obtained by ethyl acetate and limonene,

403 with 25.29 ± 0.58 and 23.89 ± 1.26 mg fucoxanthin g^{-1} extract, respectively. An increase in
404 the temperature favors mass transfer and the co-extraction of other compounds present in
405 the biomass; in addition, a degradation of fucoxanthin can occur at high temperatures.

406 In order to quantify the selectivity of each solvent, a ratio between total carotenoids and
407 total chlorophylls of each extract was calculated by a spectrophotometric method
408 (described in section 3.5.). Total carotenoids were expressed as mg of fucoxanthin
409 equivalents (FE), while total chlorophylls were expressed as mg of chlorophyll-a
410 equivalents. As detailed in Table 3, the highest selectivity of limonene was confirmed. The
411 ratio carotenoid/chlorophylls for this solvent was 3.54 ± 0.53, followed by ethyl acetate
412 with a ratio of 1.22 ± 0.18. For the rest of the solvents tested, the ratio was below the unit,
413 which means that more chlorophylls than carotenoids were extracted. The difference in
414 selectivity of limonene compared to the other solvents is in accordance with the theoretical
415 HSP, as the R_a for limonene at 40 ºC was the smallest one.

416 <Table 3 >

417 On the other hand, considering the benchmark extraction as 100% recovery of fucoxanthin,
418 the percentage of recovery of fucoxanthin was calculated for each extract (see Table 3), in
419 order to compare the extraction efficiency. As it can be seen, the most selective extraction
420 using limonene was the one with the lowest extraction yield and consequently the one with
421 the lowest recovery, 51%. However, the use of a selective solvent is of great interest when
422 developing green processes, since purification and concentration steps can be minimized, or
423 even avoided. For this reason, the exploitation of limonene as extraction solvent of

fucoxanthin centered our attention and more studies were carried out in order to improve the amount of fucoxanthin selectively extracted from the biomass.

4.4 Optimization of fucoxanthin recovery employing limonene

As confirmed experimentally, limonene was the most selective solvent for the extraction of fucoxanthin from *P. tricornutum*, using one cycle 20-min PLE at 40 ºC. However, the recovery of fucoxanthin from the biomass just reaches 51% (compared to acetone maceration). In order to increase the recovery of fucoxanthin, two different approaches were considered: (i) a multistage extraction by cumulative PLE cycles, and (ii) a dynamic extraction using a continuous flow of limonene mixed with supercritical CO_2 in the CXLs region.

(i) *Multistage PLE extraction of fucoxanthin*. In this approach, a kinetic study was performed (in duplicate) by adding extraction cycles of 10 min after the first cycle of 20 min, for a total extraction time of 110 min, without the manipulation of the biomass in the extraction cell. The extraction yield followed a constant increase after every cycle and reached 24.7% at the end of the study (see Table 4). The selectivity of the extraction measured as carotenoids/ chlorophylls ratio showed a different behavior instead; as depicted in Figure 4, after 70 min of extraction (6 PLE cycles); this ratio reaches a minimum that remains constant until the end of the study. Therefore, 70 min were selected as the extraction time to enhance the recovery of fucoxanthin. Two replicates PLE of *P. tricornutum* were performed at the selected conditions (10 MPa, 40 ºC, 1×20min + 5×10min), and the recovery of fucoxanthin and the carotenoids/chlorophylls ratio were

445 determined for both extracts. The selectivity decreased from a ratio of 3.54 ± 0.53 obtained
446 in a single-cycle extraction (Table 3) to a ratio of 1.73 ± 0.21 (Table 5) in the multistage
447 extraction of 70 min. In addition, although the extraction yield increased (from 3.86% to
448 14.23%), the concentration of fucoxanthin in the extract decreased (probably because of
449 degradation), resulting in a decrease in the recovery of fucoxanthin from 51.2% to 44.4%.
450 Therefore, a single-cycle PLE of fucoxanthin using limonene is preferred.

451 < Table 4 and Figure 4>

452 (ii) *Dynamic extraction of fucoxanthin using carbon dioxide-expanded limonene*. A second
453 approach was tested to improve the recovery of fucoxanthin extracted with pressurized
454 limonene. A kinetic study was performed for the continuous extraction of *P. tricornutum*
455 using a mixture $ScCO_2$/limonene (50:50 v/v) at 40 ºC and 30 MPa during 180 min (Table
456 4). According to the HSP theoretical approach, the mixture CO_2 + limonene (see Table 1)
457 showed the lower *Ra* among the mixtures calculated, being these conditions those selected
458 for this second approach. The study was accomplished in duplicate by collecting 7 aliquots
459 during the entire extraction time. Similarly to the multistage extraction experiment, the
460 extraction yield followed an increasing trend; reaching 21.8% at the end of the kinetic study
461 (see Table 4). As in the case of the multistage extraction, the selectivity of the CXL
462 extraction measured as carotenoids/chlorophylls ratio reached a minimum. As depicted in
463 Figure 4, the minimum ratio is obtained after 105 min of extraction and remains constant
464 until the end of the study. Therefore, three replicate extractions were carried out using 105
465 min as total extraction time. The results are presented in Table 5. The 27.54% extraction
466 yield is higher than the obtained with single-stage PLE and, although the concentration of

467 fucoxanthin in the extract decreased, the recovery of fucoxanthin reached 92.7%.
468 Nevertheless, the selectivity of the process is reduced, as the average value of
469 carotenoids/chlorophylls ratio of this extraction was 1.18. Looking at the results detailed in
470 Table 5 and Table 3, the recovery of fucoxanthin and the selectivity of this process are
471 similar to those obtained with ethyl acetate, but the extract obtained by PLE using ethyl
472 acetate is 4-fold enriched in fucoxanthin and 5-times faster than the process that uses a
473 mixture of $ScCO_2$/ d-limonene.

474 **5. Conclusions**

475 The present work demonstrates the usefulness of Hansen approach to reduce the number of
476 experiments for the selection of a selective solvent for a target compound. Only 4 bio-based
477 solvents were tested for the extraction of fucoxanthin from *P. tricornutum*, according to
478 HSP. Among them, d-limonene was the most selective. But the recovery of fucoxanthin
479 was approximately the 50% of the amount of this carotenoid present in the biomass. An
480 increase in the extraction time using multistage PLE did not improve the recovery of
481 fucoxanthin, which was increased above 90% by a dynamic extraction using a mixture of
482 SC-CO_2/d-limonene under CXL conditions. However, the dynamic extraction is more time-
483 consuming and yielded an extract with a lower concentration of bioactive than PLE using
484 ethyl acetate. Therefore, the extraction of fucoxanthin by the tested bio-based solvents set
485 up the dilemma selectivity vs. recovery, which requires information about economic,
486 energetic and environmental aspects (out of the scope of the present work) to be resolved.

As a final remark, the present work proposed two alternatives for the extraction of fucoxanthin from microalgae: (1) selective extraction of 50% of fucoxanthin from the biomass using static PLE extraction with limonene, and (2) total extraction of fucoxanthin from the biomass, together with other co-extractants, using either static PLE with ethyl acetate or dynamic extraction using SC-CO_2+limonene. The choice will depend on the particular interest of the end user.

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Figure captions

Figure 1. Structure of fucoxanthin.

Figure 2. Three-dimensional Hansen solubility parameter sphere of all solvent for evaluated. Blue dots inside the sphere dissolve the fucoxanthin and ret squares outside do not dissolve it. 1. Hexane (behind the sphere) 2. Dichloromethane; 3. d-limonene; 4. Ethyl acetate; 5. Ethyl lactate; 6. Ethanol, 7. Acetone, 8.Methanol, 9. Water.

Figure 3. Comparison of HPLC profiles (450 nm) of PLE extracts of *P. tricornutum* with different bio-based solvents at 40 ºC. Peaks marked with an asterisk (*) showed an absorbance spectrum characteristic of chlorophylls. The rest of the compounds showed absorbance spectrum characteristic of carotenoids. Extract of limonene is half concentrated than the others.

Figure 4. Monitoring of carotenoids/ chlorophylls ratio during pressurized extractions of *P. tricornutum* using limonene.

Table 1. Hansen solubility parameters and Ra value of fucoxanthin and bio-based solvent at subcritical, supercritical and CXL conditions.

Compound/solvent	Molar Volume, cm^3/mol	T, ºC	P, MPa	δ_D ($MPa^{1/2}$)	δ_P ($MPa^{1/2}$)	δ_H ($MPa^{1/2}$)	δ_T ($MPa^{1/2}$)	Ra
Target compound								
Fucoxanthin	631.10	40	10.0	18.16	4.07	5.06	19.39	-
	631.10	100	10.0	17.59	3.94	4.90	18.68	-
Subcritical conditions								
d-limonene	166.46	40	10.0	16.74	1.78	4.17	17.34	3.76
	181.90	100	10.0	14.98	1.70	3.69	15.52	5.81
Ethyl acetate	101.40	40	10.0	15.26	5.23	6.96	17.57	6.22
	114.50	100	10.0	13.11	4.92	6.05	15.25	9.10
Ethyl lactate	118.79	40	10.0	15.36	7.48	12.06	20.91	9.59
	135.59	100	10.0	13.02	7.00	10.43	18.09	11.11
Ethanol	60.66	40	10.0	15.13	8.65	18.69	25.56	15.61
	70.32	100	10.0	12.58	8.03	16.04	21.91	15.53
Supercritical and CXL conditions								
CO_2	48.36	40	30	11.91	4.68	5.12	13.78	12.52
CO_2 + d-limonene (50:50 v/v)	NC	40	30	14.33	3.23	4.65	15.40	7.73
CO_2 + Ethyl Acetate (50:50 v/v)	NC	40	30	13.58	4.95	6.04	15.67	9.25
CO_2 + Ethyl Lactate (50:50 v/v)	NC	40	30	13.64	6.08	8.59	17.22	9.92
CO_2 + Ethanol (50:50 v/v)	NC	40	30	13.52	6.66	11.91	19.21	11.82

NC : Not calculated

Table 2. Scoring of solubility of fucoxanthin, HSP and relative energy difference (RED) of different solvents employed to obtain the radius of interaction (Ro) of Hansen sphere.

Solvent number	Solvent Name	δ_D (MPa$^{1/2}$)	δ_P (MPa$^{1/2}$)	δ_H (MPa$^{1/2}$)	δ_T (MPa$^{1/2}$)	Score	RED
1	Hexane	14.90	0.00	0.00	14.90	4	1.872
2	Dichloromethane	17.00	7.30	7.10	19.82	1	0.427
3	d-limonene	17.20	1.80	4.30	17.82	2	0.998
4	Ethyl acetate	15.80	5.30	7.20	18.15	1	0.243
5	Ethyl lactate	16.00	7.60	12.50	21.68	2	0.998
6	Ethanol	15.80	8.80	19.40	26.52	3	2.303
7	Acetone	15.50	10.40	7.00	19.94	1	0.991
8	Methanol	14.70	12.30	22.30	29.41	3	3.104
9	Water	15.50	16.00	42.30	47.81	5	6.830

Table 3. Evaluation of different bio-based solvents for the selective extraction of fucoxanthin.

HSP		PLE, 10.3 MPa*			Fucoxanthin		Ratio carotenoids/ chlorophylls
Solvent	**Ra**	**T, ºC**	**Yield, %**	**mg g^{-1} extract**	**mg g^{-1} alga**	**Recovery (%)**	**mg g^{-1} extract**
Ethanol	15.61	40	$13.04 \pm 0.26^{c,d,e}$	11.93 ± 0.46^{c}	1.56 ± 0.03^{b}	86.44 ± 1.57^{b}	0.52 ± 0.02^{a}
	15.53	100	24.68 ± 1.15^{g}	6.27 ± 0.01^{a}	1.55 ± 0.08^{b}	85.99 ± 4.19^{b}	0.400 ± 0.002^{a}
Ethyl acetate	6.22	40	6.69 ± 0.33^{b}	25.29 ± 0.58^{f}	$1.69 \pm 0.07^{b,c}$	$93.98 \pm 3.99^{b,c}$	1.22 ± 0.18^{b}
	9.10	100	$12.62 \pm 0.56^{c,d}$	14.65 ± 0.75^{d}	1.85 ± 0.10^{c}	102.64 ± 5.35^{c}	0.55 ± 0.03^{a}
Ethyl lactate	9.59	40	$14.66 \pm 2.33^{d,e}$	11.60 ± 1.18^{c}	$1.69 \pm 0.19^{b,c}$	$93.80 \pm 10.60^{b,c}$	0.54 ± 0.02^{a}
	11.11	100	21.37 ± 0.09^{f}	8.73 ± 0.45^{b}	1.87 ± 0.09^{c}	103.63 ± 5.01^{c}	0.494 ± 0.003^{a}
D-limonene	3.76	40	3.86 ± 0.20^{a}	23.89 ± 1.26^{f}	0.92 ± 0.06^{a}	51.16 ± 3.21^{a}	3.54 ± 0.53^{c}
	5.81	100	15.58 ± 0.01^{e}	11.88 ± 0.55^{c}	1.85 ± 0.08^{c}	102.77 ± 4.64^{c}	$0.77 \pm 0.03^{a,b}$
Benchmark extraction, 24h**							
Acetona	8.64	25	11.24 ± 0.20^{c}	16.02 ± 0.40^{d}	$1.80 \pm 0.03^{b,c}$	100.0**	$0.684 \pm 0.003^{a,b}$

*PLE: 20 min, 1 extraction cycle. **Benchmark extraction: considered as maximum % recovery of fucoxanthin.
In each column, superscripts letters mean groups not statistically different ($p > 0.05$), as analyzed by one-way ANOVA.

Table 4. Kinetic studies on the extraction of fucoxanthin by PLE and CXL using limonene.

PLE kinetic study					
Sample	**Time (min)**	**Cumulative Yield (%)**	**mg FE g^{-1} extract**	**mg chlorophyll g^{-1} extract**	**Ratio carot./ chlor.**
1	20	4.90 ± 0.28	31.80 ± 1.25	10.86 ± 0.27	2.94 ± 0.19
2	30	7.39 ± 0.83	22.63 ± 5.04	9.61 ± 0.30	2.35 ± 0.45
3	40	9.74 ± 1.18	12.85 ± 1.98	7.60 ± 0.30	1.70 ± 0.33
4	50	11.93 ± 1.31	10.83 ± 0.68	9.11 ± 2.04	1.21 ± 0.20
5	60	13.79 ± 1.31	8.96 ± 1.29	9.71 ± 2.55	0.94 ± 0.11
6	70	16.05 ± 1.58	6.21 ± 0.35	7.70 ± 0.69	0.81 ± 0.03
7	80	18.14 ± 1.81	6.51 ± 0.32	9.52 ± 0.56	0.69 ± 0.01
8	90	20.53 ± 1.90	5.59 ± 0.71	9.03 ± 1.29	0.62 ± 0.01
9	100	22.66 ± 1.99	5.81 ± 0.52	9.47 ± 0.92	0.61 ± 0.00
10	110	24.72 ± 2.17	6.66 ± 1.11	10.87 ± 1.48	0.61 ± 0.02
CXL kinetic study					
Sample	**Time (min)**	**Cumulative Yield (%)**	**mg FE g^{-1} extract**	**mg chlorophyll g^{-1} extract**	**Ratio carot./ chlor.**
1	10	3.53 ± 0.65	40.78 ± 2.77	11.09 ± 0.11	3.68 ± 0.21
2	20	5.40 ± 0.19	37.49 ± 0.75	14.96 ± 0.21	2.51 ± 0.09
3	40	7.85 ± 0.48	24.09 ± 1.54	14.27 ± 0.70	1.69 ± 0.02
4	70	11.00 ± 1.02	9.78 ± 0.56	13.21 ± 0.48	0.74 ± 0.07
5	105	14.12 ± 1.42	4.04 ± 1.03	8.17 ± 0.79	0.49 ± 0.08
6	140	17.38 ± 1.58	3.23 ± 2.22	7.63 ± 3.55	0.40 ± 0.10
7	180	21.82 ± 2.33	2.09 ± 0.86	5.02 ± 1.02	0.41 ± 0.09

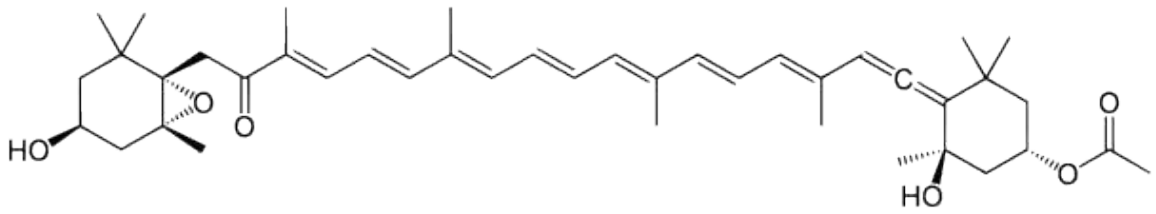
FE: Fucoxanthin equivalent

Table 5. Optimization of fucoxanthin recovery employing limonene and SC-CO_2 + limonene.

HSP		**PLE, 10.3 MPa**				**Fucoxanthin**			**Ratio carotenoids/ chlorophylls**
Solvent	**Ra**	**T, ºC**	**Time, min**	**Cycles**	**Yield, %**	**mg g^{-1} extract**	**mg g^{-1} alga**	**Recovery (%)**	**mg g^{-1} extract**
D-limonene	3.76	40	70	6	14.23 ± 0.84	5.64 ± 0.75	0.80 ± 0.06	44.41 ± 3.30	1.73 ± 0.21
			CXL, 30 MPa						
SC-CO_2 + d-limonene (50:50% v/v)	7.73	50	105	-	27.54 ± 5.39	6.22 ± 1.34	1.67 ± 0.14	92.71 ± 7.84	1.18 ± 0.15

647

648 **Figure 1.**



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652 **Figure 2**

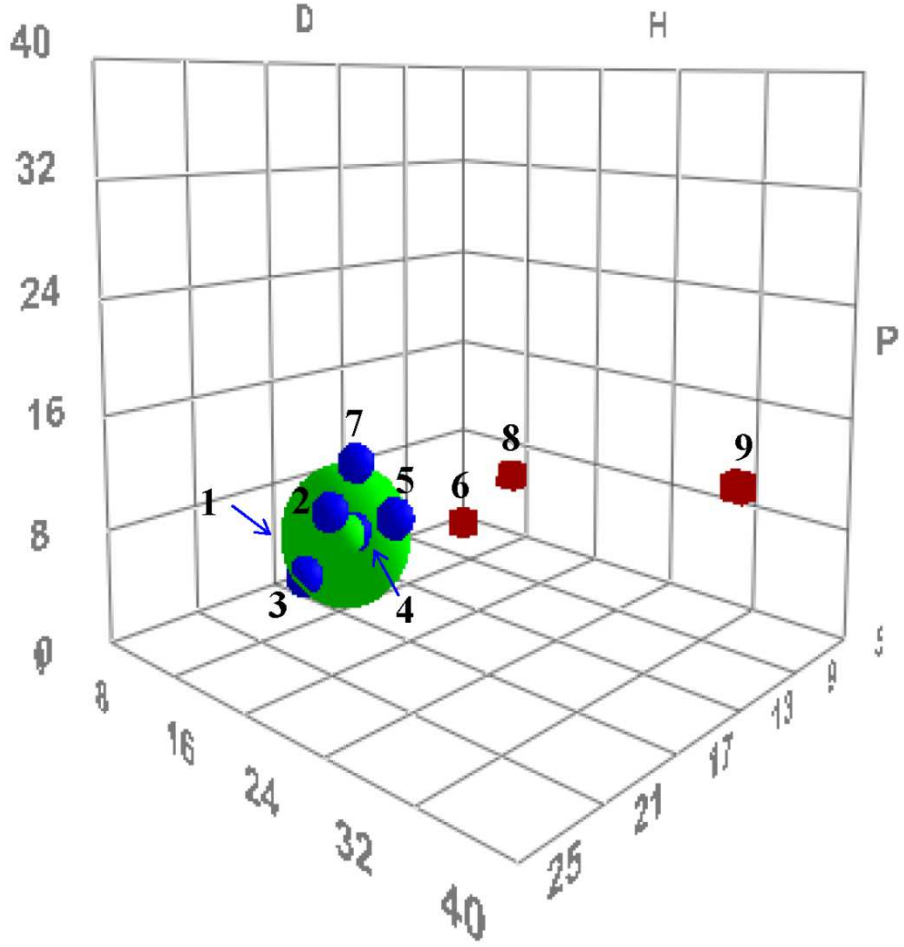

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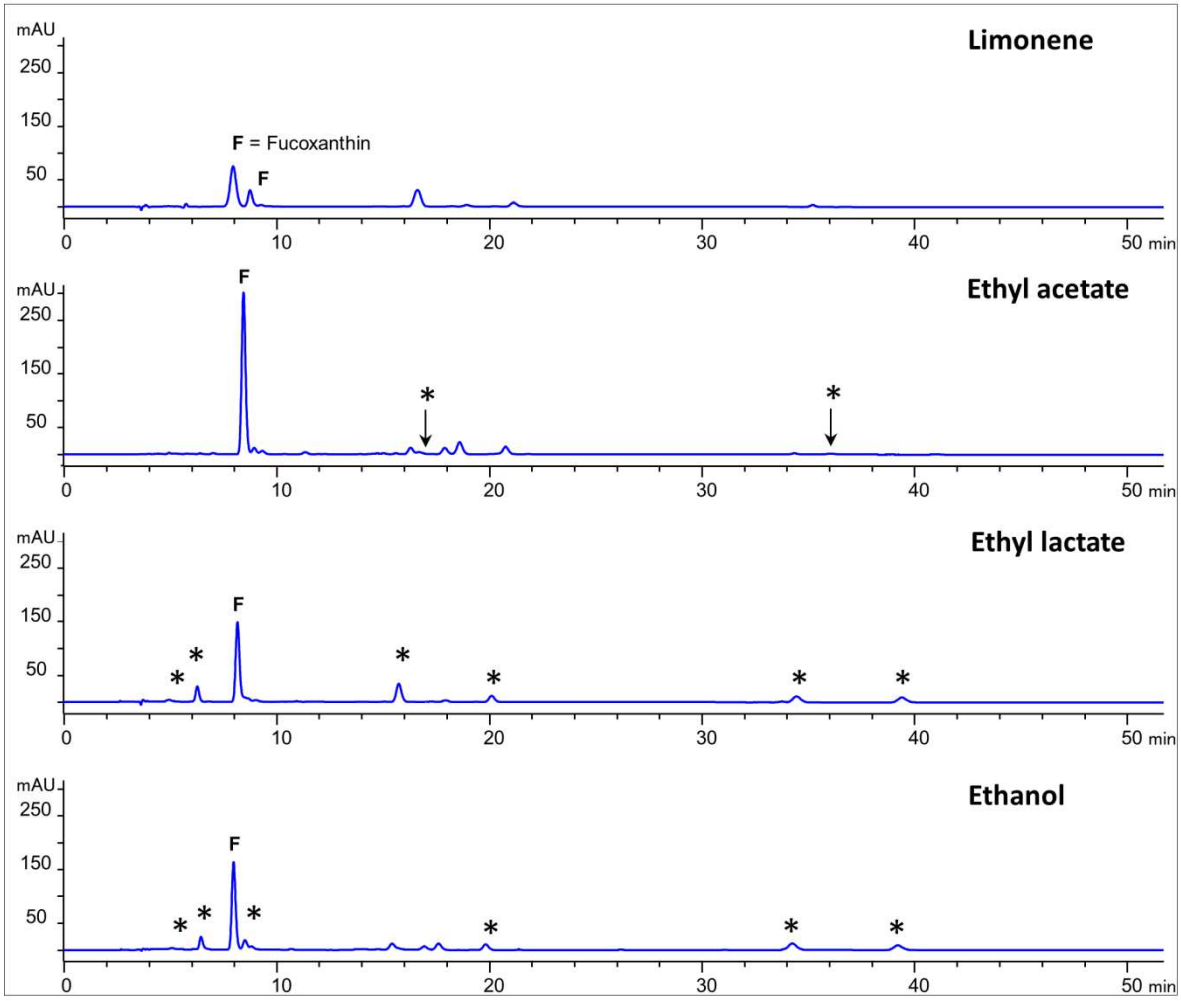
Figure 3


Limonene
mAU
250
150
50
F = Fucoxanthin
F
0
10
20
30
40
50 min
Ethyl acetate
F
*
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Ethyl lactate
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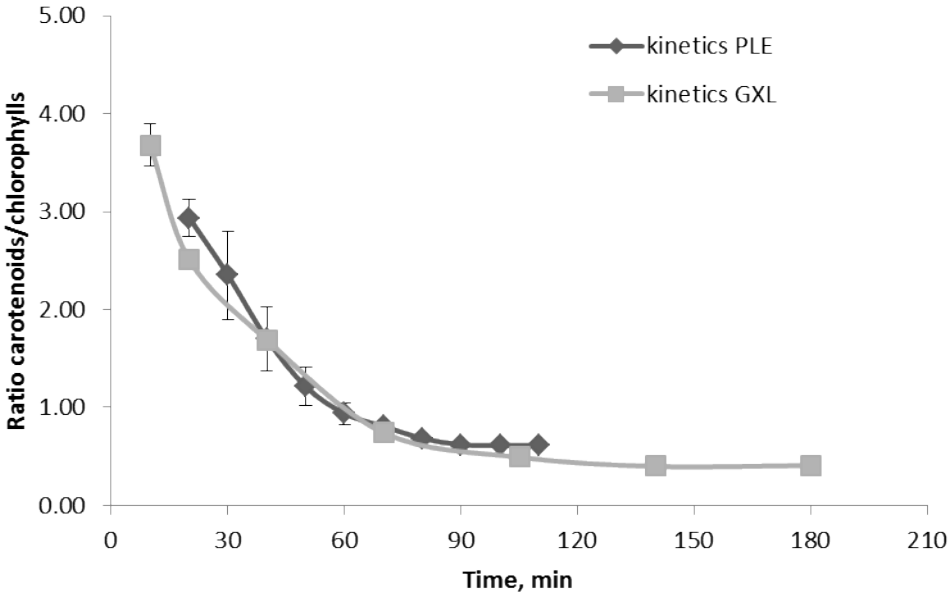
666 **Figure 4**



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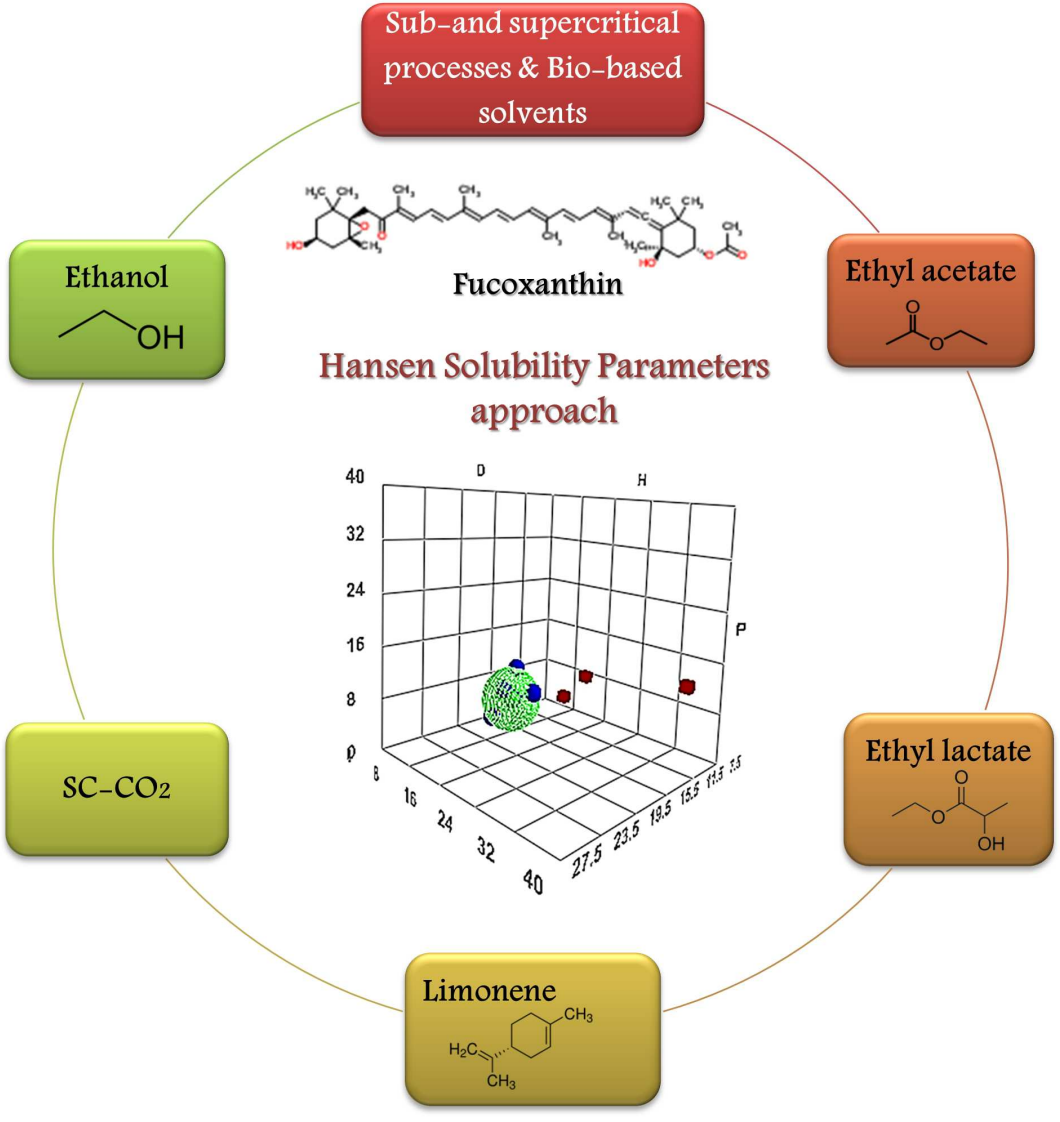
Highlights

- Selective extractions of fucoxanthin from *Phaeodactylum tricornutum* were studied.
- Hansen solubility parameters approach is a useful tool to select bio-based solvents.
- D-limonene was the most selective solvent employing pressurized liquid extraction.
- Dynamic extraction using SC-CO_2+limonene mixture was assessed.
- Ethyl acetate showed good recoveries of fucoxanthin, but it was less selective.


Sub-and supercritical processes & Bio-based solvents
Fucoxanthin
Hansen Solubility Parameters approach
Ethanol
OH
Ethyl acetate
Ethyl lactate
OH
Limonene
CH3
H2C
CH3
SC-CO2
D
H
P
40
32
24
16
8
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CAPÍTULO 5.
DISCUSIÓN GENERAL

5. DICUSIÓN GENERAL

En los últimos años, el laboratorio de Alimentómica (Instituto de Investigación en Ciencias de la Alimentación, CIAL, CSIC-UAM) se ha enfocado en profundizar en el conocimiento de algunos aspectos de los alimentos y la nutrición, tales como su bioactividad, seguridad y calidad, desde una nueva perspectiva global (Alimentómica), empleando para ello herramientas basadas en tecnologías -ómicas tales como transcriptómica, proteómica y metabolómica.

En concreto, las líneas de investigación se han centrado en i) el desarrollo de procesos con fluidos sub- y supercríticos para la obtención de extractos bioactivos de fuentes naturales y en ii) el estudio de sus efectos en la prevención y evolución de una de las enfermedades con mayor incidencia en nuestra sociedad, el cáncer de colon mediante una aproximación alimentómica.

Asimismo, los proyectos recientemente desarrollados han orientado sus esfuerzos hacia la búsqueda de fuentes naturales de compuestos bioactivos que demuestren una potencial actividad antitumoral. Proyectos como "Evaluación alimentómica de la actividad de polifenoles de origen alimentario frente al cáncer de colon empleando modelos *in-vitro* e *in-vivo* (AGL2011-29857-C03-01, 2012-2015) y "Una nueva estrategia para acercar los ensayos *in-vitro* a la realidad en humanos y su aplicación al estudio alimentómico de la bioactividad de compuestos alimentarios" (AGL2014-53609-P, 2015-2018) se han dirigido al estudio alimentómico de la actividad antitumoral de los compuestos fenólicos procedentes de extractos de romero y algas marinas obtenidos mediante el uso de procesos basados en el empleo de fluidos presurizados. La selección de las fuentes naturales y procedimientos de extracción estudiados en esta Tesis Doctoral se han detallado y justificado en el Capítulo 1 de la presente Memoria.

En un primer estudio realizado durante el desarrollo de estos proyectos se evaluó el efecto antiproliferativo de extractos de romero obtenidos mediante líquidos presurizados (agua y etanol, a temperaturas entre 100 y 200 °C) y CO_2 supercrítico. El extracto de romero obtenido mediante SFE en una única etapa empleando CO_2 a 40 °C, 15.0 MPa y 7% (p/p) de etanol como modificador, durante 300 min como tiempo de extracción ("*Single step*"), mostró una actividad

antiproliferativa superior que el resto de extractos en la línea celular de cáncer de colon HT-29, observándose una reducción de la viabilidad de aproximadamente del 45% a las 48 h de incubación con una concentración de extracto de 30 μg/mL (Valdés et al., 2014, 2013). Este extracto se caracterizó por su elevado contenido en ácido carnósico (256 mg/g de extracto) y carnosol (37 mg/g de extracto), lo cual aportó nuevas evidencias sobre la capacidad inhibitoria de la proliferación celular de estos compuestos en la mencionada línea celular. Con el fin de investigar la contribución de estos compuestos mayoritarios en la capacidad del extracto para inhibir la proliferación celular, se evaluó el posible efecto sinérgico, antagónico y aditivo *in vitro* de estos dos diterpenos fenólicos empleando patrones puros, comercialmente disponibles. Los resultados de este estudio demostraron que (i) el ácido carnósico y el carnosol tienen un efecto aditivo a las concentraciones a las que se encuentran en el extracto, (ii) que la suma de ambas actividades explicaría la mayor parte de la actividad del extracto de romero, (iii) pero que, sin embargo, su actividad conjunta es inferior a la observada del extracto, lo cual sugiere que otros constituyentes del extracto podrían estar contribuyendo a la actividad antiproliferativa (Valdés et al., 2014). Estos estudios constituyeron el punto de partida de esta primera parte de la Tesis Doctoral que se centró en el desarrollo de nuevos procedimientos de extracción dirigidos a la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol con mayor actividad antiproliferativa en la línea celular HT-29.

Para cumplir con este objetivo, se plantearon dos enfoques diferentes: 1) el desarrollo de procesos de SFE en dos etapas secuenciales y 2) el fraccionamiento supercrítico antisolvente de extractos hidroalcohólicos de romero obtenidos por PLE.

A pesar de las características bioactivas presentadas por el extracto supercrítico de romero obtenido en una única etapa (Valdés et al., 2014), el uso de etanol como modificador en el proceso de extracción genera un incremento en el rendimiento debido a la co-extracción de compuestos indeseables o menos activos, proporcionando así un extracto químicamente más complejo (Herrero et al., 2010). Tomando este hecho en consideración, se estudió la intensificación de un proceso de extracción más selectivo tipo *batch* (por lotes) a escala piloto, empleando un solo equipo. Se seleccionaron dos condiciones de proceso diferentes:

- Proceso 1: consistió en una primera etapa de extracción a 15.0 MPa, 40°C, con CO_2 puro durante 60 min, para la eliminación de aceites esenciales, preferentemente extraídos a bajas presiones, seguido por una segunda etapa (15.0 MPa, 40°C, con CO_2 y 7% (p/p) etanol como modificador durante 120 min) en las mismas condiciones que el proceso descrito como "*Single step*".
- Proceso 2: consistió en una primera etapa de extracción a 30.0 MPa, 40°C, con CO_2 puro durante 60 min, para la eliminación de de ceras y oleorresina menos polar, seguida por una segunda etapa en las mismas condiciones que la descrita para el proceso 1.

Los rendimientos de extracción obtenidos en las segundas etapas fueron significativamente mayores a los obtenidos en las primeras etapas, debido al empleo de etanol como modificador. Al combinar los rendimientos de extracción de las etapas 1 y 2 del proceso 2 se obtuvo un rendimiento de extracción mayor (7.4% p/p) que el obtenido en el proceso "*Single step*" (6.5 % p/p), en un menor tiempo de extracción (180 *vs* 300 min) y empleando un volumen reducido de modificador (etanol). En cuanto a la fracción enriquecida en compuestos fenólicos, la segunda etapa del Proceso 2 alcanzó un máximo enriquecimiento de CA (403 mg/g extracto) y CS (45 mg/g extracto).

Con el objeto de profundizar en el carácter antiproliferativo de los extractos se llevó a cabo el cálculo del porcentaje de crecimiento a partir de los datos obtenidos de los ensayos *in vitro* con la línea celular HT-29. Los resultados obtenidos indicaron que utilizando la misma concentración de extracto (30 μg/mL), los extractos obtenidos en la etapa 2 de los dos procesos inducían efectos citotóxicos, a diferencia de la acción citostática leve e intensa producida por los extractos obtenidos en la etapa 1 de ambos procesos y mediante SFE en una única etapa, respectivamente. Además, un aspecto importante a considerar de los procesos en dos etapas es la posibilidad de valorización de las dos fracciones obtenidas. En este sentido, los extractos de las primeras etapas (Proceso 1 y 2) poseían elevadas concentraciones de terpenoides volátiles (monoterpenos, terpenos oxigenados y sesquiterpenos, principalmente) que proporcionan un valor añadido a estos co-productos por sus potenciales aplicaciones cosméticas, antioxidantes,

saborizantes, medicinales, antimicrobianas (Jiang et al., 2011; Santoyo et al., 2005), o incluso para el desarrollo de envases activos para la industria de alimentos (Sirocchi et al., 2016).

Aunque la estrategia de extracción desarrollada demostró ser una buena opción para el enriquecimiento de ácido carnósico y carnosol, así como para mejorar la potencia inhibidora de la proliferación celular de los extractos, los rendimientos de extracción obtenidos por esta técnica fueron limitados. Por otro lado, el empleo de SFE (con etanol como modificador) no permitía la extracción de otros compuestos bioactivos con mayor polaridad presentes en el romero, como el ácido rosmarínico. Para mejorar estos aspectos se desarrolló una nueva estrategia de extracción a escala analítica, susceptible de ser posteriormente implantada a escala piloto.

Como se ha mencionado en el primer capítulo de esta Memoria, la extracción con líquidos presurizados es uno de los procesos verdes más empleados para la extracción de compuestos bioactivos. En nuestro laboratorio, esta técnica de extracción se ha utilizado ampliamente para obtener extractos de alto valor añadido con elevados rendimientos, permitiendo acortar los tiempos de extracción y reducir la cantidad de disolvente con respecto al soluto, en comparación con las técnicas tradicionales de extracción a presión atmosférica (Herrero et al., 2015, 2010; Plaza et al., 2012; Rodríguez-Meizoso et al., 2010)

Partiendo de los resultados obtenidos previamente (Herrero et al., 2010; Valdés et al., 2013), se empleó PLE utilizando una mezcla de etanol:agua (80:20 % v/v) a 150 °C para conseguir un extracto rico en las dos familias de compuestos presentes en las hojas de romero: i) diterpenos fenólicos, como ácido carnósico y carnosol, y ii) ácidos fenólicos, principalmente, ácido rosmarínico. El rendimiento de extracción fue del 39.9% (p/p), demostrando ser altamente superior al obtenido en el proceso de SFE previamente propuesto, debido principalmente a la selectividad, relativamente baja, de la técnica de extracción PLE.

La integración de este proceso con el fraccionamiento supercrítico antisolvente (PLE + SAF) fue la siguiente estrategia propuesta, ya que presenta ventajas para la separación y enriquecimiento de diferentes familias de compuestos, con diferentes bioactividades. Puesto que son varios los factores que afectan el comportamiento de los compuestos en la mezcla que se pretende separar, fue necesario llevar a cabo un estudio del equilibrio de fases ternario de

etanol-SC-CO_2-agua para evaluar teóricamente la selectividad del proceso y seleccionar las condiciones más favorables de operación. Los coeficientes de reparto del *refinado* (fase "rica" en agua) y del *extracto* (fase "rica" en CO_2), y a su vez, la selectividad del proceso, están altamente influenciados por i) la presión, ii) el porcentaje de masa de agua en el extracto hidro-etanólico de PLE de alimentación del sistema SAF, y iii) la relación másica de alimentación de extracto PLE a SC-CO_2. De este modo, se optimizaron estos tres factores empleando un diseño experimental a escala laboratorio.

Los resultados mostraron que la fracción obtenida en el *refinado* se enriqueció (6,76% p/p) en RA (el más polar de los compuestos estudiados) cuando el fraccionamiento se llevó a cabo a 30,0 MPa, 20% (v/v) de agua en la mezcla y 0.025 de relación de flujo másico de extracto PLE a SC-CO_2 suponiendo un aumento de 2.7 veces mayor comparada con la concentración encontrada en el extracto original de PLE. Además, en estas condiciones de fraccionamiento se obtuvo el contenido más alto de compuestos fenólicos (341 mg GAE/g extracto) y la mayor actividad antioxidante (4.16 mM equivalentes de Trolox /g extracto); sin embargo, estos extractos no presentaban un efecto antiproliferativo importante dado que tan solo indujeron una reducción del 18% de la viabilidad celular después de 24 h de incubación. Por otro lado, tal y como había predicho el estudio teórico de la selectividad, el mayor contenido de ácido carnósico + carnosol se obtuvo a 10.0 MPa, 50% (v/v) de agua en la solución de alimentación y 0.025 de relación de flujo másica de extracto inicial de PLE/SC-CO_2, logrando valores de 478.1 mg/g en la fracción *extracto*, con una recuperación de estos dos compuestos del 78.5% (p/p). La incubación de las células HT-29 con este extracto durante 24 horas redujo su viabilidad en un 83%, indicando un claro efecto citotóxico en esta línea celular.

Una de las ventajas asociadas al proceso integrado PLE + SAF es la posible valorización de las dos fracciones (*refinado* y *extracto*). En este sentido, aunque la fracción *refinado* no produjo una inhibición de la proliferación celular significativa en las células HT-29, esta fracción podría valorizarse por su concentración en ácido rosmarínico y por tanto, por su importante actividad antioxidante (Muñoz-Muñoz et al., 2013). En relación a la fracción *extracto*, a pesar de que la concentración máxima de ácido carnósico fue menor que en el proceso de SFE en dos etapas secuenciales del estudio previo (346 vs. 403 mg/ g extracto, respectivamente), la suma

global de las concentraciones de ácido carnósico y carnosol fue superior. Asimismo, el extracto optimizado presentó valores de viabilidad celular menores con relación al proceso SFE en dos etapas (39%), cuando fueron expuestos al mismo tiempo de incubación (24 h). Este estudio demostró, en las diferentes condiciones estudiadas, que no existe una dependencia directa entre la relación molar de CA/CS en los extractos y su potencia inhibidora de la proliferación celular, sugiriendo que existen otros compuestos afines a la mezcla SC-CO_2-etanol que podrían contribuir a la actividad antiproliferativa total observada.

A continuación, la optimización de las condiciones a nivel de laboratorio, permitieron llevar a cabo un aumento de la escala de extracción, para lo cual se modificó un equipo comercial de SFE y se implementó un sistema SAF a escala piloto. Se seleccionaron tres condiciones de extracción diferentes (incluyendo la condición óptima) para su validación y estudio.

Finalmente, se compararon los cuatro procesos desarrollados en la presente Tesis Doctoral, con el fin de evaluar su potencial para obtener extractos de romero (a escala piloto) con actividad antiproliferativa en dos líneas celulares de cáncer de colon con un patrón mutacional distinto, HT-29 y HCT116. En la Tabla 5.1, se presenta un resumen de las condiciones empleadas en dichos procesos: (1) PLE a escala de laboratorio; (2) extracción SFE en una sola etapa a escala piloto (SFE1); (3) Proceso 2, etapa 2 del SFE secuencial en dos etapas a escala piloto (SFE2); y (4) PLE + SAF a escala piloto (SAF1-3).

A su vez, en la Tabla 5.2 se presenta un resumen de los resultados más relevantes de este estudio comparativo. Como se puede observar, el proceso de extracción por PLE proporcionó el rendimiento de extracción más alto (38,46 g/100 g de hojas de romero seco) debido, principalmente, a la polaridad del disolvente, que implica una menor selectividad. Por otra parte, a pesar del uso de modificador y diferentes condiciones de extracción por SFE, los rendimientos de extracción de los procesos SFE1 y SFE2 fueron bajos, no siendo significativamente diferentes entre ellos. Los rendimientos de extracción de los procesos SAF (medidos en términos de % de recuperacion) fueron mayores cuando la relación de flujo de alimentación a SC-CO_2 fue la más baja. Así, se recuperó alrededor del 21% (p/p) de extracto

seco de PLE empleando las condiciones del proceso SAF1, corroborando los resultados obtenidos a escala laboratorio.

Tabla 5.1. Condiciones experimentales empleadas para la obtención de extractos de romero

Proceso	Presión (MPa)	Temp. (°C)	Relación de Flujo másico PLE/SC-CO_2	%Etanol (p/p)	Tiempo de extracción (min)
PLE	10,3	150	-	76,0**	20
SFE única etapa (SFE1)	15,0	40	-	7,0*	300
SFE dos etapas (SFE2)	30,0	40	-	0	60
	15,0	40	-	7,0*	120
SAF1	10,0	40	0,025	44,2**	180
SAF2	10,0	40	0,100	44,2**	60
SAF3	10,0	40	0,025	76,0**	180

*Etanol como modificador; **etanol en la mezcla disolvente de extracción.

Aunque se lograron mayores rendimientos de extracción utilizando PLE, el extracto obtenido presentó la actividad antiproliferativa más baja sin observarse un efecto citotóxico claro en las concentraciones estudiadas. Por otro lado, los extractos obtenidos utilizando el procedimiento PLE + SAF proporcionaron los extractos de romero más activos, mostrando valores de citotoxicidad (LC50[3]) entre 11,2 y 12,4 μg/mL en la línea celular HCT116, y entre 21,8 y 31,9 μg/mL en la línea HT-29. En general, los extractos activos de romero se caracterizaron por contener ácido carnósico y carnosol a concentraciones superiores a 263,7 y 33,9 mg/g de extracto, respectivamente. Por otro lado, es interesante señalar que, en este estudio, no se pudo establecer una relación directa entre el contenido de ácido carnósico + carnosol y la actividad antiproliferativa de los extractos. Esta observación motivó un análisis químico más detallado de los compuestos presentes en los extractos con el fin de obtener mayor información e identificar compuestos que pudieran contribuir a la actividad antiproliferativa de los extractos más activos. La caracterización química permitió identificar compuestos tales como rosmaridifenol y safficinolide, y cuya presencia exclusiva en los extractos más activos (SAF) sugiere que podrían contribuir al efecto antiproliferativo de los

[3] LC50 (50% Lethal Concentration): se define como la concentración del compuesto que causa la muerte del 50% de la población célular con respecto al control no tratado.

extractos. Por tanto, el estudio de su posible actividad antiproliferativa puede ser muy interesante en trabajos futuros.

Tabla 5.2. Rendimiento de extracción (% peso seco), concentración de ácido carnósico (CA) y carnosol (CS) y valores de LC50 en las líneas celulares de cáncer de colon estudiadas, HT-29 y HCT116.

Muestra	Rendimiento (g/100 g muestra)	mg/g extracto*			HT-29 µg/g extracto**		HCT116 µg/g extracto**	
		CA	CL	CA+CL	LC50 24h	LC50 72h	LC50 24h	LC50 72h
PLE	38,46 ± 1,99 ^{d}	104,96 ± 1,87^{c}	10,66 ± 0,06^{c}	115,63 ± 1,93^{c}	-	-	-	-
SFE1	6,74 ± 0,33 ^{a}	355,73 ± 17,44^{a}	39,23 ± 1,91^{a}	394,97 ± 17,91^{a}	38,93 ± 4,17^{c}	46,71 ± 3,02^{c}	29,11 ± 0,34^{c}	31,64 ± 2,08^{c}
SFE2	4,68 ± 0,01 ^{a}	443,28 ± 4,09^{b}	88,88 ± 5,41^{b}	532,16 ± 4,73^{b}	24,80 ± 2,03^{a,b}	29,63 ± 0,57^{a,b}	26,35 ± 0,25^{b}	23,70 ± 3,85^{b}
SAF1	20,65 ± 1,74 ^{c}	386,49 ± 1,98^{d}	64,24 ± 4,04^{d}	450,73 ± 4,70^{d}	31,93 ± 2,26^{b,c}	31,18 ± 0,83^{b}	12,39 ± 1,51^{a}	14,69 ± 1,04^{a}
SAF2	5,74 ± 0,45 ^{a}	354,92 ± 7,63^{a}	44,04 ± 0,73^{a}	398,96 ± 8,00^{a}	29,35 ± 1,37^{a,b}	29,81 ± 2,05^{a,b}	12,40 ± 0,84^{a}	15,64 ± 0,64^{a}
SAF3	15,36 ± 1,41 ^{b}	263,70 ± 8,07^{e}	33,89 ± 0,51^{e}	297,59 ± 8,57^{e}	21,76 ± 1,95^{a}	24,35 ± 0,24^{a}	11,17 ± 1,13^{a}	14,71 ± 0,32^{a}

*Concentraciones expresadas como media ± SD; **Concentraciones expresadas como media ± SE. En cada columna, los superíndices significan grupos estadísticamente diferentes ($p < 0,05$), según se analizan por ANOVA unidireccional

Las algas marrones son otra de las fuentes empleadas en la presente Tesis Doctoral. En concreto, se ha escogido el estudio del principal grupo de compuestos fenólicos presente en algas marrones, los florotaninos, que como se ha expuesto en el Capítulo 1 de la presente Memoria, suscitan gran interés como ingredientes funcionales.

Previamente a la realización del presente estudio, se llevó a cabo el desarrollo de un método de separación y caracterización de florotaninos a partir de un alga marrón empleando la técnica LC × LC–DAD–MS/MS en el laboratorio de Alimentómica. El método de extracción para el análisis de estos compuestos fenólicos, implicó el empleo de protocolos de purificación (extracción sólido–líquido) en los que predominaban disolventes orgánicos y varias etapas (Montero et al., 2014). Este hecho motivó la búsqueda de estrategias de extracción más limpias y eficaces, donde además se viera favorecida la extracción de estos compuestos.

Como se ha descrito anteriormente, la pared celular de las algas marrones está compuesta principalmente por polisacáridos (ácido algínico, alginatos y fucanos), cuya presencia limita la accesibilidad de los compuestos bioactivos, reduciendo así la eficiencia de extracción durante la aplicación de métodos tradicionales (Balboa et al., 2013). Además, los florotaninos pueden encontrarse naturalmente formando complejos con proteínas estables (Stern et al., 1996). Teniendo en consideración estas premisas, el uso de enzimas (proteasas o carbohidrasas) como pretratamiento a la extracción con fluidos presurizados se propuso como una opción que podría constituir un proceso verde integrado (EAE + PLE) interesante para obtener una mayor cantidad de compuestos fenólicos bioactivos a partir del alga marrón *Sargassum muticum*, procedente de la región de Bretaña, Francia.

Los resultados obtenidos después de llevar a cabo el estudio de distintas condiciones de hidrólisis enzimática (tiempos de reacción y relaciones enzima/alga) previa a la extracción con líquidos presurizados, mostraron que el contenido en fenoles totales, tanto en la fracción de hidrolizado (6.01–10.3 mg GAE/g extracto) como en el extracto PLE del residuo remanente tras el proceso de hidrólisis (21.9–39.2 mg GAE/g extracto), no era superior al obtenido mediante extracción con PLE sin tratamiento enzimático previo (47.6 mg GAE/g extracto). Estos resultados indicaron la necesidad de llevar a cabo la optimización del proceso de PLE empleando un diseño experimental factorial, y estudiando así el efecto de las variables i)

temperatura de extracción y ii) composición de la mezcla disolvente de extracción. Se pudo determinar que las mejores condiciones de extracción para maximizar las variables de respuesta seleccionadas (rendimiento de extracción, contenido de fenoles totales, contenido de florotaninos totales y actividad antioxidante) incluyeron el uso de una mezcla etanol: agua (95:5 % v/v) a 160 °C.

Estas condiciones óptimas sirvieron como base para el estudio del efecto de la ubicación geográfica en el contenido de florotaninos de 13 muestras de *Sargassum muticum* recolectadas a lo largo de la costa Atlántica Europea, incluyendo localizaciones en España, Portugal, Irlanda, Francia y Noruega. En este estudio se concluyó que las muestras de algas recolectadas en una de las localizaciones de Noruega presentaba los mayores contenidos en fenoles totales (148.97 mg GAE/g extracto) y florotaninos (5.12 mg PGE/ g extracto). Incluso teniendo en cuenta la elevada concentración en florotaninos de los extractos, fue necesario el uso de protocolos de purificación para su caracterización química y funcional. La identificación tentativa, empleando el método previamente desarrollado utilizando LC × LC-DAD-MS/MS (Montero et al., 2014) indicó que el extracto obtenido en esta localización de Noruega presentaba predominantemente florotaninos del tipo fuhaloles, hidroxifuhaloles y floretoles, y que aquellos que contenían entre 4 y 7 unidades de floroglucinol (PGU) eran los más abundantes. Posteriormente, se observó que este extracto, purificado y enriquecido en florotaninos (11.73 mg PGE/g extracto), ejercía un efecto citotóxico en las células de cáncer HT-29 a bajas concentraciones (LC50= 53.50 μg/mL) exhibiendo la máxima actividad inhibitoria después de 24 h de incubación.

A pesar de estos prometedores resultados, la etapa de purificación posterior a los procesos de extracción y previa a caracterización química y funcional continuaba siendo necesaria debido a la complejidad de los extractos crudos que contienen, entre otros, compuestos mayoritarios tales como lípidos, proteínas y carbohidratos, que deben ser eliminados con el fin de obtener extractos enriquecidos en florotaninos. En este sentido, para lograr una extracción más selectiva dirigida hacia la recuperación de florotaninos, se abordó el estudio de los parámetros de solubilidad de Hansen como una herramienta de diseño para seleccionar los disolventes más selectivos que, además, pudieran reemplazar/eliminar el uso de disolventes

contaminantes para la extracción de ingredientes bioactivos (Figura 5.1). Aunque este enfoque teórico se ha estudiado desde los años 50, solo recientemente se ha demostrado su versatilidad en la obtención de extractos naturales (King, 2014; Li et al., 2014; Srinivas et al., 2009). Con el fin de conducir el proceso hacia la extracción de los compuestos diana del alga marrón *Cystoseira abies-marina* (procedente del Banco Español de Algas, Gran Canaria), se evaluaron tres procesos de extracción, de los que, el uso de PLE empleando acetona:agua como disolvente de extracción (70:30 % v/v, 100 °C, 10.3 MPa, 20 min) demostró ser el más eficaz. Posteriormente, el extracto resultante se purificó como descrito en la figura 5.1 y se caracterizó químicamente (LC × LC-MS/MS, Montero et al., 2014), pudiéndose identificar tentativamente como compuestos más abundantes los florotaninos heptafloretol, heptafucol o heptafucofloretol (7PGU).

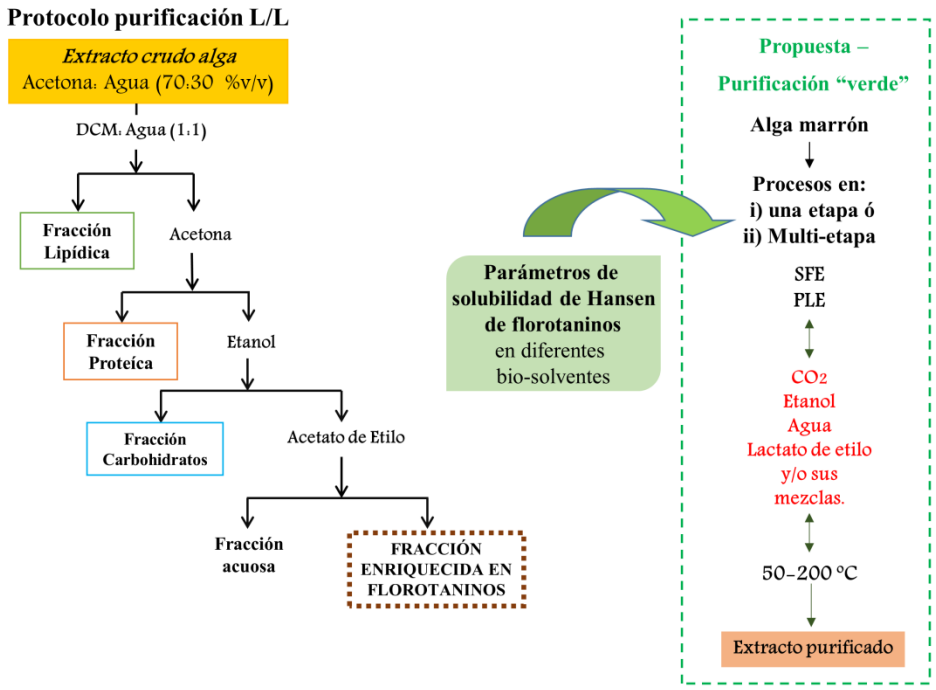

Figura 5.1. Estrategia de purificación de florotaninos propuesta.

Debido a la limitada información acerca de estos compuestos, la predicción de sus propiedades físico-químicas y de sus parámetros de solubilidad se realizó empleando métodos de contribución de grupos de tercer orden a partir de sus estructuras moleculares utilizando

reglas aditivas. De esta forma, se estimaron sus parámetros de solubilidad en los disolventes alternativos seleccionados en estado subcritico (etanol, agua, lactato de etilo) y supercrítico (CO_2 + etanol). Con el fin de evaluar teóricamente la selectividad del disolvente se empleó el parámetro *Ra*, que indica la diferencia en la solubilidad entre el bio-disolvente subcrítico y supercrítico y los solutos (heptafloretol y heptafucol) (Figura 5.2). Así, cuanto más bajo es el valor del parámetro *Ra*, mejor es el disolvente para el soluto. De acuerdo con este enfoque teórico, se demostró que el etanol puro a baja temperatura (25°C) era el disolvente más adecuado para solubilizar estos compuestos fenólicos, ya que proporcionó el valor de *Ra* más bajo, en comparación con los otros disolventes verdes.

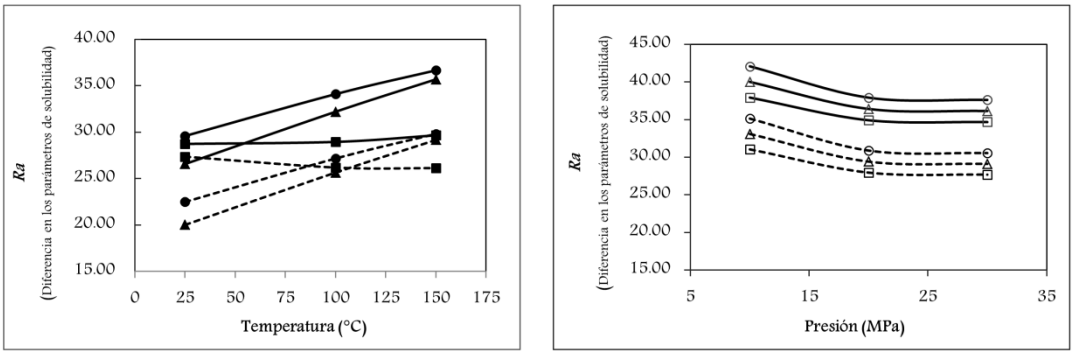

Figura 5.2. Influencia de la temperatura y la presión en el parámetro *Ra* para heptafloretol y heptafucol en diferentes (a) disolventes subcriticos a 10,3 MPa y (b) disolventes supercríticos a 40 °C. (●) Lactato de Etilo, (▲) Etanol, (■) Agua, (○) CO_2+EtOH (70:30 %v/v), (△) CO_2+EtOH (60:40 %v/v), (□) CO_2+EtOH (50:50 %v/v). Línea discontinua (- - -), Heptafloretol; línea continua (---), Heptafucol

En relación a los resultados experimentales, se observó que el etanol puro a 100°C en estado subcrítico (10.3 MPa) presentaba la mayor selectividad hacia los florotaninos (6.7 mg PGE/g extracto) en una sola etapa de extracción. Estos resultados son similares a los obtenidos empleando la mezcla acetona: agua (70:30 % v/v) a 100°C (6.4 mg PGE/g extracto) (disolvente tradicional de extracción), lo que indica que el enfoque teórico empleado es una herramienta útil para la selección de un bio-disolvente adecuado.

Por otra parte, la diferencia entre los resultados teóricos y los experimentales en relación a la temperatura de extracción empleando etanol (25 °C vs. 100 °C) se debe a los fenómenos de

transporte que tienen lugar durante el proceso de extracción y que no se consideran en el enfoque de los parámetros de solubilidad de Hansen.

La recuperación de compuestos bioactivos de fuentes naturales como las algas puede llevarse a cabo empleando también procesos multi-etapa, donde además se pueden recuperar otras fracciones con valor añadido (PUFAs, fitoesteroles, fucanos, carotenoides, proteínas, etc.), bajo el nuevo concepto de biorrefineria. En este sentido, nuestro grupo de investigación participa en el proyecto Europeo MIRACLES - Multi-product Integrated bioRefinery of Algae: from Carbon dioxide and Light Energy to high value Specialties (KBBE.2013.3.2-02, 2013-2017), cuyo objetivo es el desarrollo de procesos de biorrefinería integrada de microalgas para la obtención de múltiples productos con valor añadido, para su aplicación tanto en alimentación humana como animal (acuicultura, principalmente), así como en otros productos no alimentarios. En el marco del desarrollo de este proyecto, la participación de nuestro grupo de investigación se ha centrado en el desarrollo de procesos verdes de extracción para la obtención de compuestos bioactivos a partir de algunas variedades de microalgas entre ellas, *Phaeodactylum tricornutum*. Como se ha comentado en el Capítulo 1, esta microalga es una importante potencial fuente de fucoxantina.

De acuerdo a los resultados previos y teniendo en cuenta que la selección de disolventes es un factor clave en el desarrollo de procesos eficientes y selectivos para la extracción de compuestos bioactivos a partir de fuentes naturales, se empleó la estimación teórica de los parámetros de solubilidad de Hansen de fucoxantina en bio-disolventes para su extracción selectiva a partir de *P. tricornutum*. La aplicación de este enfoque teórico redujo notablemente la lista de bio-disolventes adecuados para su extracción a los siguientes: i) acetato de etilo, ii) lactato de etilo, ii) d-limoneno y iv) etanol. Además, se estimaron los parámetros de solubilidad de fucoxantina en SC-CO_2 y mezclas de SC-CO_2 y estos bio-disolventes, como una opción de disolventes limpios que pueden emplearse para su extracción. Los resultados teóricos revelaron que el d-limoneno a 40ºC era el disolvente más adecuado para la extracción de fucoxantina, disminuyendo su solubilidad (según el enfoque teórico de los HSP) en el siguiente orden: d-limoneno, acetato de etilo, lactato de etilo, SC-CO_2 + d-limoneno y etanol. Después de obtener la predicción teórica, los resultados experimentales empleando PLE (un ciclo de extracción,

10.3 MPa, 20 min) para la extracción de fucoxantina mostraron que, efectivamente, el disolvente más selectivo era el d-limoneno, utilizado a 40 °C como temperatura de extracción; este extracto presentó una relación de concentración carotenoides/clorofilas de 3.53, corroborando así la predicción teórica y la tendencia de solubilidad descrita por los HSP para los demás bio-disolventes. No obstante, en términos de rendimiento de extracción, el d-limoneno fue el bio-disolvente que proporcionó el valor más bajo (0,92 mg fucoxantina (FX)/g alga), mientras que otros disolventes tales como el lactato de etilo y el acetato de etilo mejoraron las recuperaciones de fucoxantina (1.69 mg FX/g alga, para ambos casos) a costa de una disminución notable de la selectividad del proceso de extracción (0.54 y 1.22, respectivamente). En consecuencia, la extracción de fucoxantina empleando los bio-disolventes presentó un dilema a considerar en el desarrollo de procesos de extracción: la elección entre una mayor selectividad o una mejor recuperación.

En este sentido, vale la pena mencionar las dos posibilidades que se desprenden de los resultados de este estudio para la extracción de fucoxantina a partir de microalgas: (1) extracción selectiva de 50% de fucoxantina de la biomasa usando extracción estática de PLE con d-limoneno a bajas temperaturas (40 °C), o (2) extracción total de fucoxantina presente en la biomasa junto con otros compuestos co-extraídos, empleando extracción estática de PLE con acetato de etilo o extracción dinámica utilizando SC-CO_2 + d-limoneno.

CAPÍTULO 6.
CONCLUSIONES

6. CONCLUSIONES

A continuación, se mencionan las conclusiones más relevantes que se derivan de la presente Tesis Doctoral, divididas de acuerdo a los objetivos específicos planteados para cada fase de trabajo:

Fase I. Uso de fluidos presurizados para la obtención de extractos de romero enriquecidos en ácido carnósico y carnosol.

1. El procedimiento de extracción supercrítica en dos etapas a escala piloto mejoró la recuperación de ácido carnósico y carnosol (aprox. 450 mg/g extracto), obteniendo extractos más activos en células de cáncer de colon HT-29, en tiempos de procesamiento más cortos en comparación con el proceso de extracción supercrítico en una sola etapa.
2. La optimización del proceso integrado PLE + SAF a escala laboratorio, demostró su potencial para la separación y obtención de dos fracciones, una enriquecida en ácido rosmarínico *(refinado)* y otra en ácido carnósico y carnosol *(extracto),* a diferentes condiciones de fraccionamiento.
3. El *extracto* obtenido a 10.0 MPa, 50% (v/v) de agua en el extracto PLE utilizado como alimentación del sistema SAF y una relación de flujo másico de extracto PLE/SC-CO_2 de 0.025, presentó el mayor contenido de carnósico y carnosol (aprox. 480 mg/g extracto) y la mayor actividad antiproliferativa, reduciendo más del 80% de la viabilidad de las células de cáncer de colon HT-29 a las 24 h de tratamiento.
4. El estudio comparativo de los cuatro procesos verdes de extracción desarrollados y la evaluación de su potencial para obtener extractos de romero con actividad antiproliferativa *in vitro* en dos líneas celulares de cáncer de colon (HT-29 y HCT116), demostró que:

4.1 Aunque se obtuvieron mayores rendimientos de extracción utilizando la técnica de extracción PLE (38,46%, p/p), este extracto proporcionó la actividad antiproliferativa más baja, sin observarse efectos citotóxicos a las concentraciones estudiadas.

4.2 Los extractos obtenidos utilizando el procedimiento PLE+SAF (SAF1-3) proporcionaron los extractos de romero más activos en ambas líneas celulares de cáncer de colon.

4.3 En general, los extractos más activos de romero se caracterizaron por presentar concentraciones de ácido carnósico y carnosol superiores a 263,7 y 33,9 mg/g de extracto, respectivamente.

4.4 Una falta de correlación positiva entre la potencia del extracto y el contenido de CA y CS, sugiere que otros componentes del extracto identificados tentativamente (rosmaridifenol y sufficinolide) podrían contribuir al efecto antiproliferativo observado de los extractos de romero.

Fase II. Obtención de extractos ricos en polifenoles y carotenoides a partir de algas marinas empleando fluidos presurizados

5. El proceso de PLE proporcionó resultados equivalentes a los obtenidos empleando un pretratamiento de hidrólisis enzimática + PLE para el aislamiento de florotaninos a partir del alga marrón *Sargassum muticum.*
6. La optimización del proceso de PLE concluyó que las mejores condiciones para maximizar el rendimiento de extracción de compuestos fenólicos, de florotaninos y su actividad antioxidante, eran: 160 °C y 95% (v/v) de etanol en la mezcla disolvente.
7. Las muestras de *Sargassum muticum* recolectadas en las localizaciones de la costa de Noruega presentaron el mayor contenido de florotaninos, las cuales, a su vez, mostraron un efecto citotóxico significativo en la línea celular de cáncer de colon HT-29, en concentraciones del intervalo micromolar.

8. La estimación de los parámetros de solubilidad de Hansen de heptafloretol y heptafucol, los florotaninos más abundantes en el alga *Cystoseira abies-marina*, mostró que etanol puro era el disolvente más selectivo para su extracción, reemplazando la mezcla acetona/agua usualmente empleada para tal fin.
9. El uso del enfoque de la estimación teórica y experimental de los parámetros de solubilidad de fucoxantina en 5 bio-disolventes, demostró que d-limoneno empleado a 40 °C durante una etapa de extracción de PLE, era el disolvente más selectivo para la obtención de extractos ricos en este carotenoide a partir de *P. tricornutum*.

En definitiva, se puede concluir que la presente Tesis Doctoral ha contribuido a generar el conocimiento necesario para el desarrollo de procesos sostenibles, basados en la química verde y el empleo de fluidos comprimidos, encaminados a la extracción de compuestos con actividad biológica a partir de diferentes fuentes naturales.

7. BIBLIOGRAFÍA

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8. ANEXOS

ANEXO A. CURRICULUM VITAE

Andrea del Pilar Sánchez Camargo es Ingeniera Química por la Universidad Nacional de Colombia (2006). A la culminación de sus estudios de grado, se desempeñó como Ingeniera de Producción para una empresa del sector de grasas y aceites comestibles y como Ingeniera de Calidad en una trasnacional farmacéutica (2006-2007). Posteriormente, se trasladó a Brasil con el fin de desarrollar sus estudios de maestría en Ingeniería de Alimentos en la Universidad Estadual de Campinas, gracias a una beca de estudios concedida por la Fundación para el Apoyo a la Investigación del Estado de Sao Paulo (FAPESP) (2008-2010). Durante la realización de estos estudios, Andrea adquirió un amplio conocimiento sobre técnicas verdes de extracción de compuestos bioactivos a partir de subproductos de la industria acuícola, que derivaron en varios trabajos científicos publicados en revistas del campo de ciencia y tecnología de alimentos.

Posteriormente, regresa a su país de origen para desempeñarse como coordinadora de proyectos de I+D desarrollados para una compañía del sector de ingredientes y aditivos alimentarios en asociación con el Instituto de Ciencia y Tecnología Alimentaria (INTAL); y como líder de investigación en la extracción de compuestos naturales con propiedades antioxidantes y antimicrobianas (2010-2013).

En abril de 2013, comienza sus estudios doctorales en Biología y Ciencias de la Alimentación en la Universidad Autónoma de Madrid gracias a una beca concedida por el Departamento Administrativo de Ciencia, Tecnología e Innovación (COLCIENCIAS – Colombia). Su tesis doctoral se ha desarrollado dentro del grupo de investigación de Alimentómica, perteneciente al Instituto de Investigación en Ciencias de la Alimentación (CIAL) del Consejo Superior de Investigaciones Científicas (CSIC), enfocándose en el desarrollo de nuevas estrategias de extracción para la obtención de compuestos bioactivos a partir de romero y algas marinas, cuyos resultados son presentados en esta Memoria.

ANEXO B. LISTA DE PUBLICACIONES

Como parte del desarrollo de la presente Tesis Doctoral se derivaron las publicaciones científicas enumeradas a continuación:

Revistas SCI

1. A. P. Sánchez-Camargo, A. Valdés, G. Sullini, V. García-Cañas, A. Cifuentes, E. Ibáñez, M. Herrero, Two-step sequential supercritical fluid extracts from rosemary with enhanced antiproliferative activity, J. Funct. Foods 11 (2014) 293-303.
2. M. Herrero, A. P. Sánchez-Camargo, A. Cifuentes, E. Ibáñez, Plants, seaweeds, microalgae and food-by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. An update, TrAC, Trends Anal. Chem. 71 (2015) 26-38.
3. A. P. Sánchez-Camargo, J. A. Mendiola, A. Valdés, M. Castro-Puyana, V. García-Cañas, A. Cifuentes, M. Herrero, E. Ibáñez, Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity, J Supercrit. Fluids 107 (2016) 581-589.
4. A. P. Sánchez-Camargo, V. García-Cañas, M. Herrero, A. Cifuentes, E. Ibáñez, Comparative study of green sub- and supercritical processes to obtain carnosic acid and carnosol-enriched rosemary extracts with *in vitro* antiproliferative activity on colon cancer cells, Int. J. Mol. Sci. 17 (2016) 2046-2064.
5. L. Montero, A.P. Sánchez-Camargo, V. García-Cañas, A. Tanniou, V. Stiger-Pouvreau, M. Russo, L. Rastrelli, A. Cifuentes, M. Herrero, E. Ibáñez, Antiproliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on North-Atlantic coasts, J. Chromatogr. A 1428 (2016) 115-125.
6. A. P. Sánchez-Camargo, L. Montero, V. Stiger-Pouvreau, A. Tanniou, A. Cifuentes, M. Herrero, E. Ibáñez, Considerations on the use of enzyme-assisted extraction in combination

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7. A. P. Sánchez-Camargo, L. Montero, A. Cifuentes, M. Herrero, E. Ibáñez, Application of Hansen solubility approach for the subcritical and supercritical selective extraction of phlorotannins from *Cystoseira eira abies-marina*, RSC Adv. 6 (2016) 94884-94895.
8. A. P. Sánchez-Camargo, F. Parada-Alfonso, E. Ibáñez, A. Cifuentes, On-line coupling of supercritical fluid extraction and chromatographic techniques, J. Sep. Sci. 40 (2017) 213-227.
9. L. Montero, A.P. Sánchez-Camargo, E. Ibáñez, B. Gilbert-López. Phenolic compounds from edible algae: bioactivity and health benefits. Curr. Med. Chem. 2016 (En prensa).
10. A. P. Sánchez-Camargo, N. Pleite, M. Herrero, A. Cifuentes, E. Ibáñez, B. Gilbert-López, New approaches for the selective extraction of bioactive compounds employing bio-based solvents and pressurized green processes, J. Supercrit. Fluids. (enviado).

Revistas no SCI

1. A. P. Sánchez-Camargo & M. Herrero. Rosemary (*Rosmarinus officinalis*) as a functional ingredient: recent scientific evidence. Curr. Op. Food Sci. 14 (2017) 13–19.

CAPÍTULOS DE LIBRO

1. A. P. Sánchez-Camargo, J.A. Mendiola, E. Ibáñez, M. Herrero (2014). Supercritical Fluid Extraction. In: Reedijk, J. (Ed.) Elsevier Reference Module in Chemistry, Molecular Sciences and Chemical Engineering. Waltham, MA: Elsevier. ISBN: 9780124095472.
2. A. P. Sánchez-Camargo, L. Montero, J.A. Mendiola, M. Herrero, E. Ibáñez (2016). Novel extraction techniques for bioactive compounds from herbs and spices. In: M. Hossain, D. Rai, N. P. Brunton (Eds.) Herbs and Spices: Processing Technology and Health Benefits, Wiley. ISBN: 9781119036616.
3. A. P. Sánchez-Camargo, E. Ibáñez, A. Cifuentes, M. Herrero (2017). Bioactives obtained from plants, seaweeds, microalgae and food by-products using preessurized liquid

extraction and supercritical fluid extraction. Comprehensive Analytical Chemistry, Vol. 76. In press. 10.1016/bs.coac.2017.01.001. Elsevier B.V. ISBN: 9780444635792.

ANEXO C.

Anti-proliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on North-Atlantic coasts.

L. Montero, A. P. Sánchez-Camargo, V. García-Cañas, A. Tanniou, V. Stiger-Pouvreau, M. Russo, L. Rastrelli, A. Cifuentes, M. Herrero, E. Ibáñez

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Anti-proliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on North-Atlantic coasts

Lidia Montero[a], Andrea P. Sánchez-Camargo[a], Virginia García-Cañas[a], Anaëlle Tanniou[b], Valérie Stiger-Pouvreau[b], Mariateresa Russo[c], Luca Rastrelli[d], Alejandro Cifuentes[a], Miguel Herrero[a,*], Elena Ibáñez[a]

[a] *Laboratory of Foodomics, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain*
[b] *LEMAR UMR 6539 CNRS UBO IRD IFREMER, Université de Bretagne Occidentale (UBO), Institut Universitaire Européen de la Mer (IUEM), Technopôle Brest-Iroise, Rue Dumont d'Urville, Plouzané 29280, France*
[c] *Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, loc. Feo di Vito, 89122 Reggio Calabria, Italy*
[d] *Dipartimento di Farmacia, Università di Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Italy*

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ABSTRACT

In the present work, the phlorotannin composition of different *Sargassum muticum* samples collected at different locations along the North Atlantic coasts as well as the bioactivities related to these components were investigated. After pressurized liquid extraction, the samples collected at the extreme locations of a latitudinal gradient from Portugal and Norway, were found to be the richest on total phenols and, particularly, on phlorotannins, containing up to 148.97 and 5.12 mg phloroglucinol equivalents g^{-1}, respectively. The extracts obtained from these locations were further purified and chemically characterized using a modified HILIC × RP-DAD-MS/MS method. The application of this methodology allowed the tentative identification of a great variability of phlorotannins with different degrees of polymerization (from 3 to 11) and structures, determined for the first time in *S. muticum*. The most-abundant phlorotannins on these samples were fuhalols, hydroxyfuhalols and phlorethols, showing also particularities and important differences depending on the geographical location. Afterwards, the antiproliferative activity of these extracts against HT-29 adenocarcinoma colon cancer cells was studied. Results revealed that the richest *S. muticum* samples in terms of total phlorotannins, i.e., those from Norway, presented the highest activity, showing a good cytotoxic potential at concentrations in the medium micromolar range.

1. Introduction

Phlorotannins are polyphenolic compounds widely recognized to be exclusive from brown seaweeds (Phaeophyceae) [1]. This particular type of polyphenols comprises a very heterogeneous group of polymeric compounds with a great chemical variability [2]. The interest of these compounds is related to their associated bioactivities, such as antioxidant [3–5], anti-inflammatory [6], anti-bacterial [7,8], antidiabetic [9] or anti-adipogenic [10], among others. Moreover, their potential anti-proliferative activity has been pointed out by several researches [11–13]. Phlorotannin content in brown algae can reach up to 15% of dry weight, depending on species, and they may be found in free form or forming complexes with different components of the cell walls, such as alginic acid [14]. From a purely chemical point of view, phlorotannins are made up of phloroglucinol (1,3,5-trihydroxybenzene) units with varying degrees of polymerization that may be linked through different bonds forming several structures and types, namely: fuhalols and phlorethols, which contain ether linkages; fucols, with phenyl linkages; fucophlorethols in which both ether and phenyl linkages are present; and eckols, that possess a benzodioxin linkage. Although their presence in brown algae is widely accepted, it is rather difficult to find studies in which the complete characterization of such complex polymeric structures is carried out. In fact, several

* Corresponding author. Tel.: +34 910 017 946; fax: +34 910 017 905.
E-mail address: m.herrero@csic.es (M. Herrero).

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approaches have been attempted for the structural elucidation of phlorotannins in their native form; for instance, Stiger-Pouvreau et al. [15] employed one- and two-dimensional nuclear magnetic resonance (NMR) (1H, heteronuclear multiple bond correlation) together with *in vivo* NMR (high-resolution magic-angle spinning, HR-MAS NMR) analyses, to structurally elucidate and fingerprint phlorotannin signals in different Sargassaceae species. Results revealed that these techniques were useful for discriminating among species, giving a differentiated profile but only determining the class of phlorotannins in the sample, without elucidating the entire structure of any compound. In a recent work carried out in our laboratory [16], a new comprehensive two-dimensional liquid chromatography coupled to DAD and tandem mass spectrometry (LC × LC-DAD-MS/MS) methodology was developed based on the coupling of a hydrophilic-interaction chromatography (HILIC)-based separation in the first dimension and an RP-based separation in the second dimension that allowed the separation and identification of more than 50 compounds in a *Cystoseira abies-marina* brown algal extract. By using this approach, phlorotannins containing from 5 to 17 phloroglucinol units were identified in this sample [16]. The application of this methodology to *S. muticum* could therefore imply a definitive step forward for the characterization of its phlorotannin composition.

S. muticum is an invasive brown macroalga widely spread along the European Atlantic coasts [17] Although native from Japan, this macroalga grows well in a variety of different environments, being in fact, one of the most readily available Sargassaceae species in Europe. Considering its availability and the fact that the presence of phlorotannins in *S. muticum* composition has been already confirmed [5,8], this seaweed has been pointed out as a potential sustainable source of bioactive compounds.

Different methods have been tested to extract phlorotannins from brown algae; the classical procedure [1] involves a solid–liquid extraction with large volumes of aqueous mixtures of ethanol or methanol for a long time. New green processes have been previously shown to be suitable for the extraction of bioactive compounds from a variety of different natural sources [18]; among them, centrifugal partition extraction (CPE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been employed, and compared to classical solid-liquid extraction, to obtain bioactive phenolic compounds from *S. muticum* [5]. Results demonstrated that PLE can be employed with advantages for obtaining extracts rich in phenolic compounds from brown algae, with high efficiency and complying with the rules of green chemistry. On the other hand, in a recent work carried out in our laboratory, enzyme-assisted extraction (EAE) was studied and compared to an optimized PLE process to try to increase the recovery of phenolic compounds from *S. muticum* [19]. This study showed that EAE did not significantly improve the results directly attainable through the use of PLE.

Thus, in the present work, the previously optimized PLE process [19] was applied to the extraction of phlorotannins from *S. muticum* samples collected at 13 different locations along the North-Atlantic coasts (Portugal, Spain, France, Ireland and Norway) with the aim to study the influence of the growing conditions on the chemical composition of the extracts. The extracts were characterized in terms of total phenol content, total phlorotannin content and antioxidant activity. Besides, a comprehensive two-dimensional liquid chromatography (LC × LC) method was optimized and applied to the richest samples to chemically characterize for the first time the native complex phlorotannin composition of *S. muticum*. Moreover, these extracts were also assayed to test their potential anti-proliferative activity against human colon cancer cells.

2. Materials and methods

2.1. Samples and chemicals

Samples of the brown alga *S. muticum* were collected from April to May 2011 in 13 different sites of five European Atlantic coast countries (Portugal, Spain, France, Ireland and Norway) as already described in Tanniou et al. [8]. The algae were rinsed firstly with filtered seawater and then with distilled water to remove the residual sediments and salts. After that, the samples were dried with absorbent paper and cut into fragments before their freeze-drying. Finally the dry material was powdered and sieved at 250 μm.

The solvents employed were HPLC-grade. Acetonitrile, ethanol, methanol and acetone were acquired from VWR Chemicals (Barcelona, Spain), whereas dichloromethane was acquired from Fluka AG (Buchs, Switzerland) and ethyl acetate from Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA).

Gallic acid, phloroglucinol, acetic acid, formic acid, 2,4-dimethoxybenzaldehyde (DMBA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma–Aldrich (Madrid, Spain). The Folin–Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany). Hydrochloric acid was obtained from Probus (Barcelona, Spain). For inhibition of cell proliferation assays, dry purified extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) at the appropriate concentrations and stored as aliquots at −80 °C until use.

2.2. Pressurized liquid extraction (PLE)

Firstly, extractions of freeze-dried and ground *S. muticum* samples from 13 different localizations along the North-Atlantic coasts were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. For each extraction, an 11 mL stainless steel extraction cell was employed to load the sample. The extraction cell bottom was loaded with 1 g of sea sand, followed by 1 g of dried brown alga being mixed with the same quantity of sea sand. Subsequently, 1 g of sea sand was added on top as dispersive agent. Before the static extraction period, an instrumentally preset warming-up time of 6 min was used. The extraction conditions applied were based on a previous optimization [19], including the use of ethanol:water (95:5) as extraction solvent at 160 °C and 10.3 MPa for 20 min. Each extraction was carried out by duplicate. After the extraction process, the ethanol was removed by evaporation (Rotavapor R-210, Buchi Labortechnik AG, Flawil, Switzerland) and finally, the extracts were freeze-dried (Labconco Corporation, MO) and kept in the darkness at −20 °C until analysis.

2.3. Phlorotannins purification procedure

In order to obtain concentrated phlorotannin extracts, a purification protocol previously reported by Stiger-Pouvreau et al. [15] was applied to the *S. muticum* samples from Norway and Portugal. The dried extracts were re-dissolved in water and submitted to a liquid-liquid extraction with dichloromethane (1:1, *v/v*) in order to eliminate the lipidic compounds and chlorophylls present in the extract, repeating this step several times until a colorless non-polar fraction was obtained. After that, successive precipitations of proteins and carbohydrates were carried out with acetone and ethanol, respectively, ending with the elimination of the organic solvent using a gentle stream of nitrogen. Finally, phlorotannins were extracted from the water fraction with three rinses with equivalent volumes of ethyl acetate. The ethyl acetate fractions

were pooled and the solvent was evaporated under a stream of nitrogen.

2.4. In-vitro determinations

2.4.1. Total phenol content (Folin–Ciocalteu method)

Total phenol content of the PLE extracts and the purified extracts were measured by the Folin–Ciocalteu method developed by Kosar et al. [20] with some modifications. Briefly, 10 μL of sample (10 mg mL^{-1} in methanol) were transferred to 600 μL of water, and then 50 μL of undiluted Folin–Ciocalteu reagent were added. After 1 min, 150 μL of 20% (*w/v*) Na_2CO_3 were added and the volume was made up to 1 mL with water. The reaction was incubated at 25 °C for 2 h and then 300 μL of the mixture were transferred to a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader Powerwave XS (Bio Tek Instruments, Winooski, VT) and compared to a phloroglucinol calibration curve (62.5–2000 μg mL^{-1}). The phenolic content was expressed as mg of Phloroglucinol Equivalents (PGE) per g extract. Moreover, total phenol content was also expressed as % dry weight of algae. All analyses were done by triplicate.

2.4.2. Total phlorotannin content (DMBA assay)

To estimate the total phlorotannin content of the algal PLE and purified extracts, the DMBA colorimetric assay was employed [6]. DMBA solution was prepared just prior to use by mixing equal volumes of 2% DMBA reagent in acetic acid (*w/v*) and 6% hydrochloric acid in acetic acid (*v/v*). A total of 50 μL of sample (5 mg mL^{-1}) was mixed with 250 μL of DMBA solution in a 96-well microplate and the reaction was conducted at room temperature for 60 min in the dark. Then, the absorbance was read at 515 nm using a microplate spectrophotometer reader Powerwave XS (Bio Tek, Winooski, VT, USA). Water was used as blank and control samples without DMBA solution were also included. A calibration curve using phloroglucinol (PG) (0.1–46.0 μg mL^{-1}) was employed to estimate the total phlorotannin content. All samples, blanks, and controls were prepared in triplicate. Data are presented as the average of triplicate analyses expressed as milligram phloroglucinol equivalents (PGE) per gram of dry extract.

2.4.3. Trolox equivalents antioxidant capacity assay (TEAC)

The antioxidant capacity of the algal extracts was estimated with the TEAC assay following the ABTS method based on the procedure described by Re et al. [21]. $ABTS^{\bullet+}$ radical was produced by mixing 7 mM ABTS and 2.45 mM potassium persulfate allowing their reaction during 16 h in the dark at room temperature. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) until an absorbance of 0.7 (±0.02) at 734 nm was achieved. 10 μL of sample (5 different concentrations ranging from 0.25 to 2 mg mL^{-1}) and 1 mL of $ABTS^{\bullet+}$ solution were mixed in an eppendorf vial and 300 μL of the mixture were transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min during 45 min in a Powerwave XS microplate spectrophotometer reader (BioTek). Trolox was used as reference standard and results were expressed as TEAC values (mM Trolox g^{-1} extract). These values were obtained from five different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All analyses were done in triplicate.

2.5. Anti-proliferative activity against human colon cancer cells

Human colon cancer cell line HT-29 was used in order to measure the anti-proliferative activity of Norway and Portugal purified extracts. HT-29 cells obtained from the ATCC (American Type Culture Collection, LGC Promochem, UK) were grown in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum, 50 U mL^{-1} penicillin G, and 50 U mL^{-1} streptomycin, at 37 °C in humidified atmosphere and 5% CO_2. Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, HT-29 cells were seeded onto 96-well culture plates at 10,000 cells cm^{-2}, and permitted to adhere overnight at 37 °C. Cells were treated with the vehicle (medium with 0.2% DMSO) or different concentrations of *Sargasssum muticum* purified extracts (from 12.5 to 100 μg mL^{-1}) for 24, 48 and 72 h. After incubation with the extracts, the medium was aspirated and 0.5 mg mL^{-1} of MTT reagent (Sigma–Aldrich) was added to the cells and incubated for 3 h at 37 °C in humidified 5% CO_2/air atmosphere. The medium was then removed, and the purple formazan crystals were dissolved in 100 μL of DMSO. The absorbance at 570 nm was measured in a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland). Results are shown as the mean ± 95% confidence interval of at least three independent experiments, each performed in triplicate. Cell viability at the beginning of the treatment (time zero) was used to calculate the following parameters related to cell proliferation: GI50 (50% growth inhibition), TGI (total growth inhibition), and LC50 (50% cell death). These parameters were calculated according to the NIH definitions [22].

2.6. Comprehensive two-dimensional liquid chromatography (LC × LC) analysis of phlorotannins

2.6.1. Instrumentation

An Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an autosampler and a diode array detector was used in the first dimension (D1) of the LC × LC setup. In order to obtain more reproducible low flow rates and gradients, a Protecol flow-splitter (SGE Analytical Science, Milton Keynes, UK) was placed between the first dimension pump and the autosampler. An additional LC pump (Agilent 1290 Infinity) was coupled to perform the second dimension (D2) separations. Both instruments were hyphenated through an electronically controlled two-position ten-port switching valve equipped with two 30 μL injection loops. An Agilent 6320 Ion Trap mass spectrometer equipped with an electrospray interface was online coupled and operated in negative ionization mode using the following conditions: dry temperature, 350 °C; mass range, *m/z* 90–2200 Da; dry gas flow rate, 12 L min^{-1}; nebulization pressure, 40 psi. The LC data were elaborated and visualized in two and three dimensions using LC Image software (version 1.0, Zoex, Houston, TX, USA).

2.6.2. LC × LC separation conditions

Samples of purified extracts of *S. muticum* from Portugal and Norway were prepared at 50 mg mL^{-1} in methanol/acetonitrile (3:7, *v/v*) from the dry extract obtained as described in Section 2.3.

D1 separation was run on a Lichrospher diol-5 (150 × 1.0 mm, 5 μm d.p., HiChrom, Reading, UK) column, following the separation conditions developed in our previous work [16]. Briefly, the mobile phases were (A) acetonitrile/acetic acid (98:2, *v/v*) and (B) methanol/water/acetic acid (95:3:2, *v/v/v*) eluted at 15 μL min^{-1} according to the following gradient: 0 min, 0% B; 3 min, 0% B; 5 min, 7% B; 30 min, 15% B; 70 min, 15% B; 75 min, 25% B; 85 min, 25% B. The injection volume was 5 μL.

D2 consisted on 78 s-repetitive gradients during the whole LC × LC separation, being modulation time of the switching valve also 78 s. In the D2 separation an Ascentis Express C_{18} (50 × 4.6 mm, 2.7 μm, d.p., Supelco, Bellefonte, CA) partially porous column with a C_{18} precolumn was employed, using as mobile phases water (0.1% formic acid, A) and acetonitrile (B) eluted according to the following repetitive gradients: 0 min, 0% B; 0.1 min, 0% B; 0.3 min, 5% B; 0.8 min, 70% B; 0.9 min, 90% B; 1.0 min, 0% B. The flow rate was

Table 1
Extraction yield (%), total phenol content (mg PGE g^{-1} extract), total phlorotannin content (mg PGE g^{-1} extract) and antioxidant activity (mmol TE g^{-1} extract, TEAC assay) of the brown macroalga *Sargassum muticum* PLE extracts according to their collection location. Values presented are mean ± sd. Superscripts mean groups not statistically different ($p > 0.05$) for each response.

Location		Extraction yield (%)	Total phenol content (mg PGE g^{-1})[A]	Total phenol content (% DWalgae)[B]	Total phlorotannins (mg PGE g^{-1})[C]	TEAC value (mmol TE g^{-1})[D]
France	F1	23.7 ± 0.3	75.43 ± 1.56^{a}	1.783 ± 0.029^{d}	3.297 ± 0.337^{c}	0.983 ± 0.018^{c,d}
	F2	17.5 ± 0.4	58.19 ± 3.31^{b}	1.016 ± 0.070^{a}	2.606 ± 0.036^{a}	0.652 ± 0.013^{a}
	F3	27.4 ± 2.5	78.55 ± 0.51^{a}	2.150 ± 0.157^{f}	3.380 ± 0.163^{c}	1.091 ± 0.017^{e}
Portugal	P1	24.9 ± 0.1	145.02 ± 3.05^{e}	3.604 ± 0.060^{h}	4.088 ± 0.239^{d}	2.101 ± 0.047^{g}
	P2	23.2 ± 0.5	119.27 ± 1.39^{f}	2.768 ± 0.068^{g}	4.127 ± 0.129^{d}	1.828 ± 0.025^{f}
Spain	S1	18.7 ± 0.1	79.29 ± 134^{a}	1.481 ± 0.030^{b,c}	2.629 ± 0.091^{a}	0.919 ± 0.023^{b}
	S2	16.2 ± 0.2	77.19 ± 1.64^{a}	1.248 ± 0.022^{a,b}	2.477 ± 0.139^{a}	0.943 ± 0.033^{b,c}
	S3	15.5 ± 0.1	80.46 ± 1.38^{a,c}	1.245 ± 0.026^{a,b}	2.567 ± 0.075^{a}	0.923 ± 0.016^{b}
Ireland	I1	21.3 ± 0.7	86.60 ± 2.44^{c}	1.844 ± 0.064^{d,e}	2.933 ± 0.090^{b}	1.106 ± 0.020^{d}
	I2	21.8 ± 0.6	78.39 ± 5.57^{a}	1.710 ± 0.156^{c,d}	2.670 ± 0.335^{a}	0.889 ± 0.011^{b}
	I3	22.3 ± 1.0	94.08 ± 0.96^{d}	2.098 ± 0.077^{e,f}	3.462 ± 0.095^{c}	1.068 ± 0.007^{e}
Norway	N1	31.5 ± 0.3	148.97 ± 0.85^{e}	4.696 ± 0.034^{i}	5.115 ± 0.145^{e}	2.297 ± 0.050^{h}
	N2	31.6 ± 1.4	146.44 ± 4.54^{e}	4.639 ± 0.274^{i}	4.839 ± 0.134^{f}	2.090 ± 0.032^{g}

[A] mg phloroglucinol equivalents g^{-1} extract.
[B] % dry weight algae.
[C] mg phloroglucinol equivalents g^{-1} extract.
[D] mmol Trolox equivalents g^{-1} extract.

3 mL min^{-1}. UV–vis spectra of the second dimension eluent were registered in the range of 190–550 nm using a sampling rate of 20 Hz, while 280 nm was the wavelength used to monitor the separations. The MS was operated under negative ESI mode. The flow eluting from the second dimension column was split before the MS instrument, so that the flow rate entering the MS detector was 500 μL min^{-1}.

2.7. Statistical analysis

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95%. One-way analysis of variance (ANOVA), together with Student–Newman–Keuls test, was employed to group samples, based on statistically significant differences. Mean values were compared using the Tukey's test and differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. Influence of growing conditions on phlorotannin content

In order to study the influence of the particular growing conditions on the chemical composition of the *S. muticum* extracts, an optimized PLE procedure was applied to 13 different algal samples collected along the North-Atlantic coasts, including Portugal, Spain, France, Ireland and Norway locations. The extraction procedure applied included the use of ethanol:water (95:5, *v/v*) as extraction solvent at 160 °C and 10.3 MPa for 20 min. The extracts obtained were firstly characterized in terms of total phenol content, total phlorotannin content and antioxidant activity. Table 1 summarizes the results obtained; as can be clearly observed, the extraction yield gives a first hint regarding the different compositions of the macroalgae studied, not only between countries and distant geographical locations but also within some areas, as in the case of the samples collected on the French coast. The highest extraction yields were obtained with the samples collected in Norway, whereas those collected in Spain possessed the lowest amount of dried matter. As for total phenols, samples collected in Norway possessed the highest amount of total phenols, followed by those harvested in Portugal. These values were significantly higher than the ones obtained for the rest of locations. *S. muticum* samples collected in France possessed the lowest amount of total phenols, less than half of those of the richest samples. Although smaller, statistically significant differences ($p < 0.05$) were also observed among the samples collected in the same country, except those collected in Spain and Norway, that could be considered statistically similar ($p > 0.05$). Next, total phlorotannin contents were estimated in the different extracts; the trend found was rather similar to that of total phenols, that is, the samples collected in Norway and Portugal were the richest. In any case, significant differences were observed between these two countries. For the samples collected from the rest of countries, the values found were closer and lower than those found in the Norwegian samples. As in the case of total phenols, intra-country variations ($p < 0.05$) were observed for the total phlorotannin contents, excepting for those from Spain and Portugal. Previous fingerprints obtained by HR-MAS NMR and FT-IR for the same samples yielded similar conclusions about inter- and intra-site variability [23].

Lastly, the antioxidant capacity of the obtained extracts was determined using the TEAC assay. A good correlation was found between the TEAC values obtained and the total phenols and total phlorotannin contents determined in the extracts. In agreement, the most active extracts were obtained from the samples collected in Norway and Portugal, whereas the rest of extracts possessed lower antioxidant capacity (Table 1). Results obtained in the present work were, in general, well in accordance with those previously presented on *S. muticum* samples [8] in terms of percentage of total phenols per g of dried algae (Table 1). Samples collected in Norway and Portugal were those with higher % of total phenols with respect to the initial dry algae employed, whereas the rest of countries possessed very similar contents (Table 1). However, in the present work, a higher content was found for Norwegian samples compared to those harvested in Portugal. These small differences observed between the two studies could be due to the different extraction process employed to achieve the phenolic-rich extracts.

Looking at the results summarized in this section, it seemed clear that the algae growing in the most extreme locations of this latitudinal gradient along the North-Atlantic coasts, i.e., Norway and Portugal, were the most interesting from a chemical composition perspective. For this reason, the samples collected in those countries (four geographical locations in total) were selected to carry out an in-depth chemical characterization as well as to study their potential anti-proliferative activity. This activity has already been related to the presence of phenolic compounds in general and phlorotannins in particular [12,13].

To do that, a purification protocol was applied as described in Section 2.3 in order to further enrich the selected extracts in

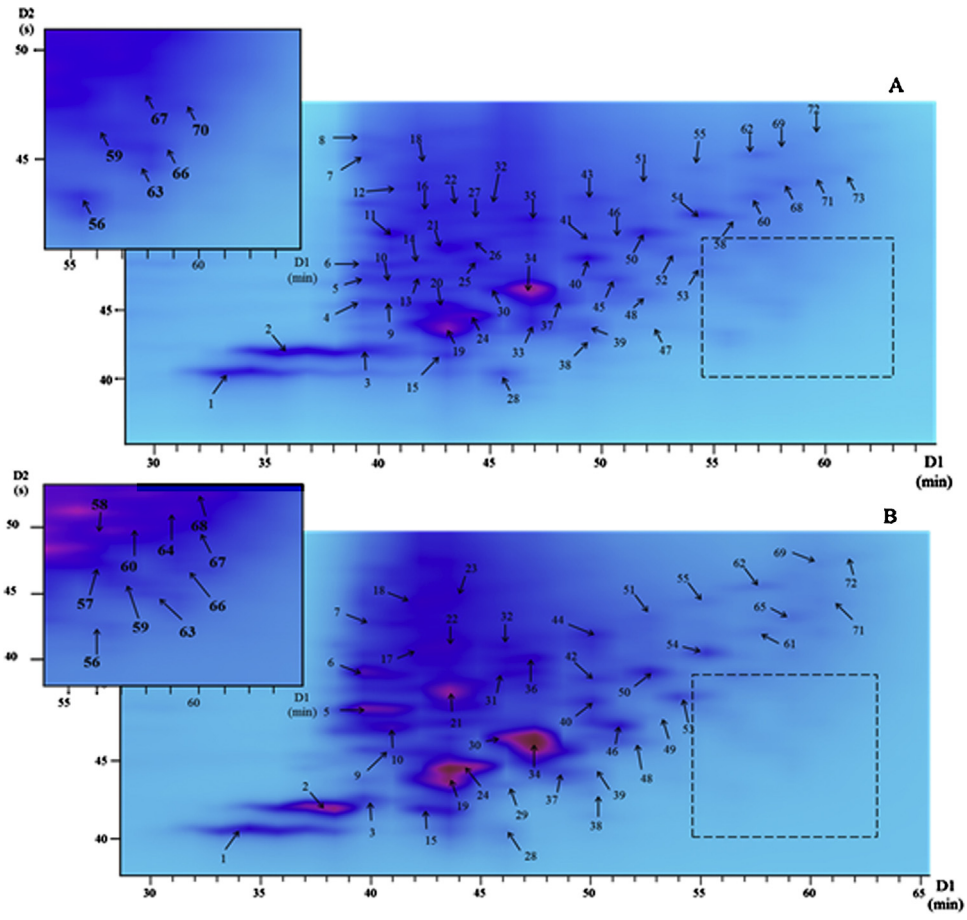

Fig. 1. 2D plot (280 nm) of the *Sargassum muticum* enriched extracts from the samples collected in Norway (A, sample N1 and B, sample N2) obtained using the optimized HILIC × RP-MS/MS method. For peak identification, see Table 2.

those compounds prior analysis. This purification protocol was properly optimized, studying the most influencing variables. To do that, different approaches were studied, including precipitation with several solvents (hexane, dichloromethane, ethyl acetate), use of SPE (different stationary phases) and molecular weight fractionation or combinations thereof. The optimum conditions were selected according to the best results observed in the two-dimensional LC analysis and involved a sample clean-up with dichloromethane followed by precipitations using acetone and ethanol finishing with an ethyl acetate extraction. After this procedure, the total phlorotannin content of the enriched extracts was more than double for Norwegian samples and around 1.5 times the initial content in Portuguese samples, being 11.730 ± 0.141, 12.461 ± 0.264, 5.906 ± 0.324 and 6.4217 ± 0.355 mg PGE g^{-1} for the samples N1, N2, P1 and P2, respectively.

3.2. Chemical characterization of phlorotannins by LC × LC-ESI-MS/MS.

Although the determination of total phlorotannins using the DMBA colorimetric method is able to provide a general estimation of the phlorotannin amount found in the extract, this assay is not able to provide any information about the chemical structure of those compounds. The structural variability known in phlorotannins (see introduction), that includes 5 chemical classes, could be therefore linked to their bioactivity. For this reason, in order to elucidate the compounds present in the most active *S. muticum* extracts, a LC × LC approach was used. We have previously presented the ability of this analytical tool to separate and tentatively identify phlorotannins of other brown algae, *C. abies-marina*, reaching degrees of polymerization of 17 phloroglucinol units (PGU) [16]. By using a combined HILIC separation in the first dimension (D1) and a RP-based separation in the second dimension (D2), 52 different phlorotannins could be effectively separated in *C. abies-marina* extracts. Considering that *C. abies-marina* is a closely related species to *S. muticum*, both belonging to the family Sargassaceae within the class Phaeophyceae, this method was employed as starting point for the optimization of the separation of the purified phlorotannins' extracts from *S. muticum* collected in Portugal and Norway. Different experimental conditions were tested in the two dimensions; D1 optimization included the use of microbore silica, diol and amino columns as well as different combination of acetonitrile and 10 mM ammonium acetate at several pH (6.6 and 8.0). The second dimension optimization was based on the study of several columns (i.e, C_{18} monolithic column, and C_{18} and PFP short partially porous columns) using different flow rates, mobile phases and analysis time. As for the combination of the two dimensions, once the conditions were optimized separately, several transfer volumes (20, 30 and 50 μL) and modulation times (1.3, 1.5 and 2.0 min) were studied.

After the optimization of all the separation conditions, the diol column was selected for the D1 HILIC. Moreover, the best separation conditions for the D2 included the use of a short C_{18} partially porous column adapting the gradient employed to the complexity and composition of the samples studied (see Section 2.6.2). Figs. 1 and 2 show the two-dimensional plots obtained (280 nm) for the samples collected in Norway and Portugal, respectively, under optimized the experimental conditions. At first sight it is

Table 2
Tentative peak assignment of the compounds separated by LC × LC-ESI-MS/MS found in the *S. muticum* samples collected in Norway.

Peak	Identification	D2 t_R (s)	Total t_R (min)	[M-H]$^-$	Main MS/MS fragments detected
1	Trifuhalol	40.30	27.97	389.0	375, 265, 245
2	Trifuhalol	41.85	30.60	389.0	375, 265, 245
3	Trifuhalol	42.00	34.50	389.1	375, 265, 245
4	NI*	45.50	34.56	448.5	415, 385, 321, 245, 196
5	NI	47.10	34.59	566.6	533, 389, 306, 244, 193
6	NI	50.75	34.65	564.3	526, 437, 373, 331, 202
7	NI	55.95	34.73	571.9	526, 449, 383, 319, 261, 193
8	NI	57.30	34.76	625.0	581, 498, 388, 258
9	Hydroxytetrafuhalol	45.30	35.86	529.8	512, 404, 389, 343, 262
10	Hydroxytetrafuhalol	47.10	35.89	529.1	513, 405, 387, 345, 264
11	NI	50.40	35.94	572.6	538, 511, 446, 318, 164
12	Pentafuhalol	53.70	36.00	637.1	621, 513, 385, 262
13	Tetrafuhalol	47.15	37.19	513.0	391, 373, 264, 245, 219
14	Hydroxytetrafuhalol	48.40	37.21	529.4	483, 465, 401, 389, 262, 245
15	Tetrafuhalol	41.60	38.39	513.4	389, 265, 245
16	Pentaphlorethol	52.05	38.57	621.5	603, 493, 357, 245
17	Hydroxytetrafuhalol	52.15	38.58	529.4	513, 389, 262
18	NI	55.25	38.32	590.5	570, 545, 466, 437, 401, 245
19	Tetrafuhalol	43.50	38.43	513.8	389, 263, 245
20	Tetrafuhalol	45.25	38.45	513.3	389, 263, 245
21	Tetrafuhalol	49.50	38.53	513.8	387, 263, 245
22	NI	52.40	38.57	683.3	648, 555, 509, 415, 387, 263, 245
23	NI	57.85	38.66	590.3	573, 547, 446, 333, 245, 195
24	Dihydroxytetrafuhalol	44.45	39.74	545.4	525, 513, 484, 403, 389, 375
25	NI	48.35	39.81	683.9	651, 557, 509, 387, 621, 245
26	NI	49.80	39.83	683.1	652, 543, 389, 302, 263, 245
27	NI	51.30	39.86	588.8	571, 522, 441, 380, 278, 246
28	Hydroxytetrafuhalol	40.20	40.97	529.5	465, 403, 389, 341, 263, 245
29	Hydroxytetrafuhalol	43.35	41.02	529.3	511, 403, 389, 263, 245
30	Pentafuhalol	46.45	41.07	637.3	621, 513, 373
31	Pentafuhalol	50.55	41.14	637.5	633, 513, 273
32	NI	52.30	41.17	807.3	775, 681, 541, 509, 385, 244
33	Pentafuhalol	44.05	42.33	637.3	621, 513, 497, 389
34	Pentafuhalol	46.30	42.37	637.4	513, 374
35	Hexaphlorethol	51.50	42.46	745.3	727, 619, 603, 371, 355, 309
36	NI	51.65	42.46	807.2	775, 757, 681, 509, 385, 245
37	Hydroxypentafuhalol	45.35	43.66	653.8	638, 527, 513, 466, 389, 263, 245
38	Dihydroxypentafuhalol	42.60	44.91	669.6	623, 527, 465, 403, 385, 341, 261
39	Dihydroxypentafuhalol	43.60	44.93	669.6	623, 543, 527, 465, 402, 385, 341, 260
40	Hydroxypentafuhalol	48.65	45.01	653.6	637, 527, 387, 245
41	Hexafuhalol	50.10	45.03	761.6	637
42	Hexafuhalol	50.40	45.04	761.3	745, 637, 498, 389, 245
43	Heptaphlorethol	53.00	45.08	869.2	851, 745, 728, 603, 245
44	Hexafuhalol	53.30	45.09	761.3	747, 637, 621, 513, 497, 245
45	Dihydroxypentafuhalol	47.15	46.29	669.8	621, 541, 527, 463, 401, 337, 271
46	Dihydroxyhexafuhalol	50.00	46.33	793.1	775, 731, 651, 527, 511, 403, 387
47	Hydroxyhexafuhalol	43.65	47.53	777.7	651, 637, 529, 511, 387, 261, 245
48	Dihydroxyhexafuhalol	46.00	47.57	793.3	775, 669, 653, 527, 403, 389, 262
49	Hydroxyhexafuhalol	47.85	47.60	777.3	763, 655, 529, 515, 388
50	NI	50.60	47.64	947.3	915, 821, 651, 527, 385
51	NI	54.00	47.70	894.2	830, 766, 625, 568
52	Dihydroxyhexafuhalol	49.15	48.92	793.7	777, 652, 589, 554, 511, 390, 311
53	Trihydroxyhexafuhalol	47.75	50.20	809.7	791, 765, 747, 667, 543, 527, 405
54	Dihydroxyheptafuhalol	51.90	50.27	917.1	873, 855, 838, 791, 775, 731, 713, 651, 513, 387
55	Octafuhalol	54.94	50.32	1009.2	994, 968, 887, 872, 747, 621
56	Dihydroxyhexafuhalol	42.80	51.41	793.3	667, 653, 529, 403, 387, 263
57	Dihydroxyhexafuhalol	47.80	51.49	794.2	774, 667, 653, 529, 403, 387, 263
58	Hydroxyheptafuhalol	51.70	51.56	901.8	857, 775, 761, 637, 511, 387
59	Dihydroxyhexafuhalol	49.50	52.83	793.9	773, 668, 653, 529, 403
60	Trihydroxyheptafuhalol	53.05	52.88	933.8	889, 793, 747, 651, 525, 385
61	Nonafuhalol	53.25	52.89	1133.9	1115, 1007, 993, 885, 869, 760, 745, 620
62	Nonafuhalol	56.15	52.94	1133.7	1117, 1009, 995, 887, 870, 853, 761, 745, 622
63	Dihydroxyheptafuhalol	45.45	52.76	917.5	874, 777, 651, 527, 387
64	Trihydroxyheptafuhalol	51.90	52.87	933.4	914, 889, 792, 748, 650, 529
65	Trihydroxyoctafuhalol	54.20	52.90	1057.2	1008, 915, 793, 652, 527, 387
66	Dihydroxyheptafuhalol	45.25	54.05	917.5	899, 874, 791, 775, 651, 527, 387
67	Dihydroxyheptafuhalol	51.75	54.16	917.7	873, 791, 777, 653, 527, 387
68	Trihydroxyoctafuhalol	54.05	54.20	1057.3	
69	Decafuhalol	56.75	54.25	1257.7	1239, 1133, 1117, 1007, 885, 624, 573, 387
70	Dihydroxyheptafuhalol	47.30	55.39	917.1	897, 873, 791, 777, 731, 653, 527, 389
71	Dihydroxyoctafuhalol	54.90	55.52	1041.3	979, 915, 901, 853, 777, 731, 651, 637, 528, 389
72	Decafuhalol	57.50	55.56	1257.7	
73	Dihydroxynonafuhalol	55.00	56.82	1165.7	1146, 1040, 1025, 917, 899, 777, 653, 637, 389

*NI, not identified.

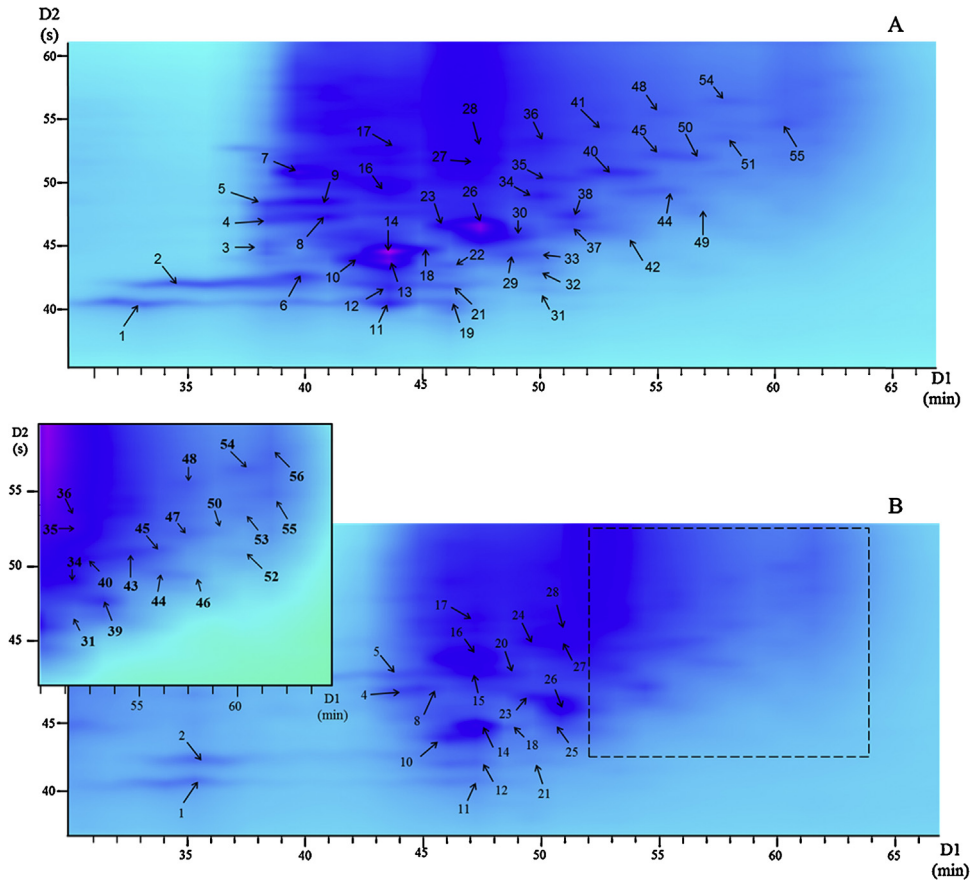

Fig. 2. 2D plot (280 nm) of the *Sargassum muticum* enriched extracts from the samples collected in Portugal (A, sample P1 and B, sample P2) obtained using the optimized HILIC × RP-MS/MS method. For peak identification, see Table 3.

possible to observe that the profiles obtained were significantly different as expected considering the different locations and associated environmental conditions such as sea surface temperature (8–10 °C for Norway and 18–20 °C for Portugal) and photosynthetically available radiation (35–40 mol photons m^{-2} day^{-1} for Norway and 40–45 mol photons m^{-2} day^{-1} for Portugal) [8]. Tables 2 and 3 summarize the data corresponding to the separated compounds in the Norwegian and Portuguese samples, respectively, as well as the tentative assignments of the peaks observed; a first comparison between samples shows important differences in terms of phlorotannin composition.

For instance, samples harvested in Norway were more complex in terms of number of components. As can be observed in Table 2, phlorotannins with a polymerization degree up to 10 units were found. Even if the separation performance achieved was quite satisfactory, the identification of phlorotannins is a very tough task due to their huge chemical variability. In these samples, fuhalol- and hydroxyfuhalol-type phlorotannins were the most-frequently elucidated components, although phlorethols with diverse degree of polymerization were also present (peaks 16, 35 and 43). These two types of phlorotannins are characterized by being formed from phloroglucinol units linked through ether bonds; the difference between phlorethols and fuhalols is the presence of one or more additional hydroxyl groups on the terminal monomer unit [24]. As shown in Table 2, different fuhalols were found in the Norwegian samples, starting from a degree of polymerization of 3 to 10, containing additional hydroxyl group(s). Moreover, other polymers were also elucidated containing up to 4 additional hydroxyl groups. Polymers containing 4–7 phloroglucinol units were the most-frequently found (see Fig. 1 and Table 2). Interestingly, although the profiles were not identical between sites in Norway, they were quite similar, being peaks 2, 19, 24 and 34 among the most intense peaks in both samples, corresponding to trifuhalol (degree of polymerization (DP) = 3, 1 hydroxyl group), tetrafuhalol (DP = 4, 1 hydroxyl group), dihydroxytetrafuhalol (DP = 4, 3 hydroxyl group) and pentafuhalol (DP = 5, 1 hydroxyl group). Sample N2 also contained other very intense peaks (peaks 5, 6) for which an assignment was not attained. In any case, the fragmentation pattern observed for the different peaks was very important for their identification, as they followed a typical fragmentation showing different losses of phloroglucinol units and hydroxyl groups, which helped to achieve a tentative identification. An example is shown in Fig. 3 with the MS/MS fragmentation pattern of dihydroxyheptafuhalol in sample N1 (peak 63). It is also important to note that the relative position of a peak in the 2D plane can be used as a tool for identification since LC × LC allows obtaining 2D patterns in agreement with differential retention in each dimension. For instance, D1 separation shows a distribution according to an increase on degree of polymerization while D2 retention implies that highly hydrophobic compounds elute later, making possible the discrimination, between doubtful identifications, based on peak position. Therefore, this enhanced identification capability of unknown peaks is one of the strongest advantages of 2D approaches over one-dimensional ones.

In the case of the Portuguese samples, less complex 2D plots were obtained (Fig. 2). In this case, the differences between the samples collected in the same country were also more marked. Anyway, peaks 14 and 26 were among the most-intense peaks in both samples, corresponding to an unidentified compound and to a pentafuhalol (DP = 5, 1 hydroxyl group), respectively. From

Table 3
Tentative peak assignment of the compounds separated by LC × LC-ESI-MS/MS found in the *S. muticum* samples collected in Portugal.

Peak	Identification	D2 t_R (s)	Total t_R (min)	[M−H]–	Main MS/MS fragments
1	Trifuhalol	40.70	31.88	389.0	263, 245
2	Trifuhalol	41.90	34.50	389.0	263, 245
3	NI	45.00	38.45	447.8	429, 385, 323, 311, 261
4	Trifuhalol	47.20	38.49	389.0	375, 265, 250
5	NI	48.15	38.50	568.4	552, 537, 443, 305
6	Trifuhalol	42.45	39.71	389.0	375, 251
7	NI	50.50	39.84	570.6	551, 511, 443, 305, 263
8	NI	47.20	41.09	526.8	507, 491, 401, 387, 357, 263, 245
9	NI	48.30	41.11	536.3	520, 475, 411, 333, 268
10	Tetrafuhalol	43.65	42.33	513.7	437, 389, 265, 251
11	Tetrafuhalol	40.30	43.57	513.0	475, 438, 391
12	NI	41.60	43.59	520.0	499, 439, 389, 319, 251
13	Tetrafuhalol	43.55	43.63	513.0	499, 437, 389, 263
14	NI	44.45	43.64	516.3	437, 427, 389, 297, 251
15	NI	48.70	43.73	685.1	651, 633, 509, 387, 245
16	NI	49.55	43.73	685.3	651, 633, 557, 509, 387, 263, 245
17	NI	52.35	43.77	590.4	572, 511, 465, 426, 325, 245
18	Dihydroxytetrafuhalol	44.65	44.94	545.7	529, 513, 389
19	Hydroxytetrafuhalol	40.30	46.17	529.7	403, 389, 277, 262, 245
20	NI	48.55	46.19	687.2	653, 579, 525, 388, 244
21	NI	41.80	46.20	531.4	513, 487, 403, 391, 341, 263, 245
22	NI	43.35	46.22	531.0	515, 486, 405, 391, 363, 307, 265, 245
23	NI	46.55	46.28	646.7	633, 529, 501, 387, 245
24	NI	50.95	46.35	646.5	633, 607, 525, 509, 387, 343, 263, 245
25	Dihydroxypentafuhalol	42.20	47.50	671.0	653, 637, 627, 544, 466, 247
26	Pentafuhalol	46.20	47.57	637.3	623, 513, 373
27	Trihydroxyhexafuhalol	51.55	47.66	809.5	791, 775, 637, 511, 387
28	NI	52.95	47.68	734.3	715, 689, 607, 566, 437, 285
29	Dihydroxypentafuhalol	44.20	48.84	669.0	651, 625, 607, 465, 403, 263
30	Dihydroxypentafuhalol	45.60	48.86	671.3	653, 637, 627, 467, 467, 405, 349
31	Hydroxypentafuhalol	41.40	50.09	653.2	633, 527, 513, 387, 263, 245
32	Hydroxypentafuhalol	42.80	50.11	653.8	637, 527, 513, 387, 263, 245
33	NI	44.20	50.14	655.3	636, 611, 529, 515, 469, 388, 341, 262, 245
34	NI	48.65	50.21	657.0	633, 621, 524, 483, 370, 263
35	NI	50.25	50.24	780.5	638, 611, 532, 388, 217
36	NI	53.05	50.28	877.6	859, 832, 797, 661, 612, 520
37	NI	46.20	51.47	661.2	612, 555, 509, 367, 263
38	NI	47.20	51.49	663.7	647, 574, 537, 505, 374
39	Dihydroxyhexafuhalol	47.70	52.80	793.1	775, 749, 731, 527, 483, 465, 385
40	Dihydroxyhexafuhalol	50.65	52.84	793.2	775, 749, 731, 527, 511, 483, 387, 245
41	NI	53.95	52.89	895.4	
42	NI	44.15	54.02	781.4	763, 701, 499, 437, 263
43	Hydroxyhexafuhalol	50.90	54.08	949.1	
44	Dihydroxyhexafuhalol	49.45	54.12	793.5	777, 730, 634, 513, 485
45	NI	51.05	54.15	948.1	775, 749, 652, 607, 528, 510, 483, 431, 389
46	Dihydroxyheptafuhalol	49.20	55.42	917.3	900, 874, 856, 714, 634, 513
47	Dihydroxyheptafuhalol	52.00	55.47	917.6	898, 873, 791, 777, 634, 513, 403
48	Dihydroxynonafuhalol	55.20	55.52	1165.3	
49	Dihydroxyheptafuhalol	48.00	56.70	919.2	900, 874, 856, 837, 714, 634, 513, 265
50	Dihydroxynonafuhalol	51.90	56.77	1165.7	
51	Dihydroxyhexafuhalol	53.20	56.80	793.1	777, 749, 653, 529, 403, 387, 263
52	Trihydroxyheptafuhalol	53.30	58.09	933.5	914, 871, 773, 667, 651, 623, 511
53	Dihydroxyoctafuhalol	54.60	58.09	1043.1	
54	Nonafuhalol	56.30	58.14	1134.9	
55	Decafuhalol	54.55	60.71	1257.7	
56	Endecaphlorethol	57.70	62.06	1365.3	

*NI, not identified.

the information summarized in Table 3 it is possible to infer that fuhalols and hydroxyfuhalols were also the predominant phlorotannins in these Portuguese samples. Different degrees of polymerization were found, from 3 to 11, whereas the additional hydroxyl groups associated to these structures were up to 4 in some cases. Although the presence of hydroxyfuhalols in *S. muticum* has been reported [25], it is worth to mention that there are no previous studies describing in such detail the phlorotannin content in *S. muticum* samples nor in any other algal sample containing a similar variability on fuhalols, hydroxyfuhalols and phlorethols composition, which gives a clear idea of the difficulty of this task. In this regard, the potential of LC × LC-MS/MS to separate and to tentatively identify such a complex mixture is demonstrated in the present work. NMR spectra (HMBC analysis) obtained with algae collected in different North-Atlantic sites allowed only the determination of phlorethols in the phlorotannin fraction of *S. muticum* [8,15]. Thus, considering the complexity of the polyphenols fraction (as demonstrated by the phlorotannins profile shown in Figs. 1 and 2), we can conclude that the pressurized liquid extraction (PLE) combined to a LC × LC-DAD-MS/MS methodology applied appears a promising tool for the complete separation and identification of phlorotannins in algal samples. This multidimensional tool offers a series of advantages that one-dimensional approaches cannot provide, thanks to the simultaneous use of different separation mechanisms. In fact, the gains obtained through this two-dimensional approach may be illustrated from the peak capacity values achieved for the separations. In this regard, a theoretical peak capacity (n_{c2D}) of 1050 was obtained for N1 sample

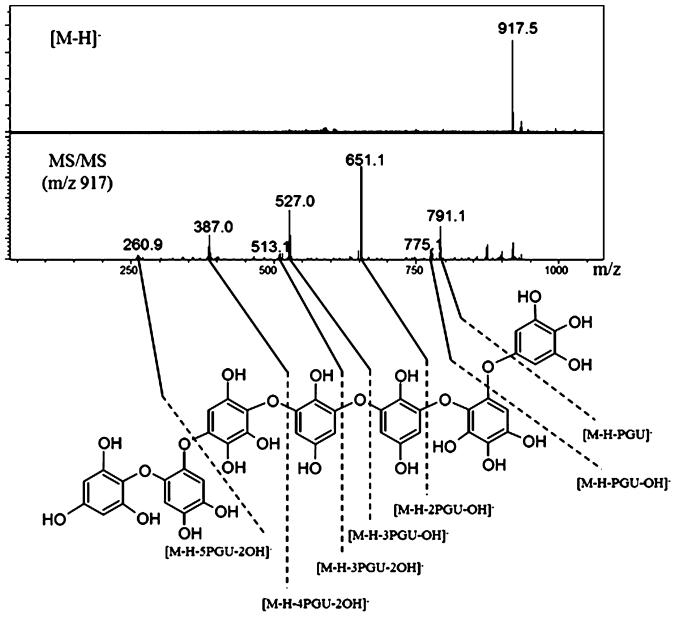

Fig. 3. MS spectrum and MS/MS fragmentation pattern of dihydroxyheptafuhalol in sample N1 (peak 63, fuhalol with DP = 7 with two additional hydroxyl groups) as well as the tentatively proposed chemical structure. PGU: phloroglucinol units.

whereas P1 separation reached a peak capacity of 906; measurements were done according to Li et al. [26] in order to consider the D2 time cycle as well as the influence of undersampling of the D1 eluate. Moreover, to stress the importance of re-optimizing the LC × LC separation according to the sample studied, the values found with the present method and that previously developed [19] were compared for the same sample (N1); results showed that a 50% increase on separation performance was obtained (n_{c2D} 699 vs 1050).

At present, very little is known about the relationship between phlorotannin structure (degree of polymerization, type of bond, branching) and bioactivity [4]. From the published data as well as from the results included in this work, it seems that the antioxidant activity of these components may be more related to their relative abundance than to their different structure [4]. Nevertheless, one should note that in Norwegian populations, a higher variety of smaller compounds compared to Portuguese populations was detected. This observation is in agreement with previous reports that demonstrated that small phlorotannins possess higher antioxidant activity than highly polymerized compounds [27]. Other reports have pointed out some discrepancies between antioxidant activity and other activities, such as hepatoprotective activity, showing that some phlorotannins that presented good antioxidant activity did not possess similar hepatoprotective activity [28]. Besides, to further study the possible relationship between phlorotannin structure and antiproliferative activity, the four purified *S. muticum* extracts (collected in Norway and Portugal) were assayed in order to observe their potential effects against a human colon cancer cell line, HT-29.

3.3. *Antiproliferative activity of phlorotannins from* S. muticum

To determine the antiproliferative effect of the phlorotannins-enriched extracts, HT-29 cells were incubated with increasing concentrations of extracts (from 0 to 100 μg mL^{-1}) for 24, 48 and 72 h and cell proliferation was analyzed by the MTT assay. As can be observed in Fig. 4, after 24 h incubation, the concentration dependence of the antiproliferative activity of the extracts was significant. The extracts demonstrated different *in vitro* antiproliferative effects on HT-29 colon cancer cells. In general, the most enriched extracts

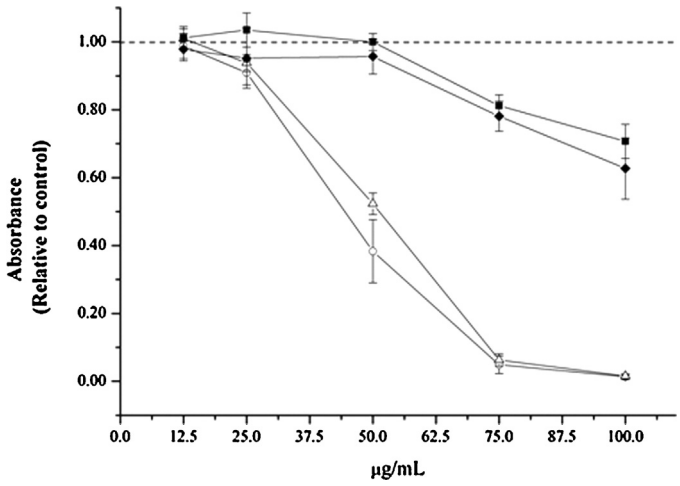

Fig. 4. HT-29 cell viability upon treatment for 24 h with different concentrations of N1 (circle), N2 (triangle), P1 (square) and P2 (diamond) extracts. Error bars are given as 95% confidence interval.

in phlorotannins (those obtained from Norway, sites 1 and 2; N1 11.730 ± 0.141 and N2 12.461 ± 0.264 mg PGE g^{-1}) exerted higher antiproliferative activity. For instance, incubation with N1 and N2 extracts at a concentration of 50 μg mL^{-1}, reduced cell proliferation by roughly 50% after 24 h, while extracts obtained from Portugal, sites 1 and 2 (P1 and P2 samples, total phlorotannin content 5.906 ± 0.324 and 6.4217 ± 0.355 mg PGE g^{-1}, respectively.) did not exert any appreciable effect at this concentration. Furthermore, cell viability was totally reduced after treatments with N1 and N2 extracts at concentrations close to 100 μg mL^{-1} at any of the assayed times, while the same concentration of P1 and P2 extracts reduced cell viability down to *ca.* 40% after 24 h incubation. In order to characterize in more detail the antiproliferative activity of these extracts, the growth inhibition (GI50), the total growth inhibition (TGI) (as an indicator for cytostaticity), and the lethal concentration (LC50) (as an indicator for the cytotoxic level of effect), were also determined at 24, 48 and 72 h incubation times. As it is shown in Table 4, the phlorotannin-enriched extracts exert different *in vitro* cytostatic and cytotoxic effects depending on the type of extract and concentration, exhibiting maximum inhibitory activity after 24 h exposure. In particular, N1 and N2 demonstrated cytotoxic potential at concentrations in the medium micromolar range. The comparison of the results obtained in the present study with those reported in literature for brown seaweeds-derived extracts is not straightforward due to limited information

Table 4
Antiproliferative *in-vitro* effect of *S. muticum* extracts on HT-29 cells. Values represent the concentrations in μg mL^{-1} of each extract that caused 50% growth inhibition (GI50), total growth inhibition (TGI), and 50% cell death (LC50). Results are shown as the mean ± SEM of three independent experiments, each performed in triplicate. Superscripts mean groups not statistically different ($p > 0.05$) for each response.

Sample	Incubation time (h)	GI_{50} (μg mL^{-1})	TGI (μg mL^{-1})	LC_{50} (μg mL^{-1})
N1	24	32.2 ± 1.7^{a}	41.7 ± 1.4^{a}	53.5 ± 0.9^{a}
	48	37.2 ± 1.4^{a}	45.5 ± 0.8^{a,b}	55.0 ± 0.2^{a}
	72	36.4 ± 4.7^{a}	46.3 ± 2.7^{a,b}	59.4 ± 4.2^{a}
N2	24	37.0 ± 2.3^{a}	46.7 ± 1.3^{a,b}	57.9 ± 0.1^{a}
	48	40.3 ± 2.5^{a}	49.2 ± 1.9^{a,b}	57.8 ± 0.2^{a}
	72	46.5 ± 1.1^{a}	56.1 ± 3.2^{b}	74.0 ± 2.6^{b}
P1	24	77.7 ± 4.4^{b}	NC*	NC
	48	72.4 ± 2.7^{b}	NC	NC
	72	81.8 ± 3.1^{b}	NC	NC
P2	24	72.0 ± 3.3^{b}	NC	NC
	48	75.8 ± 2.9^{b}	NC	NC
	72	83.8 ± 4.6^{b}	NC	NC

*NC, not calculated.

available about chemical phlorotannin composition and differences in the *in vitro* cell models used. However, the parameter values obtained in the present study seem to be close to the antiproliferative concentration levels of brown seaweeds extracts published in various reports for other *in vitro* cell studies [11,29,30]. As an example, He et al. [31] reported cell viability inhibition values of 36.9 and 60.5% for the brown seaweed *Saccharina japonica* extracts containing phlorotannins (2.19 and 1.28 mg g^{-1}, respectively) on hepatocellular carcinoma cells following a exposure time of 24 h at the concentration of 60 μg mL^{-1}. Interestingly, data summarized in Table 4 also showed that N1 extract, which contained lower phlorotannin concentration than N2 extract (see Table 1 for the values of TPC and total phlorotannins for the purified N1, N2, P1 and P2 extracts), exerted slightly higher effect on cell proliferation than N2 extract, provided by the lower values obtained for the parameters at the different incubation times. According to the chemical differences in the phlorotannin fraction observed between these extracts, these results suggest that either phlorotannins with selective bioactivity or the presence of other compounds in the extracts might be responsible for the differential antiproliferative effectiveness observed between N1 and N2. In this regard, further work is required to elucidate the active constituents responsible for this differential effectiveness between phlorotannin-enriched extracts.

4. Conclusions

In the present work, the phlorotannin composition of different *S. muticum* samples collected at different locations along the European Atlantic coast was investigated. After PLE extraction, the samples collected at the extreme locations of a latitudinal gradient along North Atlantic coasts, i.e., Portugal and Norway, were found to be the richest in total phenols and, particularly, in phlorotannins. The extracts obtained from these locations were further purified and chemically characterized using a modified HILIC × RP-DAD-MS/MS method. The application of this methodology allowed the tentative identification of phlorotannins with great chemical variability containing different degrees of polymerization and structures; fuhalols, hydroxyfuhalols and phlorethols were the most-abundant phlorotannins on these samples, showing also particularities and important differences depending on the geographical location. This is the first time that these complex structures are separated and characterized with such detail. Afterwards, the antiproliferative activity of these extracts against HT-29 adenocarcinoma colon cancer cells was studied. Results revealed that Norwegian samples of *S. muticum* presented the highest activity, showing a good cytotoxic potential at concentrations in the medium micromolar range.

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ANEXO D. Articulos de Revisión

1. Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. *M. Herrero, A. P. Sánchez-Camargo, A. Cifuente, E. Ibáñez.* **TrAC Trends Anal. Chem. 71 (2015) 26–38.**

2. Phenolic compounds from edible algae: bioactivity and health benefits. *L. Montero, A.P. Sánchez-Camargo, E. Ibáñez, B. Gilbert-López.* **Curr. Med. Chem, 2016** (En prensa).

3. Rosemary (*Rosmarinus officinalis*) as a functional ingredient: recent scientific evidence. A. P. Sánchez-Camargo, M. Herrero. **Curr. Op. Food Sci. 14 (2017) 13–19**

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Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction

Miguel Herrero *, Andrea del Pilar Sánchez-Camargo, Alejandro Cifuentes, Elena Ibáñez

Laboratory of Foodomics, Institute of Food Science Research (CIAL, CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain

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ABSTRACT

We present an up-to-date review on the use of subcritical and supercritical extraction processes to obtain functional bioactive compounds from different natural matrices, including plants, food by-products, seaweeds and microalgae. The ever-increasing demand for natural bioactive compounds is fostering the search for new underexplored natural sources for this kind of components. The use of advanced sustainable extraction techniques to extract and to purify the bioactive compounds is of the utmost importance. Pressurized liquid extraction and supercritical fluid extraction are two of this kind of technique that have already been widely employed to recover bioactives from different sources. We comment on the most relevant recent developments and improvements involving these natural sources and discuss the future evolution of the use of these two extraction processes.

Contents

1. Introduction

The ever-increasing market of functional foods is always demanding new bioactive ingredients that can be used by the food industry for the development of functional products with scientifically sustained claims. In this regard, much attention has been paid in recent years to natural compounds, such as polyphenols, carotenoids, peptides, sterols or polyunsaturated fatty acids (PUFAs), and their associated bioactivities. As an example, it has been suggested that polyphenols possess positive effects against cardiovascular diseases, neurodegeneration and cancer in different human intervention trials, thanks to their antioxidant, anti-inflammatory or anti-proliferative activities [1], although how these natural components may confer the benefits mentioned has not been completely elucidated [2].

Even if these relationships are not clear enough, the search for new natural bioactive components is a hot topic on which a lot of research effort is being focused. Among the different kinds of natural sources for bioactives, plants are probably most studied, mainly due to their easy availability and diversity. Many plant-derived natural

* Corresponding author. Tel.: +34 910 017 946; Fax: +34 910 017 905.
E-mail address: m.herrero@csic.es (M. Herrero).

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products have been suggested as sources for bioactive compounds [3], although the research has also been started on other interesting natural sources, such as macroalgae (or seaweeds) and microalgae. The marine environment is considered a rich, underexploited source of bioactive compounds [4]. Macroalgae and microalgae are probably the two groups of marine organisms that have attracted most attention for their potential as industrially feasible natural sources of bioactive compounds [5,6].

Besides these natural sources, the use of food industry-related by-products is also gaining interest, since some important residues generated during food manufacture have been revealed to possess significant amounts of bioactive compounds [7]. The use of this latter source is further promoted because it allows reduction in food wastes and makes by-products valuable, so adding important environmental benefits.

Closely related to this point, the sustainability of the processes employed to extract and to purify the bioactive compounds is of the utmost importance. Today, not only is efficiency of the extraction techniques sought by extracting bioactives with the highest possible extraction yield and associated bioactivity, but the development of environment-friendly extraction processes is also preferred over conventional extraction protocols [8]. These new developments are strongly desired to comply with the Green Chemistry principles related to extraction [8]:

1 innovation by selection of renewable resources;
2 use of alternative solvents, mainly water;
3 reduction of energy consumption using innovative technologies;
4 production of co-products instead of wastes;
5 reduction of unit operations, fostering automation of processes; and,
6 aim for non-denatured, biodegradable extracts without contaminants.

In order to comply with these requirements and to increase, even further, the "greenness" of these processes, biorefinery concepts are being developed, based on the use of natural biomass to obtain a wide array of products useful in different production fields (e.g., food, pharmaceutical or agricultural) together with the generation of energy in order to minimize or to eliminate any industrial wastes. As a part of these biorefinery approaches, suitable, efficient, fast, cheap and green extraction techniques are needed. In this regard, technologies based on compressed fluids play an important role.

Among such technologies, supercritical fluid (SCF) extraction (SFE) and pressurized liquid extraction (PLE) are the most widely employed in obtaining bioactive components from natural sources [9]. These two advanced extraction techniques provide advantages over the traditional extraction procedures that make them really attractive for efficient, scalable extraction of bioactive and functional compounds from different natural matrices. Thus, the goal of the present review is to provide an up-to-date overview on the use of PLE and SFE to recover bioactive compounds from plants, food by-products, algae and microalgae, following our previous work [10]. We highlight and describe the most relevant characteristics of each technique, and the most recent, relevant applications and developments focused on the use of these technologies for the extraction of target components at different scales.

2. Pressurized liquid extraction (PLE)

2.1. General aspects

The general term PLE involves the application of an extraction technique that has also been referred in the literature as pressurized fluid extraction (PFE), pressurized hot-solvent extraction (PHSE) or accelerated solvent extraction (ASE). In any case, this technique is based on the use of pressurized solvents at high temperatures (always below their critical points), under conditions in which the solvents are maintained in the liquid state during the extraction process. When the extraction solvent is water, this technique is also called subcritical water extraction (SWE), superheated water extraction (SHWE) or pressurized hot-water extraction (PHWE). The general principles and instrumental requirements are the same in both cases, as just the solvent employed is modified, although, in SWE, other important parameters have significant influence, as described below.

In general terms, PLE processes are faster, and require smaller volumes of solvents than traditional extraction techniques, such as Soxhlet extraction [11]. These characteristics are provided by the enhanced mass-transfer rate, the increase in solubility of the analytes and the decreases in solvent viscosity and surface tension that occur under PLE conditions. These latter properties further facilitate the extraction rate, considering that the solvent will penetrate into the matrix more easily, reaching deeper areas and increasing the surface contact, thus improving the mass transfer to the solvent. In SWE, besides these characteristics, the extraction is affected by the dielectric constant (ε) of water, which is a measure of the polarity of the solvent and is significantly reduced when water is heated at high temperatures while maintained in the liquid state. This value (room temperature, $\varepsilon \sim 80$) might be decreased to values close to those of some organic solvents, such as ethanol or methanol when heated to 200–250°C ($\varepsilon \sim 30$–25) [10]. Consequently, the use of SWE can be suggested as a useful alternative to employing organic solvents in some applications. Indeed, although not feasible for every application, SWE can be seen as the "greenest" of the PLEs.

Instrumental requirements for PLE and SWE are the same; further details and schemes can be found elsewhere [9,10,12,13]. Briefly, the instrumentation comprises a high-pressure pump to push the solvent into an extraction cell, which is maintained in an oven to control the extraction temperature, with different valves and restrictors to control the extraction pressure. Obviously, these basic requirements may be made more or less sophisticated by adding other additional parts, such as solvent-controller valves, nitrogen-purging lines, extraction cells, and vial trays for automation. The possibility of automation is a further advantage of PLE, since automation helps to reduce extraction-to-extraction variation, increasing reproducibility. Thanks to these features and to their relatively simple instrumental requirements, PLE and SWE gained popularity in recent years as extraction techniques for the isolation of natural bioactive compounds, as we describe in the following sections.

2.2. PLE of bioactives from plants

Pressurized liquids have been widely employed for the extraction of bioactive compounds from plants. This group of organisms has undoubtedly seen the largest number of applications of these techniques for the recovery of bioactives. In this section, we highlight some remarkable advancements and innovations made recently. For a more comprehensive list of PLE applications, other recent reviews are also available [9,14] {e.g. coumarins [15], flavonoids [16,17] and other polyphenols [18,19], saponins [20], phospholipids [21], inositols [22] and other bioactive compounds and active extracts [23,24] from a variety of plant materials}.

A good number of applications employ very high extraction temperatures (above 150°C), most notably when water is the extracting solvent [9]. It has been repeatedly shown that high temperatures do not always negatively affect usually labile natural bioactive compounds. In SWE, the increase in temperature decreases water polarity and increases the solubility of target components, and so their

extraction. Nevertheless, depending on the chemical nature of the components targeted, the applicability of water may be limited. In order to solve this shortcoming, adjustment of water pH has been proposed and demonstrated for the extraction of curcumin from turmeric (*Curcuma longa* L) [25]. Although curcumin has very limited water solubility, this study demonstrated that using pressurized water at 197°C with 62 g L^{-1} of buffer concentration at pH 1.6 allowed a significant increase on curcumin solubility, probably due to its protonation [25]. Thus, the pH of the extracting solvent should be closely examined as a way to optimize the extraction process. This parameter could have more importance when degradation of bioactives is observed at high temperature. For example, Co et al. [26] studied the extraction of phenolic compounds from birch bark by SWE at 80°C and 180°C. They showed that, at 80°C, the extraction reached a steady-state after 5 min. At that temperature, the extracted compounds were stable for longer extraction times. At 180°C, there were partly degraded extracts for which a multitude of new peaks was observed after high-performance liquid chromatography diode-array detection (HPLC-DAD) analysis, although the antioxidant activity of these latter extracts was higher [26].

Attainment of more active SWE extracts at the highest test temperatures has been repeatedly observed in a variety of matrices [27–31]. These observations led to study of the possibility of some reactions occurring at high temperatures during the extraction process. To confirm this hypothesis, a thorough battery of assays was developed in glycation-model systems [32] and natural samples of different types [33]. In both cases, the neoformation of antioxidant compounds derived from Maillard and caramelization reactions when proteins/amino acids and reducing sugars were present in the natural samples was confirmed during SWE at high temperatures (up to 200°C). These data suggest the need to characterize attained extracts carefully in order to assess their safety correctly, even if their antioxidant activity might be higher.

In an effort to improve the recovery of compounds from plant sources, different approaches have been studied in order to produce structural damage to the vegetable cell walls. Cellulases and hemicellulases, pectinases and proteases may be effective enzymes to carry out this task. Miron et al. [34] recently compared the extraction yield, total amount of phenols and antioxidant activity of lemon-balm extracts treated and untreated with enzymes. This study revealed that PLE alone using water at 150°C was able to produce better results than PLE in combination with enzymes for the studied variables. However, depending on the application, this and other physical treatments should not be discarded in order to weaken the vegetable cell walls and to increase the extraction of target compounds.

Most published applications are performed using commercial instruments, in which one or more static extraction cycles are performed. However, some home-made continuous extraction systems have been also developed {e.g. a continuous flow instrument applied for the SWE of polyphenols from onion [35]}. The use of this kind of system was reported to be able to reduce degradation effects and to complete the extraction of flavonols within 60 min, performing the extraction at 110°C with pressurized acidified water/ethanol 95:5 pumped at a flow rate of 4 mL min^{-1}. A further advantage of this approach was later demonstrated by the same group, by introducing an on-line particle formation and drying process based on the use of SCFs [36]. This novel patented process, called WEPO (Water Extraction and Particle formation On-line) [37] (Fig. 1), combines a dynamic SWE procedure with the production of a continuous aerosol mixing the water extract with supercritical CO_2, which is later dried by a hot N_2 current. This process allows attainment of results similar to static SWE processes but with the advantage of directly obtaining a dry powder with particles as small as 4 μm in diameter [36]. Thus, a massive gain in process time is obtained compared to the more conventional extraction and freeze-drying processes needed to achieve a dried extract using SWE.

Recently, the search for suitable clean solvents for PLE processes was widened {e.g., ionic liquids (ILs) [38], surfactants [39], bioethanol [11] and ethyl lactate [40] are some examples of novel solvents reported as environmentally acceptable options for the recovery of bioactive compounds from plants}.

Although there is no published application of their use in this review, deep eutectic solvents (DESs) have also been pointed out as possible green solvents [41], which are formed by mixing two naturally-occurring components, namely associated hydrogen-bond acceptor and a hydrogen-bond donor, usually having a much lower melting point that their individual components. DESs have been reported to be environment-friendly, economically-viable, promising alternatives to traditional volatile solvents, since they are easily prepared from natural compounds [41]. Thus, their potential for use in PLE processes is worth exploring.

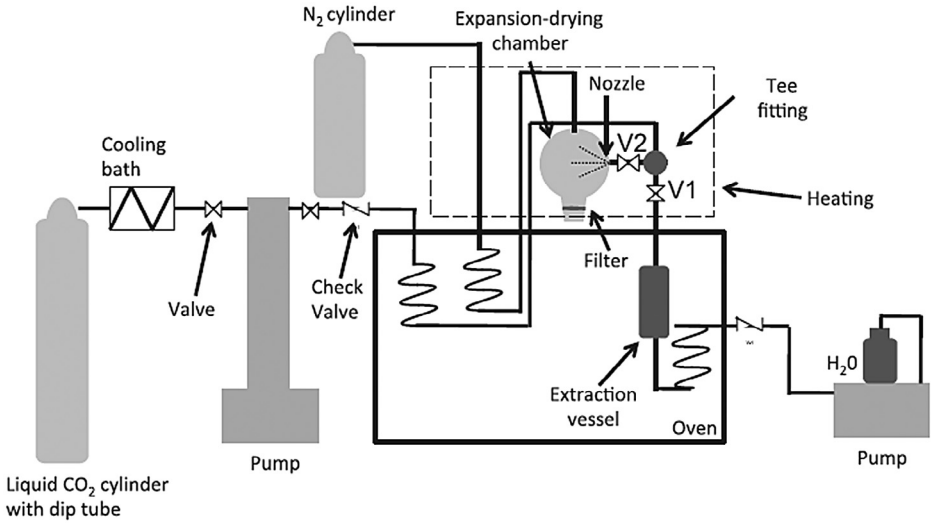

Fig. 1. WEPO (Water Extraction and Particle formation On-line) equipment. {Reproduced with permission from [36]}.

2.3. PLE of bioactives from macroalgae and microalgae

The marine ecosystem is widely considered an underexplored vast source of potential bioactive compounds useful in different areas, such as pharmaceuticals, cosmetics and food science. Many marine organisms have already been pointed out as sources for bioactive compounds [4]. The particular growing environment, sometimes involving extreme conditions, of these organisms is partly responsible for bioactive compounds that are synthesized as adaptation tools and responses to demanding life conditions, such as high salinity, radiation, temperature or availability of nutrients. Within marine organisms, macroalgae (or seaweeds) and microalgae are probably most studied in terms of potential sources of bioactives. Today, microalgae are also the focus of a hot research topic for other non-food applications, such as the production of biofuels [42,43] or carbon-dioxide capture [44]. Considering the potential of microalgae for production and overexpression of selected compounds under particular growing conditions, these organisms are commonly used as bioreactors. They possess some characteristics that make them much appreciated for industrial applications, such as the possibility of using residual nutrients or CO_2 from flue vents without the need for arable land [45]. For these reasons, microalgae have been pointed out as the focus of economically feasible biorefinery processes [46,47]. The concept of biorefinery implies all constituents generated from microalgal biomass have value for the generation of products applicable to different areas, such as food, non-food, biofuel or energy production [45]. In this regard, the use of extraction techniques with compressed fluids is of great interest as a part of the processing chain within a biorefinery, given their usefulness in obtaining high added-value products from the natural materials and the possibility of re-extracting the materials until exhaustion.

In the period covered by the present review, different applications were developed to extract several kinds of bioactive compounds from microalgae and seaweeds using PLE and SWE. Table 1 summarizes the extraction conditions employed in the most remarkable applications.

As Table 1 shows, carotenoids are the most targeted type of compound. Carotenoids have a physiological function in organisms as pigments, but possess some interesting bioactivities, such as antioxidant, anti-proliferative, anti-inflammatory, provitamin A activity, and even protection of macular degeneration [63]. Some macroalgae and microalgae are well-known producers of carotenoids (e.g., *Dunaliella salina*, a green microalga, is widely employed at industrial level for the production of β-carotene, where salinity and light are the most-relevant parameters for overproduction of this compound) [64]. Naturally synthesized *Dunaliella* β-carotene is a mixture of all-*trans*, 9-*cis*, 15-*cis* and other minor isomers. In this regard, it may be interesting to direct and to tune the growing conditions towards the production of 9-*cis* isomer, which was shown to be more active (antioxidant activity) than the corresponding all-*trans* isomer.

After comparing ethanol and hexane as extracting solvents for the isolation of carotenoids from *Dunalilla salina*, Herrero et al. [52], could not conclude that there was a positive correlation between the all-*trans*/9-*cis* isomers ratio and the antioxidant activity of the extracts. Besides, it could be demonstrated that other minor carotenoids present in the ethanol PLE extracts could also have a positive, important influence on the overall antioxidant activity of the generated extracts. In any case, it could be clearly observed that β-carotene recovery increased with the extraction temperature, the best results being attained at 160°C. This work demonstrated that high temperature may not always be detrimental to the extraction of bioactive compounds from natural matrices.

Although other microalgae, such as *Haematococcus pluvialis*, have also been a well-studied source of carotenoids (e.g. xanthophyll

Table 1
Some representative applications involving the use of pressurized liquid extraction (PLE) or subcritical water extraction (SWE) for bioactive compounds from macroalgae[M] and microalgae[m] published during the period 2006–14.

Matrix	Compounds of interest	Extraction solvent	T(°C) / P (MPa)	Mode of extraction	Sample dispersion	Extraction time (min)	Ref.
Phaeodactylum tricornutum [m]	Fucoxanthin	Ethanol	100/ 10.3	Static	Sea sand	10	[48]
Eisenia bicyclis [M]	Fucoxanthin	Ethanol/water 90:10	110 / 10.3	Static	Sea sand	5	[49]
Chlorella ellipsoidea [m]	Zeaxanthin	Ethanol	115.4 / 10.3	Static		23.3	[50]
Chlorella vulgaris [m]	Carotenoids	Acetone	50 / 10.3	Static	Sea sand	20	[51]
Dunaliella salina [m]	Carotenoids	Ethanol	160 / 10.3	Static	Sea sand	17.5	[52]
Neochloris oleoabundans [m]	Carotenoids	Ethanol	112 / 10.3	Static	Sea sand	20	[53]
Haematococcus pluvialis [m]	Carotenoids	Ethanol	50 / 10.3	Static		20	[54]
Rhizoclonium hieroglyphicum [m]	w-3 fatty acids	Chloroform/ methanol	120 / 10.3	Four cycles	Diatomaceous earth, Ottawa sand	30	[55]
F. serratus [M], *L. digitata* [M], *G. gracilis* [M], *C. fragile* [M]	Polyphenols	Ethanol/Water 80:20	100 / 6.9	Static	Silica (1:2 w/w, sample:silica ratio)	25	[56]
		Methanol/Water 70:30	90 / 6.9		and diatomaceous earth		
Anabaena doliolum [m], *Spongiochloris spongiosa* [m], *Porphyra tenera* [M], *Undaria pinnatifida* [M]	Polyphenols	Clean: Hexane/acetone (1:1 v/v)	130 / 13	Two 10 min cycles	n.i.	20	[57]
		Extraction: methanol/ water (v/v) 80:20					
Sargassum muticum [M]	Polyphenols	Ethanol/water 75:25	100 / 10.3	Static	Sea sand	20	[58]
Ascophyllum nodosum [M], *Fucus vesiculosus* [M], *Fucus serratus* [M]	Antioxidants	Ethanol/ water 80:20	100 / 6.9	Static	Silica (sample:silica ratio 1:3 (w/w)) and diatomaceous earth	n.i.	[59]
Haematococcus pluvialis [m]	Antimicrobials	Ethanol	100 / 10.3	Static	Sea sand	20	[60]
Haematococcus pluvialis [m]	Antioxidants	Water	200 / 10.3	Static	Sea sand	20	[61]
Himanthalia elongata, [M] *Synechocystis* sp. [m]	Antioxidants, antimicrobials	Ethanol	150 / 10.3	Static		20	[62]

astaxanthin) [54], seaweeds have also been targeted as sources of carotenoids. Fucoxanthin is a carotenoid typical of brown algae with important related bioactivities [49]. Because of its more polar nature, compared to β-carotene, fucoxanthin is preferably extracted by PLE using high proportions of ethanol [49]. Interestingly, it can be inferred from Table 1 that medium-high temperatures ~100–110°C provide the best extraction efficiencies, confirming once more the possibility of using high temperature with pressurized solvents to extract relatively labile compounds.

PLE-based approaches have also been employed to extract other antioxidants, such as polyphenols. In general, as can be observed in the numerous applications already developed and commented upon, PLE allows improvement of the recovery of natural bioactives compared to classical extraction approaches. However, depending on the application, this is not always the case. For example, Heffernan et al. [56] compared the ability of solid-liquid extraction (SLE) and PLE at several fixed conditions to obtain antioxidant extracts from four different macroalgae. Their results demonstrated that SLE extracts possessed higher antioxidant activity than their PLE counterparts.

In general, it is important to note that, as in any other natural matrix, the results attainable may also greatly depend on not only the species studied but also the growing conditions (environmental conditions and growing location) and collection time. For example, it was possible to produce PLE antimicrobial extracts from *Haematococcus pluvialis* collected in the red phase, whereas extracts from the same microalga collected in the green phase did not present any activity [60]. Thus, these parameters should be closely examined and studied for scaling-up and production purposes. Moreover, the need for pre-treatment prior to extraction to weaken the cell-wall structure of the targeted algae or microalgae might also have important implications from different perspectives (e.g. feasibility, costs, and process time). In this regard, the same microalga was also studied to unravel the influence of different sample pretreatments (i.e. ultrasound, freezing-thawing cycles, physical homogenization and freezing-smashing-thawing) on the extraction outcome [61]. As Fig. 2 shows, freezing-smashing-thawing treatment produced higher cellular damage, facilitating subsequent PLE.

Comparing SWE and PLE for the extraction of antioxidants, the behavior of the extracts produced is not always the same. Whereas, generally, different organic solvents may produce similar trends, water extracts have a different behavior. For example, considering the antioxidant activity of several *Chlorella vulgaris* extracts, it could be observed how the extraction yield always increased with temperature, regardless of the solvent used, as a typical response to mass-transfer enhancement. However, while extracts of organic solvents (ethanol and acetone) obtained at the lowest tested temperature (50°C) provided the best antioxidant-activity values, the most active extracts produced with water were achieved at the highest studied temperature (200°C). Possible explanations of these findings are the decreased dielectric constant of water at 200°C, which could imply that water is more suitable under such conditions for the extraction of the antioxidants present in this microalga, and, also, as previously commented upon, the chance of occurrence of Maillard reaction processes at these conditions could effectively generate neoformed antioxidant components.

2.4. PLE of bioactives from food-related by-products

The use of food-related by-products, from agricultural and food-industry sources, to obtain bioactive compounds is very interesting from the point of view of sustainability [65]. By using these materials, it is possible to produce high added-value compounds, while reusing industrial wastes and undervalued substrates, thus reducing the impact on the environment of some production processes. In this regard, the use of environment-friendly extraction techniques adds obvious advantages [7]. PLE has already been explored as the extraction technique of choice for a variety of bioactive compounds from different food-related by-products. Table 2 summarizes the most relevant recent applications. As Table 2 shows, most PLE and SWE applications devoted to extraction and recovery of bioactives from by-products focus on vegetal products. Water and ethanol are the solvents most employed to recover phenolic compounds from coffee [71], grapes [76], olive leaves [78,83] and other fruit by-products [68,70,79,80], although the conditions vary among applications (see Table 2).

One of the most interesting groups of applications developed concerns the extraction of quercetin from onion waste, whioch is based on extractiing quercetin glycosides with water at 120°C and 5.0 MPa, followed by enzymatic hydrolysis in water at 90°C and pH 5.0 [79]. To do that, a thermostable β-glucosidase from *Thermotoga neapolitana*

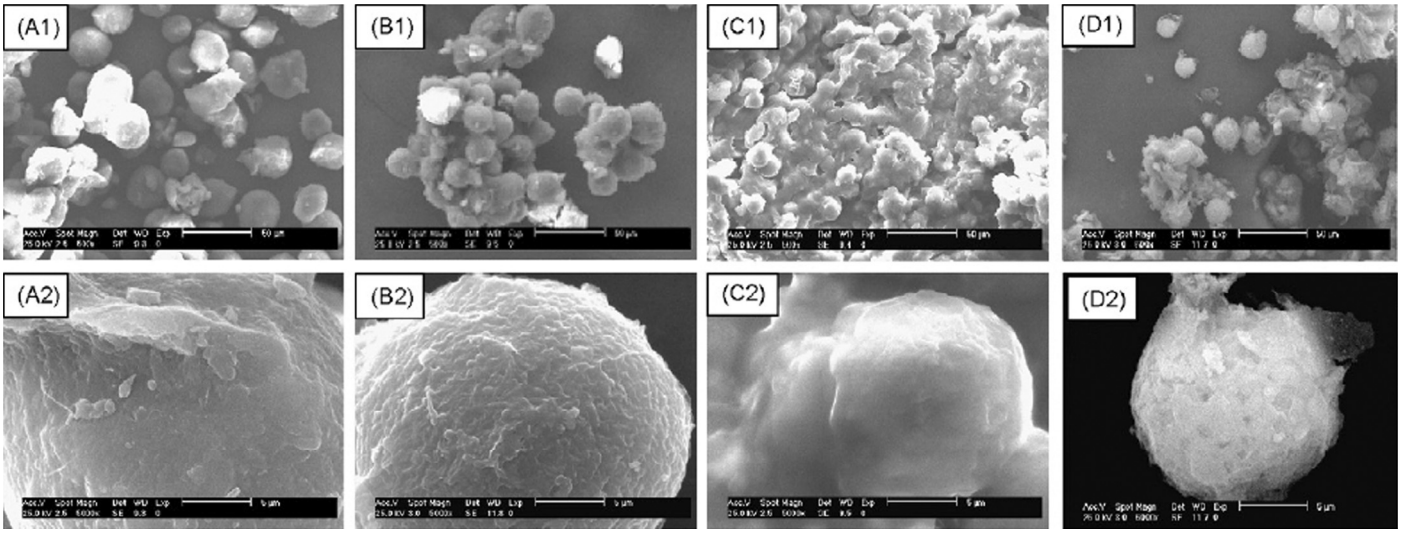

Fig. 2. SEM pictures of the *H. pluvialis* (A) before the extraction process, (B) residue after SWE at 100°C using freezing-smashing-thawing pretreatment, (C) residue after SWE at 200°C using freezing-smashing-thawing pretreatment, and D) residue after SWE at 200°C using the dispersing tool pretreatment. {Reproduced with permission from [30]}.

Table 2
Some representative applications involving the use of pressurized liquid extraction (PLE) or subcritical water extraction (SWE) for bioactive compounds from food-related by-products published during the period 2006–14.

Matrix	Compounds of interest	Extraction solvent	T(°C) / P (MPa)	Mode of extraction	Sample dispersion	Extraction time (min)	Ref.
Agaricus bisporus by-products	Sterols	Ethanol	50 / 10.3	Five 5 min cycles	Sea sand	25	[66]
Apple by-products	Flavonols	Water	120 / 10.3	Static		3	[67]
Brazilian cherry seeds	Phenolic compounds	Ethanol	70 / n.i.	Four cycles	Sodium sulfate	16	[68]
Carrot by-products	Carotenoids	Ethanol	60 / 5.2	Two 5 min cycles	Glass beads	10	[69]
Citrus unshiu peel	Hesperidin and narirutin	Water	160 / 10.0	Static	Diatomaceous earth (1:2, sample: dispersant)	10	[70]
Coffee silver skin	Antioxidants	Water	270 / 5.3	Static		40	[71]
Ginseng stems and leaves	Antibacterials	Water	190 / 5.2	Static	Diatomaceous earth (1:3, sample:dispersant)	10	[72]
Grape Pomace	Tannins	Water	150 / 10.3	Static	Quartz sand	30	[73]
Grape Pomace	Phenolic compounds	Water	100–200 / 10.3	Static	Quartz sand	5	[74]
Grape Pomace	Aromatic aglycones	Ethanol/water 50:50	90 / 10.3	Three 8 min cycles	Sea sand	25	[75]
Grape seeds	Catechins and proanthocyanidins	Water	150 / 10.3	Static		30	[76]
Green tea by-products	Lutein	Ethanol/ water 80:20	160 / 10.3	Two 5 min cycles	Quartz sand	10	[77]
Olive leaves	Phenolic compounds	Ethanol	150 / 10.3	Static	Sea sand	20	[78]
Onion waste	Quercetin	Water	120 / 5.0	Three 5 min cycles		15	[79]
Pomegranate peels	Phenolic compounds	Water	40 / 10.3	Static	Sea sand	5	[80]
Potato peels	Anthocyanins	Ethanol/water 80:20	80 / 10.0	Continuous (10 g min^{-1})		180	[81]
Potato peels	Phenolic acids	Methanol/water 90:10	160 / 6.9	Static	Ottawa sand	15	[82]

n.i: non indicated.

was used in order to release the quercetin aglycone from the extracts. This approach could even be seen as a part of a wider biorefinery concept [84], similar to that described above for microalgae. Once the success of this coupling was demonstrated, the authors further improved their process by developing a procedure for the continuous SWE and enzymatic hydrolysis of quercetin glycosides using the same enzyme immobilized in a supporting material [85]. To optimize all the variables involved, the enzymatic hydrolysis was firstly optimized using a three level central composite design considering temperature, pH and volume concentration of ethanol. Once the optimum temperature (84°C) was determined, the continuous extraction was optimized in terms of pH and concentration of ethanol. Lindahl et al. [85] showed that 5% ethanol favored the extraction whereas the pH selected was 5.5, using a continuous flow of solvent of 3 mL min^{-1}. This combination allowed significant reduction in laboratory work thanks to the simultaneous operation of the two processes involved, extraction and hydrolysis.

We already commented upon use of SWE at very high temperature in order to produce antioxidant extracts, this being once more confirmed using coffee silver skin as the natural matrix to obtain antioxidant water extracts [71]. In this case, an in-laboratory-built extractor was employed and different extraction temperatures up to 270°C were studied. Interestingly, the higher the extraction temperature employed, the higher the antioxidant activity obtained, although the chemical characterization of the extracts revealed some remarkable data. For example, above 200°C, antioxidant activity was maximized, although recovery of caffeine decreased and formation of 5-hydroxymethylfurfural was confirmed. Moreover, no 5-caffeoyl quinic acid was found in those extracts, probably due to degradation at these very high temperatures. In any case, the total amount of phenols found in the extracts increased with the extraction temperature and correlated well with the antioxidant activities found [71]. However, these results put a question mark over the neoformation of antioxidants derived from Maillard and/or caramelization reactions that could have a significant influence in the overall antioxidant activity, giving a positive response to the Folin method, as was already demonstrated in other by-products [67]. In this regard, deeper chemical characterization of those extracts could have shed more light on this issue.

In general, we can conclude that PLE and SWE may be useful extraction tools for the recovery of interesting bioactive components from by-products.

3. Supercritical fluid extraction (SFE)

3.1. General aspects

SFE is a widely considered green extraction technology that has been broadly applied for the recovery of valuable compounds from different materials, both at laboratory and industrial levels. SFE is based on the use of solvents above their critical pressures and temperatures. At those conditions, SCFs possess particular physicochemical characteristics between gases and liquids, generally acquiring higher density than a gas but maintaining similar viscosities and intermediate diffusivities [86]. For more in-depth information on SFE fundamentals, readers are referred to some excellent reviews and book chapters [86–91]. Although a variety of solvents may be employed at supercritical conditions, carbon dioxide is, by far, the most used SCF for the recovery of bioactive and valuable compounds from natural matrices. CO_2 presents several advantages, including mild critical conditions, non-toxic, non-flammable, non-explosive and, furthermore, easily available and cheap. Besides, being a gas at room conditions, CO_2 is straightforwardly eliminated from the extracts obtained during decompression, allowing the attainment of solvent-free extracts. This latter

property is of the utmost interest for the generation of bioactive compounds to be used in the food industry.

The solvent properties of SCFs may be tuned by changing pressure and temperature values, directly influencing density, making SFE a very selective technique, which is another advantage when aiming at extraction of target compounds from complex natural matrices. However, an important shortcoming of the use of supercritical CO_2 ($scCO_2$) is its low polarity. Considering the chemical nature of most natural bioactives, generally polar compounds, CO_2 alone may not be able to extract them. To cope with this issue, cosolvents (also called modifiers) are employed during extraction at small proportions (typically, 1–10%). These cosolvents are solvents with higher polarity than CO_2, expanding the range of compounds attainable by increasing the polarity of the supercritical mixture. Another practical advantage of SFE is the possibility of performing fractionation during decompression, just by using two or more decompression steps. This cascade depressurization is useful to separate components in the extract.

A lot of factors are therefore involved in an extraction process, not only those previously mentioned (supercritical solvent, nature of modifier, proportion of modifier, temperature and pressure), but also other parameters related to the sample and the extraction process, such as water content, particle size, dispersant agent, amount, SCF flow rate, extraction time or fractionation. Even the mode of extraction may be varied, from static to dynamic or mixed mode using a short static step followed by a longer dynamic step. Consequently, we can assume that optimization of a particular process may be very complicated due to the interaction of the different parameters involved. For this reason, experimental designs to determine the best extraction conditions in a particular extraction process are widely used [92], so a systematic study may be performed with a statistically supported selection of influential variables.

Regarding the instrumentation needed, it can be more or less complex, depending on the process developed. Further details and schemes can be found elsewhere [9,10,12,88]. A simple SFE extractor is composed of solvent and modifier pumps, extraction cell with temperature control, pressure restrictor and collection vessel. Alternatively, the instrument may be made more complicated by adding fractionation vessels with independent pressure and temperature controls (separators), extraction column for liquid extraction (counter-current extraction), CO_2 recycling system or refrigerated trap to collect volatiles. Likewise, the complexity of the instrument will also depend on the scale.

SFE has been widely applied to bioactives from natural sources, including those targeted in this review. Considering the great number of applications published relating to these topics, in the following sections, we describe and comment on the most-remarkable advancements made in recent years.

3.2. SFE of bioactives from plants

Several recent reviews dealt with the recovery of bioactive compounds from plants using SFE from a more or less broad perspective [9,88,93–97], and gave an idea of the great applicability of this extraction technique.

Considering the nature and the physical properties of $scCO_2$, SFE with CO_2 has mainly been used to extract non-polar bioactive compounds, such as fatty acids, sterols or carotenoids. Phytosterols are much appreciated for being natural components with an important anti-cholesterolemic activity [98]. These compounds are quite soluble in $scCO_2$. Different phytosterols have been effectively extracted at 60°C and 35 MPa, together with linoleic and linolenic acids, producing black sesame-seed extracts with neuroprotective activity in a rat model [99]. Milder conditions (40°C and 15 MPa) were found more appropriate for the recovery of β-sitosterol from sea buckthorn [100]. In the recovery of phytosterols from Kalahari melon seeds, $scCO_2$ at 30 MPa and 40°C allowed attainment of phytosterol-enriched oil that possessed 94% higher concentration of phytosterols than conventional solvent extraction [101]. SFE was also superior for the recovery of those components from pumpkin seeds compared to solvent extraction [102]. These data highlight the importance of optimizing the most relevant extraction conditions for each sample, as the particular nature of the sample may directly influence extraction of target components, even if the same kinds of compounds are sought [98].

Carotenoids are some of the most important pigments found in plants, being much appreciated within bioactive compound-related industries because of their potential bioactivities, as previously mentioned. Although PLE has been explored for the extraction of carotenoids, the non-polar nature of some of these components implies that SFE using $scCO_2$ may be the most suitable extraction technique. Carotenoids have been extensively recovered from different plant matrices using SFE [93–95,103,104]. As mentioned above, the moisture of the sample is an important parameter in SFE, as it can directly affect the composition of the extracts. A recent study compared the performance of SFE with CO_2 to recover carotenoids from pumpkin after oven-drying and after freeze-drying [105]. Although freeze-drying is generally considered a milder drying process, more adequate for labile compounds, such as carotenoids, vacuum oven-drying combined with milling allowed an eight-fold increase in extracted carotenoids compared to freeze-drying and milling. The residual moisture measured prior extraction was 8% and 12% for oven-dried and freeze-dried samples, respectively. These data highlight the importance of closely studying the physical state of the sample (moisture and particle size) in order to maximize recoveries of target components, above all, for subsequent scale-up of the process. Indeed, considering the relative complexity of this kind of SFE process when applied at industrial scale, all the parameters involved should be carefully studied so that the resulting process is economically feasible [106,107].

A possible way to increase further the recovery of target bioactive compounds was recently explored taking carotenoids as model compounds [108]. A combination of plant cell-wall glycosidases was found to be useful to increase lycopene recovery from freeze-dried tomato after SFE (50 MPa, 86°C, 4 mL min^{-1} $scCO_2$ flow). However, it was found that the resulting denser tomato microstructure and smaller particle size caused a restriction in the CO_2 diffusion, leading to a channeling effect. Interestingly, the addition of an oleaginous co-matrix (hazelnut seeds, ratio 1:1) improved the $scCO_2$ diffusion, allowing co-extraction of lipids and further increasing the lycopene solubility in $scCO_2$ and, thus, its recovery from the matrix being studied, as can be seen in Fig. 3 [108]. The resulting extracts contained three times more lycopene than control extracts using sample without any treatment.

SFE was also demonstrated to be useful for the extraction of other lipophilic compounds, such as squalene [109], tocopherols [110] or fatty acids [111]. This technique was also applied to the extraction of more polar natural bioactives, such as phenolic compounds [27,112–116] and some alkaloids [117]. In these cases, the use of cosolvents is unavoidable. Ethanol or ethanol/water mixtures are the most employed cosolvents in this kind of extraction.

3.3. SFE of bioactives from macroalgae and microalgae

The application of SFE to macroalgae and microalgae has found a good number of applications due to algae being a well-known source for a great variety of bioactive compounds. Leaving aside the use of SFE to extract lipids from microalgae for biodiesel production, carotenoids [118,119], chlorophylls [120], polyunsaturated fatty acids [121] and even polyphenols [122] have been extracted from different seaweeds and microalgae. Carotenoids, including lutein,

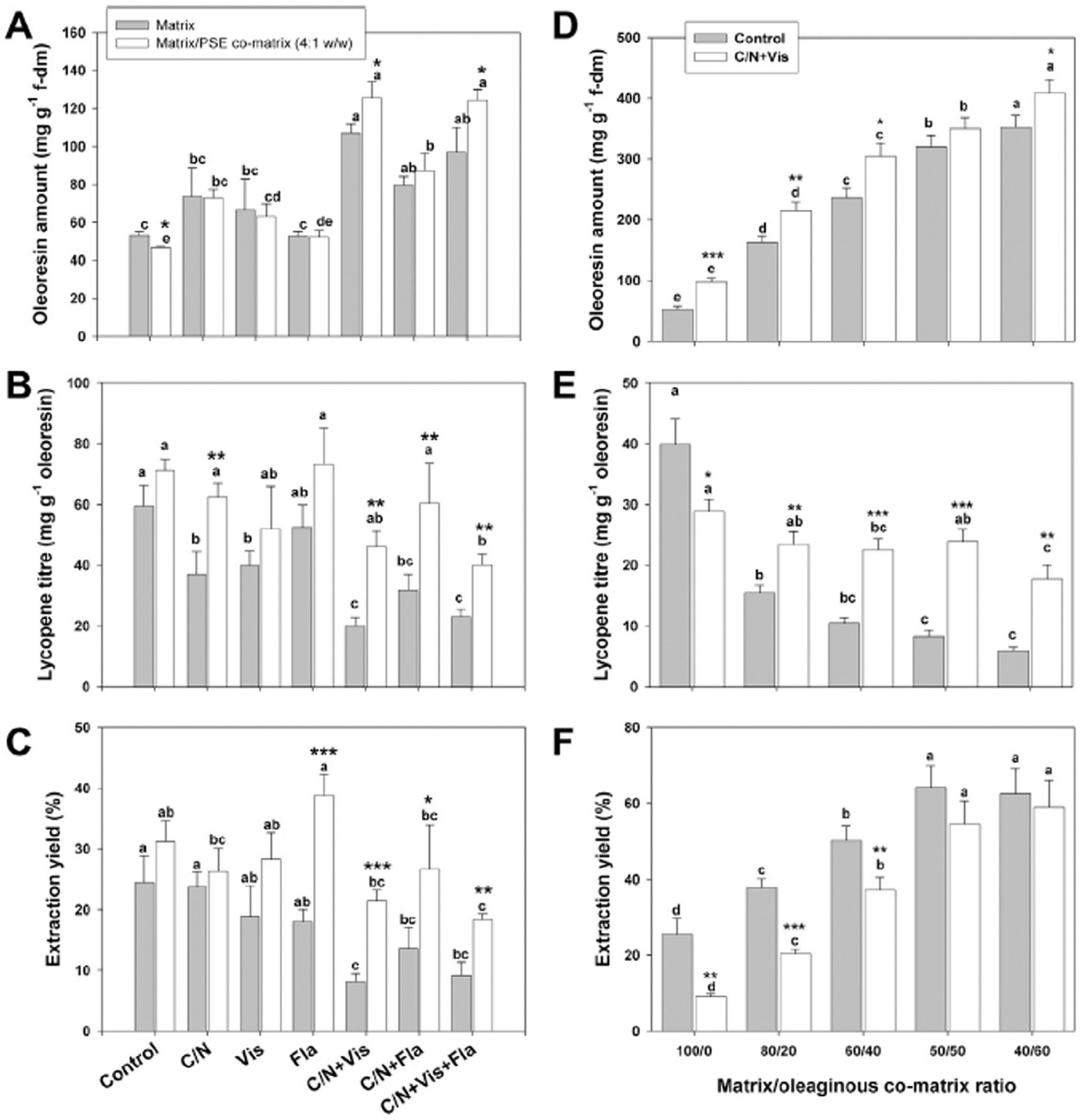

Fig. 3. Amount (A) and lycopene titer (B) of the oleoresins extracted by SC-CO_2 from control and enzyme-digested freeze-dried tomato matrices pure or blended (4:1 w/w) with an inert inorganic co-matrix (PSE co-matrix); lycopene extraction yield (C). Effect of the addition of increasing amounts of oleaginous co-matrix (roughly ground hazelnut seeds) on the weight (D) and lycopene titer (E) of the oleoresins extracted by SC-CO_2 from control and Celluclast/Novozyme + Viscozyme digested tomato matrices and on lycopene extraction yield (F). Extraction operative parameters were: pressure = 50 MPa; temperature = 86°C; SC-CO_2 flow = 4 mL min^{-1}; and, extraction time = 75 min. Data, expressed as mean ± standard deviation, are representative of three independent replicates ($n = 3$). Bars of the same series marked with different letters indicate significant differences among treatments (Holm-Sidak post-hoc test, $p < 0.05$). Asterisks describe value levels of statistical significance within each group ($^{*}p < 0.05$,$^{**}p < 0.01$; $^{***}p < 0.001$). {Reproduced with permission from [108]}.

neoxanthin, zeaxanthin, astaxanthin and β-carotene, were extracted from *Scenedesmus* sp. using CO_2 at 30 MPa and 60°C with 10% ethanol as cosolvent [123]. The addition of the modifier was critical to increase the amount of carotenoids recovered compared to the use of neat $scCO_2$, although it also implied the co-extraction of other less valuable compounds, such as chlorophylls.

On the same lines, other authors reported that the highest carotenoid-to-chlorophyll ratio in *Scenedesmus obliquus* extraction was reached at 25 MPa and 60°C using 7.7% ethanol as cosolvent [124]. Another important bioactive carotenoid present in microalgae in relevant amounts is astaxanthin. The use of ethanol as cosolvent has been repeatedly observed as necessary to achieve correct

extraction of this carotenoid by SFE from *Haematococcus pluvialis* green microalgae [125] and *Monoraphidium* sp. [126]. Subsequent acid treatment with H_2SO_4 or HCl and centrifugation were reported to be useful to eliminate chlorophylls from the astaxanthin-enriched extracts [126].

Regarding extraction of polyunsaturated fatty acids, *Scenedesmus obliquus* was shown to be the best source of α-linolenic acid among three different microalgae species [127]. Experimental results fitted to a Sovová mathematical model. The highest recovery of this compound was found at 15 MPa and 45°C during 30 min with 5% ethanol as modifier, offering similar results to Soxhlet extraction but significantly faster and environmentally cleaner. Likewise, ethanol was found to be necessary to maximize the recovery of docosahexaenoic acid (DHA) from *Schizochytrium limacinum* also using moderate temperatures but higher pressures (40°C and 35 MPa) [121]. Besides, ultrasound treatment was coupled to SFE to study the effect on the extracts obtained and on the cell-wall degradation. Interestingly, the use of the ultrasound-enhanced SFE provided similar results to regular SFE although it allowed a reduction of extraction time from 2 h to 1 h [121].

3.4. SFE of bioactives from food-related by-products

Comparing the target compounds in the different groups of applications targeted in this review, a greater proportion of works dealing with extraction of phenolic compounds by SFE from food by-products can be observed [7,128]. Residues from processing of blueberry [129] and blackberry [130], pomelo peels [131], *Prunus persica* leaves from peach production [132], grape marc [133] and even eucalyptus bark [134] have been explored for extraction of different kinds of phenolic compound by SFE. Unsurprisingly, all these applications involve use of a cosolvent, generally ethanol below 15%, although water has also been effective. One of the least straightforward developments includes the use of both solvents; the addition of 15% water or 15% ethanol for the $scCO_2$ extraction of grape marc at different temperatures, pressures and extraction times in order to recover proanthocyanidins from this by-product was studied [133]. Water was, by far, more favorable than ethanol as cosolvent. However, Da Porto et al. [133] found that a combined process in which a first extraction using 15% water as cosolvent (10 MPa, 40°C) followed by a second extraction step using 15% ethanol as modifier (10 MPa, 60°C) provided the highest recovery of proanthocyanidins, reaching phenolic yields of 68.0 g per kg of extract. This combined two-step process allowed more pronounced exhaustive extraction of the sample.

In any case, the non-polar or low polarity bioactive compounds targeted in other matrices are also targeted in plant by-products (e.g., carotenoids). In this regard, similar extraction conditions have been commonly applied for the recovery of these compounds compared to those optimized for their extraction from plant materials [135]. For example, lycopene recovery from tomato wastes could be carefully adjusted depending on the sample characteristics. Also considered should be possible isomerization processes that may occur during extraction. It was shown how, during the extraction of tomato waste, *trans*-lycopene was isomerized to *cis*-lycopene above 60°C [136]. Anyway, an increase from 60°C to 80°C did not affect the total amounts of lycopenes, only the *trans*/*cis* proportion. This kind of behavior is important when a single isomer is sought. The authors determined that the maximum *trans*-lycopene recovery (93%) was obtained at 60°C and 30 MPa by maintaining a solvent-flow rate of 0.59 g min^{-1} and keeping the moisture of the sample to a minimum (4.6%).

SFE has also found a good niche in the recovery of valuable bioactive components from fishery by-products. This kind of industrial waste may be valuable as a source of polyunsaturated fatty acids (PUFAs). A good example of application is the development of an SFE process based on the use of $scCO_2$ for the extraction of PUFAs from common carp by-products [137]. In this work, artificial neural networks (ANN) was employed to optimize different operating parameters, such as pressure, temperature, mass flow of CO_2 and extraction time, whereas the output vector was the PUFA yield obtained. In this way, after mathematical modeling and 3D response-surface methodology, the maximum PUFA extraction yield was 3.11%, obtained at operating values of 40 MPa and 60°C with a CO_2 mass flow of 0.194 kg h^{-1} for 180 min [137].

4. Conclusions and future outlook

As can be deduced from the published works included in this review, both PLE and SFE play important roles in the extraction of bioactive compounds from natural matrices. A further advantage to those already mentioned above is the possibility of scaling-up these processes, which adds interest from an industrial perspective. Nevertheless, in spite of the great number of applications developed, there is room for improvement for subcritical fluid extraction and SFE techniques.

Future developments include the coupling of processes or integration of procedures within the same process, in order to make the whole extraction more efficient. One example is the coupling of ultrasound treatment and extraction simultaneously or in sequence. There are already some interesting published works on this technology (see Fig. 4), although we expect further improvements and developments. One relates to the presence of strong cell walls in some plant and algae materials that hamper to a certain extent the efficiency of extraction. Likewise, the use of enzyme treatments with PLE or SFE could provide with better results in terms of recovery of bioactive compounds. Moreover, this latter coupling has also the advantage of being able to produce bioactive compounds in a different, but more active, chemical form than that originally present in the natural matrix, as some may be present as glycosidic or esterified forms.

Another process improvement explored is the introduction of continuous extract drying. Although some advancements have already been made in PLE to include a drying phase after extraction [30], most applications still require the use of a drying technique after extraction (e.g. freeze-drying, or vacuum evaporation). Interestingly, there exist several SCF-based drying processes that could be useful to obtain dried particles after PLE, such as those based on the precipitation of analytes from solutions [138]. Gas antisolvent (GAS), supercritical antisolvent (SAS), solution-enhanced dispersion by SCFs (SEDS) or rapid expansion of a supercritical solution (RESS) are some examples that generally differ in the contact mode between the two phases, in the phase-flow direction or in the mode (continuous or batch). Although these processes are well described, we foresee generation of new integrated processes for extraction using pressurized liquids coupled on-line to drying or particle formation. These developments may be pushed forward thanks to the design of new biorefinery concepts, including the use of pressurized fluids (subcritical and supercritical).

The development of biorefinery processes is closely related to environmental sustainability and green chemistry, as they seek the complete use of a particular biomass to be converted into value-added products and energy in order to limit or to eliminate the generation of by-products in the whole processing chain. Several biorefinery processes were recently proposed, including the use of SCFs. Microalgae are among the organisms with higher potential in this regard. For example, SFE was used to produce lipids and carotenoids from *Nannochloropsis* sp. microalgae, while the remaining biomass was fermented to produce biohydrogen [139]. Another example was developed for the production of bioactive compounds within a sugarcane-biorefinery process [140]. In this case, bioactive compounds from Brazilian ginseng were obtained, taking

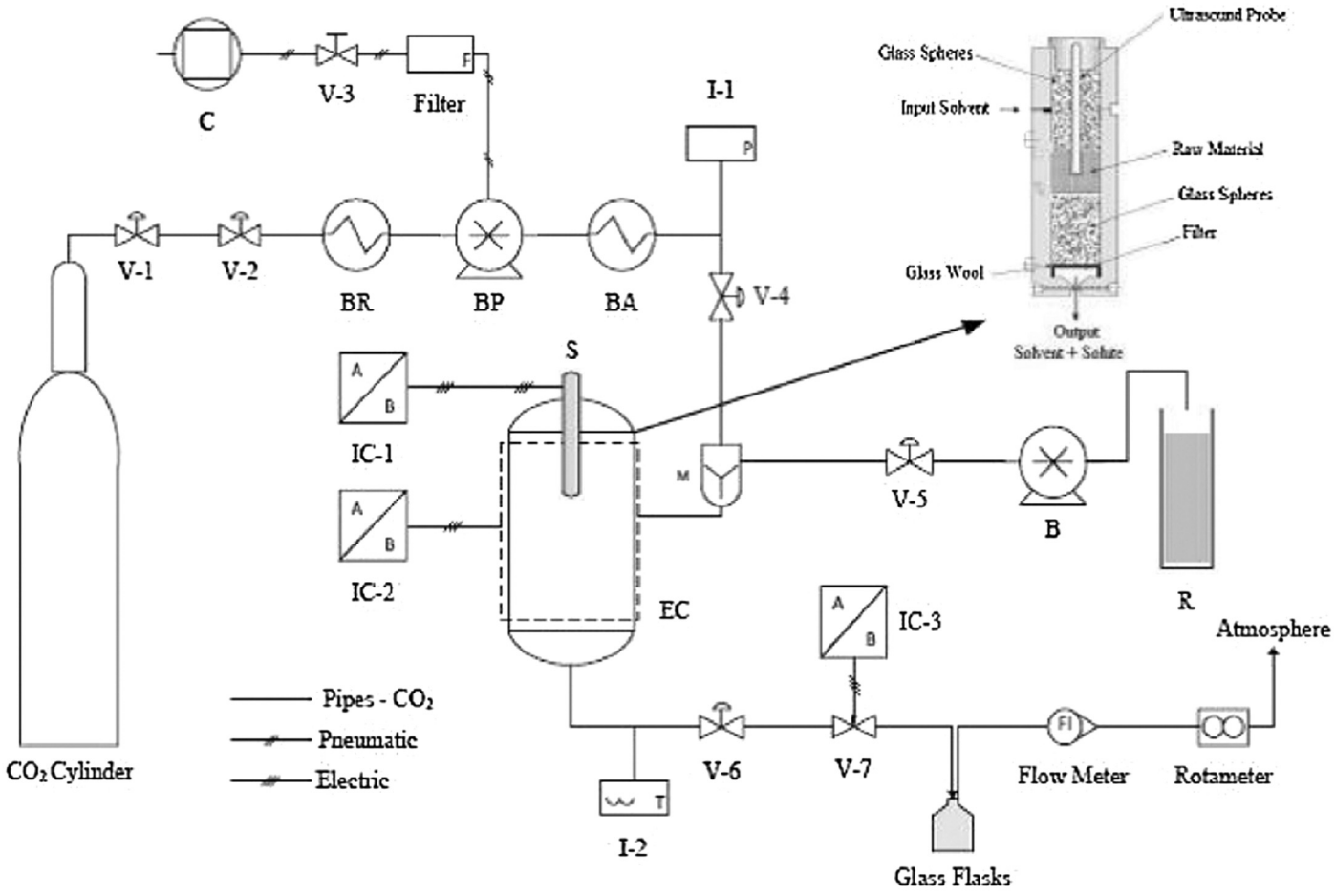

Fig. 4. SFE-US unit with co-solvents: V-1–V-6, control valves; V-7, micrometer valve; C, compressor; F, compressed air filter; BR, cooling bath; BP, pump (*Booster*); BA, heating bath; B, liquid pump (HPLC); R, fluid reservoir; M, mixer; I-1 and I-2, pressure and temperature indicators, respectively; IC-1, indicators and controllers of ultrasound power; IC-2, temperature of extraction column, and IC-3, temperature of micrometer valve; EC, extraction column; and, S, ultrasound probe. {Reproduced with permission from [130]}.

advantage of the ethanol, CO_2, heat and electricity already available from the sugarcane manufacture, reusing by-products and directly making the SFE process more feasible from the point of view of economics. This concept could be further developed even within well-established processes, such as lycopene extraction by SFE from tomato pomace and other by-products. We have observed how carbohydrates present in pomace leftovers after lycopene SFE could be used effectively for conversion into bioethanol [141]. Another possibility is integration of SFE into a coffee-production facility to extract interesting compounds from spent coffee grounds [142]. As can be deduced, the integration of subcritical fluid extraction and SFE in these types of biorefinery is really useful in obtaining high-value-added products while keeping the standards required for environmental sustainability. Thus, further developments in this field will surely be published in the near future [143].

Moreover, the future development of these techniques could closely relate to the use of new solvents. In pressurized solvents, new food-grade solvents, such as ethyl lactate, could find new applications, although ILs or DESs will probably also be further explored. Another interesting approach is based on the use of gas-expanded liquids (GXLs), which consist of a mixture between a liquid solvent and a compressible gas (usually CO_2), where the properties of the liquid phase are substantially different from those at atmospheric pressure. This strategy may be effective for the recovery of bioactive compounds from natural matrices, as demonstrated for the extraction of astaxanthin from *Haematococcus pluvialis* microalgae using CO_2-expanded ethanol [144]. These conditions should therefore also be considered when evaluating the most appropriate extraction technique to obtain bioactives from natural samples.

As a general conclusion, we can state that natural matrices, such as plants, macroalgae and microalgae, and food-related by-products are interesting materials for the recovery of bioactive compounds using subcritical fluid and SCF approaches. Although these techniques have already been extensively employed for this aim, as shown in this review, further developments and improvements are possible, always from the perspective of green extraction principles [8]. Thus, we expect a great amount of research in this area in the coming years, in not only the development of new extraction processes but also the improvement of existing processes to convert them to more sustainable options. Lastly, we should not underestimate the potential for these extraction techniques to be applied at the industrial level to be part of more complex biorefinery concepts. This subfield will most probably become more relevant within the future practical applications of techniques of subcritical fluid extraction and SFE.

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De: Luca RASTRELLI [mailto:rastrelli@unisa.it]
Enviado el: lunes, 27 de febrero de 2017 9:47
Para: elena.ibanez@csic.es
CC: kskalicka@pharmacognosy.org; nabavi208@gmail.com
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Phenolic compounds from edible algae: bioactivity and health benefits

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Phenolic compounds from edible algae: bioactivity and health benefits

Lidia Montero[a,b], Andrea del Pilar Sánchez-Camargo[a], Elena Ibáñez[a]*, Bienvenida Gilbert-López[a]

[a]Laboratory of Foodomics, Bioactivity and Food Analysis Department, Institute of Food Science Research CIAL (UAM-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain

[b]Laboratory of Food Chemistry, Dipartimento di Agraria (QuaSic.A.Tec.), Università Mediterranea di Reggio Calabria, Reggio Calabria, loc. Feo di Vito, 89122

Reggio Calabria, Italy.

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*Corresponding author

Laboratory of Foodomics, Bioactivity and Food Analysis Department

Institute of Food Science Research, CIAL (UAM-CSIC)

C/ Nicolás Cabrera 9, Campus UAM Cantoblanco

28049 Madrid, Spain

e-mail: elena.ibanez@csic.es

ABSTRACT

Algae have been consumed for many years in Asian countries; at present, its consumption around the world either as food ingredient or directly as food commodity has increased considerably mainly due to the Asian influence and its cuisine, but also because the growing interest on the beneficial health effects of algae.

Although several biological activities have been attributed to different algae compounds such as pigments, lipids, polysaccharides, fiber, proteins or vitamins, in the present review we will highlight the different phenolic compounds present on seaweeds and their important role in the functional and health effects, paying special attention to the antioxidant, antiproliferative, anti-obesity and antidiabetic activities.

Keywords: bioactive, macroalgae, polyphenols, phlorotannins, seaweed, review.

1. INTRODUCTION

Marine algae, also known as seaweeds, have been considered as the "plant-based food of the future" [1] because their composition comprises a wide interesting range of nutrients and micronutrients, mainly lipids and polyunsaturated fatty acids (PUFAs), pigments as carotenoids and chlorophylls, proteins, polysaccharides and phenolic compounds [2, 3]. For this reason, seaweeds are being considered a source of health promoting compounds.

Functional foods have been defined as "the food that besides its nutritious effects, has a demonstrated benefit for one or more functions of the human organism, improving the state of health or well-being or reducing the risk of disease" [4]. Commonly, functional foods are designed including in their composition compounds responsible of the health promotion, which are known as functional ingredients; in this sense, algae can be considered as a rich source of functional food ingredients [5].

Asian countries have a long tradition on the use of marine algae as food. Data show that the daily consumption of algae in this countries reaches between 8.5 and 10 g alga/day in Korea and Japan, respectively [6]. Nowadays, the increasing information of algae benefits, as well as the population movements between countries and cultures have been the responsible of the growing interest on algae also in Western countries.

From a biological point of view, seaweeds are macroalgae, pluricellular photosynthetic aquatic organisms. There is a huge diversity of macroalgae species, and they are usually classified in three categories: green algae (*Chlorophyceae*), red algae (*Rhodophyceae*), and brown algae (*Phaeophyceae*), according to the composition of pigments responsible of their color [7].

In this review, the importance of one of the functional ingredients of seaweeds, the phenolic compounds, and their implication on the human health are reviewed, mainly focusing on the reported antioxidant, antiproliferative, anti-obesity and antidiabetic activities.

1.1 Phenolic compounds in marine algae

Phenolic compounds are considered as one of the most important natural antioxidants present in fruits, vegetables, cereals, legumes, species and plants such as coffee, tea or *Lamiaceae* plants [8]. Chemically, polyphenols can be divided in several classes such as phenolic acids, flavonoids, tannins, stilbenes and lignans [9].

Although usually phenolic compounds have been associated to plants, algae are also a rich source of polyphenols. Among the different classes of algae, brown algae are considered the ones with higher amount of these interesting compounds [10]. In fact, there is a variety of phenolic compounds called phlorotannins that have been only described in the composition of brown algae [11-14].

Phlorotannis present a structural function in algae due to their contribution on the formation and fortification of the cell wall. Besides, as secondary metabolites of algae, other functions have been attributed to these compounds, for instance, the protection of phlorotannins from UV radiation, their heavy metal chelation or the antibacterial antifouling and anti-herbivory activity [15].

From the chemical and structural point of view, phlorotannins are complex polymeric phenolic compounds of phloroglucinol units, linked to each other by different bonds. Depending on the linkages of the phloroglucinol units, phlorotannins can be classified in: fucols (phenyl linkages), phloretols (ether bonds), fuhalols (ether bonds and additional hydroxyl groups), fucophlorethols (with ether and phenyl linkages) and eckols and carmalols (dibenzodioxin linkage) [12]. Moreover, the complexity of these polymers increase since some brown algae present halogenated phlorotannins and bromo-, chloro- and iodo- phlorotannins have been detected [16].

As the number of phloroglucinol units growths, the complexity of their structural and diversity increases greatly [17]. This level of complexity makes the identification and chemical characterization of phlorotannins a difficult challenge. Different analytical techniques have been employed to separate and identify phlorotannins from different brown algae, such as HPLC-DAD-MS [11, 18, 19], UHPLC-DAD-MS [10, 20] or even comprehensive two dimensional liquid chromatography coupled to MS (LCxLC-MS/MS). This technique has been successfully employed to separate phlorotannins with different chemical structure and degree of polymerization from *Cystoseira abies marina* and *Sargassum muticum* brown algae [21, 22].

Although phlorotannins are the main phenolic compounds studied in seaweeds, the content, detection and quantification of other kind of phenolic compounds has been widely tested [23-26]. However, the chemical characterization of algal phenolic compounds other than phlorotannins is not as deeply detailed and it is difficult finding works that chemically characterize the composition of the polyphenol fraction. Machu *et al.* (2015) determined the content of gallic acid, hydroxybenzoic acid, catechin, epicatechin, catechin gallate, epicatechin gallate and epigallocatechin gallate in four brown algae (*Laminaria japónica*, *Eisenia bicycli*, *Hizikia fusiformi* and *Undaria pinnatifida*), two red algae (*Porphyra tenera* and

Palmaria palmata) and one green alga (*Chlorella pyrenoidosa)* [27]. The different chemical structures of phenolic compounds studied in algae are shown in **Figure 1**.

The flavanol content in algae has been also studied [28, 29], as well as the content of phenolic acids [29-31], flavonoids [32] and other phenolic compounds as galloyl glucose [33] in brown, green and red algae. In spite of this, not many information can be found about the composition of phenolic compounds.

< **Figure 1**>

1.2 Extraction of phenolic compounds of marine algae

The increasing interest on finding new functional ingredients to improve health is closely linked with the study of appropriate, cost-effective and environmental friendly extraction procedures to isolate these ingredients [34]. In this regard, different green extractions techniques have been employed for the extraction of phenolic compounds from seaweeds. For instance, microwave assisted extraction (MAE) [35], pressurized liquid extraction (PLE) [22, 36, 37], ultrasound assisted extraction (UAE) [38], supercritical fluid extraction (SFE) [39, 40] and enzyme assisted extraction (EAE) [37, 41] are among the most promising processes.

1.3 Bioactivities of phenolic compounds of marine algae.

As mentioned above, phenolic compounds present on algae have been the focus of many studies in order to test the different biological activities that these interesting compounds may possess. In this section, a brief description of some of them is presented.

1.3.1 Anti-Alzheimer activity

Some phlorotannins -in particular eckols- from brown algae have demonstrated to possess acetylcholinesterase and butyrylcholinesterase inhibitory activity [42, 43]. These two enzymes present an increased activity in the Alzheimer disease (AD), inducing a cholinergic deficit and therefore a decline in the cognitive system which contributes to the development and progression of AD [44].

The activity of eckols from *Ecklonia cava* [42] and *Eisenia bicyclis* [43] showed an inhibitory enzyme activity higher than traditional and currently anti-AD drugs. Therefore, the potent inhibitory enzyme activity studied in eckols indicates these phlorotannins as a potential treatment for AD.

1.3.2 Antiinflammatory activity

Inflammation is a response of the immune system and it has an important role in many diseases. One of the main factors responsible of inflammation is the nitric oxide (NO) generated by NO synthases, including the inducible nitric oxide synthase (iNOS) and, besides, by mediators of inflammation such as COX-2. The overproduction of NO in macrophages may cause an inflammation and an autoimmune disorder.

Phenolics from algae, in particular an extract from the red alga *Callophyllis japonica* [45] and brown algae *Ecklonia cava* [46] have shown effect against the inflammatory processes, reducing the iNOS and COX-2 activity and, therefore, inhibiting the NO production [45].

On the other hand, the anti-inflammatory effect of certain phlorotannins from the brown algae *Eisenia arborea* have been tested *in-vivo* using ICR mice with positive and similar results [47].

1.3.3 Anti-allergic activity

Allergic reaction is characterized by a number of symptoms that appear during the acute phase of the allergic process. These symptoms appear by chemical or immunological activation of mast cells that cause a massive release of mediators like histamine. Besides, another mechanism involved in allergic reactions is the release of inflammatory mediators (prostaglandins, leukotrienes) by the action of enzymes present on the arachidonate cascade, mainly phospholipase A2 (PLA2), cyclooxygenase (COX) and lipoxygenase (LOX) [48].

Extracts of phlorotannins and purified phlorotannins from different brown algae have been studied for their anti-allergic effect. This effect can be related to a strong inhibition of mast cells degranulation induced by phlorotannins that leads to a reduction of histamine mediator; moreover, phlorotannins seems to have an inhibition effect on the enzymes involved in allergic reaction (PLA2, COX and LOX) [48-51].

Phlorotannins of high molecular weight, that is, with higher degree of polymerization, have shown to present stronger inhibitory effect on histamine release than medium molecular weight phlorotannins or the phloroglucinol monomer [49].

It is worth to mention that in some anti-allergic tests, the studied phlorotannins showed a similar or even stronger suppressed degranulated effect than epigallocatechin gallate, the most common natural inhibitor of granulation [50, 51].

1.3.4 Hepatoprotective effect

The protection against liver damage is closely related to the antioxidant activity. The studies on the hepatoprotective effect of algae phenolic compounds are focused on their antioxidant protection in the essential hepatic antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) or glutation peroxidase (GPX) [52, 53].

Phenolic acid fractions of green algae *Halimeda monile* [52] and *Halimeda opuntia* [53] were evaluated for their hepatoprotective effect in rats with liver damage induced by CCl_4. Significant increase in the enzyme activity levels of SOD, CAT and GPX between animals treated with the algal extract and animals without treatment were demonstrated. On the other hand, phlorotannin from *Eisenia bicyclis* showed an *in-vitro* protective activity against hepatotoxicity induced by oxidative stress in HepG2 cells [54].

In the following sections of this manuscript, four of the most important and studied functional activities related with the seaweed polyphenols will be extensively reviewed: antioxidant, antiproliferative, anti-obesity and antidiabetic activities.

2. ANTIPROLIFERATIVE ACTIVITY

According to the International Agency for Research on Cancer, the specialized cancer agency of the World Health Organization (WHO), in 2012 cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths. However, the concern is increasing even more because the number of new cases is expected to rise by about 70% over the next two decades. Among men, the top 5 most diagnosed cancer types in 2012 were lung, prostate, colorectal, stomach, and liver cancer. On the other side, among women the top 5 most diagnosed cancer types were breast, colorectal, lung, cervix, and stomach cancer [55]. In this sense, the high contents of bioactive substances in seaweeds with great potential as antitumoral drugs have caused an emerging interest in the biomedical area [56, 57].

Polyphenol-rich extracts, isolated phlorotannins and bromophenols compounds have been described as important inhibitors of cancer cells proliferation and some interesting review works have disclosed their potential pharmacological applications [57, 58]. In the **table 1**, some recent research works about the *in vitro* anti-proliferative activity of seaweeds extracts on some cancer cells lines are presented. The antitumoral properties of the polyphenols present in the genus *Cystoseira* have been recently studied and, for instance, aqueous extract derived from brown *Cystoseira crinita* has shown great

antiproliferative activity on breast adenocarcinoma (MCF7) (in a dose dependent manner) [59]. In this study, the concentrations for 50% of maximal inhibition of cell proliferation (named as GI50) were 17.9 ± 0.60 and 1.9 ± 0.05 μg mL^{-1} for the aqueous extract and cisplatin (a reference drug), respectively. The extract was also tested in lung (A549) and colon (HCT15) cancer cell lines, obtaining less promising results [59]. Ahn *et al.* (2015) found that the ethanolic extract and isolated phlorotannins from *Ecklonia cava* have cytotoxic effects on A2780 and SKOV3 ovarian cancer cells. Dieckol, a major active compound in *E. cava,* induced the apoptosis of SKOV3 cells and suppressed tumor growth without any significant adverse effect in the SKOV3-bearing mouse model [60].

Integrated processes (e.g. enzyme assisted extraction, pressurized liquid extraction, ultrasound assisted extraction and their combinations) and some purification methods such as liquid-liquid extraction and solid phase extraction have been studied to obtain phlorotannins-enriched extracts [61, 62]. For example, the crude phenolic fraction from the enzyme hydrolized extract (CphF) of *Ecklonia cava* showed the best results of antiproliferative activity for murine colon cancer cell line (CT26) with a GI50 value of 5.1μg mL^{-1}. The antiproliferative effect of CphF is believed to be associated with apoptotic cell demise in CT26 confirmed by the nuclear staining experiment. In addition, in this study is suggested that the antiproliferative activity of the extract may be due to its high amount of polyphenol content [63]. Montero *et al.* (2016) described similar observations for purified hydroalcoholic extracts from samples of *Sargassum Muticum* collected at five different locations along the North Atlantic coasts. Results revealed that Norwegian samples, which presented the highest total phenols (148.97 ± 0.85 mg phloroglucinol equivalent (PGE) g^{-1}) and phlorotannins (11.730 ± 0.141 mg PGE g^{-1}) contents, exerted the highest anti-proliferative activity against HT29 adenocarcinoma colon cancer cells. Those extracts contained mainly phlorethols and hydroxyfuhalos, a type of phlorotannins that contain ether linkages and additional hydroxyl groups, as mentioned before [22]. On the other side, Yang *et al.* (2010) evaluated the anti-proliferative activity of the phlorotannins extract derived from the edible brown algae *Laminaria japonica* Aresch on human hepatocellular carcinoma cells (BEL-7402) and on murine leukemic cells (P388). IG50 values obtained after incubation for 48 h were 120 μg mL^{-1} and >200 μg mL^{-1}, respectively. Moreover, the anti-proliferative effects of the kelp extracts were positively correlated with the total phlorotannins content in the extracts [64].

< **Table 1**>

3. ANTIOXIDANT ACTIVITY

The interest in natural antioxidants for health benefits and improved shelf life of foods has intensified their research from different sources [57, 69-71]. The term ***oxidative stress*** is the result of an imbalance between pro-oxidant and antioxidant homeostasis that leads to the generation of toxic reactive oxygen species (ROS) and their excessive production and accumulation can induce apoptosis and cause damage, especially to cellular proteins, polyunsaturated fatty acids, and DNA [72, 73] . In this sense, ROS are significant contributors to the origin and progression of cancer [74] and of neurodegenerative diseases, mainly Alzheimer's and Parkinson's diseases [75].

In particular, phenolic compounds are considered as one of the most important classes of natural antioxidants and the main property associated to them is the antioxidant activity, which can be of benefit in reducing oxidative reactions harmful to health [27, 76, 77]. Polyphenols from terrestrial plants are derived from gallic and ellagic acid, whereas the algal polyphenols (phlorotannins) are derived from polymerised phloroglucinol units (1,3,5-trihydroxybenzene), which are typically isolated from brown algae [14, 77]. Phlorotannins constitute an extremely heterogeneous group of molecules (structure and polymerization degree heterogeneity) that may be linked through different bonds forming several structures and types, as mentioned in the introduction.

Green and red algae possess comparatively lower concentration of phenols compounds than brown algae, and therefore the last one have been more extensively studied [23, 25, 76] . Polyphenol content has typically been analyzed by Folin-Ciocalteu method, which is based on the reaction of a colored reagent (phosphotungstic-phosphomolybdic compounds) with the easily oxidized phenolic functional group [78]. Some recent review papers have summarized the polyphenol content of different edible algae species, which can represent up to 25% of the dry weight of brown algae (and up to 50% w/w in the extract) [27, 76, 77, 79]. However, as occurs with other chemical constituents, the composition of phenolic compounds qualitatively might vary depending on the species as well as on the environmental conditions and location of the seaweed [76]. In addition, the extraction method and the solvents employed for the polyphenols isolation exert a huge influence on their solubility [34]. For further information about this topic, Plaza *et al.* (2014) and Herrero *et al.* (2015) have presented interesting up-to-date reviews on advanced extraction process to obtain polyphenols from seaweeds and marine foods [3, 62]. On the other hand, Stern *et al.*

(1996) developed other method based on the ability of 2,4-dimethoxybenzaldehyde (DMBA) to react with 1,3 and 1,3,5–substituted phenols (i.e. phlorotannins) as a measure to quantify more accurately the phlorotannins content [80]. A recent study on ten dominant brown seaweeds of the occidental Portuguese coast showed values between 75 and 969 mg Kg^{-1} phloroglucinol units (dry matter), being *Fucus spiralis* ranked first, followed by three *Cystoseira* species [81]. Currently, the most studied algae species are *Ecklonia, Fucus, Cystosiera* and *Sargassum* (which belong to the brown algae group), mainly due to their high quantity in polyphenols compared with the other species of algae [76].

The literature on the beneficial health properties of marine algal polyphenols – from a human physiological viewpoint- is scarce, and a little research has been published related to the antioxidant role of algal polyphenols in preventing diseases linked to oxidative stress [69, 82]. The antioxidant activity detected in marine algae also have been related of biological activities such as antibacterial, cytotoxic, hepatoprotective and anti-proliferative activities [52, 53, 57, 69], which are described in this review.

The antioxidant activity of phenolic compounds from marine algae has been demonstrated actively by employing *in vitro* assays in the majority of published research works. Assessments of the antioxidant capacity of a phenolic compound or extract can be distinguished in two major groups: **a) Methods based on single electron transfer (SET)** such as Trolox equivalent antioxidant capacity (TEAC), ferric reducing ability (FRAP), copper reduction (CUPRAC), and 2,2-diphenylpicrylhydrazyl (DPPH) radical-scavenging-capacity assays, and those based on **b) hydrogen atom transfer (HAT),** including oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP) assays [27, 76, 82, 83] . Due to the differences in the mechanism of the reaction, the combination of different assays has been very useful to compare with the behavior of other kind of antioxidants [14, 76, 83]. High values of total phenolic content have been very often correlated with a high antioxidant activity [23, 27, 84], but sometimes the relationship between them is not linear. Several algae extracts with low phenolic content have shown higher antioxidant activity than expected, suggesting that other compounds are also responsible for the action such as ascorbic acid, proteins or peptides, carotenoids as fucoxanthin, sterols, sulfated polysaccharides or Maillard reaction products [76, 85].

In this sense, isolated phlorotannins such as eckol, dieckol, phlorofucofuroeckol A and 8,8′-bieckol from the Japanese brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* were assessed and showed from 2 to 10-fold higher antioxidant activity than catechin, α-tocopherol and ascorbic acid [86]. Furthermore, in the same study, those phlorotannins showed a potent inhibition of phospholipid

peroxidation at 1 M in a liposome system. Also, polymeric phlorotannins belonging to fucol and fucophlorethol classes exhibited greater antioxidant activity than phloroglucinol [87]. Recently, Balboa *et al.* (2013) have compiled the EC_{50} values for the DPPH radical scavenging activity of pure compounds isolated from brown algae in different research works performed between 2007 and 2013. EC_{50} is referring the amount of antioxidant necessary to scavenge by 50% the initial radical. As a general trend, pure phloroglucinol derivatives are more potent than standard antioxidants [76]. In addition, bromophenols (EC_{50} = 7.5–24.7 μM) are more active than BHT ($EC_{50, BHT}$ =81.8 μM) [58].

Today, the Far East food have taken an important place to the diet of many west countries (North America, Western Europe, Latin America) and some studies have associated its dietary patterns with the disease prevention [69, 88]. The three most important seaweed used as food are several species of *Porphyra* (whose common name in Japan is nori), *Laminaria* (kombu) and *Undaria* (wakame) [89]. In the table 3.1 it is summarized the phenolic content and the different *in vitro* methods of the antioxidant activity for those edible seaweeds, as well as the location from which they were harvested and extraction method employed. As mentioned before, the quantity of phenols present in the algae extract depends on both the place of collection and the extraction method employed. Solvents most commonly used to extract phlorotannins have been water and aqueous mixtures of ethanol and acetone [34]. Besides, the total phenolic content trend to be higher for brown algae (*Laminaria and Undaria*) than for red algae (*Porphyra*). Due to the variety of methodologies for measuring antioxidant activity, it is difficult to compare between species. However, the EC_{50} for DPPH radical scavenging capacity activity for *Laminaria saccharina* and *Laminaria digitate* is lower in the order of magnitude that *Porphyra leucosticta* and *Porphyra purpurea*, which can be related by the higher phenol content in the brown algae extracts. In addition, those edible algae, besides having a high content of phenolic compounds, have traditionally been consumed because of its high nutritional content as source of proteins, carbohydrates, vitamins and mineral salts [89].

< **Table 2**>

4. ANTI-OBESITY ACTIVITY

Obesity is a condition in which there is an excessive accumulation of lipid in the adipose tissue leading to adverse effects on health, including increasing the risk of diabetes, hypertension or arteriosclerosis.

Obesity is a major component of the metabolic syndrome, which is characterized by the combination of multiple metabolic disorders, namely obesity, dyslipidemia, insulin resistance, hypertension and hyperglycemia [95].

The anti-obesity effects of dietary phytochemicals are mediated by regulation of various pathways, including: lipid absorption, intake and expenditure of energy, regulatory effect on lipid metabolism (increasing lipolysis, decreasing lipogenesis), and differentiation and proliferation of preadipocytes [95].

4.1. Lipase inhibitory effect

Pancreatic lipase is a key enzyme for triglyceride absorption in the small intestine. This enzyme is secreted from the pancreas and hydrolyses triglycerides into glycerol and fatty acids. Gastrointestinal lipase inhibitors hinder fat digestion and absorption. Thus, pancreatic lipase inhibitors are considered to be valuable therapeutic agents for treating diet-induced obesity in humans [96]. In this sense, Bitou *et al.* (1999) screened 54 marine algae for pancreatic lipase inhibitory activity. Interestingly, 27 of these algae species showed lipase inhibitory activity, in either methanol or ethyl acetate extracts. Polyphenols present in the extracts, especially from algae belonging to phylum *Phaeophyta* (brown algae), were in a high extent responsible of the inhibitory activity [97].

Phlorotannins from several seaweed species have been also shown to possess lipase inhibitory activity, as detailed in **Table 3**. Several phloroglucinol derivatives isolated from *Eisenia bicyclis* inhibited pancreatic lipase activity in vitro with 7-phloroeckol (IC_{50} = 12.7 μM) showing the strongest inhibition, followed by fucofuroeckol A (IC_{50} = 37.2 μM) [96]. Dieckol from *Ecklonia cava* also exhibited pancreatic lipase inhibitory activity, but with lower IC_{50} value of 350.4 μM. [98]

Ethanol extracts and dried powders of the tropical red algae *Kappaphycus alvarezii*, *Kappaphycus striatus* and *Eucheuma denticulatum* were analyzed for their effect on lipase activity using a turbidimetric method. Extracts of *K. alvarezii*, *E. denticulatum* and *K. striatus* inhibited lipase activity by 85, 83 and 92 %, respectively, while the dried powders inhibited the enzyme by 61, 60 and 67 %, respectively. These positive results could be attributed to the polyphenols present in the seaweeds [99].

4.2. Inhibitory effect on adipocyte differentiation and proliferation

Adipocytes play a central role in the maintenance of lipid homeostasis and energy balance, by storing triglycerides and releasing free fatty acids in response to changing energy demands. 3T3-L1

preadipocytes cells are currently used as an *in vitro* model for the study of obesity, because such cells accumulate triglycerides upon differentiating in culture [100]. Some recent works showing the anti-adipogenesis activity of phenolic compounds from algae are presented in **Table 3**.

A polyphenol extract, as well as the individual phlorotannins dieckol [101] and 7-phloroeckol [102] from *E. cava* have been reported to exhibit potential inhibition of adipocyte differentiation and adipogenesis in 3T3-L1 cells, in a dose-dependent manner. In a more recent study, triphlorethol-A, eckol and dieckol from *E. cava* also showed inhibition of adipocyte differentiation in 3T3-L1 cells and were able to suppress lipid accumulation in adipocytes [103].

Phloroglucinol, eckol, dieckol, dioxinodehydroeckol, and phlorofucofuroeckol A isolated from *Ecklonia stolonifera* inhibited adipogenesis in a dose-dependent manner in 3T3-L1 cells, being phloroglucinol and eckol more potent than the other compounds. This anti-adipogenic activity can be related to the reduction in expression levels of several adipocyte marker genes, including proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα). The results suggested that the molecular weight of the phlorotannin is an important factor affecting its ability to inhibit adipocyte differentiation and modulate the expression levels of adipocyte marker genes [104]. Similarly, 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, dieckol and phlorofucofuroeckol A isolated from *Eisenia bicyclis* showed anti-obesity activity by suppressing the differentiation of 3T3-L1 adipocytes in a dose–dependent manner. Among the five compounds, 6,6′-bieckol markedly decreased lipid accumulation and expression levels of PPARγ, C/EBPα, sterol regulatory element binding protein (SREBP-1c), and fatty acid synthase and acyl-coA carboxylase [105].

Aqueous methanolic extract of another brown alga, *Ascophyllum nodosum,* containing 220 mg phlorotannins/ g extract, depressed glycerol-3-phosphate dehydrogenase (GPDH) activity in 3T3-L1 adipocytes. Reduction of GPDH activity indicated a slow down on fatty acids and triacylglycerols' synthesis. It also reflected the inhibition on differentiation in the early differentiating preadipocytes and lipogenesis in the mature adipocytes. [106].

In addition to showing inhibitory activity against adipocyte differentiation, several naturally-occurring compounds have displayed apoptotic effects on maturing pre-adipocytes, inhibiting in consequence the formation of adipose tissues. Diphlorethohydroxycarmalol (DPHC) isolated from *Ishige okamurae* efficiently induced apoptosis in 3T3-L1 pre-adipocytes [107].

4.3. Regulatory effect on lipid metabolism

Recently it has been reported that *E. cava* polyphenol-rich extract (containing dieckol, 2,7″-phloroglucinol-6,6′-bieckol, pyrogallol-phloroglucinol-6,6′-bieckol and phlorofucofuroeckol A) ameliorates hepatic lipid metabolism, oxidative stress, and inflammation in high-fat diet (HFD)-induced obese mice [108]. Administration of the extract produced a reduction in the body weight gain of obese mice. Levels of circulating triglycerides and total cholesterol were also reduced, thus suggesting that extract supplementation has potential in the prevention of dyslipidemia-induced atherogenesis in obese mice. Moreover, extract supplementation in HFD-induced obese mice suppressed hepatic lipid accumulation by reducing lipogenesis and stimulating fatty acid β-oxidation. Furthermore, glutathione peroxidases and catalase were activated and consequently reduced hepatic lipid peroxidation. Reduction of pro-inflammatory markers such as TNFα and IL-1β was also observed, which is in agreement with the results found by Park and coworkers, (2012) [109].

In a different study, polyphenol extract and dieckol from *E. cava* also suppressed body weight gain and reduced plasma triglycerides, total cholesterol and low-density lipoprotein (LDL) cholesterol levels in HFD-induced obese mice. The anti-hyperlipidemic effect can be related to the inhibition of 3-hydroxyl-methyl glutaryl coenzyme A reductase activity, which is in the metabolic pathway that produces cholesterol and other isoprenoids [101].

A clinical trial including a freshwater algae infusion (ProAlgaZyme) for 10 weeks in the diet of 60 overweight and obese people offered some beneficial effects against dyslipidemia. Total cholesterol, LDL-cholesterol, triglycerides, C-reactive protein and fasting blood glucose levels were reduced, accompanied by a significant increase in HDL-cholesterol levels. A reduction on body weight and body fat was also observed [110].

< **Table 3** >

5. ANTIDIABETIC ACTIVITY OF SEAWEED POLYPHENOLS

Diabetes mellitus is a group of chronic diseases which can be attributed to hyperglycaemia, a condition characterized by a high concentration of glucose circulating in the blood. Diabetes mellitus can be divided in two basic groups: (i) type 1 or insulin-dependent, which results from a deficient secretion of insulin and can be efficiently controlled by administration of insulin, and (ii) type 2, or non-insulin-dependent,

characterized by insulin resistance. Hyperglycemia-induced oxidative stress contributes to the damage and death of pancreatic β-cells, which dysfunction plays a key role in pathogenesis of type 1 and 2 diabetes [111].

Edible seaweed phlorotannins have exhibited various anti-diabetic mechanisms such as inhibition of starch-digesting enzymes (α-glucosidase and α-amylase), protein tyrosine phosphatase 1B (PTP 1B) enzyme inhibition, modulation of glucose-induced oxidative stress and reduction of glucose levels and lipid peroxidation in diabetic mice [112]. However, only few investigations have been done at the cellular level to study the potential of polyphenols isolated from marine algae against diabetes [113].

5.1. *In vitro* inhibition of α-amylase and α-glucosidase enzymes

Phlorotannins extracted from *Alaria marginata*, *Fucus distichus*, [114] *Ascophyllum nodosum* [115], *Eisenia bicyclis*, *Ecklonia cava*, *Ishige okamurae* [113], *Sargassum ringgoldianum* or *Sargassum paten* [116], for instance, showed antidiabetic activity *in vitro* by the inhibition of α-amylase and α-glucosidase (as detailed in **Table 4**), two carbolytic enzymes involved in serum glucose regulation. α-Amylase is located on the pancreas and catalyzes the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen, and a variety of oligosaccharides; further, α-glucosidase, located in the brush-border surface membranes of intestinal cells, degrades disaccharides into simple sugars, which are finally absorbed by the intestine. Therefore, inhibition of these two enzymes is considered a key tool in controlling hyperglycemia.

Bromophenols extracted from *Symphyocladia latiuscula*, *Odonthalia corymbifera* and *Polysiphonia morrowii* red algae exhibited inhibitory activity against *Saccharomyces cerevisiae* α-glucosidase (**Table 4**). The inhibitory activity increased with the degree of bromination and decreased with the degree of methyl-substitution [117]. Bis(2,3,6-tribromo-4,5-dihydroxybenzyl) ether was the most potent α-glucosidase inhibitor reported in the study, with an IC_{50} of 0.03 μM, while 3-bromo-4,5-dihydroxybenzyl methyl ether, with an IC_{50} > 1000 μM, was the weakest inhibitor. In addition, it seems that the number of phenyl units in the molecules also affect the enzyme inhibition activity, being those with diphenyl units much more active than the compounds with one phenyl unit [58]. Bis(2,3-dibromo-4,5-dihydroxybenzyl) ether isolated from the red alga *Polyopes lancifolia* showed inhibitory activity against *S. cerevisiae* α-glucosidase (IC_{50} of 0.098 μM) and *Bacillus stearothermophilus* α-glucosidase (IC_{50} of 0.120 μM) [118]. 2,4-Dibromophenol and 2,4,6-tribromophenol from red seaweed *Grateloupia elliptica* also inhibited α-glucosidase from different organisms, but their activity was lower [116].

Generally, seaweed extracts and isolated compounds exhibited more inhibitory potency towards α-glucosidase activity compared to α-amylase in several studies. This is desirable since excessive inhibition of α-amylase activity has been suggested to cause abnormal fermentation of undigested carbohydrates by the colonic microbiota [116].

< Table 4 >

5.2. *In vitro* inhibitory effect on PTP 1B enzyme

Protein tyrosine phosphatase 1B enzyme is involved in the regulation of insulin cell signaling. Inhibition of PTP 1B increases insulin sensitivity and therefore it has been suggested as a target mechanism in the treatment of diabetes mellitus type 2 [119]. Different phenolic compounds have been reported as PTP 1B inhibitors in red, green and brown algae (**Table 4**).

Red algae: Four bromophenol derivatives from red algae *Rhodomela confervoides*, 2,2′,3,3′-tetrabromo-4,4′,5,5′-tetra-hydroxydiphenyl methane (1), 3-bromo-4,5-bis(2,3-dibromo-4,5-dihydroxybenzyl) pyrocatechol (2), bis(2,3-dibromo-4,5-dihydroxybenzyl) ether (3) and 2,2′,3-tribromo-3′,4,4′,5-tetrahydroxy-6′-ethyloxy-methyldiphenylmethane (4) showed significant inhibitory activity against PTP 1B (IC_{50} were 2.4, 1.7, 1.5 and 0.84 μM, respectively) [120]. A series of bromophenol derivatives have been synthesized based on these bromophenol structures and evaluated as PTP 1B inhibitors.

Green algae: A novel vanillic acid derivative ($C_{25}H_{16}O_8$) and its sulfate adduct ($C_{25}H_{16}O_{12}S$) isolated from the green algae *Cladophora socialis* have shown inhibitory activity against PTP 1B. Both compounds possess a unique biphenyl functionality, being 3.71 and 1.70 μM their respective IC_{50} values against PTP 1B [121].

Brown algae: Phlorofucofuroeckol A, dieckol, 7-phloroeckol and eckol (IC_{50} = 0.56, 1.18, 2.09 and 2.64 μM, respectively) isolated from brown algae *Ecklonia stolonifera* and *Eisenia bicyclis* showed strong *in vitro* inhibitory effect on PTP 1B, 5 or 10-fold stronger than the positive control ursolic acid (IC_{50} = 10.82 μM) [122]. Dioxinodehydroeckol (IC_{50} = 29.97 μM) and phloroglucinol (IC_{50} = 55.48 μM), on the other hand, were less active than ursolic acid.

5.3. Modulation of glucose-induced oxidative stress

Hyperglycemia can cause glucose to undergo autooxidation to generate intermediates that lead to the formation of reactive oxygen species (ROS), nitric oxide (NO), peroxynitrite (ONOO−), and advanced glycation end products (AGE), which cause various complications of diabetes such as nephropathy, retinopathy, and neuropathy [112].

In vitro studies reported that dieckol from *Ecklonia cava* [123] and diphlorethohydroxycarmalol (DPHC) from *Ishige okamurae* [124] showed protective effects against high glucose-induced oxidative stress in human umbilical vein endothelial cells (HUVECs). Both phlorotannins inhibited lipid peroxidation, intracellular reactive oxygen species (ROS), and nitric oxide (NO) levels induced by high glucose treatment of HUVECs.

5.4. *In vivo* anti-diabetic effects in diabetes mellitus mice models

Polyphenol-rich methanol extract of *Ecklonia cava* reduced plasma glucose levels in streptozotocin-induced type 1 diabetic rats [113]. In addition, a dieckol enriched extract of *E. cava* suppressed lipid peroxidation and improved not only glucose levels but also glycosylated hemoglobin and plasma insulin levels in C57BL/KsJ-db/db mice (diabetes type 2 model) [125]. In other study the ingestion of methanol extracts of *Ecklonia stolonifera* reduced plasma glucose levels and produced a decrease in lipid peroxidation in a genetically non-insulin-dependent diabetic mice model KK-A^y [126]. The decrease in glucose level was dependent on the inhibition of maltase by *E. stolonifera* extract. HPLC-MS analysis of the extract suggested that active compounds were phlorotannins.

The anti-diabetic effects of polyphenol-rich methanol extract of *I. okamurae* were investigated in C57BL/KsJ-db/db mice [127]. After including the extracts in the mice diet, a reduction on plasma glucose level was observed and insulin resistance was improved. The amelioration of hyperglycemia can be related to changes in hepatic glucose metabolism, as an enhancement of glucokinase activity was observed, while glucose-6-phosphatase and phosphoenolpyruvate carboxykinase enzymes reduced their activities in treated mice, compared to the control group.

Polyphenolic extract from *A. nodosum* improved fasting blood glucose level in diabetic mice and decreased blood total cholesterol compared to the untreated group [116]. Other study focused on seaweed polyphenols revealed that an ethanolic extract of *Ulva rigida* significantly reduced blood glucose concentration in Wistar diabetic rats [128, 116]. *S. ringgoldianum* methanolic extracts also reduced blood glucose concentration in streptozotocin-induced diabetic mice [129].

Ethanolic extract of red algae *Rhodomela confervoides* anti-hyperglycemic effect was investigated on streptozotocin-induced diabetes male Wistar rats fed with high fat diet. A reduction on blood glucose level was observed after treatment with the extracts, effect that can be a consequence of the inhibition of PTP B1 enzyme exerted by bromophenols present in the extract [120].

CONCLUSIONS

In the present work, some of the most important bioactivities of phenolic compounds from algae have been reviewed with the objective of providing the reader with an overview on the possibilities offered by these interesting marine plants. Thus, antioxidant, antiproliferative, anti-obesity and antidiabetic activities of phenolic compounds present on seaweeds are described focusing on the compounds and mechanisms behind these bioactivities. Considering the huge amount of algae species not yet explored, it is easy to infer that this will be in the future an even more important field of discoveries in the search of alternative therapies to improve health or prevent the extension of important global worldwide diseases.

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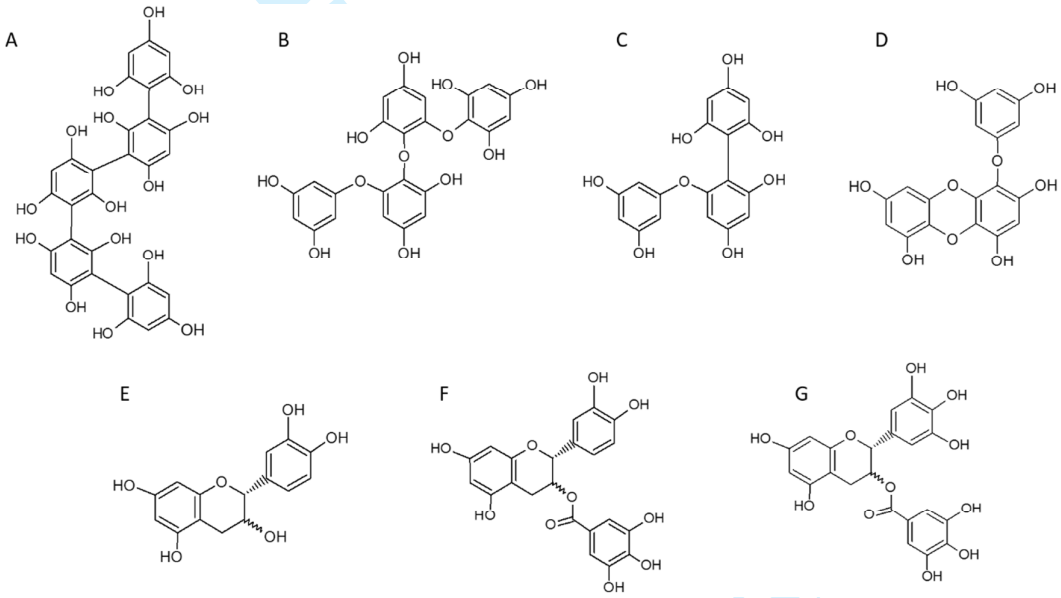
Figure captions

Figure 1. Examples of chemical structures of some phenolic compounds detected in algae, phlorotannins (pentafucol (A), tetraphloretol (B), fucophlorethol (C), eckol (D)) and flavanols ((epi)catechine (E), (epi)catehcin gallate (F), (epi)gallocatechin gallate (G)).

Figures

Figure 1



Tables

Table 1. Some examples of recent research works of extracts from seaweed with *in vitro* anti-proliferative activity.

Seaweed	Location	Type of extract	Disease related	Cell Line tested	GI50 ($\mu g\ ml^{-1}$)	Reference
Cystoseira crinita	Tunisia	Aqueous extracts	Breast adenocarcinoma	MCF7	17.9 ± 0.60	[59]
Cystoseira compressa	Tunisia	Methanolic extract subsequent extraction with Ethyl acetate	Colon cancer cells Lung cell carcinoma	HCT15 A549	27 ± 0.1 33 ± 0.2	[65]
Cystoseira sedoides	Tunisia	Aqueous extracts	Colon cancer cells	HCT15	10.5 ± 0.20	[59]
Ecklonia cava	South Korea	Ethanolic extract	Ovarian cancer cells	A2780 SKOV3	84.3 ± 0.016 99.6 ± 0.050	[60]
Ecklonia cava	South Korea	Crude phenolic fraction from enzyme hydrolized extract	Murine colon cancer cells Mouse melanoma cells	CT26 B16	5.1 29.3	[63]
Eucheuma cottonii	Malaysia	Methanolic extract	Breast adenocarcinoma	MCF7 MDA-MB- 231	25 ± 0.1 50 ± 0.4	[66]
Laminaria japónica	China	Ethanolic (85%) extract	Murine leukemic cell carcinoma Hepatocellular carcinoma cell	P388 BEL-7402	120 >200	[64]
Laurencia obusta	Tunisia	Methanolic extract, subsequent purification, using C18 cartridges	Lung cell carcinoma Colon cell carcinoma Breast adenocarcinoma	A549 HCT15 MCF7	50 35.95 20.5	[67]
Sargassum muticum	Norway	Ethanolic (95%) extract with subsequent purification.	Human colon cancer cells	HT29	32.2±1.7	[22]
Turbinaria turbinata	Mexico	Dichloromethane:methanol (7:3) extracts	Human laryngeal cancer cells Human cervical cancer cells Human nasopharyngeal cancer cells	Hep-2 HeLa KB	93.2 ± 2.3 84.8 ± 1.1 29.8 ± 0.9	[68]

GI50: Concentration that provide 50% growth inhibition

Table 2. Some examples of recent research works of extracts from *Porphyra, Laminaria* and *Undaria* and their *in vitro* antioxidant activity tests .

Seaweed	Location	Type extraction, Solvent used, conditions	Total Phenolic content ($mg \cdot g^{-1}$ GAE)	*In vitro* antioxidant activity	Reference
Porphyra tenera	USA	S-L extraction, distilled water, 80°C,10 min	18.2 ± 0.6	PCL: 0,13 µmol AA·g^{-1}	[27]
Porphyra leucosticta	France	ASE, dichloromethane:methanol (1:1, v:v), 75ºC,1500 psi	2.05 ± 0.01	$EC_{50,}$ DPPH: 8.58 mg ml^{-1}	[90]
Porphyra purpurea	France	ASE, dichloromethane:methanol (1:1, v:v), 75ºC,1500 psi	1.32 ± 0.06	$EC_{50,}$ DPPH:12.08 mg ml^{-1}	[90]
Porphyra tenera	Korea	S-L Extraction, 70% ethanol, 37ºC.	30.18±0.41	DPPH (100 mg ml^{-1}): 6.84±0.40	[91]
Laminaria saccharina	Ireland	S-L Extraction,60% methanol, 95ºC	75.6	EC_{50}, DPPH = 0.48 mg ml^{-1}; EC_{50}, H_2O_2 = 0.31 mg ml^{-1}; FRAP = 4 mg TE g^{-1}	[92]
Laminaria digitata	Ireland	S-L extraction,60% methanol, 95ºC	64.0	EC_{50}, DPPH_ = 0.62 mg ml^{-1}; EC_{50}, FRAP = 3 mg T g^{-1}	[92]
Laminaria japonica	Japan	S-L extraction, 80% methanol, 70°C, 60 min	14.9 ± 0.1	PCL: 0,21 µmol AA·g^{-1} TEAC: 1.512 ± 0.191 mmol TE g^{-1}	[27]
Undaria pinnatifida	Spain	SWE, Water, 200ºC, 1500 psi, 10 min	67.11 ± 6.00	$ORAC_{FL}$: 1522.692 ± 150.928 µmol TE g^{-1}	[93]
Undaria pinnatifida	Spain	50% Acidified Methanol	NI	FRAP = 17.57 µg TE g^{-1} TEAC = 1.81 µM TE g^{-1}	[94]

PCL: Photochemiluminescence: AA: Ascorbic acid, TE: Trolox Equivalent, ASE, Accelerated Solvent Extraction, SWE: Subcritical Water Extraction: NI No indicated

Table 3. Anti-obesity activity of phenolic compounds from edible seaweeds.

Algae strain	Phenolic compounds responsible of bioactivity	Activity	References
27 algae species of *Phaeophyta*, *Chlorophyta*, and *Rhodophyta* phyla	Polyphenols and other compounds	Pancreatic lipase inhibitory activity	[97]
Eisenia bicyclis	7-phloroeckol	Pancreatic lipase inhibitory activity (IC_{50} = 12.7 μM)	[96]
	Fucofuroeckol A	Pancreatic lipase inhibitory activity (IC_{50} = 37.2 μM)	
	Eckol	Pancreatic lipase inhibitory activity (IC_{50} = 76.6 μM)	
	Dieckol	Pancreatic lipase inhibitory activity (IC_{50} = 99.3 μM)	
Ecklonia cava	Dieckol	Pancreatic lipase inhibitory activity (IC_{50} = 350.4 μM)	[98]
Kappaphycus alvarezii	Ethanol extract	Pancreatic lipase inhibitory activity (85% inhibition, 3.8 mg mL^{-1} extract)	[99]
	Dried powder	Pancreatic lipase inhibitory activity (61% inhibition, 3.8 mg mL^{-1} extract)	
Eucheuma denticulatum	Ethanol extract	Pancreatic lipase inhibitory activity (83% inhibition, 3.8 mg mL^{-1} extract)	
	Dried powder	Pancreatic lipase inhibitory activity (60% inhibition, 3.8 mg mL^{-1} extract)	
Kappaphycus striatus	Ethanol extract	Pancreatic lipase inhibitory activity (92% inhibition, 3.8 mg mL^{-1} extract)	
	Dried powder	Pancreatic lipase inhibitory activity (67% inhibition, 3.8 mg mL^{-1} extract)	
Ecklonia cava	Dieckol	Inhibition of adipocyte differentiation and adipogenesis in 3T3-L1 cells. Dose: 0-200 μg mL^{-1}.	[101]
Ecklonia cava	7-Phloroeckol	Inhibition of adipocyte differentiation and adipogenesis in 3T3-L1 cells. Dose: 5-50 μM.	[102]
Ecklonia cava	Triphlorethol-A	Inhibition of adipogenesis in 3T3-L1 cells. (49% inhibition, 20 μM).	[103]
	Eckol	Inhibition of adipogenesis in 3T3-L1 cells. (57% inhibition, 20 μM).	
	Dieckol	Inhibition of adipogenesis in 3T3-L1 cells. (46% inhibition, 20 μM).	
Ecklonia stolonifera	Phloroglucinol	Inhibition of adipogenesis in 3T3-L1 cells. (92% inhibition, 100 μM).	[104]
	Eckol	Inhibition of adipogenesis in 3T3-L1 cells. (58% inhibition, 50 μM).	
	Dieckol,	Inhibition of adipogenesis in 3T3-L1 cells. (15% inhibition, 50 μM).	
	Dioxinodehydroeckol,	Inhibition of adipogenesis in 3T3-L1 cells. (32.5% inhibition, 100 μM).	
	Phlorofucofuroeckol A	Inhibition of adipogenesis in 3T3-L1 cells. (95% inhibition, 50 μM).	
Eisenia bicyclis	6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, dieckol,	Inhibition of adipocyte differentiation and adipogenesis in 3T3-L1 cells (60% inhibition, 50 μg mL^{-1}).	[105]

	phlorofucofuroeckol A		
Ascophyllum nodosum	Aqueous methanolic extract	Inhibition of GPDH (20% inhibition, 75 μg mL^{-1})	[106]
Ishige okamurae	Diphlorethohydroxycarmalol (DPHC)	Induced apoptosis in 3T3-L1 pre-adipocytes	[107]
Ecklonia cava	Extract containing dieckol, 2,7″-phloroglucinol-6,6′-bieckol, pyrogallol-phloroglucinol-6,6′-bieckol, phlorofucofuroeckol A	Ameliorated hepatic lipid metabolism, oxidative stress, and inflammation in high-fat diet-induced obese mice	[108]
Ecklonia cava	Extract containing phloroglucinol, eckol, phlorofucofuroeckol A, dieckol, 8,8'-bieckol	Reduces obesity and glucose levels by anti-inflammatory actions and improvement of lipid metabolism	[109]
Ecklonia cava	Dieckol	Anti-hyperlipidemic effect and -hydroxyl-methyl glutaryl coenzyme A reductase inhibition in high-fat diet-induced obese mice	[101]
Fresh water algae	Infusion ProAlgaZyme	Reduction on body weight, body fat, total cholesterol, LDL-cholesterol, triglycerides, C-reactive protein and fasting blood glucose levels in overweight and obese people.	[110]

Table 4. Antidiabetic effects of phenolic compounds from edible seaweeds.

Algae strain	Phenolic compounds responsible of bioactivity	Activity	References
Alaria marginata	Methanolic extract fractionated with ethyl acetate	α-amylase (IC_{50} = 15.66 µg/ mL) and α-glucosidase (IC_{50} = 63.28 µg/ mL) inhibitory activity	[114]
Fucus distichus	Methanolic extract fractionated with ethyl acetate	α-amylase (IC_{50} = 0.89 µg/ mL) and α-glucosidase (IC_{50} = 13.98 µg/ mL) inhibitory activity	[114]
Ascophyllum nodosum	Methanolic extract rich in phlorotannins	α-amylase and α-glucosidase inhibitory activity	[115]
Eisenia bicyclis	1-(3′, 5′-dihydroxyphenoxy)-7-(2″, 4″, 6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin	α-amylase inhibitory activity (89.5% inhibition at 1 mM)	[113]
	Eckol	α-amylase inhibitory activity (87.5% inhibition at 1 mM)	
	Dieckol	α-amylase inhibitory activity (97.5% inhibition at 1 mM)	
Ecklonia stolonifera,	Phlorofucofuroeckol A	α-glucosidase inhibition (IC_{50} = 1.37 µM)	[122]
Eisenia bicyclis	Dieckol	α-glucosidase inhibition (IC_{50} = 1.61 µM)	
	7-Phloroeckol	α-glucosidase inhibition (IC_{50} = 6.13 µM)	
	Eckol	α-glucosidase inhibition (IC_{50} = 22.78 µM)	
	Dioxinodehydroeckol	α-glucosidase inhibition (IC_{50} = 34.60 µM)	
	Phloroglucinol	α-glucosidase inhibition (IC_{50} = 141.18 µM)	
Ecklonia cava	Dieckol	α-amylase (IC_{50} = 660 µM) and α-glucosidase (IC_{50} = 240 µM) inhibitory activity	[113]
Ishige okamurae	Diphlorethohydroxycarmalol (DPHC)	α-amylase (IC_{50} = 530 µM) and α-glucosidase (IC_{50} = 160 µM) inhibitory activity	[113]
Sargassum ringgoldianum	Methanolic (80%) extract	α-amylase (IC_{50} = 180 µg/ mL) and α-glucosidase (IC_{50} = 120 µg/ mL) inhibitory activity	[116]
Sargassum paten	2-(4-(3,5-dihydroxyphenoxy)-3,5-dihydroxyphenoxy) benzene-1,3,5-triol	α-amylase (IC_{50} = 3.2 µg/ mL) inhibitory activity	[116]
Symphyocladia latiuscula	2,3,6-Tribromo-4,5-dihydroxybenzyl alcohol	α-glucosidase inhibitory activity (IC_{50} = 11 µM)	[117]
	Bis(2,3,6-tribromo-4,5-dihydroxybenzyl) ether	α-glucosidase inhibitory activity (IC_{50} = 0.03 µM)	
Odonthalia corymbifera	2,3-Dibromo-4,5-dihydroxybenzyl alcohol	α-glucosidase inhibitory activity (IC_{50} = 89 µM)	[117]
	Bis(2,3-dibromo-4,5-dihydroxybenzyl) ether	α-glucosidase inhibitory activity (IC_{50} = 0.098 µM)	
	4-Bromo-2,3-dihydroxy-6-hydroxymethylphenyl 2,5-	α-glucosidase inhibitory activity (IC_{50} = 25 µM)	

	dibromo-6-hydroxy-3-hydroxymethylphenyl ether		
	4-Bromo-2,3-dihydroxy-6-methoxymethylphenyl 2,5-dibromo-6-hydroxy-3-methoxymethylphenyl ether	α-glucosidase inhibitory activity (IC_{50} = 53 μM)	
Polysiphonia morrowii	3-Bromo-4,5-dihydroxybenzyl alcohol	α-glucosidase inhibitory activity (IC_{50} = 100 μM)	[117]
	3-Bromo-4,5-dihydroxybenzyl methyl ether	α-glucosidase inhibitory activity (39.5% inhibition at 1000 μM)	
Polyopes lancifolia	Bis(2,3-dibromo-4,5-dihydroxybenzyl) ether	Inhibition against α-glucosidase from *B. stearothermophilus* (IC_{50} = 0.12 μM) and *S. cerevisiae* (IC_{50} = 0.098 μM)	[118]
Grateloupia elliptica	2,4,6-tribromophenol	Inhibition against α-glucosidase from *B. stearothermophilus* (IC_{50} = 130.3 μM) and *S. cerevisiae* (IC_{50} = 60.3 μM)	[116]
	2,4-dibromophenol	Inhibition against α-glucosidase from *B. stearothermophilus* (IC_{50} = 230.3 μM) and *S. cerevisiae* (IC_{50} = 110.4 μM)	[116]
Rhodomela confervoides	2,2′,3,3′-tetrabromo-4,4′,5,5′-tetra-hydroxydiphenyl methane	Inhibitory activity against PTP 1B enzyme (IC_{50} = 2.4 μM)	[120]
	3-bromo-4,5-bis(2,3-dibromo-4,5-dihydroxybenzyl) pyrocatechol	Inhibitory activity against PTP 1B enzyme (IC_{50} = 1.7 μM)	
	bis(2,3-dibromo-4,5-dihydroxybenzyl) ether	Inhibitory activity against PTP 1B enzyme (IC_{50} = 1.5 μM)	
	2,2′,3-tribromo-3′,4,4′,5-tetrahydroxy-6′-ethyloxy-methyldiphenylmethane	Inhibitory activity against PTP 1B enzyme (IC_{50} = 0.84 μM)	
Cladophora socialis	Vanillic acid derivative, $C_{25}H_{16}O_8$	Inhibitory activity against PTP 1B enzyme (IC_{50} = 3.71 μM)	[121]
	Vanillic acid derivative sulfate, $C_{25}H_{16}O_{12}S$	Inhibitory activity against PTP 1B enzyme (IC_{50} = 1.70 μM)	
Ecklonia stolonifera, Eisenia bicyclis	Phlorofucofuroeckol A	Inhibitory activity against PTP 1B enzyme (IC_{50} = 0.56 μM)	[122]
	Dieckol	Inhibitory activity against PTP 1B enzyme (IC_{50} = 1.18 μM)	
	7-Phloroeckol	Inhibitory activity against PTP 1B enzyme (IC_{50} = 2.09 μM)	
	Eckol	Inhibitory activity against PTP 1B enzyme (IC_{50} = 2.64 μM)	
	Dioxinodehydroeckol	Inhibitory activity against PTP 1B enzyme (IC_{50} = 29.97 μM)	
	Phloroglucinol	Inhibitory activity against PTP 1B enzyme (IC_{50} = 55.48 μM)	
Ishige okamurae	Diphlorethohydroxycarmalol (DPHC)	Protective effect against glucose-induced oxidative stress in HUVECs	[124]
Ecklonia cava	Dieckol	Protective effect against glucose-induced oxidative stress in	[123]

		HUVECs	
Ecklonia cava	Methanolic extract	Reduced plasma glucose levels in streptozotocin-induced type 1 diabetic rats	[113]
Ecklonia cava	Ethanolic extract enriched in dieckol	Reduction of lipid peroxidation. Improvement of plasma glucose and insulin levels in C57BL/KsJ-db/db diabetic mice.	[125]
Ecklonia stolonifera	Methanolic extract	Decrease in plasma glucose levels and lipid peroxidation levels in diabetic mice model KK-A^y.	[126]
Ishige okamurae	Polyphenol-rich methanol extract	Reduction of plasma glucose levels and improvement of insulin resistance in C57BL/KsJ-db/db diabetic mice.	[127]
Ascophyllum nodosum	Polyphenolic extract	Improved fasting blood glucose level and decreased blood total cholesterol and glycated serum protein levels in streptozotocin-induced diabetic mice	[116]
Ulva rigida	Ethanolic extract	Reduced blood glucose concentration in Wistar diabetic rats	[128]
Sargassum ringgoldianum	Methanolic (80%) extract	Reduced blood glucose concentration in streptozotocin-induced diabetic mice	[129]
Rhodomela confervoide	Ethanolic extract	Reduced blood glucose concentration in Wistar diabetic rats	[120]

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Current Opinion in Food Science

Rosemary (*Rosmarinus officinalis*) as a functional ingredient: recent scientific evidence

Andrea del Pilar Sánchez-Camargo and Miguel Herrero

Nowadays, research on bioactive substances of plant has earned a growing interest in industries such as pharmaceutical and food processing, due their beneficial health effects. Rosemary is one of the most studied Mediterranean herbs, thanks to its active compounds, which belong mainly to the class of secondary metabolites. Several rosemary components, mainly, carnosic acid and carnosol, have demonstrated different biological activities, such as anti-inflammatory, anti-cancer, as well as beneficial effects against neurological and metabolic disorders. In this regard, this review summarizes and discusses recent remarkable advances on the use of rosemary as a potential functional ingredient in order to provide beneficial health benefits against various human chronic diseases. Besides, the recent scientific evidence related to its safety and bioavailability has been critically discussed.

Address
Laboratory of Foodomics, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain

Corresponding author: Herrero, Miguel (m.herrero@csic.es)

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Introduction

Rosemary (*Rosmarinus officinalis* L.) is an aromatic plant belonging to the *Lamiaceae* family which is widely consumed as a food ingredient, particularly as a culinary spice. This plant, commonly used in the so-called Mediterranean diet, is able to significantly contribute to the organoleptic characteristics of the food in which is included. However, rosemary has also been traditionally used in folk medicine to treat several health disorders; this has finally triggered a great deal of research that has focused on this plant and its main chemical components which could be responsible for the beneficial health properties attributed to this species. The main bioactivities related to rosemary are anti-inflammatory, chemopreventive, anti-proliferative, antimicrobial, protective towards neurological disorders as well as decreasing the risks related to obesity, diabetes and metabolic syndrome, among others [1••,2••,3•,4••]. The main compounds responsible for those bioactivities are the polyphenols present on this plant [4••]. Among them, two main groups of components with varying polarities may be found, namely, phenolic acids and phenolic diterpenes. In the first group, rosmarinic acid is highlighted as one of the most abundant, whereas in the second group, carnosic acid (CA) and carnosol (CS) are pointed out. In any case, the chemical pattern in rosemary is complex, and other minor components including rosmanol, epiisorosmanol, epirosmanol, rosmadial, carnosol quinone, methyl carnosate as well as some flavonoids could have a significant influence on some of the activities related to rosemary extracts and fractions. Moreover, other components present on the essential oil, including 1-8-cineol, camphor, α-pinene or borneol have also been related to some bioactivities, mostly antioxidant and antimicrobial activities [4••].

The existence of these bioactivities has prompted the commercialization of different rosemary-based products and extracts that may be nowadays easily purchased, although their actual *in-vivo* activity in humans is not still properly documented. A strong scientific-driven approach may potentially provide sufficient evidence to consider rosemary or some of their components as useful functional ingredients that could eventually be applied for the production of new functional foods. However, important aspects have to be closely examined to that aim, including comprehensive chemical characterization, bioavailability or metabolization-related issues. In the present review, the main bioactivities associated to rosemary and its components are described and the latest advances (2006–2016) on their knowledge commented. Table 1 shows some recent examples. Moreover, some important safety and bioavailability issues related to the use of this plant and its components are also critically discussed.

Beneficial effects of rosemary

Anti-inflammatory activity

Different researches have covered the study of the anti-inflammatory activity of rosemary extracts and components, confirming previous observations regarding the activity presented by rosemary leaves. In order to reveal the particular mechanisms of action that may operate behind this activity, different individual components of

Table 1

Some examples of recent relevant bioactivities (2015–2016) found for rosemary extract in the literature

Bioactivity	Rosemary extract preparation	Cellular/animal model	Extract concentration/ dosage tested	Outcomes	Reference
Anti-inflammatory	- Rosmarinic acid standard (RAS) - Methanolic rosemary extract (MRE): 0.47 mg g $^{-1}$ RA	Male Wistar rats	RAS: 10, 25 and 50 mg kg^{-1} MRE: 10 and 25 mg kg^{-1}	- RAS and MRE (both at 25 mg kg^{-1}) reduced paw oedema at 6 hours by over 60%, exhibiting a dose–response effect. - In the liver ischemia–reperfusion model, RAS (25 mg kg^{-1}) led to a significant reduction in the serum concentration of transaminases (AST and ALT) and LDH. - In the thermal injury model, RAS (25 mg kg^{-1}) reduced multi-organ dysfunction markers by modulating NF-jB and metalloproteinase-9.	[5]
	Supercritical rosemary extracts - Rosemary A: 180 mg g^{-1} CA, 16 mg g^{-1} CS - Rosemary B: 256 mg g^{-1} CA, 38 mg g^{-1} CS	Human THP-1 monocytes	5 μg mL^{-1}	- Rosemary B showed the highest anti-inflammatory activity, demonstrated by the important reduction of TNF-α, IL-1β and IL-6. - The supercritical extract is more active than the equivalent amount of CA and CS compounds tested separately.	[6]
Anti-proliferative	Carnosic acid standard (CAS)	Human hepatocellular carcinoma HepG2 cells	0–100 μM	- CAS significantly inhibits growth and induces apoptosis in HepG2 cells (in a dose-dependent manner), due the mitochondrial dysfunction and deactivation of Akt.	[7]
	Carnosic acid standard (CAS)	Human hepatocellular carcinoma HepG2 cells	0–100 μM	- CAS inhibited HepG2cells proliferation in both a dose-dependent and time-dependent manner. - CAS-induced autophagic cell death was closely linked to negative regulation of the Akt/mTOR pathway.	[8•]
Metabolic disorders (obesity)	Rosemary extract (RE) (80% CA)	Male C57BL/6J mice	0.14 and 0.28% w/w RE	- RE reduced body weight gain, percent of fat, plasma ALT, AST, glucose, insulin levels, liver weight, liver triglyceride, and free fatty acid levels	[9]
Neurological disorders	Carnosic acid standard (CAS)	Human SH-SY5Y neuronal cells	1 μM	- CAS protects SH-SY5Y cells against 6-Hydroxydopamine (6-OHDA) induced apoptosis through the autophagy pathway.	[10]
	Carnosic acid standard (CAS)	Male Wistar rats	20 mg kg^{-1} body weight of CA	- CAS improved the locomotor activity and reduced the apomorphine-caused rotation in 6-OHDA-stimulated rats.	[11•]

TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β.

rosemary have been closely studied. Among them, CA and CS are the most examined components [3•,4••]. It has been observed that the activity of these phenolic diterpenes may be on the level of gene regulation, as they were demonstrated to activate the peroxisome proliferator-activated receptor gamma (PPARγ). Moreover, these components suppressed the formation of pro-inflammatory leukotrienes in intact human polymorphonuclear leukocytes and inhibited the activity of 5-lipoxygenase [12]. Other already described mechanisms of action for CS rely on the reduction of lipopolysaccharide stimulated NO production in cells treated with this substance. However, not only these components may be solely responsible for the anti-inflammatory activity of rosemary; in fact, rosmarinic acid has also been studied. This pure compound was recently tested in different rat models of local

(carrageenan-induced paw oedema) and systemic inflammation (liver ischemia-reperfusion and thermal injury models) producing a notable activity on both. Rosmarinic acid administered at 25 mg kg^{-1} efficiently reduced paw oedema at 6 hours by over 60% exhibiting a dose-response effect, whereas the same amount was also able to reduce multi-organ dysfunction markers by modulating NF-κB (nuclear factor κB) and metalloproteinase-9 in the thermal injury model [5].

Although working with individual components may be useful for mechanistic studies, the use of rosemary extracts is more related to its potential use as functional ingredients. For this reason, several studies have dealt with the assessment of the anti-inflammatory activity of different rosemary extracts [13]. It has even been observed how rosemary extracts enriched in CA and CS may be more active than the equivalent amount of those compounds tested separately, suggesting a synergistic or additive effect with other components present in the extracts in lesser extent. Particularly, a rosemary extract produced a stronger inhibition of pro-inflammatory cytokines secretion reducing the TNF-α (tumor necrosis factor-α), IL-1β (interleukin-1β) and IL-6 secretions in two cell models of THP-1 human macrophages activated with lipopolysaccharide and with human oxidized low-density lipoproteins, simulating a general inflammatory response and an atherosclerotic-related inflammation, respectively [6].

Anti-cancer activity

The anticancer effects of rosemary extract and its major phenols have been widely studied in the last years and several interesting reviews have addressed this issue [1••,2••,14–16]. Studies employing *in-vitro* models of leukemia [17,18], breast [19,20], lung [21], liver [21,22], pancreas [23,24], prostate [21,25,26], colon [27–30], cervical [31] and ovary [32] cancer cell lines have recently been carried out. Anticancer activity of rosemary extracts can be classified according to its ability to protect against the main three stages of cancer development: initiation (chemopreventive activity), promotion (anti-proliferative activity) and progression (anti-invasive or anti-metastatic activity). The chemopreventive activity of rosemary extracts has been related to its antioxidant properties, in particular with its capacity to scavenge free radicals, which can protect against ROS-induced oxidative damage to lipid, proteins, and DNA [1••,2••,33]. Besides to the protective effects derived from the response to oxidative stress, there are also several studies suggesting that CA and CS may exert an anti-proliferative activity [34]. For instance, a recent study performed on HepG2 liver cancer cells showed that CA possessed anti-proliferative activity mainly caused by its capacity to destabilize the mitochondrial membrane which leads the subsequent release of pro-apoptotic proteins into the cytoplasm. Once into the cytoplasm, those proteins are able to activate other proteins, such as caspase-3, which can promote programmed cell death. Furthermore, CA reduced the phosphorylation of Akt, which was partially inhibited by insulin, an activator of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway [7]. This last signaling pathway is involved in the proliferation, growing and survival processes of cancer cells, and its inactivation can provoke cell death by apoptosis. Those results have also been corroborated in another study with the same cell line (HepG2) [8•]. In spite of this, there is no a particular or universal signaling pathway consistently followed by the main polyphenols present in rosemary extracts for the killing of different types of cancer cells. Indeed, CA may induce apoptosis, cell cycle arrest, autophagy, and inhibition of cellular proliferation on various types of carcinomas. Recently, Bahri et al. have carried out an interesting summary of different molecular pathways involved in the anti-proliferative effect of CA in several types on cancer, including the dose and the specific effect of this compound on target cancer cells [1••]. The antimetastatic activity of rosemary extracts also has been deeply studied; metastasis is a complex process by which cancer cells are able to move from the original site of the tumor and to form tumors in other tissues. In order to invade, epithelial cancer cells need to penetrate through the basement membrane and to disorganize the extracellular matrix (ECM) [35]. In this context, proteases play an important role since they can either degrade or process the ECM components and thereby support cancer cell invasion. Some proteins such as the matrix metalloproteinase (MMP-2 and MMP-9) and the urokinase plasminogen activator (uPA) are responsible for the degradation of several ECM components and play important roles in the process of human colon cancer invasion and metastasis. In this regard, it has been demonstrated that CA is able to reduce the invasive capacity in Caco-2 colon cancer cell model by a reduction of MMP-2, MMP-9 and uPA [35]. Moreover, the effects of CA on the metastatic characteristics of B16F10 melanoma cells have been evaluated revealing that CA suppressed the adhesion of B16F10 cells, as well as the secretion of MMP-9, uPA, tissue inhibitor of metalloproteinase (TIMP)-1, and vascular cell adhesion molecule (VCAM)-1 [36]. Regarding CS, other studies showed that this component can also inhibit the cellular adhesion of different cancer cell lines such colon, ovary and breast, having a dose-dependent effect [37].

Beneficial effects against metabolic syndrome and related disorders (obesity and diabetes)

Metabolic syndrome is a complex condition involving several disorders including obesity, dyslipidemia, impaired glucose metabolism, hypertension or atherosclerosis, which, in combination, increase the risk of developing diabetes mellitus and cardiovascular diseases. As this condition is strongly related to diet, the use of

functional foods and ingredients able to alleviate to a certain extent the mentioned disorders is extremely interesting. Several published reports have linked rosemary with different positive effects on parameters related to metabolic syndrome. Those studies are based on both *in-vitro* and *in-vivo* investigations [4••,38••]. A variety of mechanisms are involved, although there is still no complete clarification of how rosemary may exert the observed effects, as there are even contradictory findings [4••]. In general, it has been demonstrated that different rosemary extracts may decrease body weight as well as cholesterol and triacylglyceride levels as well as increase fecal lipid excretion when administered to different rat models during several weeks [39–41]. Results comparison is difficult caused by extensive genetic differences among animal models including obese, diabetic and hypercholesterolemic animals, as well as among the extracts tested and amount administered. When an extract containing 40% CA was fed (0.5% of diet) to lean and obese rats, it was possible to observe differences on the regulation of key metabolic sensors, suggesting that obese animals were more resistant to the rosemary extract, although a body weight loss was also observed in this case [39]. Anyhow, CA was always pointed out as the main component present on the studied extracts. This has led to the design of experiments in which rosemary extracts with different CA contents are fed to animals for comparison [9]. Mice following a high fat diet supplemented with those different extracts following a chronic dietary intake during 16 weeks showed reduced body weight gain, percent of fat, plasma ALT, AST, glucose, insulin levels, liver weight, liver triglyceride, and free fatty acid levels. Moreover, rosemary also decreased the levels of advanced glycation end products as well as lipid accumulation in hepatocytes. On the other hand, fecal lipid excretion was enhanced and the liver GSH/GSSG ratio was increased. Authors concluded that CA was the major active substance in the extract which was demonstrated to be useful to ameliorate obesity and metabolic syndrome induced by a high fat diet in mice [9]. To gain more insight on the potential molecular mechanisms of action of CA against obesity, readers are referred to an excellent recent review [1••].

Beneficial effects against neurological disorders

Rosemary has been proposed as an effective agent against several neurological degeneration diseases. However, in this case, CA and CS have been mostly tested, being crude rosemary extracts by far less studied. Different effects mainly caused by their antioxidant and anti-inflammatory actions may be behind those activities. One of the most worrisome neurological disorders nowadays is Alzheimer's disease, which is implied on 60–80% of dementia cases in elders [3•]. At present, it is widely accepted the "amyloid hypothesis" based on the accumulation of amyloid-β (Aβ) peptides and their aggregation into neurofibrillary tangles (NFTs), leading to the production of reactive oxygen species (ROS) and inflammatory cascades, which collectively cause neuronal cell death. Both CA and CS have been shown to protect neuronal cells by scavenging ROS [42]. This protection could be most probably caused by the activation of the Keap1/Nrf2 pathway [3•]. Keap1 is a regulatory protein associated to the transcriptional factor Nrf-2 that bind the antioxidant response elements (ARE), which are directly related to the induction of several genes for antioxidant enzymes, including Phase II enzymes. Thus, rosemary diterpenes activity would directly promote the synthesis of antioxidant protecting enzymes, having a positive influence on neuronal homeostasis. Other more recent works have primarily focused on the direct Aβ toxicity. For instance, it has been shown how CA may protect SH-SY5Y human neuroblastoma cells from Ab-induced toxicity through the induction of autophagy, activating AMPK and efficiently reducing toxic protein accumulation and inhibiting oxidative stress and mitochondria dysfunction [43]. However, this is not the only mechanism of action described; CA was able to suppress activation of caspase cascades by reducing the intracellular oligomerization of exogenous Aβ42/43 monomer in the same type of cells treated with 10 μM of that compound, reducing apoptosis [44]. Likewise, both neuronal and astroglial cells treated with CA suppressed the production of Aβ40/42/43 release increasing APP (amyloid precursor protein)-cleavage through the induction of an α-secretase [45].

Similar antioxidant effects were shown to be effective in Parkinson's disease models. CA prevented cell death by a redox-related mechanism that depended on Nrf2 signaling pathway in a paraquat model of SH-SY5Y cells [46]. Moreover, this compound has demonstrated a potential for neuroprotection *in-vivo* and *in-vitro* against 6-hydroxydopamine-induced neurotoxicity by inducing antioxidant enzymes and inhibiting cell apoptosis at the molecular level. Rats fed 10 mg kg^{-1} CA during 3 weeks before 6-hydroxydopamine exposure showed increased levels of antioxidant enzymes that improved the locomotor activity and reduced the apomorphine-caused rotation in 6-hydroxydopamine-stimulated rats [11•]. Moreover, pretreatment with 1 μM CA was able to attenuate 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells, promoting autophagy thanks to the interaction of parkin and Beclin1 proteins [10] and reduced α-synuclein accumulation in a dose-dependent manner in a transgenic OW13 *Caenorhabditis elegans* model of Parkinson's disease that expresses human α-synuclein in muscle cells [47]. Thus, the available evidence at present suggests that these compounds, particularly, CA, could have a good potential in the prevention of different neurological disorders, including Parkinson's and Alzheimer's diseases among others, through a variety of complex and frequently interrelated mechanisms of action [48••].

Safety and bioavailability issues

The use of rosemary as a functional ingredient may have an important potential closely related to the different bioactivities that some constituents of this species may provide (Table 1). In this regard, considering the concentration of bioactives in the plant, an extremely high amount of it should be included in the food in order to exert, in principle, the mentioned effects. For this reason, it is necessary to efficiently extract those components from the natural matrix so that enriched ingredients are generated. Different advanced environmentally friendly extraction techniques have shown good capabilities to produce active rosemary extracts, including pressurized liquid and supercritical fluid extraction [49]. However, even if the extracts are prepared from an edible natural source, their potential toxicity should be comprehensively evaluated prior human consumption. This fact is important as during the extraction procedures, new components, not natively present in the plant, may be produced, which poses an important food safety issue. Furthermore, during the extraction, some unwanted compounds and/or contaminants might be concentrated, also implying a potential safety problem. In any case, this possibility will depend on the particular process and conditions applied for extraction. For instance, the European food safety authority (EFSA) sustained an opinion pointing out the lack of safety concerns for several solvent-based rosemary extracts [50], authorizing their use as food additives (E-392).

Another very important aspect to consider when evaluating the possibility of using rosemary as functional ingredient is related to its bioavailability and metabolism. There are few published data regarding the bioavailability of rosemary components. It has been observed that CA and CS present in a rosemary extract were transported through a Caco-2 monolayer model, although their absorption was slow [51]. Similar results were obtained using obese and normal Zucker rats [52]. In that work, as much as 26 different metabolites derived from the rosemary extract administration were tentatively identified in the gut content, liver and rat plasma. Interestingly, trace quantities of carnosic acid-derived metabolites were also found in the brain [52]. In any case, more information about the metabolism of the involved compounds, including their metabolic fate *in-vivo* is needed to establish if the tested compounds are actually able to reach the target tissues in realistic conditions. Moreover, there is a lack of information on how those potentially bioactive components may interact with the rest of the food in the diet; this information is also relevant to understand their true potential as functional compounds helping to prevent diseases in human beings.

Conclusions

There is increasing scientific evidence sustaining that rosemary and some of its components may exert different interesting bioactivities, as highlighted in the present review, mainly from *in-vitro* experiments and animal models (see Table 1). From a purely scientific point of view, this is a great starting point for the design of new functional foods that may help the consumer to efficiently reduce the risks to suffer several health issues, such as neurological disorders, metabolic syndrome and obesity, or even cancer. However, there is still not sufficient evidence on its true potential activity in humans. In fact, the translation from *in-vitro* experiments to *in-vivo* reality is quite difficult caused by important aspects that remain broadly overlooked, such as bioavailability, metabolism, chemical modifications, matrix effects, influence and interaction with other food components as well as the influence of the gut microbiome. In the case of animal models, their metabolism is significantly different than in humans and their genetic variability much lower. These are the main reasons why the available promising results should not be considered as definitive in humans.

In order to increase the certainty on the true activity of rosemary extracts and their components in human beings, several aspects should be studied in detail, including: (i) to investigate the potential bioactivity of the metabolites that are generated during food digestion; (ii) to closely consider their bioavailability as well as the modifications that occur caused by metabolism; (iii) to consider the influence of the gut microbiome; (iv) to use realistic concentrations, performing *in-vitro* assays using the metabolites and catabolites that could theoretically reach the target tissues *in-vivo* at concentrations attainable after normal feeding, and; (v) to confirm the observations using *in-vivo* studies. To carry out these tasks, researchers may take advantage of the technology gains recently incorporated including metabolomics approaches, microbiomics, gastrointestinal digestion simulation tools as well as the use of humanized animal models. Future advancements made in these topics will help to demonstrate if rosemary may unambiguously be identified as a multi-activity functional ingredient.

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- • of special interest
- •• of outstanding interest

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ANEXO E. Capítulos de Libro

1. Supercritical Fluid Extraction. A. P. Sánchez-Camargo, J.A. Mendiola, E. Ibáñez, M. Herrero (2014). In: Reedijk, J. (Ed.) Elsevier Reference Module in Chemistry, Molecular Sciences and Chemical Engineering. Waltham, MA: Elsevier. ISBN: 9780124095472
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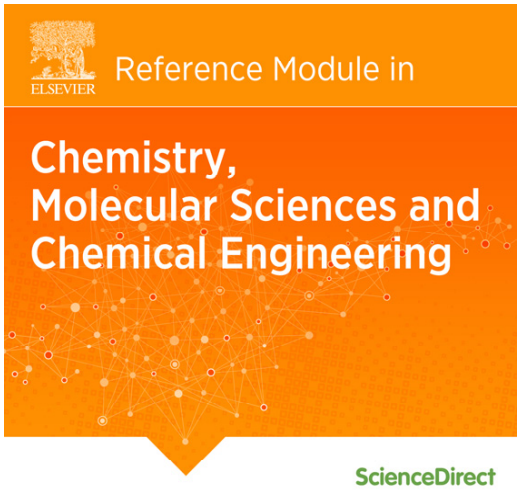

Sánchez-Camargo A.P., Mendiola J.A., Ibáñez E. and Herrero M. (2014) Supercritical Fluid Extraction. In: Reedijk, J. (Ed.) Elsevier Reference Module in Chemistry, Molecular Sciences and Chemical Engineering. Waltham, MA: Elsevier. 30-Jun-14 doi: 10.1016/B978-0-12-409547-2.10753-X.

Supercritical Fluid Extraction

AP Sánchez-Camargo, JA Mendiola, E Ibáñez, and M Herrero, Institute of Food Science Research (CIAL, CSIC), Madrid, Spain

Introduction

At present, there is an increasing interest in developing processes and methodologies able to comply with the green chemistry principles. Among them, extraction techniques have received a great deal of attention since new approaches are needed to solve some important drawbacks associated to the use of conventional techniques involving the extensive use of toxic organic solvents and high energy usage while providing low selectivity and low extraction yields. These shortcomings can be partially or completely overcome by using newly developed advanced extraction techniques, which are faster, more selective toward the compounds to be extracted and, on the top of it, more environmentally friendly. In fact, by using the advanced extraction techniques, the use of toxic solvents is highly limited or greatly reduced.

This is especially true for supercritical fluid extraction (SFE), a technique based on the use of solvents at temperatures and pressures above their critical points. SFE can be a fast, efficient, and clean method for the extraction of compounds of interest from different matrices while being also an appropriate reaction media, among other important applications, as it will be demonstrated throughout this article.

Fundamentals of SFE

SFE is based on the use of a fluid at pressures and temperatures beyond its critical point, in order to achieve significant physical changes that will modify its capabilities as a solvent. Although the first experimental works with supercritical phenomena and with supercritical extraction started back in the nineteenth century, the increase on the interest of this technique as a potential alternative to conventional solvent-based extraction techniques is quite recent. Charles Cagniard de la Tour observed, in 1822[1,2] for the first time, changes in solvents at certain values of pressure and temperature. More than 40 years passed until Thomas Andrews presented the first definition of the term 'critical point' in 1869.[3] Some years later, the first application of this knowledge to extraction was introduced by Hannay and Hogarth[4] who reported how solids could get dissolved in solvents at pressures above their critical point. These early works started to show the important implications occurred in a substance that is submitted to pressure and temperature conditions beyond its critical points, mainly derived from important physical changes that are directly responsible for their possible applications in SFE. In the following section, these physical properties are described in more detail.

The Critical Point: Physical Peculiarities

The critical point (determined by the critical pressure and temperature) is a particular property of a substance; when these values are reached, some changes are induced that effectively modify its physical properties. As can be seen in **Figure 1** (pressure–temperature

phase diagram), when the temperature of a solvent is increased at the same time with its pressure and the critical point is reached, a homogeneous supercritical fluid is obtained in which no distinction can be found between phases.

As can be observed in Figure 1, supercritical fluids have mixed properties between those of liquids and those of gases; for instance, the viscosity is similar to a gas, whereas its density is close to values found for liquids. On the other hand, its diffusivity is intermediate between that of liquids and that of gases. Other important properties are also modified in a supercritical fluid (surface tension, solvent strength, etc.) and will be responsible of the properties as a solvent that the fluid will present. Besides, changes in temperature and pressure beyond their critical points will also modify mainly density, effectively changing the solvent capabilities and permitting the achievement of a high degree of selectivity, as it will be described later. For a more in-depth description of all the physical modifications produced in a supercritical fluid, readers are referred to other book chapters and review articles.[5,6] In practice, a wide group of compounds might be used as supercritical fluids provided that they are submitted to the appropriate temperature and pressure conditions, from water to organic solvents, among others. In Table 1, some of the mostly used supercritical fluids and their corresponding critical values are shown. As it can be observed, the critical values greatly change from a substance to another. It is clear that attaining the correct conditions may be very expensive hindering the practical applicability of some of them at pilot and industrial scales. Besides, it is also important to note that some of these substances are not safe. Considering the always increasing awareness for the development of environmentally respectful processes, the use of solvents that demand extremely high amounts of energy to be placed into a supercritical state and those that may not be perfectly safe or that are toxic cannot be justified at all. For these reasons, most of SFE applications developed nowadays seek to gain advantage of the mild critical temperature and pressure values of carbon dioxide (Table 1). Moreover, CO_2 is a green solvent that is considered a generally recognized as safe solvent for the food industry and is cheap and easily available. Besides, the use of this fluid is not against the limitations established at present for processes generating CO_2, as the carbon dioxide employed is not produced ad hoc, but just recycled or collected from other industrial processes. Thus, the use of CO_2 in SFE processes is a way to reuse this important industrial by-product. Another important advantage that increases even more the interest on the use of this compound is that CO_2 is a gas at room conditions. That means in practice that after the extraction process, when the pressure is relieved, the CO_2 is automatically evaporated leaving a perfectly solvent-free extract. On the other hand, the main shortcoming related to the use of supercritical CO_2 is its very low polarity. Consequently, its ability to extract high- or medium-polarity compounds is rather limited.

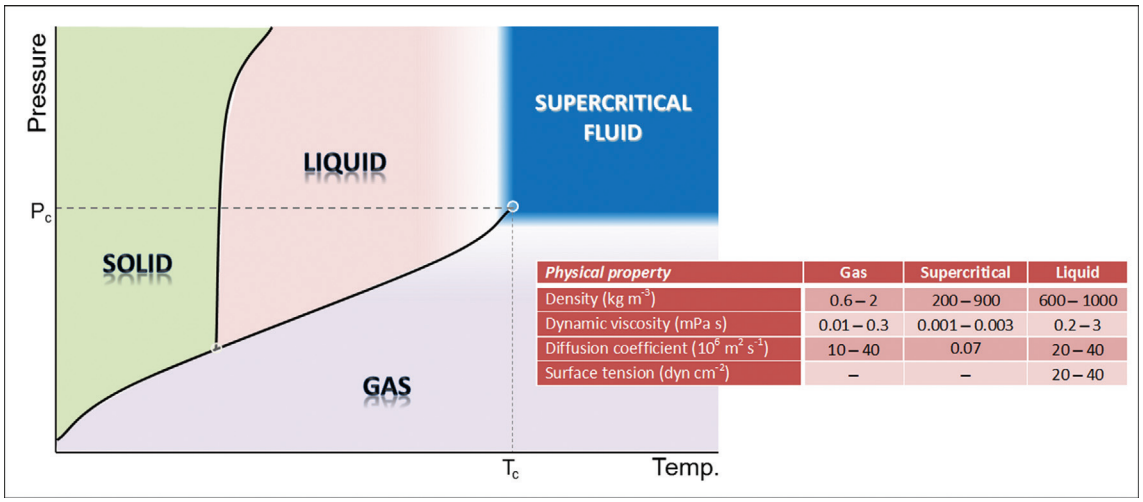

Figure 1 Typical pressure–temperature phase diagram for a given fluid and main physical properties of fluids in the gas, liquid (at room conditions), and supercritical phase. P_c, critical pressure; T_c, critical temperature.

Table 1 Critical properties of some of the most employed fluids used in supercritical fluid extraction

Fluid	*Critical value*			
	Solubility parameter δ_{SFC} ($MPa^{1/2}$)	*Density ($kg\ m^{-3}$)*	*Temperature (°C)*	*Pressure (MPa)*
Carbon dioxide	15.34	470	31.2	7.38
Water	27.61	322	101.1	22.05
Methanol	18.20	272	−34.4	8.09
Ethylene	11.86	200	10.1	5.11
Ethane	11.86	200	32.4	4.88
n-Butene	10.64	221	−139.9	3.65
n-Pentane	10.43	237	−76.5	3.37

To overcome this issue, another solvent may be employed together with CO_2 at very low proportions, in order to increase the polarity of the supercritical fluid. This added solvent is commonly termed modifier or entrainer. Ethanol or methanol mixed below a 10% of total CO_2 employed is frequently used as modifiers. In the following section, the most influencing parameters during an SFE process, including the use of modifiers, are detailed.

Parameters Affecting the Extraction Process

Although the selection of the supercritical solvent to be employed may be envisioned as the most influencing parameter in the extraction, there are a number of important parameters that will significantly affect the solvent strength and the mass transfer processes generated during the extraction and, thus, the outcome of an extraction process. In this section, the most important parameters are briefly described and commented.

Raw Material

The raw material is herein defined as the sample to be extracted. For SFE applications, either solid or liquid samples might be employed, although in each case, the considerations given and the instrumentation needed are slightly modified. Considering solid samples, the physical state of the sample may have a strong influence. Particle size and porosity will have a great impact on mass transfer by increasing the surface contact although the humidity of the sample may also hamper the extraction process. In general, the use of dried samples allows attaining better results, although exceptions exist. The correct parameters have to be experimentally set. If the sample size is too small, the formation of preferential channels inside the extraction cell is possible. To avoid this problem, dispersion agents may be used to produce homogeneous extractions.

In the case of liquid samples, countercurrent extractions are commonly employed to increase contact between the sample and the supercritical fluid. In these applications, the liquid sample is introduced in the upper part of a packed extraction column, whereas the supercritical CO_2 is introduced from the bottom. By correctly selecting the introducing point (height) and the inert column packing material that increases the surface contact, the mass transfer may be optimized.

Solubility (Pressure and Temperature)

Extraction pressure and temperature are probably the most influencing parameters in terms of solubility of a substance in the supercritical fluid. In general, it can be said that higher density of the supercritical fluid will be obtained through an increase in pressure and will lead to an enhanced solubility of sample components. On the other side, an increase on temperature will decrease the density (for a given pressure) although it will also promote the transfer of solutes from the sample to the supercritical fluid due to the increment on their vapor pressure. Thus, the selection of the temperature and pressure values to be employed in a process should be carefully made according to the aim of the process and the targeted compounds. For natural complex samples, the use of experimental designs that allow the statistical observation of the influence of the different parameters involved as a function of one or more response variables is frequent. Response surface methodology or simplex centroid designs are often selected.

Polarity/Use of Modifiers

As it has been already mentioned, CO_2 is the most widely employed supercritical fluid nowadays, although its low polarity limits somehow its application to the extraction of low-polar/lipophilic compounds. In order to increase the range of potential applications, a modifier might be employed together with the supercritical CO_2. Typically, organic solvents such as methanol and ethanol are employed as modifiers, at concentrations below 10% related to the amount of CO_2 used for the extraction, although water has been also employed in some applications. This way, it is possible to increase the solubility of sample components with higher polarity. Under these conditions, the physical state of the solvent mixture is more complex, above all, because the modifiers might not be in their supercritical state and, thus, different phases may be coexisting during the extraction procedures. Other modifiers have been also used to help in the extraction of very low-polarity components, such as oils mixed with CO_2 at very low proportions. Lastly, it has to be noted that when using modifiers, the possibility of attaining solvent-free extracts is lost because these solvents are not gases at room conditions.

Solvent-to-Feed Ratio

The solvent-to-feed ratio to be employed has a critical importance on the supercritical process. Once the pressure and temperature conditions have been defined, it is important to study the effect of the solvent-to-feed ratio or the influence of the CO_2 flow rate. This flow rate should be high enough to maximize the extraction yield but also low enough to allow good contact with the sample in order to minimize the amount of CO_2 employed and, thus, the operational costs. As it can be deduced, this parameter is particularly important when extracting liquids under countercurrent conditions, as in those cases, the ratio will define the contact time allowed between the sample and the supercritical CO_2.

Instrumentation for SFE

Nowadays, there exist a wide range of commercial instruments from benchtop to industrial scales to carry out SFEs. However, it is common to find applications based on in-house-made equipment. The basic instrumentation needed to build an SFE instrument will slightly vary depending on the application, solid or liquid extraction. In Figure 2, the basic components of an SFE extractor are depicted.

The first part of any extractor is devoted to the extraction process itself, composed of a temperature-controlled extraction cell or column able to withstand the high pressures needed to perform the extractions, a CO_2 pump, and a modifier pump. In the case of liquid samples, the extraction column is frequently equipped with different ports for the introduction of the sample at variable heights. In this latter case, another additional pump is needed to introduce the sample into the system. The CO_2 pump is the one setting the pressure inside the extraction chamber, so that the supercritical CO_2 is always under the desired conditions, which is maintained using a restrictor or a back pressure regulator. The second part of the extractor is focused on extract recovery. It may be composed of a collection cell or several fractionation vessels in order to perform cascade depressurization.

This basic equipment may be further developed into more complicated systems, for instance, by installing a system for CO_2 recycling or by a variety of devices depending on the scale of the extractor. More details can be found elsewhere.[6,7]

Applications

Food Science

SFE has been widely used in food science; in fact, the first industrial application was the extraction of caffeine from coffee beans by Zosel.[8,9] Since then, a high variety of samples, types of materials, target compounds, and procedures have been published.

Two clear trends coexist in the applications of supercritical fluids to food science: removal of unwanted compounds and extraction of valuable compounds. Both operating trends will be discussed in the following sections.

Removal of unwanted compounds

When dealing with the removal of unwanted compounds, SFE can be used with different approaches: to remove external toxic compounds from different raw materials and to eliminate or reduce unwanted compounds naturally present in the sample. In some cases, both the extraction residue and the extract can be used in different applications. Some examples of each approach are discussed.

Removal of external contaminants

This is probably the main use of SFE as a sample preparation method. One of the main areas of application of SFE in the last few years has been in food pollutant analysis, mainly pesticide residues and environmental pollutants.[10] A common characteristic of

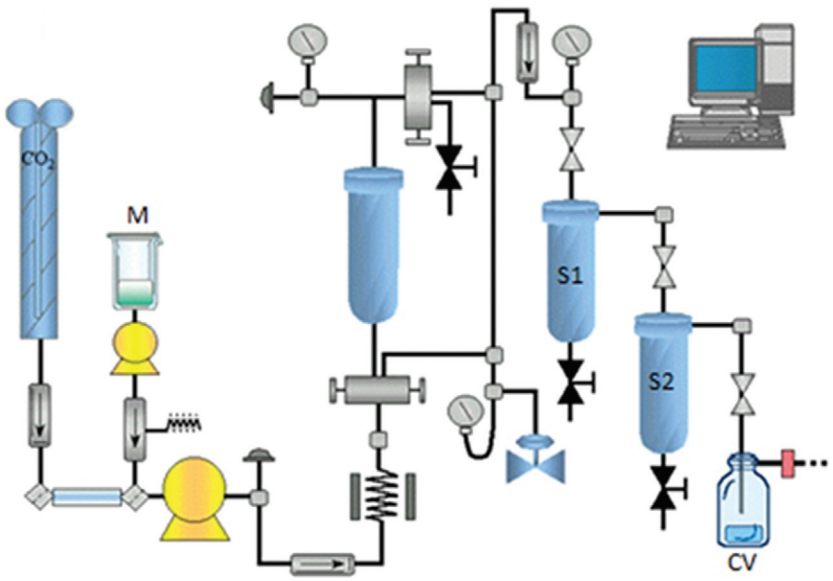

Figure 2 Basic instrumentation needed for a supercritical fluid extraction equipment. M, modifier; S1, separator 1; S2, separator 2; CV, collection vessel.

these works is the extremely high selectivity of SFE in the isolation of the low-polarity pesticides; this fact makes SFE probably the technique of choice to isolate pesticides from low fat food.[11] In fact, in the last years, SFE is being used as a sample preparation method for multiresidue analysis; for example, Valverde et al.[12] developed a method to analyze 22 pesticides by GC-ECD/NPD from rice, wild rice, and wheat; in their work, CO_2 at 20 MPa and 50 °C was used in combination with methanol as a modifier, and results were compared with those of classical extraction using ethyl acetate as an extracting solvent, with the use of SFE having better results than the conventional approach.

Besides pesticides, some other examples of pollutants that can be extracted in foods and other matrices by SFE are Polycyclic Aromatic Hydrocarbons (PAHs),[13] halogenated dioxins and biphenyls (PCBs),[14,15] veterinary drugs,[16,17] etc. An interesting application by Choi et al.[17] has been the extraction of polar and nonpolar fluoroquinolone antibiotics (enrofloxacin, danofloxacin, and ciprofloxacin) in pork by using Na_4EDTA and sea sand in combination with CO_2 at 80 °C, 30 MPa, and 30% methanol. The interest in controlling the presence of drug residues in livestock products has raised important public health concerns (related to toxic effects, development of resistant strains of bacteria, allergic hypersensitivity reactions, etc.) and environmental (air, water and land contamination) and industrial (cheese or yogurt production, etc.) problems.

Removal of naturally occurring toxins

Several kinds of toxins can be present in food depending on their origin, namely, mycotoxins, algal toxins, and plant toxins. In many cases, these toxins are large polar compounds that cannot be extracted by supercritical fluids, but not always. Some examples are the isolation of toxins from *Acorus calamus*[18] or from *Podophyllum hexandrum* rhizomes,[19] where SFE provided much higher recoveries of some toxins, using neat CO_2, than conventional Soxhlet extraction.

Removal of unwanted compounds from food matrices

Sometimes, compounds found naturally in foods are not toxic, but they decrease the overall quality of the food; this is the case of the presence of free fatty acids in several oils such as olive oil,[20] soybean oil,[21] and yuzu oil,[22] which are related to the quality of the fruits prior to oil extraction. Deacidification processes can be conducted by countercurrent SFE with advantages compared with conventional chemical processes providing two fractions, the deacidified oil in the raffinate fraction and the free fatty acids and volatile compounds in the separators.

Removal and use of both fractions

The very first example of this process is the removal of caffeine from coffee[9]; in this example, both fractions are used: decaffeinated coffee and caffeine. Nowadays, not only coffee but also tea[23] and other herbs like mate herb[24] can be used as source of caffeine. In both cases, mild pressures combined with temperatures close to 60 °C must be used to increase the extraction ratio. Another example is the removal of odorant volatile compounds from winemaking inactive dry yeast preparation.[25] Inactive dry yeasts are used as supplements to enhance wine fermentation, but during the inactivation of yeast, several odorant compounds are synthesized; the use of 20 MPa, 60 °C, and ethanol as cosolvent provided an inactive dry yeast preparation free of odorant compounds and an extract rich in 'toasted' flavor that could be used in bakery products.

Another example using liquid matrices together with countercurrent extraction is the fractionation of wine to obtain three valuable fractions: dealcoholized wine, ethanol, and wine. First, the recovery of aroma from wine was attained in a countercurrent packed column (white and red wines were investigated) using very low CO_2/wine ratios. Then, the aroma-free wine recovered from the bottom of the extraction column was dealcoholized by applying different extraction conditions. The results obtained from these studies permit the design of a two-step countercurrent CO_2 extraction process at 9.5 MPa and 40 °C, in which the different CO_2/wine ratios employed in each step lead to the recovery of aroma or the removal of ethanol. One example of countercurrent extraction apparatus can be seen in **Figure 3**.

A similar approach has been also used for the fractionation of essential oils,[26] recovery of used oils,[27] extraction of tocopherols from oil production by-products,[28] or recovery of alkoxyglycerols from shark liver oil.[29]

Extraction of functional food ingredients

From plants

One of the most widely studied applications of the use of supercritical fluids is obtaining functional food ingredient from plants. Notably, there is an important increase in the number of published works in the last decade about the use of supercritical fluids for the recovery of bioactive compounds, mainly with antioxidant activity. Aromatic plants, fruits, legumes, and seeds have been used as sources of natural antioxidant compounds. **Table 2** summarizes the more remarkable studies published in the last 5 years (2009–13) for the SFE of bioactive compounds from plants.

An important application is the SFE of essential oil from medicinal herbs. Essential oils have been traditionally employed in the manufacture of foodstuffs, cosmetics, cleaning products, herbicides, fragrances, and insecticides. Depending on the location and the community knowledge, several of these plants have been used in traditional medicine as diuretics, expectorants, and digestives, among other uses.[52,53] Essential oils have a complex composition containing a few dozen to several hundreds of components, especially hydrocarbons (terpenes and sesquiterpenes) and oxygenates (alcohols, aldehydes, ketones, acids, phenols, oxides, lactones, acetals, ethers, and esters). Besides their fragrance, the mixture of compounds confers several bioactivities (e.g., antimicrobial and antioxidant). Among the most well-known advantages of SFE toward the extraction of essential oils is the use of low

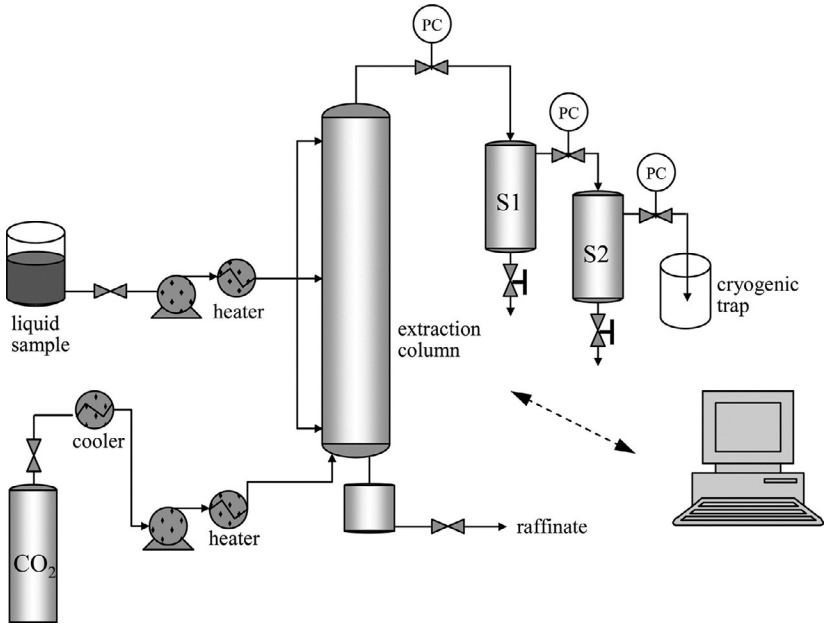

Figure 3 Experimental CC-SFE device. Reprinted with permission from Vázquez, L.; Fornari, T.; Señoráns, F. J.; Reglero, G.; Torres, C. F. Supercritical Carbon Dioxide Fractionation of Nonesterified Alkoxyglycerols Obtained from Shark Liver Oil. *J. Agric. Food Chem.* **2008**, *56* (3), 1078–1083. Copyrights (2008) American Chemical Society.

temperatures that preserve the integrity of the sample. Recently, Fornari et al.[53] reviewed the advances in the SFE of essential oils and accomplished an analysis of the effect of matrix and process conditions.

As can be observed from the information presented in **Table 2**, the bioactives extracted belong to a wide range of compound classes, from polar phenolic compounds to carotenoids, alkaloids, and other pigments and essential oils. As mentioned, in order to extend the polarity range of compounds extracted, ethanol and methanol have been used as modifiers. Usually, quantities of up to 20%[37,47,49] have been employed, although percentages as low as 2–5% have shown to be useful to extract, for instance, polyphenols and terpenoids.[30,54]

Other less-polar bioactive compounds can be potentially recovered by using small amounts of modifiers or even using pure CO_2 at higher pressures. Compounds such as carotenoids, with low polarity, generally need to be extracted using high pressures due to their low solubility in CO_2. These components are basically interesting by their antioxidant activities and coloring properties at the same time. Results of the study of SFE of carotenoids from pumpkin (*Cucurbita maxima*)[55] showed that the total amount of carotenoids extracted increased by increasing pressure from 25 to 35 MPa and temperature from 40 to 70 °C. The highest pressure tested (35 MPa) presented the highest yield (109.6 mg g^{-1}), with a 73.7% recovery. In fact, 60 MPa of pressure was employed for the extraction of lutein and zeaxanthin from *Hemerocallis disticha*. Also, the addition of a cosolvent to SC-CO_2 was proven to improve the extraction efficiency.[35] Although so far, the antioxidant activity is the most studied feature of the extracts obtained by supercritical fluids, other biological activities such as anti-inflammatory, antiviral, antibacterial, cytotoxic, and antiproliferative activity against cancer cells are started to be explored.[36,39,40,44,50,51] Santoyo et al.[50] evaluated the antiviral properties of supercritical CO_2 extracts obtained from thyme species (*Thymus vulgaris*, *Thymus hyemalis*, and *Thymus zygis*) against the herpes simplex virus type 1 at different stages during virus infection. Results indicated that when cells were pretreated with the thyme extracts, an important reduction of virus infectivity was achieved, *T. zygis* extract being more effective than the other thyme species. Meanwhile, Valdés et al.[44] studied the effect of rosemary extracts rich in polyphenols (rosmarinic acid, carnosol, and carnosic acid) obtained using SFE (15 MPa, 40 °C, and 7% ethanol as modifier) on the gene expression of human SW480 and HT29 colon cancer cells. This study showed that rosemary extracts, more specifically carnosol-/carnosic acid-enriched extracts, showed the strongest effect on the proliferation of both cell lines.

Considering the great variations among bioactive compounds and the huge number of plant species, recently, Azmir et al.[52] adapted from Farnsworth et al.,[56] a strategy to build up a standard and integrated approach to screen out these compounds with potential benefits for human health. Selection of plant species, evaluation of toxicity, preparation of sample (extraction) and elemental analysis, biological testing, isolation of active compounds, and *in vivo* analysis are among the steps proposed before marketing the bioactive compounds. The extraction step is critical, and a large number of factors have to be properly adjusted in order to optimize the process; as mentioned in the preceding text, the use of experimental designs is of great help in order to minimize the number of experiments needed to determine the optimum extraction conditions. Taguchi, Box–Behnken, or central composite experimental designs have been used, among others, for the optimization of response variables involved in the SFE extraction of bioactives from plants.[57] Ramandi et al.[58] applied a full factorial design for screening the extraction of fatty acids from *Borago officinalis L.* flowers before optimization using a central composite design. Temperature, pressure, volume of modifier, and

Table 2 Remarkable recent published works (2009–13) dealing with the use of SFE for the extraction of bioactive components from plants

Source	*Bioactive compound of interest*	*Related functional bioactivities*	*Extraction conditions*			*References*
			Solvent	*P (MPa)/T (°C)*	*Extraction time (min)/ mode*	
Amaranth seeds	Squalene, tocopherols	Antioxidant activity	CO_2 + ethanol	65/40	180/Dynamic	30
Baccharis dracunculifolia leaves	Artepillin C	Antioxidant activity	CO_2	40/60	20 + 260/ static + dynamic	31
Camellia sinensis	Fatty acids and antioxidants	Antioxidant activity	CO_2	32/45	90/Static	32
Ginger (*Zingiber officinale R.*)	Phenolic compounds	Antioxidant activity	CO_2 Propane	CO_2: 25/60 Propane: 10/60	180/Dynamic	33
Green tea Leaves	Caffeine	Stimulant	CO_2 + ethanol	23/65	120/Dynamic	34
Hemerocallis disticha	Lutein, zeaxanthin	Antioxidant activity	CO_2	60/80	30 + 30/static + dynamic	35
Magnolia officinalis	Honokiol and magnolol	Antioxidant, anti-inflammatory activities	CO_2	40/80	60 + 40/static + dynamic	36
Mangifera indica leaves	Phenolic compound	Antioxidant activity	CO_2 + ethanol	40/55	180/Dynamic	37
Mitragyna speciosa	Alkaloids	NI	CO_2 + ethanol	30/65	45/Dynamic	38
Olive leaves	Phenolic compounds	Cytotoxic activity	CO_2 + ethanol	15/40	120/Dynamic	39
Oregano leaves (*Origanum vulgare*)	Essential oil	Anti-inflammatory activity	CO_2	30/40	NI/dynamic	40
Persea indica	Diterpene ryanodanes	Insecticidal antifeedant activity	CO_2	20/50	660/Dynamic	41
Pumpkin (*Cucurbita maxima*)	Carotenoids	Antioxidant activity	CO_2 + ethanol	25/80	60/Dynamic	42
Rosemary (*Rosmarinus officinalis*)	Phenolic compounds	Antioxidant activity	CO_2	30/40	300/Dynamic	43
Rosemary (*Rosmarinus officinalis*)	Carnosic acid, carnosol, rosmarinic acid	Antiproliferative colon cancer cells activity	CO_2 + ethanol	150/40	300/Dynamic	44
Rosemary (*Rosmarinus officinalis*)	Volatile compounds, carnosic and carnosol	Antioxidant activity for use in edible oils	CO_2 + ethanol	15/40	180/Dynamic	45
Rosemary + spinach leaves (50%)	Phenolic diterpenes and carotenoids	Antioxidant activity	CO_2	30/40	300/Dynamic	46
Satureja hortensis L.	Phenolic compounds	Antioxidant activity	CO_2 + ethanol	45/40	60/Dynamic	47
Spearmint (*Mentha spicata L.*)	Essential oil	Antioxidant activity	CO_2	9/35	30/Static	48
Strawberry (*Arbutus unedo*)	Total phenolics	Antioxidant activity	CO_2 + ethanol	60/48	60/Dynamic	49
Thyme (*Thymus vulgaris*, *vulgaris*, *Thymus hyemalis*, *Thymus zygis*)	Thymol, carvacrol, borneol, linalool	Antiviral activity	CO_2	30/40	480 Min/ dynamic	50
Usnea barbata L.	Usnic acid	Antibacterial activity	CO_2	30/40	NI/dynamic	51

NI, not indicated.

static extraction time were selected as factors to study their influence on the yield of the extracted oil. Caldera et al.[59] optimized the SFE of antioxidants (carnosol and carnosic acid) from rosemary (*Rosmarinus officinalis L.*). 2^3 full factorial design was used to select important variables before optimization of the selected factors through the Box–Behnken design. Three factors (temperature, pressure, and static extraction time) were studied in this experiment. Extraction pressure, dynamic extraction time, and modifier volume were the factors studied to maximize the recovery of essential oils from *Myrtus communis* leaves,[60] whereas extraction pressure, temperature, and time were the parameters selected in the extraction of essential oils from *Garcinia mangostana*.[61] In this latter case, total extraction yield and radical scavenging activity of the extracts were chosen as response variables, and the composition and amount of cosolvent used as modifier were kept constant.

From marine products

The high biodiversity of the marine environments makes the ocean an extraordinary source of high-value compounds that can be obtained from algae, microalgae, and other marine-related organisms such as crustaceans and fish and their by-products.[62,63] Table 3 summarizes the most relevant literature recently published (from 2009 to 2013) dealing with the recovery of valuable compounds from marine sources using SFE.

Marine sources, especially fish oil and fish by-products, provide the major natural dietary source of ω-3 PUFAs (polyunsaturated fatty acids), mainly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), which have been associated to a lower incidence of cardiovascular diseases due to their potential biological properties, such as anti-inflammatory, antithrombotic, and antiarrhythmic.[79,80] Recently, using the fish oil with low ω-3 fatty acid content (10%) from *Pseudoplatystoma corruscans*, Lopes et al.[70]

Table 3 Remarkable recent published works (2009–13) dealing with the use of SFE for the extraction of bioactive components from marine products and by-products

Marine source	*Bioactive compound of interest*	*Related functional bioactivities*	*Extraction conditions*			*References*
			Solvent	*P (MPa)/T (°C)*	*Extraction time (min)/mode*	
Arthrospira platensis (*Spirulina platensis*)	Fatty acids, γ-linolenic	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2/ethanol (1:1)	30/40	90/Dynamic	64
Brazilian red-spotted shrimp waste (shell and tail)	ω-3 PUFAs, astaxanthin	Antioxidant activity, anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2 + ethanol	30/50	20 + 100/static + dynamic	65
Brazilian red-spotted shrimp waste (heads, shell and tail)	ω-3 PUFAs, astaxanthin	Antioxidant activity, anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2	40/60	20 + 200/static + dynamic	66
Chlorella vulgaris	Lutein	Antioxidant activity	CO_2 + ethanol	40/40	45/Dynamic	67
Chlorella vulgaris C-C	Polyphenols and flavonoids	Antioxidant and anticancer activity	CO_2 + ethanol	31/50	20/Static	68
Fish by-products (off cuts from hake, orange roughy and salmon, and livers from jumbo squid)	ω-3 PUFAs	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2	25/40	90/Dynamic	62
Fish by-product (*Indian mackerel skin*)	ω-3 PUFAs	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2	35/75	180/10 Static cycles of 18 min	69
Fish oil (*Pseudoplatystoma corruscans)*	ω-3 PUFAs	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2	20/40	30/Static + dynamic	70
Haematococcus pluvialis	Astaxanthin	Antioxidant activity for use in edible oils	CO_2 + ethanol	50/75	60 + 150/static + dynamic	71
Monoraphidium sp. GK12	Astaxanthin	Antioxidant activity	CO_2 + ethanol	20/60	60/Static	72
Nannochloropsis oculata	Lipids, zeaxanthin	Anti-inflammatory, reduce risk of certain cardiovascular diseases, antioxidant activity	CO_2 + ethanol	35/50	NI/dynamic	73
Northern shrimp by-products (heads, shell, and tail)	ω-3 PUFAs	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2	35/40	90/Dynamic	74
Sargassum muticum	Phlorotannins	Antioxidant activity	CO_2 + ethanol	15.2/60	90/Dynamic	75
Scenedesmus almeriensis	Lutein and β-carotene	Antioxidant activity	CO_2	40/60	300/Dynamic	76
Schizochytrium limacinum	Fatty acids DHA	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2 + ethanol	35/40	30/Urea complexation + static	77
Striped weakfish (*Cynoscion striatus*) wastes	PUFAs	Anti-inflammatory, reduce risk of certain cardiovascular diseases	CO_2	30/60	150/Dynamic	78

NI, not indicated; PUFA, polyunsaturated fatty acid.

studied the possibility, under different temperatures and pressures, of fractionating the Triacylglycerides (TAGs) with respect to EPA and DHA and demonstrated that the fractionation is improved by using fish oil with lower ω-3 fatty acids content as the basis.

The applicability of SFE technology to add value to fish industry waste products has been also demonstrated by using different fish by-products and some marine invertebrate as raw materials to obtain ω-3 PUFAs. Yamaguchi et al.[81] reported for the first time the application of SFE to crustacean waste. These authors extracted mainly triglycerides from the Antarctic krill and analyzed the effects of temperature (40–80 °C) and pressure (25.5 MPa) on oil extraction with SC-CO_2. Later, Hardardottir and Kinsella[82] studied the extraction of lipids from rainbow trout in a range of pressures and temperatures of 13.8–34.5 MPa and 40–50 °C, respectively. Also, the addition of 10% ethanol as the cosolvent was evaluated, showing a significant increase in the solubility of the lipids in SC-CO_2. Tanaka and Ohkubo[83] reported data from SC-CO_2 extraction of carotenoids and lipids from salmon roe. These researchers used pressures and temperatures ranging from 9.8 to 31.4 MPa and from 40 to 80 °C, respectively. Authors observed that at constant temperature, the oil extraction yield increased with the pressure; the highest oil recovery (about 60%) was achieved under the maximum conditions tested. In general, they observed that the low-molecular-weight triglycerides were extracted easily at low pressures and triglycerides of high molecular weight were readily extracted at high pressures. Another interesting work recently developed by Sánchez-Camargo et al.[65] studied the effect of the addition of ethanol on the extraction yields of lipids and astaxanthin from red-spotted shrimp (*Farfantepenaeus paulensis*) waste. Results showed that the extraction yields increase considerably with the increase in the amount of ethanol in the solvent mixture, reaching maximum recoveries of 93.8% and 65.2% for lipids and astaxanthin, respectively, when employing 15% ethanol. Besides, increasing the amount of ethanol resulted in the increase in the concentration of the ω-3 fatty acids in the lipids of the extract.

Macroalgae, microalgae, and cyanobacteria have been also used as natural sources for the extraction of lipids and antioxidants, namely, carotenoids, isoflavones, polyphenols, and flavonoids.[84] Due to their polarity, these compounds have been traditionally extracted using organic solvents. However, most of the applications presented in **Table 3** employed a certain amount of a cosolvent (ethanol or methanol) to modify the polarity of the SC-CO_2.

For instance, Wang et al.[71] extracted carotenoids (astaxanthin) from *Haematococcus pluvialis* and studied its antioxidant potential in sunflower oil. An increasing cosolvent amount resulted in an improved astaxanthin yield at 40 MPa and 65 °C. Since carotenoids volatility is very low, the use of modifiers is generally recommended instead of increasing the pressure above 50 MPa. The addition of the extract to sunflower oil showed a significant increase in the oxidation stability of the sample at low temperatures, resulting in a higher inhibitory effect on the peroxide formation. On the other hand, the use of high amounts of modifier (up to 50%) was tested to obtain fractions enriched with γ-linolenic acid from the cyanobacteria *Arthrospira platensis* (*Spirulina platensis*); using CO_2-expanded ethanol at 30 MPa, 40 °C, and a ratio CO_2/ethanol 1:1 in the optimum, a recovery up to 35.3% was achieved.[64]

One recent interesting area of research is the SFE of phenolic compounds (phenols and flavonoids) from marine sources. For instance, Wang et al.[68] used SFE to extract the active components (flavonoids as antioxidants) from a novel microalga, *Chlorella vulgaris* C-C. Authors compared SC-CO_2 at 31 MPa and 50 °C, using 50% aqueous ethanol mix as modifier, and ultrasound-assisted extraction (UAE) with 50% aqueous ethanol and reported that polyphenol and flavonoid contents obtained under SFE conditions were 29.1- and 3.7-fold higher than those obtained using UAE, respectively. This resulted in a higher antioxidant activity and better inhibition of lung cancer metastasis.

From food by-products

Food industrial processing generates a large quantity and variety of by-products and wastes ranging from manure to packing residuals; this fact has raised important environmental concerns mainly related to their disposal and/or elimination. A strong research has emerged toward the development of suitable alternatives for these by-products, aimed to create high-value products. Their conversion into valuable materials by, for instance, the extraction of high-value compounds can provide enormous benefits from an environmental and economic point of view. SFE has been widely used, among other applications, to add value to agricultural and food by-products[85–87] that have been employed as sources of bioactive compounds (**Table 4**).

The main bioactive compounds extracted by SFE from agricultural and food by-products have been not only polyphenols and carotenoids with antioxidant properties but also fatty acids, phytosterols, and essential oils. Polyphenols extraction is generally carried out by using ethanol as the cosolvent in amounts ranging between 10% and 20%, although extraction using up to 60% has been reported.[101] Most of the published works about polyphenol extraction measured the efficiency of the extraction of these bioactive compounds using the Folin–Ciocalteu methodology, thus expressing their outcomes as total phenolic content; however, some studies measure the levels of specific compounds such as resveratrol,[90] kaempferol glycosides,[101] and chlorogenic acid.[100] Recently, olive processing by-products,[40,95] vineyard,[90,91] and winemaking residues[89] have been recognized as potential sources of polyphenols with high antioxidant activity. Peralbo-Molina and Luque de Castro[108] reviewed the potential of these residues from the Mediterranean agriculture and agrifood industry.

Regarding carotenoids, lycopene is the most studied pigment and antioxidant extracted from food by-products; it is the most abundant carotenoid in tomatoes, accounting for more than 80% of the pigments present in fully red ripe fruits.[104,105,109] SFE extraction of carotenoids has been mainly carried out from tomato by-products (skins, seeds, and tomato paste waste), although it has been also extracted from watermelon, pink guava, and apricot by-products and carrot press cakes.[109,110] Extraction temperature is a critical variable affecting extraction efficiency of SC-CO_2 extractions. While high temperatures can improve the extraction of some carotenoids, it can also induce thermal degradation or isomerization of the compounds during extraction.[106] As for the pressure, values in the range of 20–40 MPa provided the best recoveries of carotenoids such as lycopene and β-carotene. The interaction between temperature and pressure is certainly important in order to maximize carotenoids' extraction yield when

Table 4 Remarkable recent published works (2009–13) dealing with the use of SFE for the extraction of bioactive components from food by-products

Food by-product	*Compounds of interest*	*Related functional bioactivities*	*Extraction conditions*			*References*
			Solvent	*P (MPa)/T (°C)*	*Extraction time (min)/mode*	
Banana peel	Carotenoids, fatty acids, phytosterols, triterpenes	NI	CO_2	30/50	220/Dynamic	88
Grape bagasse	Polyphenols	Antioxidant activity	CO_2 + ethanol	35/40	10 + 340/static + dynamic	89
Grape by-products (seed, stem, skin, and pomace)	Resveratrol	Antioxidant activity	CO_2 + ethanol	40/35	180/Dynamic	90
Grape seed	Proanthocyanidins	Antioxidant activity	CO_2 + ethanol	30/50	60 Min/dynamic	91
Guava seeds (*Psidium guajava*)	Phenolic compounds	Antioxidant activity	CO_2 + ethanol	30/50	30/Static × 4 cycles	92
Jabuticaba (*Myrciaria cauliflora*)	Polyphenols and anthocyanins	Antioxidant activity	CO_2 + ethanol	30/60	NI/dynamic	93
Melon seeds	Phytosterol-enriched oil	NI	CO_2	40/80	30 + 180/static + dynamic	94
Olive oil mill waste	Phenolic compounds	Antioxidant activity	CO_2	35/40	60/Dynamic	95
Orange (*Citrus sinensis L. Osbeck*) pomace	Flavonoids, phenolic acids and terpenes	Antioxidant activity, antimicrobial activity	CO_2 + ethanol	30/50	300/Dynamic	96
Palm kernel cake	Palm oil	NI	CO_2	41.36/70	60/Dynamic	97
Peach (*Prunus persica*) almond	Oleic and linoleic acid	LDL cholesterol reduction	CO_2 + ethanol	30/50	150/Dynamic	98
Red pepper (*Capsicum annum L.*) by-products	Vitamin E and provitamin A	Different protective effects	CO_2	24/60	120/Dynamic	99
Spent coffee grounds and coffee husks	Caffeine and chlorogenic acid	Antioxidant activity	CO_2 + ethanol	30/60	Spent coffee grounds, 270/dynamic Coffee husks, 150/dynamic	100
Tea seed cake	Kaempferol glycoside	Antioxidant activity	CO_2 + ethanol	45/80	150/Dynamic	101
Tea stalk and fiber wastes	Caffeine	Stimulant	CO_2 + ethanol	25/65	180/Dynamic	102
Sugarcane residue	Octacosanol, phytosterols	Hypocholesterolemic effect	CO_2	35/60	360/Dynamic	103
Tomato juice	Lycopene	Antioxidant activity	CO_2	35/40	5 + 180 or 360/static + dynamic	104
Tomato peel and seeds	Lycopene	Antioxidant activity	CO_2	40/90	180/Dynamic	105
Tomato skin	Lycopene	Antioxidant activity	CO_2 + ethanol + olive oil + water	35/75	NI/dynamic	106
Wheat bran	Alkylresorcinols	Antioxidant activity	CO_2	40/80	215/Dynamic	107

NI, not indicated

SC-CO_2 is used as the solvent; however, some studies affirm that the effects of temperature are more significant compared to pressure, for example, in maximizing lycopene recovery.[105,109] Due to the low solubility of certain carotenoids in CO_2, the type of modifier and its percentage in the mix with CO_2 are crucial parameters affecting carotenoids' extraction yield. Ethanol and some edible oils like almond, peanut, hazelnut, olive, and sunflower seed oil have been used as cosolvents.[109] The effect of the addition of ethanol, water, and olive oil as different cosolvents on the lycopene extraction yield from tomato skin from a tomato processing plant was investigated by Shi et al.[106]; the recovery of lycopene increased when the cosolvent was increased from 5% (w/w) to 15% (w/w), in the following order: olive oil (58.2%) > ethanol (51.7%) > water (48.8%).

Pharmaceutical

Pharmaceutical industries are facing important challenges nowadays, mainly related to the development of production processes with very low environmental impact; in particular, they are urged to reduce the use of volatile organic compounds in drug

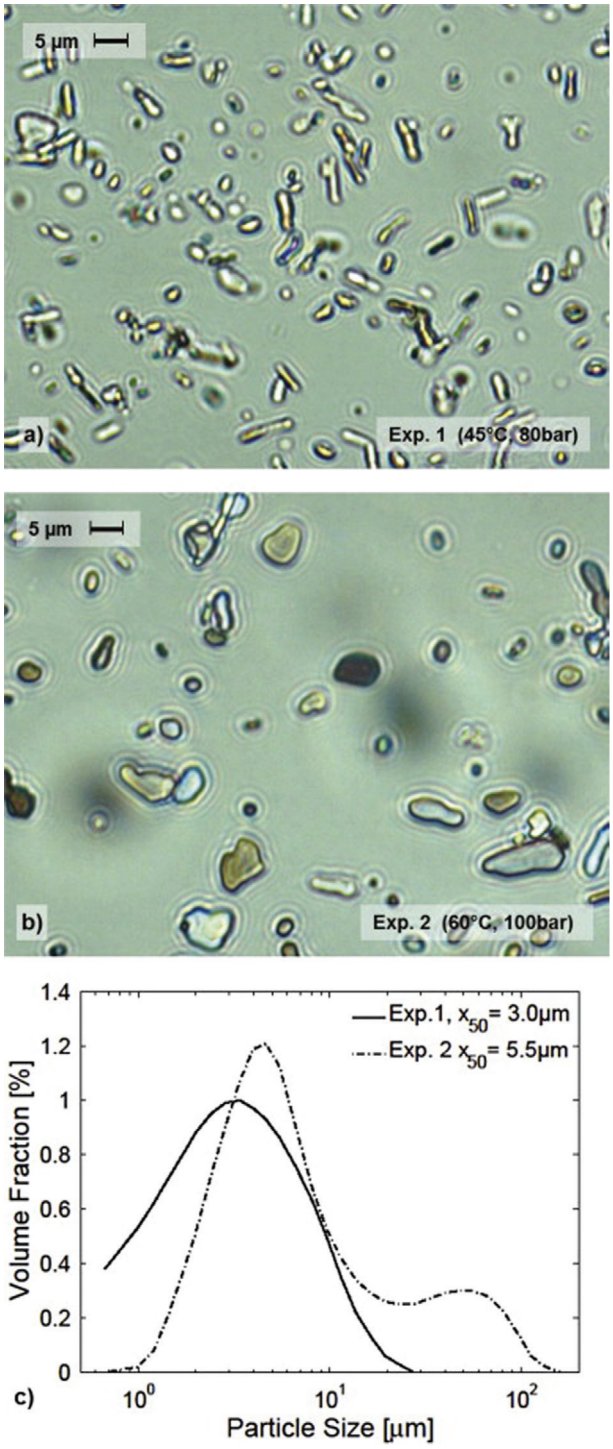

Figure 4 Crystallization upon supercritical fluid extraction of emulsions (SFEE): (a) and (b) phenanthrene crystals obtained at different operating conditions, (c) corresponding particle size diagram. Reprinted with permission from Kluge, J.; Joss, L.; Viereck, S.; Mazzotti, M. Emulsion Crystallization of Phenanthrene by Supercritical Fluid Extraction of Emulsions. *Chem. Eng. Sci.* **2012**, *77*, 249–258. Copyrights (2008) Elsevier.

synthesis/manufacturing and to avoid residues in the finished product. In general terms, the main use of supercritical fluids in pharmaceutics deals with the extraction of bioactive compounds from a mixture (purification from reactions, quantification of active enantiomer, extraction from natural matrices, etc.) or with the extraction of the matrix itself. In this case, crystallization and particle formation have undergone an enormous development in recent years.[111] Other benefits of supercritical fluid technologies, strictly related to the aforementioned new paradigm in pharmaceutics, are linked to the reduced complexity of the process, which stems from a reduction of the number of steps, and to the improved process understanding and control.[112] Despite all the

advantages that supercritical fluids can provide to the pharmaceutical industry, extraction is only a minor field in this area; other uses of supercritical fluids are described for their interest although they are not specifically related to SFE:

- Particle generation and coprecipitation: In the pharmaceutical industry, fine particles (μm or nm) with uniform narrow size range are of particular interest. Various supercritical processes for particle formation include the following:
  (i) Rapid expansion of supercritical solutions (RESS): it involves a fast depressurization of saturated supercritical fluid–drug solution through a heated nozzle into a low-pressure vessel that produces a rapid nucleation of the substrate in form of very small particles.[113]
  (ii) Supercritical antisolvent precipitation: a solution composed of a solute and a solvent is injected into the antisolvent (supercritical fluid). While the solvent and the antisolvent are miscible, the solute is quasi-nonsoluble in the mixture, and consequently, the mixture is supersaturated and solute particles precipitate.[114]
  (iii) Particles from gas-saturated solutions: it is a process similar to RESS, but in this case, the substances are not soluble in the supercritical fluid, but they are melted forming a dispersion; then, the Joule–Thomson effect associated to depressurization cools the dispersion and small particles are obtained.[115]
  (iv) Aerosol solvent extraction system: drug and polymer are dissolved or dispersed in an organic solvent, which is sprayed into a supercritical phase; the organic solvent, soluble in the supercritical gas phase, is extracted resulting in the formation of solid microparticles of drug and polymer.[116]
  (v) Solution-enhanced dispersion by supercritical fluids (SEDS): it allows simultaneous dispersion, solvent extraction, and particle formation. The drug solution meets the supercritical carbon dioxide in a coaxial nozzle of the SEDS apparatus, producing a supersaturated solute. The turbulent, high-velocity flow speeds both mixing and particle formation. The supercritical carbon dioxide disperses and mixes the drug solution, acting as an antisolvent at the same time.[117]
- Coformulation of drug and excipient is one of the emerging concepts in the pharmaceutical industry; in this case, some of the aforementioned techniques are used to prepare formulations with drug and polymer[118] or drugs into liposomes.[119] Attending to the extraction capabilities of supercritical fluids and its use in the pharmaceutical industry, one of the main areas of interest is in solvent removal. Residual solvent removal by supercritical fluids exploits the great diffusivity of the compressed gas and the easy evaporation of organic solvent into the supercritical phase. The efficiency of the process is a function of the solid/solvent and the solvent/supercritical fluid affinity.[112] For example, Kluge et al.[120] proved that crystallization from oil in water emulsions may be used as a purification step; they used SFE to remove the solvent and control crystallization rate of phenanthrene. In this process, the solvent is extracted before the onset of crystallization; therefore, different methods of solvent extraction, such as dilution with water or SFE, affect the process primarily by providing different initial conditions for the crystallization step. SFE-processed emulsions showed a low residual solvent content, especially in comparison with simple dilution of the system. This causes a higher supersaturation of the oil phase, thus accelerating the self-nucleation of droplets. Both effects are in good agreement with the observation that smaller particles have been obtained at the higher suspension density (see **Figure 4**). This process has been named as SFE of emulsions.

SFE can also be used in a combined process of solvent removal and sterilization of drugs. The process described by Howell et al.[121] demonstrated that it is possible to inactivate difficult to kill spores while removing, in the same process, organic solvent. The process was carried out directly from dispensing vials containing a drug, a biological indicator, and one sterilant (peracetic acid) and using SC-CO_2 as the extracting agent. Recovery of drug and analysis of two drugs treated by the process (acetaminophen and paclitaxel) showed no increase in degradation products. After processing, no residual peracetic acid was detected. The process operates at a temperature of about 37 °C (±2 °C) and pressure of about 8 MPa and has a full cycle time of less than 90 min. While much remains to be done before this process could be commercially applicable, the procedure is promising, especially for the preparation of drugs that are easily susceptible to hydrolysis in the presence of water.

Other Applications

Heavy metal recovery

SFE is a promising technique for metal recovery. Chelation combined with solvent extraction is one of the most widely used techniques for separation of metal ions from solid and liquid samples; however, these solvent extraction procedures are usually time- and labor-intensive. In addition, solvent extraction techniques require a large amount of organic solvents and often create environmental problems. In recent years, there has been an increasing interest in extracting metal ions by using SFE. When CO_2 is used to extract chelated complexes, CO_2 and the chelating agent can be easily separated by simply lowering the pressure of the system.[122] Nejad et al.[122] optimized the extraction of some lanthanides by SFE using bis(2,4,4-trimethylpentyl) dithiophosphinic acid (Cyanex 301) as a chelating agent and tributyl phosphate (TBP) as coextractant. They used a fractional factorial design, 2^{5-1} for process optimization considering five experimental factors: amount of Cyanex 301, flow rate, temperature, pressure, and amount of TBP, pressure being the most significant factor. Their results showed that La^{3+}, Ce^{3+}, and Sm^{3+} ions could be quantitatively extracted from the solid matrix by using the following conditions: amount of Cyanex 301, 0.14 g; flow rate, 4 ml min^{-1}; temperature, 40 °C; pressure, 25 MPa; and amount of TBP, 30 μl.

The possible combination of (food residues and heavy metal) extraction has been demonstrated by Albarelli et al.[123] These authors analyzed the effects of SC-CO_2 on banana peels for copper adsorption. SC-CO_2 was used for antioxidant recovery and in an

emerging biomass treatment to increase the efficiency of the subsequent heavy metal-removal step. Adsorption studies showed similar behaviors for fresh and extracted samples, demonstrating that banana peels can successfully be used for the adsorption of copper ions after being subjected to SFE for antioxidant recovery, enabling a promising alternative process chain focused on the integral use of banana peels.

Biopesticides production

The interest for biopesticides has been growing rapidly since the awareness for sustainability, climate change, and organic farming has risen dramatically. Biopesticides, according to the US Environmental Protection Agency (USEPA), include naturally occurring substances and microorganisms that control pests and pesticidal substances produced by plants containing added genetic material. The production of biopesticides is included in the philosophy of green chemistry, a current within the chemistry, which seeks safer products with cleaner processes; in this sense, supercritical fluids can provide important advantages.[124]

Supercritical fluids are used at different stages and in different approaches in the production of biopesticides. In this article, the focus will be on the application of SFE to biopesticides, but readers can refer to Martín et al.[124] for other uses of supercritical fluids for biopesticides. There are two main families of biopesticides that are commonly extracted by supercritical fluids, pyrethrins, and azadirachtins:

- Pyrethrins are the most widely used natural domestic insecticides, extracted from pyrethrum flowers (genus Chrysanthemum), and are composed mainly of pyrethrin, jasmolin, and cinerin. The first application of SFE to obtain pyrethrins was patented in 1981[125]; in general, better results are obtained at low temperatures and mild pressures. In a recent study, Cai et al.[114] compared the results obtained by using hexane and supercritical CO_2; their results showed that the main chemical compounds in pyrethrum flower extracts were β-farnesene, β-cubebene, ethyl palmitate, and ethyl linoleate, besides six pesticidal active compounds of pyrethrins (cinerin I, jasmin I, pyrethrin I, cinerin II, jasmin II, and pyrethrin I). The supercritical extract was very similar to the one obtained with n-hexane, still containing waxes and oil, which could be eliminated by cascade depressurization.
- Azadirachtins are tetranortriterpenoids obtained from the tree *Azadirachta indica* (neem), formed by a group of closely related compounds including azadirachtin, salannin, gemudin, and nimbin. They are very active as insecticides but have very low toxicity for vertebrates. In fact, Chen et al.[127] found that the synergism of azadirachtin, oil, and other active components in neem SFE extracts could increase the bioactivity against insects. The extraction of one of those azadirachtins, nimbin, was optimized by Zahedi et al.[128] who found that optimal conditions to extract nimbin from neem seeds were 40 °C and 20 MPa, with methanol as cosolvent (10%).

Besides these well-known pesticides, there are several essential oils extracted by supercritical fluids that are being assayed as pesticides. Extracts of thyme (*T. vulgaris*) and savory (*Satureja hortensis*) obtained at 12 MPa and 50 °C have proven insecticidal activity comparable to traditional pesticides.[129,130] But not only insecticide activity of essential oils obtained by SFE has been assayed; Liang et al.[131] compared the acaricidal effect of traditional extracts (hydrodistillation and organic solvent extraction) and SFE (18.0 MPa at 40 °C using ethanol as cosolvent) of *Artemisia absinthium*. The supercritical extracts exhibited stronger antifeedant effects than the traditional ones (up to eight times more active) with moderate selective phytotoxic effects.[132]

Future Trends

In this article, we have tried to present the most recent applications of SFE in different fields, including not only the extraction of valuable compounds from different natural raw materials such as plants, marine products, and agricultural by-products but also new and recent advances in different areas such as food science, pharmaceutics, and environmental science. The information is provided as a tool for readers to develop new processes at lab and pilot scale, to discover new ways for sample preparation, and to learn how to deal with SFE optimization and how to tune the different parameters involved in the process and to be able, at the end, to contribute to the development of future emerging technologies able to fulfill the requirements of green chemistry processes. Bearing this in mind, new emerging technologies, for instance, the use of supercritical fluids in particle formation, sterilization, heavy metal removal, or biopesticides production, have been included.

Even if SFE is now a real option for product development, mainly those related to new foods, food ingredients, or pharmaceutical products, there is still a long way to go to be able to implement and demonstrate the sustainability and eco-friendliness of a particular SFE process; to do so, different tools to evaluate the environmental impact of the different procedures are needed, like those based on life cycle analysis. Moreover, more focus is needed in terms of economic considerations of SFE processes at large scales.

Even though in this article, applications based on the use of supercritical CO_2 (plus some modifiers) are mainly presented for their interest and applicability, the future trends in the SFE field point out to the use of a wider range of experimental conditions (including sub- and supercritical conditions) and a higher number of solvents such as supercritical ethane, near-critical dimethyl ether, and gas-expanded liquids or combinations of ionic liquids and supercritical fluids. Readers are referred to Refs. 6,133,134 for more information on new solvents and approaches.

Finally, it is expected that there is an important development of green processing platforms based on the use of green solvents such as supercritical CO_2 and water, multiunit operations consisting of raw material pretreatment, reactions, extraction, and biofuel conversion, etc. For a really interesting revision of this important field of research, readers are referred to the review of Catchpole et al.[135] where recent developments in integrated processing using supercritical fluids for bioseparations are presented.

Together, all the ideas presented in this article and in many other interesting reviews and papers suggested that supercritical fluid technology can be used toward the real development of process sustainability, providing new answers to the most challenging demands posted today.

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Dear Authors

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Authors: Andrea P. Sánchez-Camargo, Lidia Montero, Jose A. Mendiola, M. Herrero, E. Ibáñez

Affiliation: Laboratory of Foodomics, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, 28049 - Madrid, Spain.

Editors of the book:

1. **Dr. Mohammad Billal Hossain**
   Research Officer
   Teagasc Food Research Centre, Ashtown
   Dublin15, Ireland
   Email: Mohammad.hossain@teagasc.ie
   Phone:00353851730719

2. **Dr. Dilip Rai**
   Department of Food Biosciences
   Teagasc Food Research Centre
   Ashtown, Dublin 15
   Email: dilip.rai@teagasc.ie
   Phone: 00353876625552

3. **Dr. Nigel Brunton**
   School of Agriculture and Food Science
   University College Dublin, Belfield, Dublin 4
   Email: nigel.brunton@ucd.ie
   Phone: 003537162807

Yours Sincerely

Mohammad Hossain

Dr. Mohammad Billal Hossain

Novel extraction techniques for bioactive compounds from herbs and spices

Andrea P. Sánchez-Camargo*, Lidia Montero*, Jose A. Mendiola, M. Herrero, E. Ibáñez

Laboratory of Foodomics, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, 28049 - Madrid, Spain.

* both authors contributed equally to this work

Corresponding author: Elena Ibañez

e-mail: elena.ibanez@csic.es

TEL: +34 910 017 956

FAX: +34 910 017 905

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1. Introduction

At present there is an enormous interest in the search of bioactive compounds from natural origin. The increasing number of publications demonstrating the efficacy of such compounds to promote human health has undoubtedly raised the interest of researchers and companies. Among the different sources of bioactive compounds, the most widely known are plants, herbs and spices and agricultural by-products; although also marine sources have shown a great potential (Ibañez, Herrero, Mendiola & Castro-Puyana, 2012).

But not only is important to select the right natural source of an specific bioactive compound or product; also processes involved in their production should be carefully optimized to improve the efficiency of the process and to minimize both, losses of bioactivity and harm to the environment.

Developed societies are becoming increasingly concerned about the environment and its preservation. Since the early 90's, the Green Chemistry movement has lead the change towards a more sustainable and environmentally benign chemistry industry and has explored new ways to reduce or eliminates the use or generation of hazardous substances from chemical products and processes and to improve all types of chemical products and processes by reducing impacts on human health and environment. As defined by Anastas and Warner (1998) "Green Chemistry is the use of chemistry techniques and methodologies that reduce or eliminate the use or generation of feedstocks, products, by-products, solvents, reagents, etc. that are hazardous to human health or the environment". Green Chemistry technologies involve all types of chemical processes and focuses in three main aspects that dominate the twelve principles of Green Chemistry: waste, hazard (health, environmental and safety) and energy (Anastas & Warner, 1998).

Under this framework, it is easy to understand the need of development of new and advanced extraction techniques able to solve important drawbacks associated to the use of conventional techniques. Characteristics of traditional extraction processes usually employed to extract bioactives (mainly solid-liquid extraction) involve the extensive use of toxic organic solvents and high energy usage while providing low selectivity and low extraction yields. These deficiencies can be partially or completely overcome by using newly developed advanced extraction techniques which are faster, more selective and more environmentally friendly.

In this chapter, an overview of the principles and applications of advanced extraction techniques, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), enzyme-assisted extraction (EAE) and combinations of them are presented focused on the extraction of bioactives from herbs and spices. It is our goal to show the huge potential of these techniques towards the extraction of high added-value compounds (with improved bioactivity) while complying with the Green Chemistry goals.

2. Pressurized Liquid Extraction (PLE)

2.1. Description of the technique

Pressurized liquid extraction is based on the use of pressurized solvents at high temperatures (always below their critical points), under conditions in which the solvents are maintained in the liquid state during the extraction process. Under these conditions solvents acquire different properties compared to ambient pressure that imply several advantages. This technique is also commonly known as pressurized fluid extraction (PFE), pressurized hot solvent extraction (PHSE) or accelerated solvent extraction (ASE). A great variability of solvents might be employed for the extractions, although nowadays, the use of the environmentally greenest possible solvents is always sought. Among them, water attracts special attention due to the modification of its physicochemical properties when submitted to high temperatures and pressures. In this case, the technique is even renamed to subcritical water extraction (SWE), superheated water extraction (SHWE) or pressurized hot water extraction (PHWE). In any case the main principles and instrumental requirements are the same regardless the solvent employed.

The basic instrumental requirements to perform PLE are not too complicated. The main parts of an instrument are: pump, extraction cell, pressure valves, oven and collection vessel. The pump is needed to introduce the solvent inside the extraction cell as well as to push the extract out, once the extraction is finished. This pump should be able to achieve the desired pressure (normally, between 35 and 200 bar) into the extraction cell. The solvents employed for the extraction should be oxygen-free in order to avoid oxidation of the bioactives as well as to prevent cavitation in the pump. To do that, degassing by ultrasounds or helium purge are commonly employed. The extraction cell should have two on/off valves in order to be able to keep the conditions stable regarding the pressure set for the extraction. Usually, the extraction cell is made of stainless steel

and is suitable for very high pressures. The extraction cell is placed inside an oven, which controls the applied temperature. The maximum working temperature in most instruments is around 200 ºC. Lastly, a collection vessel is needed. Nevertheless, apart from these basic instrumental requirements, the equipment employed might be more sophisticated. For instance, if dynamic extractions instead of static processes are aimed, more accurate pumps might be needed in order to maintain a precise flow rate during the whole extraction procedure. Besides, in this case, a heating coil should be included inside the oven so that the solvent reaches the extraction cell at the expected temperature. A device to vent the whole system is also common in most PLE instruments; a nitrogen circuit can be included in this regard allowing the complete purging after the extraction as well as to assure that all the extracting solvent has reached the collection vial once the extraction is finished. In spite that there exist several commercial options, lab-made instruments are also quite common. Further information on how to build a PLE extraction system may be found in Turner and Ibáñez (2011).

The main advantages related to PLE come from the particular extraction conditions employed, that is, high temperature and pressures. The main requirement of any PLE extractor is to ensure an extraction pressure high enough to keep the solvents in the liquid state in spite of the very high temperatures that may be used. Under these conditions, the mass transfer rate is greatly favored due to the increase of solubility of the analytes in the sample matrix as well as thanks to a decrease on the viscosity and surface tension of the solvent that further enhance mass transfer. Thus, faster extraction processes with lower volumes of solvents are needed. When the extraction takes place using water as solvent, the same phenomena occurs. However, in this latter case other physical changes are produced. These changes are related to the water dielectric constant. The dielectric constant (ε) can be used as a measure of the polarity of a solvent. This parameter is very significantly affected by the temperature when the water is maintained in liquid state. In fact, although the dielectric constant of water at room temperature is around 80, being by far the most polar solvent, this value decreases to ε= 25-30 (at ca. 200-250 ºC) when the temperature is increased; that is, similar to those presented by some organic solvents, such as ethanol or methanol. Thus, the change on this property when using water implies that the superheated water may acquire new physical and solvent properties that may be very interesting to develop new extraction processes for medium to low polarity compounds, avoiding the use of organic solvents.

Thus, the use of SWE can be suggested as a useful green alternative to the employment of organic solvents in some applications. Indeed, although not feasible for every application, the use of SWE can be therefore seen as the “greenest” one within the pressurized liquid extractions.

In PLE, the solvent is the most critical factor since it will directly influence the type of extractable compounds. Solvent selection depends on the particular aim of the extraction process; the most adequate solvent considering the nature and characteristics of the target analytes should be selected, generally considering that like dissolves like. Moreover, environment has a great influence on this selection as the greenest possible solvent should be always employed.

Once the solvent or solvent mixture is selected, the temperature is the most influencing parameter in any PLE process. As a general rule, when extracting natural matrices, higher extraction temperatures will provide with higher extraction yields. It has been repeatedly observed how the use of very high temperatures is not always detrimental for the recovery of bioactives from natural matrices. However, due to the nature of those components and to their lability, it has to be considered that too high extraction temperatures could potentially lead to the degradation of compounds. For this reason, the use of experimental designs to optimize the extraction temperature is always recommended, monitoring as a response variable the presence of bioactives in the extracts.

Extraction pressure may also have an important impact on the process. However, different researches have shown that once the pressure is enough to maintain the solvent liquid, its effect is quite limited. For this reason, pressures of 50-100 bar are usually employed. These values are enough to keep the liquid state at the usually employed temperatures. Moreover, theoretically, the pressure might exert a rupture effect on the sample being extracted, further increasing the mass transfer rates. Concerning the extraction time, this variable, even though important, has a smaller influence. Extraction time refers to the time in which the solvent is effectively in contact with the sample being extracted at the set temperature and pressure. It is also called static extraction time. This latter term refers to static extractions in which a given volume of solvent is left in contact with the sample at the desired extraction conditions for a specific period of time. In general, relatively short static extraction times (5-20 min) are applied for the extraction of bioactives from natural matrices. Longer times do not generally produce better results as an equilibrium between sample components and solvent is reached.

Other possibility is to run dynamic extractions in which solvent is flowing through the sample at fixed extraction conditions and at a fixed flow rate (Saldaña & Valdivieso-Ramírez, 2015). Most common commercial instruments only work in static mode, meaning that most dynamic extraction applications are based on the use of lab-made equipment.

Other variables that should be also considered include the possibility of running more than one static extraction cycle in order to further exhaust the sample. This approach is also common for the extraction of bioactives from plant materials (Luthria, 2008; Howard & Pandjaitan, 2008). A variation of this multiple extraction processes using the same sample is the employment of sequential extractions that are based on repetitive extractions in which the temperature is raised gradually from one step to the next, so that different extract compositions are obtained.

Other considerations, common to other extraction processes, deal with the sample size, moisture content or dispersing agent employed, among others, and should be optimized appropriately for each particular application.

2.2 Applications of PLE to herbs and spices

PLE has been used to extract a wide range of different compounds from herbs and spices, such as phenolic compounds, essential oils, anthraquinones, alkaloids, or even, pesticides.

As mentioned, in order to obtain the best extraction efficiency, many of the applications carry out an optimization of the main PLE parameters that affect the extraction process. Nevertheless, besides the extraction parameters of the PLE process, the type, chemical structure and stability of the target compounds should be carefully studied.

Phenolic compounds

PLE has been used for the extraction of phenolic compounds from a huge number of vegetable matrices. Extraction conditions selected depend on the content and type of the phenolic compounds present in the sample.

As mentioned above, temperature is the most important parameter affecting both, extraction yield and stability of the compounds. Even if extraction yield increases with a raise in temperature, however, many of the phenolic compounds are thermolabile, and this fact should be carefully considered. For example, coumarins from *Cnidii fructus* or *Heracleum leskowii* degrade above 150ºC (Ga et al., 2013; Skalicka-Woźniak &

Glowniak, 2012). Therefore, temperature should be selected balancing the extraction efficiency and the stability of the compounds of interest (Skalicka-Woźniak & Glowniak, 2012). Temperatures from 50 to 100 ºC usually allow an increase on the extraction yield, while when temperature raise above 100 ºC, the recovery of analytes is generally limited (Runnqvist et al., 2010).

Other than temperature, extraction time and static cycles are parameters that are commonly studied to improve the extraction efficiency. For example, Gao et al. studied the effect of the extraction temperature, extraction time and static cycles for the isolation of bioactive constituents (coumarins) from *Cnidii fructus* (Gao et al., 2013). The optimization of the extraction parameters was carried out by response surface methodology (RSM); degradation of coumarins, as mentioned, was observed at temperatures 150ºC. Extraction conditions selected as the best compromise between maximum yield and maximum recovery of bioactive coumarins were: 122 °C, 5 min and two extraction cycles.

Another key factor that has to be always optimized is the selection of the extraction solvent employed to extract the phenolic compounds from the matrix. The use of a solvent with a high affinity and specificity for the target compounds may provide a more effective and selective extraction, avoiding the co-extraction of unwanted compounds present on the matrix (Skalicka-Woźniak & Glowniak, 2012).

The most used solvents in PLE extraction of herbal phenolic compounds are methanol, ethanol and their mixtures with water. The selection of these solvents depends mainly on the range of polarities of the phenolic compounds, their chemical structure and stability.

A study of the solvent effect on hydroxyanthraquinone extraction from *Rumex crispus* was developed by Wianowska et al. (2014a). In this plant, hydroxyanthraquinones are found mainly in the glycoside form, while the aglycone form represents only a small proportion of the total phenolic content. Due to the presence of sugar units in the glycoside forms, these kinds of compound are expected to be more soluble in water than the aglycone; therefore, the extracting solvent selected should be a methanol/water mixture rich in water (aglycones are better extracted with methanol due to their less polar nature). However, in this study, mixtures with high water content gave rise to extracts with high aglycone concentration. This result might be due to the hydrolytical degradation of the glycosides into their aglycone form, enhanced by the high

temperatures employed in the PLE process. Hence, in this case, to obtain an extract without degradation, methanol was employed as extracting solvent.

Ionic liquids (ILs) have been also suggested as environmentally friendly solvents with interesting properties for the extraction of bioactive compounds from herbs and spices; their main features are their thermal stability and their capacity to solubilize compounds of a wide range of polarities (Zhang, Sun, He, Lu & Zhang, 2006). Although the use of ILs in PLE is very recent and there are only few publications, this approach seems to be very successful. One example is the use of the ionic liquid-based pressurized liquid extraction (ILs-PLE) of rutin and quercetin from different herbs (*Flos sophorae Immaturus, Crateagus pinnatifida Bunge, Hypericum japonicum Thunb and Folium Mor*) (Wu, Chen, Fan, Elsebaei & Zhu, 2012). The optimized ILs-PLE provided higher extraction yields than the traditional extractions processes.

Essential oils

The extraction of essential oils from herbal materials by PLE has been successfully applied with advantages over traditional extraction methods such as distillation, ultrasound extraction or Soxhlet extraction (Chienthavorn, Poonsukcharoen & Pathrakorn, 2011). In general, temperatures between 60 and 100 ºC, and extraction times from 5 to 30 min seem to be the more convenient for the PLE extraction of essential oils (Nur Ain, Zaibunnisa, Zahrah & Norashikin, 2013; Wianowska, 2014b; Kukula-Koch et al., 2014; Liu et al., 2012; Eikani, Golmohammad, Amoli & Sadr, 2013; Pouralinazar, Yunus, & Zahedi, 2012).

In some cases, essential oils obtained by PLE can contain compounds that are not extracted by conventional extraction techniques. For instance, PLE extract of *Iris dichotoma Pall* contains a significant quantity of long-chained fatty acids esters, while the same extracts obtained by hydrodistillation contain mainly simple terpenes (Kukula-Koch et al., 2014).

Considering that the composition of essential oils is very complex and includes lipophilic and hydrophilic components, it is expected that the composition of PLE extracts will mainly depend on solvent polarity (Miron, Plaza, Bahrim, Ibáñez, & Herrero., 2011). Frequently, mixtures of ethanol/water are used in order to include the whole range of polarities of the essential oil components, being the extraction of lipophilic compounds enhanced when an hydroalcoholic solvent rich in ethanol is employed; on the other hand, extracts rich in hydrophilic analytes are achieved when

higher amounts of water are present in the extracting solvent mixture (Cho, El-Aty, Choi, Kim, & Shim., 2013). Although other organic solvents such as n-hexane, acetonitrile, ethyl acetate or dichloromethane have been studied for essential oil extraction by PLE (Cho et al., 2007; Wianowska et al. 2014b), as mentioned previously, the use of ethanol and water as extracting solvents have the main advantage of being green, GRAS (generally recognized as safe) and food-grade solvents.

Alkaloids

The main compounds responsible not only for the characteristic pungency of the pepper but also for its bioactive effects are alkaloids as piperine, chavicine, piperidine, and piperetine (Butt et al., 2013; Liu, et al. 2015).

PLE has been employed for the extraction of piperine and piperidine from commercial peppers as a way of evaluating their quality (De Mey et al., 2014). Considering that piperine and piperidine have a completely different solubility in water, a selective extraction of each compound was possible by employing dichloromethane at 70 ºC for the extraction of piperine, and water at 50 ºC for the complete recovery of piperidine. The effect of the physical state of the sample (powder, cracked peppercorns or peppercorns) also was studied. The recovery of piperine from whole peppercorns was much lower that with the grinded sample; it seems clear that non-treated material did not allow enough contact with the solvent and therefore, lower yields are achieved.

3. Supercritical Fluid Extraction (SFE)

3.1. Description of the technique

Supercritical fluid extraction is based on the use of solvents at temperatures and pressures above their critical points. The term “critical point” was coined by Thomas Andrews, who further elucidated the meaning of Cagniard de la Tour's état particulier (Berche et al., 2009).

Physical properties of supercritical fluids are between a gas and a liquid. For instance, the density of a supercritical fluid is similar to a liquid while its viscosity is similar to a gas and its diffusivity is placed between gas and liquid. Thermal conductivities are relatively high in supercritical fluids and have large values near the critical point. Surface tension is close to zero in the critical point, being similar to gases and much smaller than for liquids. Many other physical properties such as relative permittivity, solvent strength, etc., highly related to density, show large gradients with pressure

above the critical point. Changes in those properties are crucial when dealing with extraction since they are related to changes in solubility and mass transfer ratios.

One of the most valuable characteristics of SFE is the highly reduced (often to zero) employment of toxic organic solvents. In this sense, SFE using green solvents has been suggested as a clean alternative to hazardous processes and thus, SFE has found its growing niche. Hereof, carbon dioxide is the solvent most commonly used to extract bioactives from natural sources. In fact, CO_2 has a series of interesting properties for bioactives extraction:

- It is cheap
- Its critical conditions are easily attainable (30.9 ºC and 7.38 MPa)
- It is an environmentally friendly solvent that, besides, is considered GRAS for its use in the food/pharma industry.

At supercritical conditions, CO_2 presents a high diffusivity whereas its solvent strength and density can be easily modified by tuning the temperature and pressure applied. Another important characteristic of this technique, when using supercritical CO_2, is the possibility of attaining solvent-free extracts. Once the extraction procedure is finished, the depressurization of the system allows CO_2 turning into gas and leaving the matrix while the compounds extracted from the matrix, and solubilized in the CO_2 at high pressures, remained in the collection vessel. These properties are responsible for the extended use of supercritical CO_2 for extraction of bioactive compounds

Although the discovery of the critical phenomena is attributed to Charles Cagniard de la Tour in 1822 (Berche, Henkel, & Kenna, 2009), supercritical fluid extraction was first introduced as an alternative extraction method in 1879 by Hannay and Hogarth. However, it was not until around 1960 that this extraction method started to be thoroughly investigated as an alternative to conventional extraction methods, such as SLE and LLE, requiring large amounts of hazardous chemicals such as chlorinated solvents. This technique has been already employed to extract a wide variety of interesting compounds from very different herbal materials (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013; Mendiola, Herrero, Cifuentes, & Ibañez, 2007). The widest application of supercritical fluids is in the extraction field, especially when using carbon dioxide. The first patent dealing with supercritical fluid extraction was by Messmore in 1943 (Messmore, 1943), although the first industrial application was developed by Zosel in 1978 (Zosel, 1978).

The main drawback of $SCCO_2$ is its low polarity, problem that can be overcome employing polar modifiers (co-solvents) to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest. For example, the addition of relatively small percentages (1–10%) of methanol to carbon dioxide expands its extraction range to include more polar analytes. The modifiers can also reduce the analyte–matrix interactions improving their quantitative extraction (Björklund, Sparr-Eskilsson, Paul, Alan, Colin, 2005). Despite some other solvents have been proposed for SFE (namely: propane, butane, dimethyl ether, etc.) none of them fulfill as well as CO_2 the principles of Green Chemistry and Green Engineering (Anastas et al., 2003).

Along with SFE, Gas Expanded Liquid Extraction (GXL) has been recently proposed as a clean alternative to obtain bioactive compounds from vegetal matrixes. A GXL is a mixed solvent composed of a compressible gas (such as CO_2) dissolved in an organic solvent (Scurto, Hutchenson & Subramaniam, 2009). In some cases, the gas solubility enhancement in GXLs is up to two orders of magnitude relative to the same organic solvent at ambient conditions. Basically GXL extraction consists of SC-CO_2 mixed with a high amount (50-90 %) of solvent (i.e. ethanol) at low pressure and temperature to avoid one single supercritical phase formation. Moreover, GXLs have the advantage of requiring mild working pressures, reducing the energy consumption hence the cost of the process. Similar to supercritical fluids, GXLs have shown to improve mass transfer by decreasing interfacial tension, reducing viscosity, and increasing diffusivity (Akien & Poliakoff, 2009; Reyes, Mendiola, Ibanez, & del Valle, 2014). When using compressed CO_2 to expand less volatile liquid phases, the vapor-liquid equilibrium is such that the vapor phase is dominated by CO_2. The presence of CO_2 in the vapor phase provides safety benefits in the case of oxygen based oxidation reactions by reducing the tendency to form explosive hydrocarbon/O_2 mixtures in the vapor phase. As drawback of GXL, it should be noted that GXL do not completely eliminate the organic solvent, as SFE using neat CO_2 does (Scurto et al., 2009).

Several parameters are involved in the extraction of bioactives from herbal sources by SFE. Among them, it is necessary to precisely control the effect of the extraction temperature, pressure, percentage and type of modifier addition, amount of sample to be extracted as well as its particle size and use of dispersing agents. The first parameters are more related to the solubility of the target compounds in the supercritical fluid, since changes on the extraction temperature and pressure will have a strong influence on the solvent properties, such as density. Although supercritical solvents have diffusivity in

390 the matrix higher than liquids, a decrease in the sample particle size generally produces
391 an increase in the extraction yield obtained, mainly due to the increase in the surface
392 contact between sample and solvent, thus increasing mass transfer. Nevertheless, in
393 some applications the use of dispersing agents (e.g. sea sand, glass beads, diatomaceous
394 earth) or hydromatrix to absorb liquid from the sample can be useful.
395 SFE can be performed in solid and liquid matrices; whereas for solid samples, the
396 equipment has an extraction vessel of a given internal volume, for liquid samples (oils,
397 juices, etc.), the extraction plant uses an extraction column in which the extraction is
398 performed in countercurrent mode. Countercurrent extraction is performed introducing
399 the sample in the system from the top of the column and the pressurized solvent from
400 the bottom side; in this process, the components distribute between the solvent and the
401 liquid sample which flows countercurrent through the separation column. Depending on
402 the separation factor between components to be extracted, the desired contact time
403 between the solvent and the sample can be varied by adjusting the height of the sample
404 introduction into the extraction column or modifying the performance of the separation
405 column, in terms of height and diameter, or of the packing material (structured/random,
406 packing dimensions, surface area, etc.) (Brunner, 1994; Brunner, 2005).
407 For a more detailed description of the equipment used for SFE, readers are referred to
408 Herrero et al. (2006).
409
410 *3.2 Applications of SFE to herbs and spices*
411 SFE has been widely studied for the recovery of bioactive compounds from herbs and
412 spices; in this sense, several recent reviews can be found dealing with this
413 application (Herrero et al., 2013; De Melo, Silvestre & Silva 2014; Fornari, Vicente,
414 Vázquez, García-Risco, & Reglero, 2012).
415 Among the type of matrices that have been extracted from herbs and spices, roots,
416 rhizomes, leaves, bark, flowers, fruits and seeds have been studied (de Melo et al.,
417 2014); being their content and composition in bioactive compounds variable and
418 dependent on factors as location, harvesting time, cultivation practice, soil nutrients and
419 climatic conditions (Pereira, Gualtieri, Maia, & Meireles, 2008). Considering the nature
420 and the physical properties of SC-CO_2, SFE has been mainly used to extract non-polar
421 bioactive compounds, such as fatty acids, triglycerides, phytosterols, terpenoids,
422 tocopherols and carotenoids, among others (de Melo et al., 2014). Table 1 summarizes

some remarkable studies published in the last five years (2010-14) for the SFE of bioactive compounds from herbs and spices.

Phenolic compounds

Herbs and spices have been widely used as source of phenolic compounds, due to their functional properties that have been extensively used in pharmaceutical formulations or as additive in food products (Embuscado, 2015). SFE of phenolic compounds usually require the addition of ethanol and methanol as modifiers of the polarity of SC-CO_2 due to their inherent polar nature. Usually, quantities of up to 20% (Ansari & Goodarznia, 2012; Santoyo, Jaime, García-Risco, Lopez-Hazas & Reglero., 2014; Camila, Pereira & Meireles, 2010) have been employed, although percentages as low as 2 - 5% have shown to be useful to extract, for instance, polyphenols and terpenoids (Kraujalis & Venskutonis, 2013). Some studies have demonstrated that spices and herbs such as rosemary, oregano, sage, hyssop and thyme, extracted with SFE possess high content of phenolic compounds with an antioxidant activity comparable to the activity of BHA (butyl hydroxy anisol) (Babovic et al., 2010). Although so far the antioxidant activity is the most studied feature of these extracts, other biological activities such as anti-inflammatory, antiviral, antibacterial, cytotoxicity and anti- proliferative against cancer cells are beginning to be explored (Cheah, Heng & Chan, 2010; Ocaña-Fuentes, Arranz-Gutiérrez, Senorans & Reglero, 2010; Valdés, et al., 2012; Santoyo et al., 2014). In this sense, Valdes *et al.* (2012), studied the effect of rosemary extracts rich on polyphenols (rosmarinic acid, carnosol, carnosic acid), obtained using PLE and SFE, on gene expression of human SW480 and HT29 colon cancer cells. The study demonstrated that carnosol/carnosic acid-enriched extracts showed the strongest effect on the proliferation of both cell lines. Meanwhile, Santoyo *et al.* (2014) evaluated the antiviral properties of supercritical CO_2 extracts obtained from thyme species (*Thymus vulgaris, Thymus hyemalis and Thymus zygis*) against the herpes simplex virus type 1 (HSV-1) at different stages during virus infection. Results indicated that when cells were pre-treated with the thyme extracts, an important reduction of virus infectivity was achieved; being *T. zygis* extract more effective than the other thyme species.

Essential oils

SFE of essential oils from herbs and spices have an increasing importance considering their wide use in the manufacture of foodstuffs, cosmetics, cleaning products,

fragrances, herbicides and insecticides and the advantages that SFE can provide (Fornari et al., 2012).

Nowadays, the research involving SFE of essential oils from herbs and spices is based on three different approaches: a) the use of SC-CO_2 as alternative replacement of steam distillation and n-hexane as extraction agents, in order to improve its extraction yield and to avoid environment and health hazards (Shao et al., 2014; Przygoda, & Wejnerowska, 2015; Pavlić, Vidović, Vladić, Radosavljević, & Zeković, 2015; Bagheri et al., 2014); b) the enhancement of the concentration of the compound(s) responsible of the bioactivity and/or of the organoleptic properties by developing integrated SFE processes including additional steps (e. g., sample pre-treatment or fractionation) (Arranz, Jaime, de las Hazas, Reglero & Santoyo, 2015; Gañán & Brignole., 2013; de Oliveira et al., 2014; Santoyo et al., 2014; Gañán, Dambolena, Martini & Bottini, 2015; Sánchez-Camargo et al., 2014); and c) the exploration of new bioactive compounds from distinctive herbs species and testing of their bioactivities (Maran & Priya, 2015). In many of the aforementioned studies, the use of the experimental design has been of great help in order to minimize the number of experiments needed to determine the optimum extraction conditions.

Considering the great variations among bioactive compounds and the huge number of plant species, recently Azmir *et al.* (2013) adapted a strategy from Farnsworth, Akerele, Bingel, Soejarto and Guo (1985) to build up an standard and integrated approach to screen out these compounds. Selection of plant spices, evaluation of toxicity, sample preparation and extraction, elemental analysis, biological testing, isolation of active compounds and *in-vivo* analysis are among the steps proposed before marketing of bioactive compounds.

Table 1. Some remarkable recent published works (2010–2014) dealing with the use of SFE for the extraction of bioactive components from herbs and spices

Source	Bioactive compound of interest	Extraction conditions			Related functional bioactivities	Reference
		Solvent	P (MPa)/T (°C)	Extraction time (min) / Mode		
Ginger (*Zingiber officinale R.*)	Phenolic compounds	CO_2 Propane	CO_2: 25/60 Propane: 10/60	180/dynamic	Antioxidant activity	1
Green Tea Leaves	Caffeine	CO_2 + ethanol (1-3% w/w)	23/65	120/dynamic	Stimulant	2
Magnolia officinalis	Honokiol and Magnolo	CO_2	40/80	60 + 40/ static + dynamic /	antioxidant, anti-inflammatory activities	3
Oregano Leaves (*Origanum vulgare*)	Essential oil	CO_2	30/40.	NI/dynamic	Anti-inflammatory activity	4
Rosemary (*Rosmarinus officinalis*)	Phenolic compounds	CO_2	30/ 40	300/dynamic	Antioxidant activity	5
Rosemary (*Rosmarinus officinalis*)	Carnosic acid, Carnosol, Rosmarinic acid	CO_2 + ethanol (7% w/w)	150/40	300/dynamic	Antiproliferative colon cancer cells activity	6
Rosemary (*Rosmarinus officinalis*)	Volatil compounds, carnosic and carnosol	CO_2 + ethanol (5-10% w/w)	15/40	180/dynamic	Antioxidant activity for use in edible oils	7
Rosemary + spinach leaves *(50%)*	Phenolic diterpenes and carotenoids	CO_2	30/40	300/dynamic	Antioxidant activity	8
Spearmint (*Mentha spicata L.*)	Essential oil	CO_2	9/35	30/static	Antioxidant activity	9
Thyme *(Thymus vulgaris, vulgaris, Thymus hyemalis, Thymus zygis)*	Thymol, carvacrol, borneol, linalool	CO_2	30/40	480 min/dynamic	Antiviral activity	10

NI: No indicated

4. Ultrasounds Assisted Extraction (UAE)

4.1.Description of the technique

Ultrasounds Assisted Extraction uses acoustic cavitation to cause disruption of cell walls, reduction of the particle size and enhancement of contact between the solvent and the target compounds. It is very versatile, due to the possibility of using several solvents of different polarities. Moreover, it allows fast extractions, which mean a key point to avoid degradation of labile compounds. Besides, it uses low amounts of solvents and it is cost-effective, therefore the development of UAE methods could represent a key point in Sustainable Development (Picó, 2013).

In UAE only a small portion of the ultrasound spectrum is used, namely power ultrasounds. Power ultrasounds, having frequencies between 20 kHz and 100 MHz, are well-known to have significant effects on the rate of various physical and chemical processes such as cleaning, degassing, solubilization, homogenization, emulsification, sieving, filtration, and crystallization. Power ultrasound involves the mechanical and chemical effects of cavitation. When a liquid is irradiated by ultrasounds, micro-bubbles form, grow, and oscillate extremely fast, and eventually collapse powerfully if the acoustic pressure is high enough. These collapses, occurring near a solid surface, generate micro-jets and shock waves that result in cleaning, erosion, and fragmentation of the surface. Micro-discharges due to high electrical fields generated by deformation and fragmentation of the bubbles and the formation of radicals could be the responsible of the observed chemical effects (Ötles, 2009). The uses of ultrasound are broadly distinguished in two groups: high and low intensity. Low-intensity ultrasound is involved in non-destructive analysis, to provide information on the physicochemical properties (e.g. in fruits it is used to study firmness, ripeness, sugar content, and acidity). On the other hand, high-intensity ultrasound – low frequency (16–100 kHz) high power (typically 10–1000 W/cm^2) – can modify food properties physically or chemically. High-intensity ultrasound is used, among other applications, to speed up and to improve the extraction efficiency (Picó, 2013).

The selection of the best extracting solvent for UAE depends on its physical properties (surface tension, viscosity and vapor pressure) which may affect the cavitation intensity in a liquid phase. The cavitation intensity increases for solvents with low vapor pressure, high viscosity and high surface tension, although the cavities are more easily formed with a solvent that has a high vapor pressure, low viscosity and low surface tension. Thus, solvents with high surface tensions, densities, and viscosities generally

have a higher threshold for cavitation but more harsh conditions once cavitation begins (Vardanega, Santos & Meireles, 2014).

The use of ultrasounds in extraction processes has two main requirements: a liquid medium (at least 5% of the overall medium) and a source of high energy vibrations (ultrasounds) (Vardanega et al., 2014). The vibrational energy source is called transducer, which transfers the vibration (after amplification) to the so-called sonotrode or ultrasonic probe, which is in direct or indirect contact with the processing medium. The two ultrasound apparatus most commonly used for extraction are the ultrasonic cleaning bath and the more powerful probe system. For small extraction volumes, an ultrasound horn with the tip submerged in the fluid can be sufficient. Large volumes of fluids have to be sonicated in an ultrasound bath or in continuous or recycled-flow sonoreactors (Ötles, 2009). While most of the research effort in UAE has concentrated on ultrasound itself, some studies have also examined the coupling between ultrasound and other techniques; examples will be provided in Section 7 of this chapter.

UAE of bioactive compounds is increasingly efficient at directly transferring knowledge into technology for commercial development. This novel process can extract analytes under a concentrated form (low volumes of solvent) and free from any contaminants or artifacts. The new systems developed so far clearly demonstrated the advantages of UAE in terms of yield, selectivity, operating time, energy input, and even preservation of thermolabile compounds (Ötles, 2009).

4.2.Applications of UAE to herbs and spices

As mentioned, ultrasound has been recognized for potential industrial application in the phyto-pharmaceutical extraction industry for a wide range of herbal extracts. The mechanical effects of ultrasound together with the disruption of biological cell walls to release their content provide important benefits in extractive processes (Vilkhu, Mawson, Simons & Bates, 2008). Due to this advantages, ultrasound-assisted extraction (UAE) has been used in many applications in food research.

Phenolic compounds

Among bioactive compounds, phenolics have been the target of numerous applications using UAE (Rathod & Rathod, 2014; Both, Chemat & Strube, 2014; Xia, Shi, & Wan, 2006; Hossain et al., 2012; Chemat, Humma & Khan, 2011).

Several studies have demonstrated that the extraction yield of phenolic compounds depends strongly on the extraction technique, solvent polarity, and temperature (Sultana, Anwar & Ashraf, 2009; Lapornik, Prošek & Wondra, 2005). For this reason, the optimization of the variables involved in UAE by response surface methodology (RSM) has gained interest in order to reduce the number of experimental runs, cost, and time. Barbero *et al*. (2008) studied the effect of the ultrasound-assisted variables as type of solvent, extraction temperature, extraction time, amount of sample and solvent volume on the extraction of capsaicinoids from hot cayenne pepper. Authors found that at the optimal conditions (methanol, 50ºC and 10 min of extraction time), 100% of the capsaicinoids were recovered and the amount of sample and the solvent volume were not relevant parameters (Barbero, Liazid, Palma & Barroso., 2008). Ghasemzadeh et al. (2014) also found that ultrasonic power (80–150 W), methanol percentage in the solvent mixture (40–80%) and extraction temperature (40-80ºC) significantly affect the extraction yield of catechin, naringin and quercetin from curry leaves (Ghasemzadeh, Jaafar, Karimi & Rahmat, 2014). Optimal conditions were obtained using an ultrasonic power of 145.52 W at 55.9°C with 80% methanol; extracts achieved showed important anticancer and antioxidant activities. UAE was also employed to extract phenolic compounds (coumaric and 2-hidroxycinnamic acid, among others) from bay leaves (*Laurus nobilis L.*). The effects of several experimental factors, such as sonication time (20-60 min), solid/liquid ratio (1:4-1:8 g mL-1) and concentration of ethanol in the solvent (0-70%) on extraction of phenolic compounds were evaluated through a randomized complete factorial design (3^3). The optimal extraction conditions were solid/liquid ratio 1:12 (g mL^{-1}), and time of sonication 40 min with an ethanol concentration of 35%.

Essential oils

Some herbs and spices like artemisia (Da Porto, Decorti & Kikic, 2009), peppermint (Shotipruk, Kaufman & Wang, 2001), laurel (*Laurus nobilis* L.), rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.) and oregano leaves (*Oreganum majorana*) have been submitted to UAE to improve essential oil extraction (Roldán-Gutiérrez, Ruiz-Jiménez & de Castro, 2008). Roldan-Gutierrez et al. (2008) stated that UAE is more effective than steam distillation and superheated water extraction for aroma compounds' extraction. UAE provides extracts with an aroma closer to the natural, due to the higher concentration in the most volatile fraction. Also, Morsy

(2015) studied the influence of UAE followed by hydrodistillation of *Elettaria cardamomum L* seeds. Authors concluded that sonication power and sonication time highly influenced the yield and quality (high-terpinyl acetate/1, 8-cineole ratio) of the obtained essential oil. The optimal operating conditions include water-to-dried seeds ratio of 12, sonication at 10% of the maximal power (30W) and a sonication time of 30 min prior to hydrodistillation for another 30 min; the process is considerably shorter (30 min vs 6 h) than the traditional hydrodistillation employed for full extraction of essential oil from cardamom seeds. Moreover, UAE has been coupled to under vacuum distillation to obtain flavor compounds from spearmint leaves (Da Porto et al., 2009). Results showed that the combination of these processes provided extracts with higher flavoring strength due to the increased concentration of desirable oxygenated compounds (from 5 to 8 times) compared to hydrodistillation. Extraction yields of flavor volatiles increased from 0.01–0.02% by hydrodistillation to 0.04–0.13% by ultrasound coupled to vacuum distillation.

5. Microwave Assisted Extraction (MAE)

5.1.Description of the technique

Microwave Assisted Extraction uses microwave radiation that causes motion of polar molecules and rotation of dipoles to heat solvents and to promote transfer of target compounds from the sample matrix into the solvent (Ötles, 2009). Just like UAE, MAE allows fast extractions with small amount of extraction solvents; avoiding degradation of thermolabile compounds and providing green extracts from herbal resources.

Microwaves are non-ionizing electromagnetic waves located within the frequency band of 300 MHz to 300 GHz (between the radio-frequency range and the infrared at the higher frequency) in the electromagnetic spectrum; 2450 MHz frequency is generally used in domestic microwave ovens and for extraction purposes (Routray & Orsat, 2012). In MAE, the sample is heated by using microwaves, at typical powers of 700 W for a short time. Compared to traditional extraction techniques, the use of microwaves allow the decrease of extraction times, significantly limiting the amount of solvent needed.

MAE was first described in 1986 (Ganzler, Salgo, & Valkó, 1986); in this work, a domestic microwave was used as sample preparation technique previous to chromatography. A variety of compounds from seeds, foods and feeds were extracted; the process was found to be more efficient than classical Soxhlet or LLE. The

application of microwave irradiation to the extraction of compounds from biological samples has been a more recent development.

A microwave system consists normally on three basic parts (Routray & Orsat, 2012):

- Magnetron: the microwave source. It consists of a vacuum tube with a central electron-emitting cathode of highly negative potential surrounded by a structured anode that forms cavities, which are coupled by the fringing fields and have the intended microwave resonant frequency.
- Wave guide: transmission lines and wave guides can be used for guiding the electromagnetic waves. In the food industry and domestic oven applications, the most common are standing wave devices, where the microwaves irradiate by slot arrays (that cut the wall currents) or horn antennas (specially formed open ends) of waveguides.
- Applicator or oven.

Solvent is one of the most important factors in MAE extraction. In general, the higher the dielectric constant of the solvent, the higher its capacity to absorb microwave energy, which can lead to faster rate of heating of the solvent with respect to the herbal material (Routray & Orsat, 2012). Combination of solvents leads to varying selectivity and microwave heating capacity. For extraction of thermolabile compounds, a solvent combination with relatively low dielectric properties can be used to ensure that the solvent temperature will remain lower to cool-off the solutes once are liberated into the solvent. In this case, the microwave energy interacts preferentially with the plant matrix (Kaufmann & Christen, 2002). Even ionic liquids can be used as potential solvents for microwave-assisted extraction of vegetable compounds. The anions and cations of ionic liquids have been described as having influence on the extraction of polyphenolic compounds, and the ionic liquids with electron-rich aromatic π-system were found to enhance the extraction yield (Du, Xiao, Luo, & Li, 2009).

In general, samples for MAE are homogenized and mixed with a solvent and the suspension irradiated at a frequency greater than 2000 MHz for short periods of time. Heating is usually repeated several times with periods of cooling in between to prevent boiling. Efficiencies seen with this technique approach those of classical Soxhlet extraction but can be performed much more rapidly. Further modifications of the technique mimic sweep co-distillation or steam distillation with air sweeping the surface of the heated sample and being collected by a condenser that protrudes through the oven

housing (Worsfold, Townshend & Poole, 2005). MAE techniques have also been developed using closed-system microwave heating that implies an increase of pressure due to the heating at temperatures higher than the boiling point of the solvent; thus, extraction takes place in similar condition as PLE.

There are two approaches of applying microwave energy, namely, the mere bulk heating of a mixture through the use of absorbing containers and solvents (oven-type apparatus with samples in closed-vessel conditions), and the more refined, although demanding, selective heating of the target materials. These two approaches led to the development of two main types of laboratory extraction instruments.

By using the selective heating it is possible to operate safely and much more efficiently under open-vessel conditions (i.e., atmospheric pressure); under these conditions, operating temperatures remain low. Once fitted with an appropriate reflux column, the system is auto-controlled in terms of temperature. A technology combining microwave and Soxhlet extraction was designed in 1998 (Garcia-Ayuso, Sanchez, Fernandez de Alba & Luque de Castro, 1998). This extraction technique, called microwave-assisted Soxhlet extraction, uses two sources of energy, namely microwaves, applied on the extraction chamber of a modified Soxhlet apparatus, and electrical heating applied on the distillation flask.

5.2 Applications of MAE to herbs and spices

In the last years, MAE has been increasingly employed for the extraction of bioactive compounds in herbs and species due to the quality of the final extracts (Heng, Tan, Yong & Ong, 2013). The most interesting bioactive compounds susceptible to be extracted with MAE are phenolic compounds, essential oils, alkaloids and saponins.

Phenolic compounds

The recovery of phenolic compounds by MAE is being widely studied nowadays, due to the high extraction yields that this technique is able to provide. Indeed, yields obtained by MAE usually are as high as those achieved using conventional processes, but with shorter extraction times (Flórez, Conde & Domínguez, 2015).

The main objective in MAE extraction of phenolic compounds is to obtain high extraction yields without affecting the stability of bioactive compounds. Thus, it is important to optimize all the parameters affecting the extraction process, that is, extraction solvent, extraction temperature, microwave power, irradiation time, liquid to

688 solid ratio, extract cycle and, in some cases, extraction pressure (Fang, Wang, Hao, Li
689 & Guo 2015).
690 As in all extraction processes, extraction of target compounds is largely affected by the
691 type of solvent; methanol, ethanol and their mixtures with water in different proportion
692 have been described as optimal solvents for MAE recovery of phenolic compounds
693 from different herbs. Besides, the material to solvent ratio is another parameter to study
694 in order to obtain a complete extraction of the target compounds while employing the
695 minimum quantity of solvent possible (Liu et al., 2012). This ratio may vary between 5
696 mL g^{-1} and 30 mL g^{-1} for the recovery of phenolic compounds of different herbs.
697 For the extraction of phenolic compounds such as flavonols, flavones, flavanones or
698 phenolic acids, usually moderate temperatures (between 50 and 80 ºC) are employed to
699 preserve the stability of the bioactive (Fang et al., 2015; Ruan, Xiao & Li, 2014;
700 Bekdeşer, Durusoy, Özyürek, Güçlü & Apak., 2014; Liu et al., 2012).
701 In terms of extraction time, as a general rule, recoveries increase with the increase of the
702 extraction time. But exposing the phenolic compounds to the microwaves and the heat
703 for long times can degrade the bioactive compounds, and therefore, most of the
704 applications of MAE for the extraction of phenolic compounds use extraction times
705 between 2 and 25 min. These extraction times are, by far, much shorter than the times
706 required in the conventional extraction techniques.
707 Another important factor in MAE is the microwave power. By increasing power it is
708 possible to enhance the yield, however, as occurs with the temperature, high power
709 could give rise to a degradation of some compounds; for this reason, relatively low
710 powers (180-600 W) are used to achieve an herbal extract with intact phenolic
711 compounds (Liu et al., 2012).
712
713 **Essential oils**
714 MAE is considered one of the best options for the extraction of herbal plants essential
715 oils due to the high extraction efficiency and the good quality of the obtained essential
716 oil (Costa, Gariepy, Rocha & Raghavan, 2014). In fact, essential oil's extraction is the
717 widest MAE application for herbs and spices.
718 In this particular application it is worth mentioning the technique known as solvent-free
719 microwave extraction (SFME). SFME consists on the extraction of the compounds of
720 interest from a fresh sample locked into a microwave reactor without the addition of any
721 solvent or water. This technique decreases the hydrolysis, transesterification and

oxidation reactions during the extraction. Therefore, the oxygenated compounds, responsible of the odoriferous quality of the essential oils, are not affected by the process and they appear on the extract in a higher quantity, giving rise to an extract with better quality and properties (Orio et al., 2012).

Filly et al. (2014), conducted the scaling up of a SFME method, from laboratory to pilot scale, for the extraction of essential oils from rosemary. Optimum conditions selected were: irradiation power of 3 kW, 30 min at 100 ºC, 75 L pilot microwave reactor. The results revealed extraction yields and quantity of oxygenated compounds (0.50 and 36.2%, respectively) comparable to laboratory scale experiments (0.54 % and 32%, respectively). Furthermore, the reduction of costs compared to a conventional hydrodistillation (HD) was proved, since the expended energy and time were largely reduced (4.5kW h per gram of essential oil and 120 min for HD and 0.25 kW h per gram of essential oil and 30 min for SFME). These data may open good perspectives for the application of this method at industrial scale.

Microwave assisted hydrodistillation technique (MAHD) is another technique derived from MAE; it is carried out employing water as exclusive solvent for the extraction. This technique has been widely employed for the extraction of essential oils from herbs and spices such as *Bunium persicum* Boiss (Mazidi, Rezaei, Golmakani, Sharifan & Rezazadeh, 2012), *Pimenta dioica* (Jiang, Feng, Li & Wang, 2013), *Mentha piperita L* (Gavahian, Farahnaky, Farhoosh, Javidnia & Shahidi, 2015), *Coriandrum sativum L.* (Sourmaghi et al., 2015), *Curcuma longa L.* and *Carum carvi* (Akloul, Benkaci-Ali & Eppe, 2014), or several Lamiaceae family herbs (*Origanum majorana L., Mentha pulegium L.* and *Melissa officinalis L*) (Petrakis et al., 2014).

Alkaloids

Compared to conventional methods for alkaloids' extraction (heat reflux extraction (HRE), infusion extraction (IE) or ultrasound assisted extraction (UAE)), MAE has demonstrated important advantages in terms of yield and extraction time. Liang et al., (Liang et al, 2014) evaluated all the parameters that affect the extraction of alkaloids from *Portulaca oleracea* by MAE, concluding that temperature was the main factor influencing the process. In general, the recovery of alkaloids improves with an increase of temperature (Zhou, Liu, Wang, & Di, 2012).

Due to the basic nature of alkaloids, in some cases, the solvent selection includes the addition of acidified solutions to the extracting solvent in order to enhance their

selective recovery (Jiang et al., 2009). For example, Zhou et al., (2012) tested the addition of different proportions of hydrochloric acid in a mixture of methanol/water (90:10, *v/v*) for the extraction of alkaloids from *Chelidonium majus L.* The addition of the optimized quantity of acid (0.5 % HCl) provided an enhanced extraction of the target compounds.

MAE extraction of alkaloids from herbs and species not only has been focused on bioactive alkaloids (Liang et al., 2014; Zhou et al., 2012), but also on toxic alkaloids like yucanotine and related aconite alkaloids (Song et al., 2012).

Saponins

Different studies have been conducted on the extraction of saponins by using MAE. Examples are the MAE extraction of steroids saponins from the medical herb *Paris polyphylla*, which optimal conditions were: an irradiation power of 300 W, liquid to solid ratio of 20 mL/g using a mixture of ethanol/water, 70:30 (v/v), 50ºC and 15 min (Xiao, Yuan, & Li., 2014). MAE conditions for the extraction of triterpene saponins from *Pulsatilla turczaninovii* were 500 W of microwave power at 80ºC during 3 min, employing the same extraction solvent and the same solid to liquid ratio. As can be seen, main differences rely on the extraction temperature and time. Although the extraction of saponins is enhanced at high temperatures, however, the percentage of saponins extracted starts to decrease when the high temperature is maintained for a long time, due to thermal degradation of the compounds. In the above mentioned applications, the condition employed are different but compatible with the thermal stability of saponins since one of them employs a moderate temperature (50ºC) during a longer time (15 min) while the other carries out the extraction at high temperature (80ºC) but in a short time (3 min). Both strategies protect the bioactive compounds allowing their efficient extraction.

Nevertheless, the temperature to be employed during the extraction not only depends on the thermal stability of the compounds, but also on the boiling point of the solvent. In this sense, vacuum microwave assisted extraction (VMAE) can decrease the extraction system pressure, and therefore the boiling temperature of the extraction solvent can be lower, thus allowing the extraction process to be performed at more adequate temperatures. As an example, Hiranvarachat, Devahastin & Soponronnarit (2015) (studied the extraction kinetic of the saponins from the herbaceous plant *Centella asiatica* comparing MAE and VMAE extractions. The solvent selected was ethanol.

VMAE carried out at 60 kPa obtained higher quantity of saponins thanks to the effect of the vacuum pressure, which reduce the boiling point of ethanol from 78º C, at atmospheric pressure, to 65 ºC at 60 kPa. However, lower pressures (40 and 20 kPa) showed lower extraction efficiencies related to a lower diffusivity of the saponins with the decrease of the solvent boiling point. A schematic diagram of the system employed in this study is shown in Figure 1.

6. Enzyme Assisted Extraction (EAE)

6.1.Description of the technique

In spite of the ever increasing demand for new environmentally friendly advanced extraction methods, due to the particular nature of plant materials, the use of enzymes to assist the extraction of bioactives is gaining attention. Plant cells possess a strong cell wall that is usually structured in a three dimensional matrix formed by polysaccharides and glycoproteins. Among the variety of cell wall constituents, cellulose, hemicellulose, pectin and lignin are the most common groups. These structures provide a physical barrier that hinders or difficult the extraction of the linked components. For this reason, the use of enzymes to effectively weaken cell wall structures is rather useful also in combination with other extraction techniques, as it will be described below.

Enzymes catalyze hydrolysis reactions of the cell wall components with a huge degree of specificity, regioselectivity and usually under mild reaction conditions, which further favors the recovery of bioactive compounds. Thus, depending on the material to be extracted, cellulases, hemicellulases, pectinases or mixtures thereof are employed. As a result, the cell walls are effectively weakened and their porosity is increased, thus facilitating the extraction of the target components.

Consequently, in order to develop effective EAE processes, the first information that should be obtained is the composition of the cell walls of the plant material to be extracted. This knowledge will allow the precise selection of the most appropriate enzymes. Once the enzyme, or enzyme cocktail, is selected, different parameters should be considered including enzyme charge, reaction temperature, reaction time, pH of the reaction medium as well as other considerations related to the physical state of the sample. In general, a reduced particle size will help to increase the efficiency of the enzyme treatment, increasing surface contact. Regarding the rest of parameters, these will be strongly related to the enzyme or enzymes to be used, as each enzyme possesses

usually narrow working ranges in terms of temperature and pH. Thus, if a mixture of enzymes is to be used, compromise values should be selected in order to have all the reactions working. Reaction time is also an important parameter; since not all the enzymes possess fast hydrolysis rates, long reaction times are common. In fact, a 2 h treatment may be perfectly regarded as quite short, which is normally lengthy compared to other extraction techniques.

Once the reaction is finished, the solid matter is separated from the solution by centrifugation. Other processes may require enzyme inactivation, generally by increasing the temperature. Although there are applications on the use of enzymes alone for the extraction of bioactives, this technique is more convenient if coupled to other advanced extraction techniques, such as PLE or SFE, as the damage caused to the cells will definitely help increasing the penetration of the solvents into the matrix, thus enhancing the mass transfer rate and favoring the recovery of the target compounds.

6.2 Applications of EAE to herbs and spices

EAE has been mainly applied to herbs and spices to obtain oleoresins, essential oils, spice emulsion, spice essence, encapsulated spices, powders, etc., due to the high yield and quality of the products obtained (Chandran, Amma, Menon, Purushothaman & Nisha, 2012).

Although, as mentioned in this book chapter, many different extraction processes are currently used for essential oils and oleoresins recovery from spices, recently the application of enzymes during the extraction process has been gaining attention due to its advantages in terms of yield and greenness. As mentioned, enzymes have to be selected based on their action towards the cell wall components and the chemical composition of the spice material (Chandran et al., 2012). Considering the composition of the primary cell wall of plants (Jordan, 2012), cellulases, hemicelullases and pectinases have been commonly used (Miron, Herrero, & Ibáñez, 2013; Azmir et al., 2013). Besides of the type of enzyme selected, other factors such as pH, reaction time, spice/enzyme ratio and temperature should be adjusted to find the optimal reaction conditions (Sowbhagya, Srinivas, Purnima & Krishnamurthy, 2011). Table 2 presents some recent applications of EAE of essential oils and oleoresins from herbs and spices. Chandran et al. (2012) reported the use of a mixture of cellulase, β-glucananse, pectinase and xylamase to increase the yield of essential oil from 0.9% to 1.8% in black pepper and 1.9% to 2.5% in cardamom (Chandran, Amma, Menon, Purushothaman, &

857 Nisha, 2012). Also, application of enzymes during the extraction of ginger has been
858 reported to increase the yield of essential oil and gingerol recovery in 5% and 90%,
859 respectively (Nagendra chari, K. L., Manasa, D., Srinivas, P., & Sowbhagya., 2013).
860 Similarly, Naidu *et al*. (2012) concluded that the use of EAE is very useful to obtain
861 higher yield of vanilla extract and superior quality of vanilla flavor, avoiding the
862 traditional laborious and time-consuming curing process (Naidu et al., 2012). Mixtures
863 of enzymes have been also employed to enhance the extraction yield and biological
864 activities of essential oils; for instance, *Thymus capitatus* and *Rosmarinus officinalis*
865 leaves were submitted of an enzymatic pre-treatment to isolate the essential oils by
866 hydrodistillation. Results showed that the combination of cellulase and hemicellulase
867 promote 109% and 20% increase in the essential oil yields, for *T. capitatus* and *R.*
868 *officinalis*, respectively. The combination of both enzymes also induced an increase of
869 38% in the amount of carvacrol, the main component in *T. capitatus.* In terms of
870 associated bioactivities, essential oils obtained from hemicellulase treated samples were
871 the most effective (Hosni et al., 2013).
872

873 **Table 2**. Some remarkable recent published works dealing with the use of EAE of
874 bioactive components from herbs and spices.
875

Source	Bioactive compound of interest	Pre-treatment Enzyme	Extraction technique	Reference
Vanilla planifolia	Vanilla extract (Vanillin)	Viscozyme®, Tea leaf enzyme extract (TLEE)	S-L Extraction (Ethanol)	1
Black pepper	Essential oil	Cellulase, β-glucanase, pectinase, and xylanase	Steam distillation	2
Cardamom	Essential oil	Cellulase, β-glucanase, pectinase and xylanase	Steam distillation	2
Cumin seeds (*Cuminum cyminum*	Essential oil	Cellulase, Hemicellulase, Protease, Pectinase Viscozyme®	Steam distillation Hydrodistillation	3
Ginger (*Zingiber officinale* R.)	Oleoresin (Gingerol)	α-amylase, viscozyme, cellulase, pectinase	S-L Extraction (Ethanol, Acetone)	4
Lemon balm (*Melissa officinalis*)	Phenolic compounds	Cellulase, endo-1,4-β-xylanase, pectinase	Pressurized Liquid Extraction (PLE)	5
Turmeric	Oleoresin Curcuminoid Volatile oil	α-Amylase, Glucoamylase, Xylanase + Cellulase	S-L Extraction (Acetone)	6
Thyme (*Thymus capitatus* L.)	Essential oil	Cellulase, hemicellulose	Hydrodistillation	7
Rosemary (*Rosmarinus officinalis*L.)	Essential oil	Cellulase, hemicellulase,	Hydrodistillation	7
Bay leaves (*Laurus nobilis L.)*	Volatile oil Hydrodistilled leaf residues extracts	Cellulase, hemi-cellulase, xylanase	Hydrodistillation S-L Extraction	8

876 S-L: Solid - Liquid
877
878
879

7. Integration of different extraction techniques

As mentioned in the introduction, nowadays the idea of obtaining valuable products of high quality and bioactivity while improving the efficiency of the process and reducing the operation costs and the impacts on human health and environment is of outmost importance. Although the different extraction techniques that have been discussed throughout this chapter can be considered by themselves a good approach, the possibility of integrating some of them in the same process can be even more effective to achieve the mentioned goals of effectiveness, greenness and sustainability. Thus, by using this approach, purification of valuable components from different raw materials and sequential extraction of different compounds from the same sample can be achieved (Boyadzhiev, Dimitrov & Metcheva, 2006).

Below, some of the most important integrated novel extraction processes developed since 2012 for the extraction of compounds of interest from herbs and spices are presented.

Ultrasound-microwave assisted extraction (UMAE)

Ultrasound-microwave assisted extraction (UMAE), also named ultrasonic-microwave synergistic extraction (UMSE), is a new technique that combines ultrasounds and microwaves and, thus, the mechanisms of action of both techniques: the vibration-cavitation of USE and the high energy of MAE (Zeng et al., 2015). As a result, an enhancement on the mass transfer mechanism is observed, caused by a high momentum and energy that induce the rupture of the plant cell and a faster release of the compounds of interest from the matrix to the extraction solvent (Chan, Yusoff, Ngoh & Kung, 2011). This combined technique has been described as a powerful and efficient technique achieving high yields and purities with short extraction times and low solvent consumption (Lianfu & Zelong, 2008; Chen et al., 2010). Consequently, UMAE accelerates the extraction procedure, and besides, improves the selectivity and simplifies the extraction operation (Xiao, Si, Tong & Li, 2012).

Since 2012, UMAE methodology has been successfully applied to the extraction of bioactive compounds from herbs; for example, Xiao et al. (2012) developed a new UMAE method for the extraction of alkaloids from *Picrasma quassioides*. The study and optimization of all the parameters affecting extraction yield during UMAE extraction (extraction temperature and time, liquid/solid ratio and ultrasonic power) was carried out. They concluded that the correct selection of the solvent is crucial to obtain

914 an optimal extraction process. Besides, the solvent/solid ratio and the extraction time
915 had an important effect on the extraction yield. Optimal conditions in terms of process
916 efficiency were as follows: water/methanol (90:10, *v/v*) as extracting solvent, 21 mL/g
917 of liquid/solid ratio, 13 min extraction time.
918 Ionic liquids have been also applied in UMAE technology under the name of ionic
919 liquid-based ultrasonic/microwave-assisted extraction (IL-UMAE) for the efficient
920 extraction of bioactive compounds in herbs. Particularly, IL-UMAE has been
921 successfully used for the extraction of steroid saponins from *Dioscorea zingiberensis C.*
922 *H. Wright* (Wang et al., 2014). When ILs are involved on the extraction, their
923 characteristics should be also optimized; that is, type of IL, length of alkyl chains and IL
924 concentration. In this work, several ILs were tested according to the characteristics of
925 the target compounds. The length of the alkyl chain has influence on the extraction
926 since the increase of the carbon chain decreases the extraction rate, whereas the
927 extraction yield of saponins increase with the concentration of IL. However, at certain
928 point, further increasing of the IL concentration lead to a slight decreasing tendency on
929 the yield, due, probably, to the fact that a higher concentration could be translated in a
930 higher viscosity, and therefore the penetration ability of the solution in the matrix is
931 reduced. Finally, the optimum parameters were set at the use of 1-ethyl-3-
932 methylimidazolium tetrafluoroborate ([EMIm]BF4) solution at concentration of 0.5
933 mol/L, with a solvent to material ratio of 15 mL g^{-1}, 500 W of microwave irradiation
934 and 8 min of extraction time.
935
936 **Enzyme-based ultrasound-microwave-assisted extraction (EUMAE)**
937 The combination of EAE and UMAE as an integrated extraction process is still not very
938 common; however, some pioneer works have been published very recently dealing with
939 the use of this integrated process for the extraction of, for example, the phenolic
940 compound oricinol glucoside from the rhizomes of the herb *Curculigo orchioides*
941 *Gaertn* (Liu, Hu, & Wei, 2014). The first step of this process consisted on the
942 incubation of the powdered sample with the enzyme solution. Cellulase (3 mL) was the
943 enzyme selected to hydrolyze and degrade the plant cell wall; incubation was carried
944 out during 10 h at 45 ºC. For the next step, factors involved in UMAE extraction
945 process were optimized; the process selected was as follows: the residue obtained after
946 enzyme incubation was redissolved in a mixture of ethanol/water (70:30, *v/v*) with a

solvent to material ratio of 8 mL/g. The extraction was carried out at 50ºC for 15 min at 400 W of irradiation.

Extraction time and temperature were the most influencing factors on the extraction yield, being the time the most important parameter; for instance, a small change on the extraction time provided significant changes on the orcinol glucoside extraction. Compared to other new and classical extraction methods, such as hot water extraction, Soxhlet, MAE, USE or even UMAE, EUMAE under the optimum conditions, a better yield of the target compounds was achieved.

Supercritical fluid extraction-pressurized fluid extraction (SFE-PLE)

Considering that SFE and PLE are two of the most important novel extraction techniques for bioactives isolation; their combination in a sequential or in-situ extractions configuration has an interesting future applicability.

Osorio-Tobón, Carvalho, Rostagno, Petenate & Meireles (2014) developed a home-built equipment that could be employed for both, SFE and PLE (Figure 2). They used this equipment for the sequential extraction of curcuminoids from the rhizomes of *Curcuma longa L.* The integration of both processes consisted on a first deflavoration of the rhizomes with SFE and, in the same vessel, the extraction of the residue by PLE. To optimize the PLE process, parameters tested were: temperature (between 60-80ºC) and pressure (between 10-35 MPa). Results showed that although, as expected, temperature had the main impact on the process, the combined effect temperature-pressure was also important. Authors described that between 60 to 70ºC, the increase of pressure caused a negative effect on the curcuminoid extraction. This fact could be due to a decrease of the active surface of the raw material at higher pressure that could lead to a smaller surface contact between solvent and the target compounds by the effect of the compacted material. Nevertheless, when the temperature raised to 80ºC, the effect of the temperature and pressure on the yield was complex and variable, and a final increase on the extraction yield was observed due to the high mass transfer rate induced by the high temperature. Finally, the operation conditions were set at 60ºC and 10 MPa, allowing to reduce the extraction time 2 or 2.5 times compared to conventional extractions. Besides, the economic study revealed that this SFE-PLE methodology is an attractive and economically feasible process.

A different use of the PLE-SFE combination was carried out by Golmakani, Mohammadi, Sani & Kamali (2014). In this application a PLE extraction of the

antioxidant compounds of *Scutellaria pinnatifida* followed by a SFE clean-up was developed. For this purpose, authors used a modified SFE apparatus to develop a PLE process; the introduction of a switching valve after the pump allowed changing the solvent alternatively in the different extraction steps (Figure 3). Hence, the implemented process started with the PLE extraction with methanol followed by a rinse of the PLE system (residual solvent, cell, tubing and back pressure regulator) with supercritical CO2 at the end of each extraction. Conditions of temperature, pressure, static and dynamic extraction time and flow rate of this combined process were optimized in order to obtain the maximum extraction yield, total phenolic, total flavonoid and antioxidant activity.

Supercritical fluid extraction assisted by ultrasound (SFE- UAE)

In order to evaluate the benefits of this integrated process, the extraction of capsaicinoids and phenolic compounds from *Capsicum frutescens L.* was tested (Santos, Aguiar, Barbero, Rezende & Martínez, 2015). The extraction of capasaicinoids and phenolic compounds was carried out combining both processes on an ultrasound-assisted supercritical fluid extraction unit (Figure 4).

In this work, authors studied the global yield and the SFE kinetic of the extraction in order to compare the use of SFE-UAE with a SFE extraction without ultrasound irradiation. Two parameters affect the global yield, that is, the US power and the application time of US. Results showed that at short times (60 min) and low US power (200 W) there are not statistically significant differences on the extraction yield between both techniques. However, a positive effect on the extraction yield of the SFE-UAE was achieved when higher power was applied (360 W). Accordingly, the irradiation time and the ultrasound power used in both processes were statistically significant for the extraction yield. Once optimized, parameters for the SFE-UAE were set at 360 W and 60 min.

Regarding the kinetics, authors demonstrated that the application of ultrasounds to the SFE had a positive effect since at the end of the process the extraction yield obtained by SFE-UAE was 35% higher than the yield achieved by SFE alone. This increase was related to the application of ultrasound irradiations that promote the rupture of the cells, and therefore the accessibility of the solvent to the inner structures of the matrix, besides of the acoustic streaming and the mechanic vibration produced by the ultrasonic

irradiations. Consequently, the extraction rates and the efficiency of the extraction were greatly improved. At this point, it is worth to mention that the main phenomenon involved on the UAE, cavitation, cannot be considered when ultrasounds are applied together with SFE because the formation of bubbles is not possible in absence of phase boundaries.

Besides of the applications in which the SFE and UAE extraction take place at the same time, some works integrated both techniques sequentially. This is the case of the fragmentation extraction of non-polar and polar compounds from *Coriandrum sativum* L. seeds (Zekovíc et al., 2015). The objective of this strategy was to carry out three sequential extractions consisting on a first SFE extraction step for obtaining essential oils, followed by an UAE with a mixture ethanol/water (70:30, *v/v*) for the recovery of moderately polar polyphenols, and ending with another UAE with water for the extraction of the more polar fraction. Conditions selected for the essential oil extraction by SFE were the following: 100 bar of pressure at 100ºC with a CO_2 flow rate of 0.194 Kg/h, for 4h, setting 15 bar and 23ºC at the separator. For the UAE the selected conditions were: a solvent to sample ratio of 10 mL/g, a frequency of 40 KHz and 140 W of ultrasound power carried out at 30ºC. Furthermore, the particle size of the sample had a huge influence on the extraction yield. It was demonstrated that smaller particle size give rise to an increment on the extraction yield of essential oils on the SFE extraction, and to a linear increment of total phenol content and total flavonoid content during UAE.

8. Conclusions and Future Outlook

In the present chapter we have tried to demonstrate the usefulness of novel extraction techniques for extracting valuable compounds, with important biological activities, from different herbs and spices. Advantages of these new technologies (PLE, SFE, UAE, MAE, EAE and combined processes) have been underlined mainly focused on the green chemistry perspective, as sustainable and viable procedures to substitute more conventional extraction techniques. For all of them, description of the technology, main parameters involved in the process and the latest applications in the field of bioactives (mainly centered in phenolics, essential oils and alkaloids) have been presented and discussed.

Although all these novel processes have been recognized as advantageous from an environmental point of view, sustainability and eco-friendliness of a particular process

is a goal that has to be approached through the application of analysis tools, such as life-cycle analysis (LCA). Real measures of the environmental impact and costs associated to these technologies would allow the selection of the best processes to be implemented at large scale.

Other interesting fields of research that can help improve even further the above mentioned processes have been suggested in this chapter. The use of new solvents, such as ILs, and integrated processes open the door to the development of more efficient, cost-effective, clean and sustainable processes. In this sense, combination of ILs with supercritical fluids can allow to integrate reaction and extraction processes into the same system; that is, carrying out a reaction in the most favorable phase (the ionic liquid) while the reaction products can later be extracted into the supercritical phase. This is just an example, as many others suggested in this chapter, of the possibilities offered by the combination of novel processes using green solvents (supercritical carbon dioxide, water, ethanol, ILs, GXLs, etc.) in which multiple operation conditions can be optimized and performed in the same platform thus minimizing production costs. Operations that can be integrated include raw material pre-treatment, reactions (biocatalysis, transesterification), extraction, biofuel conversion, and, in global, biorefinery of raw materials in their main and valuable constituents. To do so, combination of processes including use of enzymes, disruption methods such as US and intensification methods like US applied to SFE, can provide new solutions for the sustainable production of, for instance, bioactives from herbs and spices.

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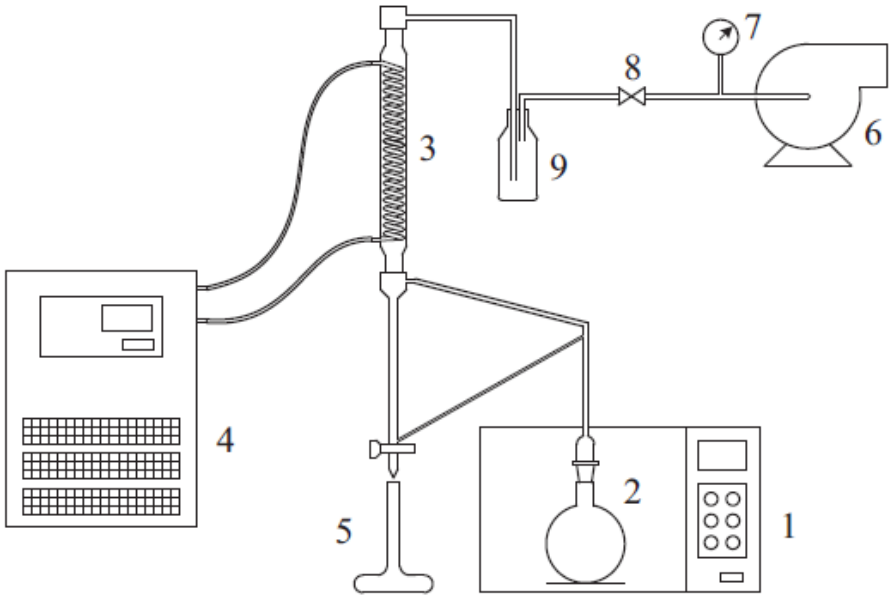
Figure Legends

Figure 1. Schematic diagram of the vacuum microwave assisted extraction (VMAE) and associated units. 1: domestic microwave oven; 2: round-bottom flask; 3: external condenser; 4: cooling water system; 5: graduated cylinder; 6: vacuum pump; 7: pressure gauge; 8: pressure-control valve; 9: vapour trap. Reprinted from Hiranvarachat et al., 2015, with permission from John Wiley and Sons.

Figure 2. Schematic diagram of the home-built equipment designed to perform SFE PLE to deflavor and recover curcuminoids from turmeric. R1: CO_2 reservoir; R2: extracting solvent reservoir; P-1: CO_2 pump; C: compressor; BC: HPLC pump; B-1: thermostatic bath; B-2: heating bath; LE: extraction cell; TC: temperature controllers; FC: collector flask; M: manometers; RT: glass float rotameter; TV: flow totalizer; V: blocking valves; MV: micrometric valve with a heating system; BP: back pressure regulator. Reprinted from Osorio-Tobón, et al. 2014, with permission from Elsevier.

Figure 3. Schematic diagram of PLE system: 1: CO_2 tank; 2: molecular sieve filter; 3: ss 2 μm pore size filter; 4: carbon dioxide transfer pump; 5, 8, 13: two-way needle valves; 6: water vessel; 7: high-pressure piston pump; 9: three ways valve; 10: preheating coil; 11: extraction cell; 12: thermostated oven; 14: back-pressure regulator; 15: sample collection vessel. Reprinted from Golmakani et al., 2014, with permission from Elsevier.

Figure 4. Diagram of the SFE + US unit: V-1, V-2, V-3, V-4 and V-5: control valves; V-6: micrometer valve; SV: safety valve; C: compressor; F: compressed air filter; CF: CO_2 filter; B1: cooling bath; P: pump; B2: heating bath; I-1 and I-2: pressure indicators; I-3: temperature indicator; IC-1, IC-2 and IC-3: indicators and controllers of ultrasound power, temperature of extraction column and temperature of micrometer valve, respectively; U: ultrasound probe; R: flow totalizer; F: flow meter; EC: extraction column and internal configuration of the extraction bed of 295 mL for SFE + US used in the kinetic experiments. Reprinted from Santos et al., 2015, with permission from Elsevier.



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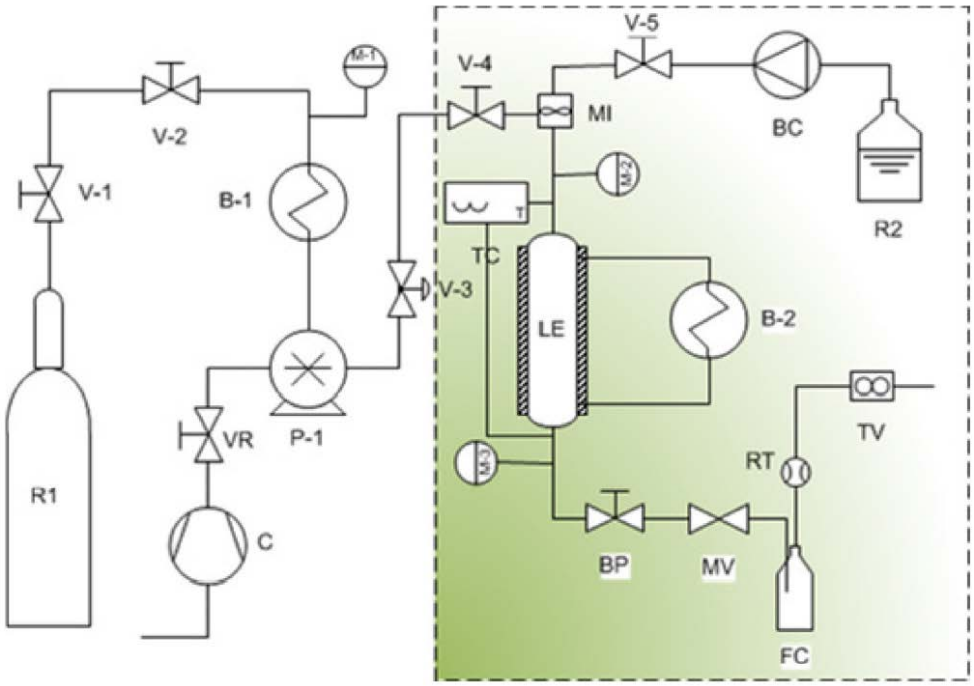

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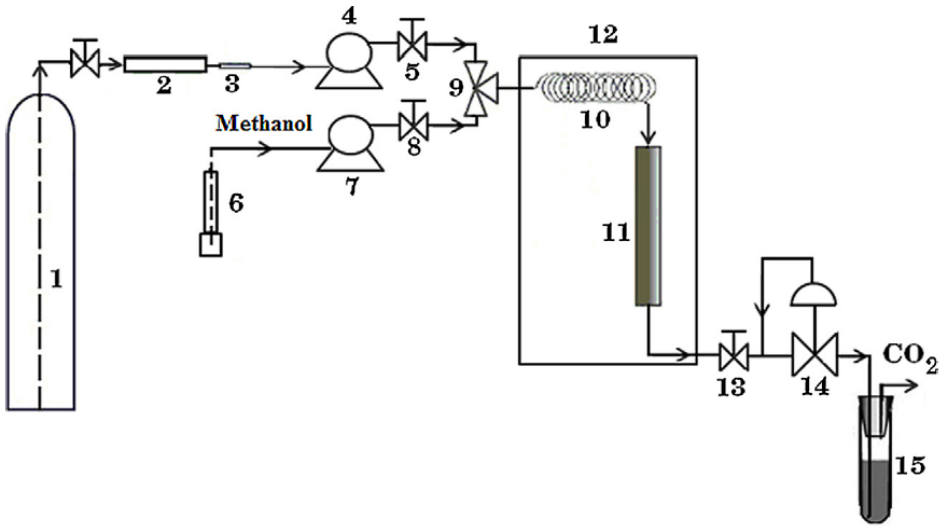

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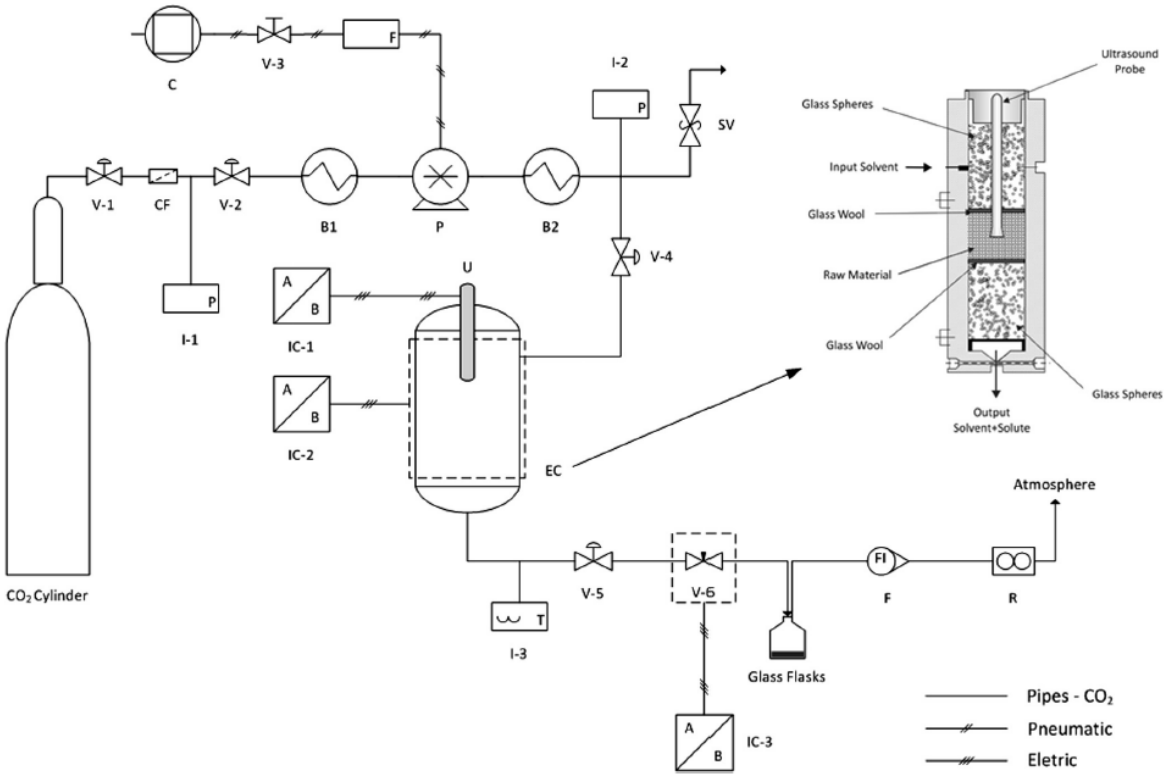
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P
I-1
CO2 Cylinder
EC
Ultrasound Probe
Glass Spheres
Input Solvent
Glass Wool
Raw Material
Glass Wool
Glass Spheres
Output Solvent+Solute
Atmosphere
V-5
V-6
FI
F
R
T
I-3
Glass Flasks
IC-3
Pipes - CO2
Pneumatic
Eletric

Figure 4.

Bioactives Obtained From Plants, Seaweeds, Microalgae and Food By-Products Using Pressurized Liquid Extraction and Supercritical Fluid Extraction

Andrea del Pilar Sánchez-Camargo, Elena Ibáñez, Alejandro Cifuentes and Miguel Herrero[1]

Institute of Food Science Research (CIAL-CSIC), Madrid, Spain

[1]*Corresponding author: E mail: m.herrero@csic.es*

Chapter Outline

1. INTRODUCTION

The extraction of bioactive compounds from natural sources is a hot research topic for the food and related industries. This is probably due to the development of the functional foods sector. A functional food is normally defined

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as a product that is able to provide with an additional physiological benefit besides the energy and nutritional requirements that every food must offer [1]. Usually, this additional health benefit is gained through the addition of a functional ingredient, which should be the responsible of the claimed bioactivity. There are a lot of examples of functional ingredients already in use in commercial products, such as polyunsaturated fatty acids [2], plant sterols and stanols [3], peptides [4] or polyphenols [5], to name a few. From a consumer perspective, these functional ingredients added to foods are clearly preferred to have a natural origin. These reasons have led to the extensive search for new natural sources of bioactive compounds that is being experimented in the last years [6,7]. In this regard, this book chapter deals with some of the most important natural sources for these components, plants, microalgae and seaweeds and agro-food by-products. At this point, the technologies applied to obtain a particular component or group of components from a natural source gain importance. Traditional methods generally imply the use of high volumes of organic, and often toxic, solvents. However, today's focus is completely different in this regard due to the fact that these methods have a significant impact on the environment [8]. As a result of increasing ecological conscience and sustainable development, conventional aggressive techniques are being replaced by more environmentally friendly processes. The European Union, as well as other international organizations, is fostering actions to stimulate the transition towards circular economy, in which exhaustive recycling and reuse in every step of the production chain is sought [9]. This is also closely related with the principles of green chemistry which are mainly aimed to reduce wastes and to a more efficient use of energy and resources [10]. Derived from these, six specific principles for the green extraction of natural products were pointed out [11], including innovation by selection of varieties and use of renewable plant resources; use of alternative solvents and principally water or agro-solvents; reduce energy consumption by energy recovery and using innovative technologies; production of coproducts instead of waste to include the bio- and agro-refining industry; reduce unit operations and favour safe, robust and controlled processes; and aim for a nondenatured and biodegradable extract without contaminants. The direct application of these principles leads to the development of biorefinery approaches. Biorefinery implies the development of integrated processes for the conversion of biomass into energy and a variety of products, mainly biofuels and added-value coproducts, in a sustainable approach [12]. As it can be deduced, some of the aforementioned principles are associated to the natural source of choice, but others are closely related with the extraction technique employed. To comply with those requirements, efficient, cheap and safe extraction techniques are needed. Among the advanced extraction methods that are nowadays utilized, compressed fluids-based extraction procedures are the most widely employed.

2. COMPRESSED FLUIDS-BASED EXTRACTION PROCESSES

There are a number of techniques based on the use of compressed fluids that have in common a higher efficiency compared to conventional atmospheric pressure processes, as well as involving operational conditions allowing the solvents acquiring physical–chemical properties not attainable otherwise. Moreover, under certain conditions, these techniques comply with the green chemistry principles. Pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) are the two most employed techniques based on the use of compressed fluids to obtain bioactive compounds from natural sources.

2.1 Pressurized Liquid Extraction: Principles and Operation

PLE involves the extraction using solvents at high temperatures and pressures high enough to maintain the solvent in liquid state. In any case, these values should be below their respective critical temperature and pressure. This technique is also referred in the literature as accelerated solvent extraction, pressurized fluid extraction or pressurized hot-solvent extraction. As a consequence of the application of those particular extraction conditions, the resulting extraction processes are generally faster compared to conventional ones, besides requiring significantly less amounts of solvents. The change in the physicochemical properties of solvents when submitted to high temperatures and pressures means that mass transfer rates are enhanced, at the same time solubility of analytes is increased and solvent surface tension and viscosity are decreased. This allows the solvent to penetrate deeper and easier into the solid matrix being extracted. All these factors produce a significant enhancement on the extraction rates. Solvent selection is a parameter of utmost importance, not only regarding the target analytes but also to consider this technique as environmentally friendly. For this reason, the use of generally recognized as safe (GRAS) solvents, such as ethanol, ethyl lactate or even D-limonene, is clearly preferred. However, the greenest possible approach involving PLE involves the use of water as extracting solvent. Readers are referred to chapter: Pressurized Hot Water Extraction of Bioactives by Plaza and Turner [12a] of this book for an in-depth description of the use of water as extracting solvent under pressurized conditions.

Among the parameters that should be carefully considered when dealing with PLE extractions, extraction temperature is by far the most influencing. As mentioned, an increase in temperature favours the disruption of intermolecular forces (i.e., van der Waals forces, hydrogen bonds and dipole attractions) in the sample matrix. Extraction pressure is, obviously, also very important, as it should be high enough to keep the solvent in liquid state. However, beyond that point (which will depend on the specific solvent and the temperature applied), even though the pressure might exert a rupture effect on the sample being extracted, several works showed that pressure did not significantly influence the obtained results once it is high enough to

maintain the solvent in the liquid state [6,7,13,14]. For this reason, pressures between 5 and 10 MPa are typically employed. Once extraction temperature and pressure are selected, extraction time has to be considered. The effective extraction time is the period in which the solvent is in close contact with the sample matrix at the required or selected extraction conditions (i.e., selected extraction temperature and pressure). For this reason, some commercial instruments apply a preheat time to allow the system to equilibrate at the selected conditions. In general, the extraction time needed to exhaust a particular sample will depend on several parameters; one of the most critical is the type of extraction. Static extraction, defined as the process in which a certain volume of solvent, under the desired conditions of pressure and temperature, is maintained in contact with the sample for a given time, is the most common. Under those conditions, once the equilibrium between sample components still in the matrix and those already solubilized in the solvent is reached, the efficiency of the extraction procedure will not be increased beyond this point. Thus, an appropriate optimization protocol of static extraction time is needed to maximize the extraction of analytes avoiding other degradation-related processes. Generally, static extraction times between 5 and 20 min are employed for bioactive extraction from natural sources. The other operation mode involves the use of dynamic extractions; in this case, heated and pressurized solvent will be flowing into the extraction cell continuously. Although this mode could be more favourable for the complete extraction of the sample matrix as the formation of equilibrium is avoided, higher volumes of the solvent may be necessary. Moreover, in this latter case, solvent flow rate has to be studied and will directly influence the needed time. An approach more commonly described in the literature than using dynamic extraction is the use of short repetitive static extraction cycles. The application of several consecutive extraction cycles may efficiently help to exhaust the sample matrix being extracted. Other parameters that should be controlled in PLE processes include the sample particle size, as this influences the available surface contact and, thus, the mass transfer rate. The introduction of dispersants together with the sample in the extraction cell is sometimes employed to favour the uniform distribution of the solvent and to maximize the extraction yield. Diatomaceous earth and sea sand are among the most frequently employed.

The instrumental requirements needed to carry out a PLE process are, in principle, simple (Fig. 1A): (1) a pump that introduces the solvent into the system and helps to push the extract out once the process is finished. The pump should be capable of maintaining the selected pressure; (2) an extraction cell, where the extraction physically takes place. Therefore, it has to be adapted for high pressures and to be equipped with at least two on/off valves to be able to keep the extraction conditions stable; (3) an oven, where the extraction cell is placed so that it can be heated to the desired value. The maximum working temperature in most instruments is around 200°C; and lastly, (4) a collection

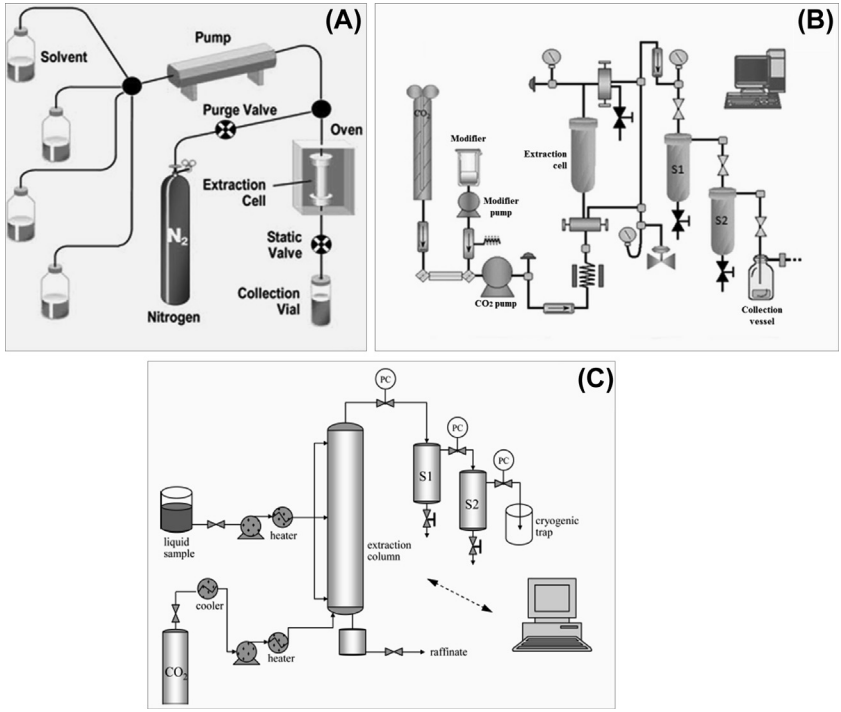

FIGURE 1 Schematic diagrams of the basic instrumentation needed to operate pressurized liquid extraction (A), supercritical fluid extraction (SFE) for solid samples (B) and SFE for liquid samples (C) systems. S1, separator 1; S2, separator 2. *(A) Adapted from Dionex Thermo Scientific. (C) Adapted with permission from L. Vázquez, T. Fornari, F. J. Señoráns, G. Reglero, C. F. Torres, Supercritical carbon dioxide fractionation of nonesterified alkoxyglycerols obtained from shark liver oil. J. Agric. Food Chem. 56 (3) (2008) 1078−1083. Copyright 2008 American Chemical Society.*

vial. Nevertheless, from this starting point, the instrumentation employed might be more or less sophisticated, depending on the process requirements. The PLE instrument may be equipped with heating coils for solvent heating in dynamic extractions. It can also have a nitrogen circuit that helps to vent all the solvent from the lines after extraction. There are a number of commercial instruments available in the market, although different applications have been also presented with laboratory-made instruments. In both cases, it has to be considered that, given the operating pressures and temperatures usually employed, corrosive-resistant materials have to be used.

2.2 Supercritical Fluid Extraction: Principles and Operation

The use of supercritical fluids for extraction involves the use of a fluid at the pressure and temperature beyond its critical point. Once beyond the critical point, the fluid acquires significantly different properties as a solvent, as a

consequence of different physical changes that are produced. Indeed, when the temperature of a solvent is increased at the same time that its pressure and the critical point is reached, a homogeneous supercritical fluid is obtained in which no distinction can be found between phases (Fig. 2). As a result, supercritical fluids possess mixed properties between those of gases and those of liquids. While their viscosity is similar to a gas, its density is close to values found for liquids. Moreover, its diffusivity is intermediate between liquids and gases. Other important properties are also modified in a supercritical fluid such as surface tension and solvent strength, which will contribute to their unique solvating properties. Besides, at supercritical conditions, density is modified, effectively changing the solvent capabilities and permitting the achievement of a high degree of selectivity for the extraction [15,16]. Although different solvents may be employed as supercritical fluids for extraction, carbon dioxide is by far the most widely used for bioactives extraction from natural sources. This is because it (1) has mild critical temperature and pressure (critical conditions = 31.2°C and 73.8 bar); (2) is considered GRAS for the food industry; (3) is cheap and easily available; (4) allows reusing CO_2 generated in other industrial processes, thus complying with some of the principles of green chemistry; and (5) is gas at room temperature. This latter property is greatly important since it allows obtaining a solvent-free extract after depressurization.

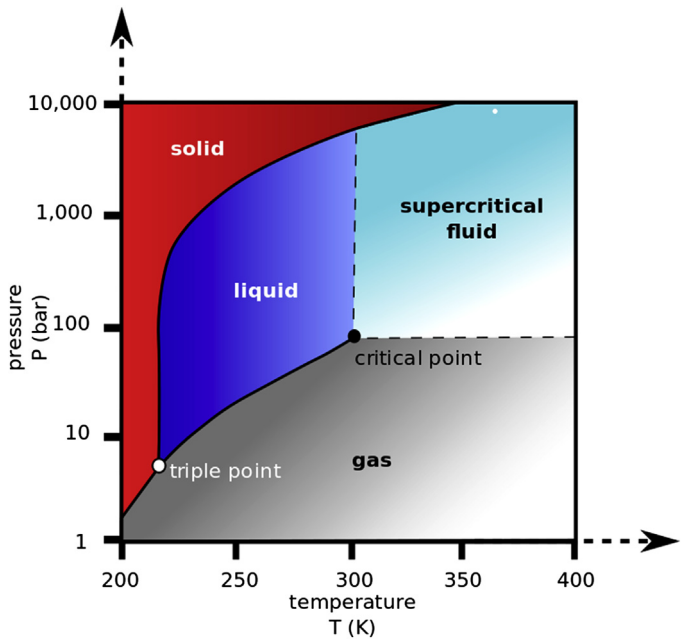

FIGURE 2 Carbon dioxide pressure–temperature phase diagram.

Among the parameters affecting the extraction by SFE, operating pressure and temperature are the most critical. These two parameters combined will determine the solubility of a substance in the supercritical fluid. The selection of the temperature and pressure to be employed in a process should be carefully made according to the aim of the process as well as the targeted compounds, considering that higher density of the supercritical fluid will be obtained through an increase in pressure and will lead to an enhanced solubility of sample components. Nevertheless, an isobaric increase in the extraction temperature will produce a decrease in the solvent density, although it will also produce an increment in the vapour pressure of the analytes present on the matrix, promoting mass transfer. In spite of the aforementioned advantages of supercritical CO_2, the main limitation on its application is its low polarity that constrains its use to the extraction of very-low-polarity components. SFE extracts obtained from natural matrices are typically mixtures of family compounds such as triglycerides, fatty acids, terpenoids, phytosterols, tocopherols, carotenoids, tocotrienols and phenolics. In Fig. 3, examples from each family are presented.

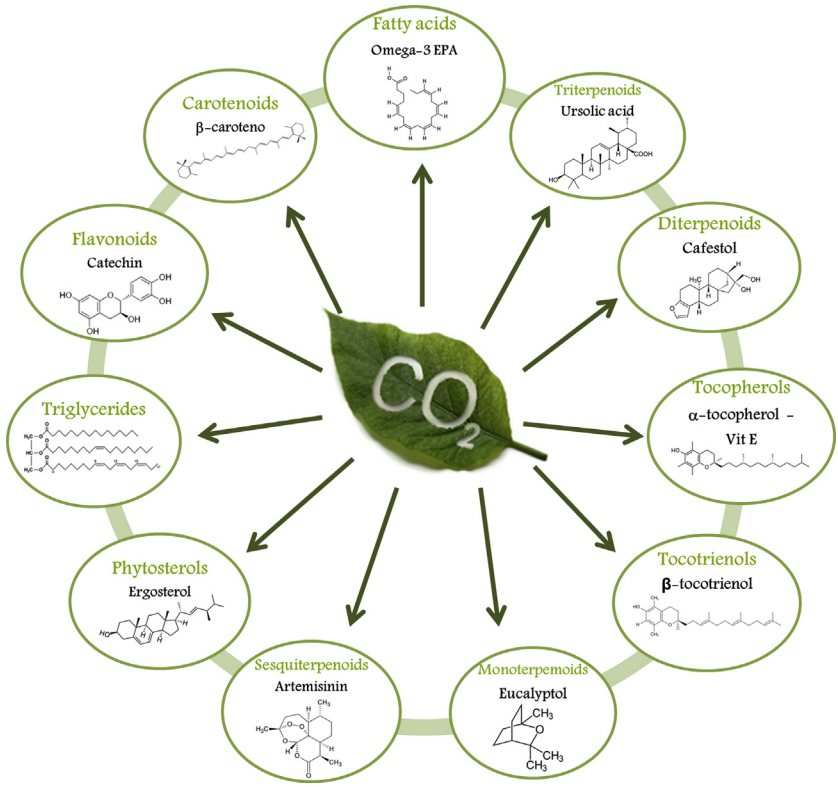

FIGURE 3 Examples of the main families of compounds found in the extracts obtained employing supercritical fluid extraction from natural sources.

To extend the range of applications of this technique, the use of modifiers is needed. A modifier is an organic solvent which is employed at a given proportion (5%−10%) together with the supercritical CO_2, resulting in an increase in overall polarity. This way, other applications may be developed involving the extraction of more polar components, which are frequent in the field of natural bioactive compounds. For this reason, the selection of the modifier as well as the proportion in which it is used should be carefully optimized to achieve an efficient extraction.

In contrast to the operating modes of PLE, SFE usually involves dynamic extraction, at least partially. Some applications are developed so that a first static period is performed for a given time, followed by a prolonged dynamic extraction phase. In those applications as well as in those purely dynamic, the supercritical fluid flow rate is an important parameter. This flow rate has to be controlled to use the lowest possible solvent but maximizing the mass transfer. This factor is even more important in the extraction of liquid materials under countercurrent conditions, as in those situations, the flow rate (or solvent-to-feed ratio) will directly define the contact time allowed between the sample and the supercritical CO_2. Other important operational factors to be considered are sample physical state (mainly particle size, porosity and moisture content) and the need to use physical dispersing agents together with the sample to avoid channelling.

Regarding the instrumentation needed, as in PLE, it should be able to withstand high pressures (as high as 50 MPa or even higher). The equipment needed is different depending on if the application deals with solid or liquid samples (see Fig. 1B and C, respectively). These differences are mainly related to the extraction cell, which will be replaced by an extraction column in a liquid samples extractor. In any case, the initial requirements are a tank for the extracting solvent, usually CO_2, a pump to pressurize the gas to the desired extraction pressure and a restrictor or valve to maintain the high pressure inside the system. An additional pump is needed when organic modifiers are to be employed. In case of solid samples, a high pressure extraction cell is needed, whereas for liquids extraction a countercurrent column is used. The countercurrent column typically possesses different levels for feed introduction (at different heights), whereas the solvent is introduced from the bottom of the column. This way, the components distribute between the solvent and the liquid sample which flows countercurrently through the separation column. Finally, a trapping vessel (or separation cells, also called fractionation cells) for the recovery of the extracts is required. Different trapping methods are employed including solid trapping, liquid trapping or cool trapping, among others. In pilot or industrial systems, collection of the extracted solutes is done by rapidly reducing the pressure, increasing the temperature or both. Particularly interesting, depressurization after the extraction can also be performed in cascade using several fractionation or separation cells, considering that each separation

vessel could have a particular temperature and pressure to have some of the extracted compounds precipitated and separated selectively.

3. EXTRACTION OF BIOACTIVES FROM NATURAL SOURCES

3.1 Extraction of Bioactives From Plants

Plants are probably the most widely studied natural sources for the extraction of bioactive compounds with potential uses in the food, nutraceutical and pharmaceutical industries. The use of both PLE and SFE has been extensively reported in a variety of applications [6,13,17]. In general, extraction conditions are variable depending on the matrix as well as on the target components. The experimental designs to statistically assess the influence of the different extraction parameters and to select the optimum extraction conditions in agreement to some response variables are very useful [18,19].

PLE has been extensively used for extracting bioactives such as flavonoids [20], anthocyanins [21], phenolic acids and other polyphenols [22], saponins [23] or essential oils [24] from a variety of plant sources. The solvent, or solvent mixture, is selected depending on the application, although ethanol, methanol, water and their mixtures are the most frequently employed. The addition of acid has been demonstrated as useful to increase the recovery of some components, such as polyphenols from black chokeberry [25]. Even though extraction temperature highly depends on the specific application, the use of high temperatures (around 150°C) is frequent, as mass transfer kinetics is improved. Moreover, when considering water as extracting solvent, very high temperatures (even higher than 200°C) have been repeatedly reported to increase bioactivity of the attained extracts, mainly antioxidant activity. However, it should be considered that not only an improvement of the extraction process could be taking place, but also reactions that could lead to the formation of new nonnative compounds that might have a positive influence on the overall antioxidant activity of the generated extract. A more in-depth discussion about the reactions promoted at high water temperature can be found in chapter: Pressurized Hot Water Extraction of Bioactives by Plaza and Turner [12a] of this book. Regarding the extraction process employed, most applications are based on the use of static extraction processes using commercial instruments. Static extraction time usually ranges from 5 to 20 min. Relatively fast extractions are performed when more than one static extraction cycle is performed.

Other interesting approaches have been based on the continuous or simultaneous PLE extraction and transformation of bioactive compounds. For instance, the possibility of extracting sinapine from rapeseed meal and its conversion to sinapic acid and canolol has been studied with the aim to efficiently obtain these latter bioactive compounds [26]. PLE using 60% methanol at 200°C for 20 min was shown to be more than 300% more

efficient than Soxhlet extraction during 10 h for the direct extraction of total phenols. However, the addition of NaOH to the extraction solvent was found to be critical for the conversion of sinapine to canolol. The best extraction−conversion conditions include the use of 100% methanol at 200°C with the addition of 1% NaOH during just 5 min [26].

On the other hand, SFE has been mainly employed for the recovery of low-polarity components from plants. Indeed, the extraction of compounds such as fatty acids, phytosterols [27], carotenoids [28], alkaloids [29] or relatively low-polarity phenolic compounds [30], essential oils [31], among others [32], have been described. As previously explained, the selection of the operating pressure and temperatures is the most influencing parameter, controlling both CO_2 density and solubility. However, given the particular nature of supercritical CO_2, process temperature normally ranges between 40 and 65°C. Pressures are more variable, ranging from 10 to 40 MPa.

Among the other influencing parameters, moisture content is an important factor, above all, when dealing with the extraction of less polar bioactive compounds from plants. For instance, significant differences were observed between pumpkin samples for the extraction of carotenoids, containing 8% and 12% of moisture [33]. Oven-dried samples (with lower residual moisture content) produced an eightfold increase in carotenoids recovery compared to freeze-dried samples containing 12%. Details and exhaustive lists of particular applications of SFE of bioactive components from plants may be retrieved from the references shown in Table 1.

Process intensification is a way to optimize systematically the energy use, capital or other benefits through the development of efficient techno-economical systems. The application of this approach is very interesting from a green chemistry perspective, as it would be useful to reduce wastes and energy consumption. The combination of SFE and PLE under process intensification approaches may have several advantages: firstly, both techniques operate at high pressure, sharing a significant amount of instrumental requirements; besides, the use of supercritical CO_2 and organic solvents, or even water, allows to selectively target different components from a given natural matrix. For instance, multipurpose equipment has been used to consecutively extract different rosemary compounds using SFE and subcritical water extraction [55]. In the first stage, typical components of rosemary essential oil, 1,8-cineole and camphor were obtained at 40°C and 30 MPa using supercritical CO_2 with a solvent-to-feed mass ratio of 2.5 g CO_2/g rosemary. Later on, temperature was raised up to 172°C at 1.1°C/min at 10 MPa using water as solvent, producing enrichment on the nonvolatile fraction, with extraction yields of 2.5% and 18.6% in each step, respectively [55]. The use and optimization of similar approaches for other plants may increase process efficiency and decrease operating costs, thus improving the extraction of bioactive compounds of different chemical nature.

TABLE 1 Summary of the Recent (2006–16) and Relevant Reviews Published Regarding the Extraction of Bioactive Compounds Using PLE and SFE From Different Natural Matrices

Year	Title	Related Process	References
2006	Supercritical CO_2 extraction and purification of compounds with antioxidant activity	SFE	[34]
	Pressurized hot water extraction of bioactive or marker compounds in botanicals and medicinal plant materials	SWE	[35]
	Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food by-products, algae and microalgae: a review	SWE, SFE	[36]
2007	Supercritical fluid extraction in plant essential and volatile oil analysis	SFE	[37]
	Use of compressed fluids for sample preparation: food applications	SFE, PLE	[38]
2008	Extraction of functional substances from agricultural products or by-products by subcritical water treatment	SWE	[39]
2009	Design and scale-up of pressurized fluid extractors for food and bio products	SFE, PLE, SWE	[40]
	Application of supercritical CO_2 in lipid extraction – a review	SFE	[41]
2010	Supercritical fluid extraction: recent advances and applications	SFE	[42]
	Pressurized hot water extraction (PHWE)	SWE	[43]
2011	Pressurized liquid extraction as a green approach in food and herbal plants extraction: a review	PLE	[44]
	Supercritical carbon dioxide extraction of molecules of interest from microalgae and seaweeds	SFE	[45]
2012	Steps of supercritical fluid extraction of natural products and their characteristic times	SFE	[46]
	Techniques to extract bioactive compounds from food by-products of plant origin	PLE, SFE	[47]
	Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed	PLE	[14]

Continued

TABLE 1 Summary of the Recent (2006–16) and Relevant Reviews Published Regarding the Extraction of Bioactive Compounds Using PLE and SFE From Different Natural Matrices—cont'd

Year	Title	Related Process	References
2013	Compressed fluids for the extraction of bioactive compounds	PLE, SFE	[7]
	Supercritical fluid extraction of plant flavors and fragrances	SFE	[48]
2014	Extraction behavior of lipids obtained from spent coffee grounds using supercritical carbon dioxide	SFE	[49]
	Recovery of biomolecules from food wastes – a review	SFE, PLE	[50]
2015	Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction	SFE, PLE	[6]
	Pressurized hot water extraction of bioactive	PLE	[13]
	Pressurized fluid systems: phytochemical production from biomass	SWE, PLE	[51]
2016	Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: a review	SFE, PLE	[8]
	Subcritical water extraction of bioactive compounds from plants and algae: applications in pharmaceutical and food ingredients	SWE	[17]
	Extraction of oil and carotenoids from pelletized microalgae using supercritical carbon dioxide	SFE	[52]
	Supercritical fluid extraction of bioactive compounds	SFE	[53]
	Application of non-conventional extraction methods: toward a sustainable and green production of valuable compounds from mushrooms	SWE, SFE	[54]

PLE, pressurized liquid extraction; *SFE*, supercritical fluid extraction; *SWE*, subcritical water extraction.

Although process intensification and process integration are quite related, the first is based on the use of the same equipment, whereas the second may be achieved by the combination of different unit operations. A typical example related to the topic of this section is the coupling of PLE extraction and fractionation aided by supercritical fluids. This approach has been already employed for different plants. Firstly, a proper optimization of the PLE process towards the extraction of the target compounds should be done. Later on, PLE extracts attained under optimum conditions are fractionated using supercritical antisolvent fractionation (SAF). By using this technique, it is possible to selectively precipitate relatively polar compounds from a PLE extract by establishing continuous contact of that extract with supercritical CO_2 at pressurized conditions. This way, the supercritical fluid dissolves the less polar compounds and the less polar solvent in the mixture, whereas the nonsoluble more polar compounds are precipitated and may be recovered separately. This approach may be used to produce the enrichment of both polar and less polar bioactive compounds; in the first case, the interesting fraction will be the precipitated components [56], whereas in the second, the interesting compounds will be concentrated in the CO_2^+ solvent fraction [22].

3.2 Extraction of Bioactives From Seaweeds and Microalgae

Seaweeds and microalgae are an underexploited source for different interesting bioactive compounds. Although some components from these organisms are being used at present in the food and pharmaceutical industries, there are still thousands of different species that have not been studied so far. Microalgae are the focus of a lot of research effort in the field of biofuels and CO_2 capture. However, the production of microalgae for food is also a commercial reality. The fact that these microorganisms may have their chemical composition modified in agreement with the growing conditions under which they are produced enhances even more their interest, as they can behave as biofactories for the production of particular bioactive compounds. On the other hand, macroalgae or seaweeds are also under study. Some species are considered invasive organisms, and thus, their valorization is of great impact.

As the use of both types of organisms is on a rise, different extraction strategies based on the use of PLE and SFE have recently been developed to obtain bioactive compounds from microalgae and seaweeds. Table 1 includes some of the review papers already published summarizing those applications. These applications include the use of SFE to extract carotenoids from micro- [52] and macroalgae [57] and poly-unsaturated fatty acids (PUFAs) [58] or the use of PLE for the extraction of bioactive carbohydrates (fucoidan) from *Saccharina japonica* [59] or phlorotannins from *Sargassum muticum*, to name some examples [19].

Taking into consideration their nature, the effect of the high water content of microalgae and seaweeds on the extraction process is extremely important.

As the removal of water of harvested algae is an expensive procedure, an interesting approach is to determine the maximum amount of water that does not hamper the extraction of the target bioactive compounds. This kind of study was carried out with *Dunaliella salina* microalgae, an important source of carotenoids. Different sample moisture contents were studied and their influence on the SFE of carotenoids using CO_2 was assessed [60]. Interestingly, authors concluded that 23% moisture content helped to extract a higher content of carotenoids, mainly β-carotene without negatively influencing the extraction process. An alternative to directly use microalgae paste harvested after growing (containing 70%−80% water) has been proposed, consisting of the use of low-cost adsorbents as support inside the extraction cell [61]. Different silica gels, chitosans and active carbon were tested; the best results were obtained using chitosan-based adsorbents allowing a high carotenoid recovery from *Neochloris oleoabundans* microalgae. Other works have combined the study of the moisture content to different cell disruption mechanisms [62]. Differently from plants, microalgae often possess strong and thick cell walls that have to be disrupted by some chemical, enzymatic or mechanical procedures before extraction to enhance the recovery of bioactive components.

Leaving aside traditional one-step extraction protocols, PLE and SFE are being studied as potential unit operations to be employed in biorefinery processes involving not only microalgae but also seaweeds. For instance, a downstream platform of process intensification has been set up for the extraction of bioactive compounds from the microalga *Isochrysis galbana* using several pressurized solvents [63]. Sequential extraction of the same initial raw material included the application of SFE with neat supercritical CO_2, followed by an intermediate extraction using CO_2-expanded ethanol followed by extractions using pressurized ethanol and water. Low−medium polarity components were recovered in the first two steps, mainly carotenoids (fucoxanthin), whereas more polar compounds were extracted by PLE. Fig. 4 shows a scheme of the overall optimized process.

3.3 Extraction of Bioactives From Food By-Products

The reduction of wastes is a topic of utmost importance for sustainability. During the different food-related and agro-food-related activities, a number of by-products are generated. Traditionally, some of these by-products could be reused for feed manufacturing or energy generation. However, today it has become obvious that some of these by-products and wastes are still rich sources of bioactive compounds. Thus, the further use and valorization of these by-products may be a good way of producing other high-added-value products at the same time that a significant reduction on wastes is obtained. Following the requirements and particularities stated earlier for each technique, different SFE and PLE approaches have been developed for the

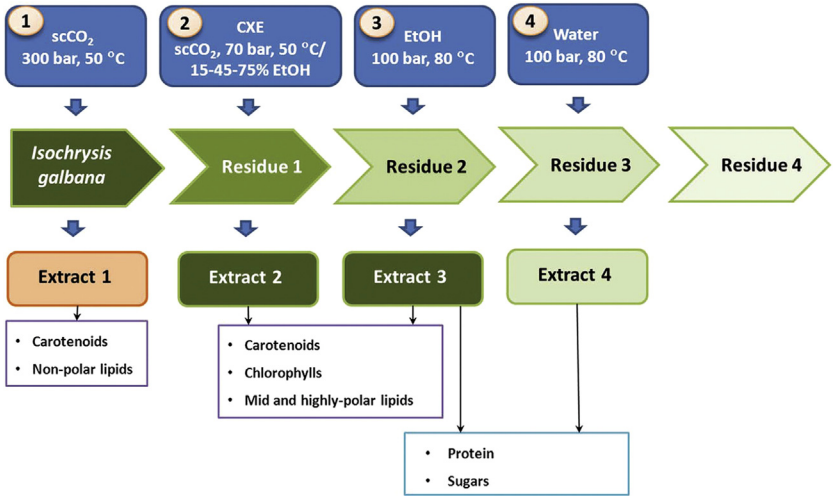

FIGURE 4 Scheme of the integrated sequential extraction process of bioactive compounds from *Isochrysis galbana*, following biorefinery approach. *Adapted with permission from B. Gilbert-López, J.A. Mendiola, J. Fontecha, L.A.M. van den Broek, L. Sijtsma, A. Cifuentes, M. Herrero, E. Ibáñez, Downstream processing of Isochrysis galbana: a step towards microalgal biorefinery, Green Chem. 17 (2015) 4599–4609, http://dx.doi.org/10.1039/C5GC01256B.*

recovery of different substances from agro-food by-products. Some of them regard to the reutilization of coffee by-products [49], winemaking-related wastes [8], olive oil [64] or fruit industries by-products [65], among others [23,66]. Readers are referred to the excellent reviews listed in Table 1 to gain more insight on particular applications and extraction conditions.

Due to the intrinsic nature of food by-products, these have been studied inside biorefinery approaches using compressed fluids-based extraction techniques, such as PLE and SFE. For instance, olive oil industry may produce four times more wastes than oil. For this reason, several approaches have been optimized to further valorize some of the generated by-products, such as olive mill waste. Several sequential SFE processes were studied based on the use of neat supercritical CO_2 and supercritical CO_2 with ethanol as modifier to produce fraction enriched on polyphenols, PUFAs, mono-unsaturated fatty acids and squalene [67]. Moreover, this approach has the additional advantage of producing an efficient drying of the remaining biomass which is also beneficial for further processing.

The combined used of PLE and SFE has been also studied for by-products extraction with the aim to recover bioactive components. SFE was selected as the first step in a sequential extraction of mango peel waste which was followed by an extraction with pressurized ethanol. Pressure and temperature were maintained during the whole sequential procedure. Nonpolar flavonoids and carotenoids present in mango peel by-product were preferentially

extracted in the supercritical CO_2 stage, whereas polar polyphenols were extracted in the second step based on PLE [68]. Interestingly, other developments have varied the order in the sequential extraction [69]; for the valorization of *Euterpe edulis* industrial residues, a PLE procedure was optimized comprising different pressures and temperatures as well as solvent mixtures. Once the best extraction conditions were selected, the solvent optimized (acidified mixture of ethanol and water) was employed as modifier in a second SFE step. By using this approach extracts enriched with anthocyanins were successfully obtained.

As for the other described natural sources, the integration of processes and/or the development of intensified processes dealing with the extraction of bioactive compounds from food by-products are/is a hot topic. Among the most promising intensified processes are those involving the coupling of extraction and drying, considering that this latter step is usually regarded as very energy demanding. One of the most notable developments in this sense is based on the coupling of PLE and supercritical antisolvent (SAS) precipitation using the same multipurpose equipment to extract flavonoids from onion wastes [70]. SAS takes advantage of the effect of a supercritical CO_2 flow which is mixed with a polar solution (PLE extract) through a valve. During mixing and after sudden depressurization, the supercritical CO_2 is quickly dissolved in the organic solution causing the precipitation of the compounds present in the polar solution thanks to the antisolvent effect. Conceptually, the application of this technique is similar to that already explained for the SAF. After optimization, the most suitable PLE conditions for the extraction involved the use of ethanol at 40°C and 12 MPa during 20 min under static conditions, whereas the pressure was reduced at 10 MPa for the dynamic extraction/precipitation step that lasted 200 min. These conditions allowed the attainment of 4% of dried microparticles from dried onion peels with high contents of quercetin. The use of this kind of approach is not only interesting from an energetic point of view, but also helps to maintain the integrity of the bioactive components since dried particles are readily produced avoiding oxidation. A scheme of the apparatus developed for this application is shown in Fig. 5.

4. CONCLUSIONS AND FUTURE OUTLOOKS

As can be deduced from the information provided in this chapter, the use of compressed fluids-based extraction techniques to obtain bioactive compounds from different natural matrices is broadly extended. There is a wealth of information regarding the extraction conditions required to obtain chemically different bioactives from a wide variety of samples. Moreover, the basic requirements for the use of PLE and SFE, including the influence of the different factors related to these processes, are well known. However, there is still a long way to go in the application of these techniques in agreement with the latest trends in this field.

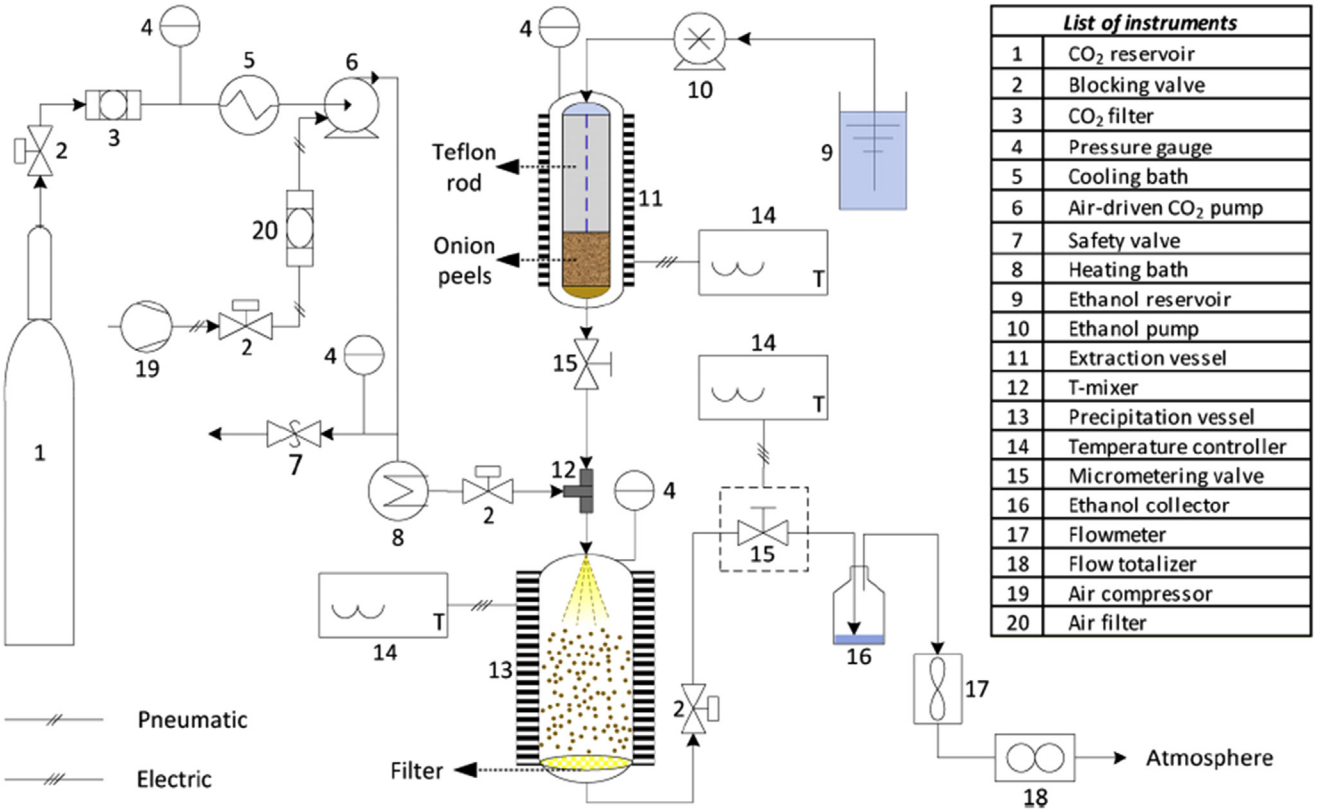

FIGURE 5 Schematic flowchart of the equipment for performing the extraction and particle formation on-line (EPFO) intensified process for onion peels extract. *Adapted with permission from G.L. Zabot, M.A.A. Meireles, On-line process for pressurized ethanol extraction of onion peels extract and particle formation using supercritical antisolvent, J. Supercrit. Fluids 110 (2016) 230–239. http://dx.doi.org/10.1016/j.supflu.2015.11.024.*

Looking at the application of these techniques alone, the development of new extraction processes will be probably related to the use and study of new solvents. Even if the displacement of supercritical CO_2 as the preferred solvent in SFE processes is not foreseen, under the PLE perspective, the picture is different. In fact, the use of new food-grade environmentally respectful solvents in PLE processes will be increased. Some applications have already presented the possibility of attaining bioactives from different natural sources using novel solvents such as ethyl lactate [71] or D-limonene [72]. Although the use of ionic liquids (ILs) and deep eutectic solvents (DESs) has already been explored in some applications [73], the use of these novel solvents in pressurized conditions may provide with new interesting applications. DESs are obtained by mixing two naturally occurring components, namely, hydrogen bond acceptor and hydrogen bond donor, which can be associated with each other by means of hydrogen bond interactions. Considering that DESs are easily prepared starting from natural compounds, these compounds may be promising economically viable and environmentally friendly alternatives to traditional volatile organic solvents in different applications. Thus, it is expected that in the following years, more applications of PLE processes based on the use of ILs and DES will be presented.

Future developments of PLE- and SFE-based processes will surely be related to the application of process integration and process intensification, with further integration of the developed approaches in biorefineries. Potential combined processes may integrate other assisted techniques with PLE and/or SFE, thus producing enhanced processes in terms of efficiency. This is the case of ultrasound-assisted extraction or enzyme-assisted extraction. Some advancements have already been done in these fields, although coupling has to be still optimized in most cases. In any case, the application of these techniques may significantly help to disrupt the natural matrices being extracted, thus increasing extraction yields and facilitating the recovery of the target bioactive compounds. Another interesting coupling is related to the integration of extraction and drying processes. As it has been described in this chapter, some applications have been studied. Drying and selective precipitation techniques based on supercritical fluids, such as gas antisolvent, SAS, solution-enhanced dispersion by supercritical fluids and rapid expansion of a supercritical solution are well-known processes with some applications, mainly in the field of pharmaceuticals micronization. However, their direct application to natural extracts drying and particle formation on line coupled to extraction procedures is more complicated and requires significantly more development and study. However, due to the intrinsic advantages that these coupled processes may provide, the development of this kind of approaches may be highly favourable for commercial applications.

In conclusion, although the use of PLE and SFE for bioactives extraction can be already considered a mature field, there is still significant room for new developments that can significantly increase the interest of these

techniques. Moreover, their characteristics in terms of sustainability and eco-friendliness are the key value to consider compressed fluids-based extraction mechanisms as very good candidates for their integration in more complex biorefinery approaches.

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